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ORIGINAL ARTICLE

Transfection of the H-RAS gene produces genomic instability in the MOLT-4 cell line

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Abstract

Objective: *The aim of this study was to evaluate the effect of the normal and mutated H-RAS gene on genomic instability and apoptosis in MOLT-4 cells.* **Material and methods:** *The MOLT-4 cells were cultured and DNA was extracted. Then, the region that compromises both codons (12 and 13) of the exon 1 in the H-RAS gene was amplified by PCR and sequenced. The pSV2neo, pSV2neo-Ras^{wt}, and pSV2neo-Ras^{mut} plasmids were extracted from the DH5-alpha strain, sequenced, and transfected in MOLT-4 cells. Finally, the number of chromosomes was analyzed by means of karyotype and the number of apoptotic bodies was evaluated using acridine orange with propidium iodide.* **Results:** *No mutations were found on the H-RAS gene in the region of the 12 and 13 codons of exon 1 in the MOLT-4 cells. On the other hand, the sequences of the pSV2neo-Ras^{wt} and pSV2neo-Ras^{mut} plasmids matched with the normal and mutated versions of H-RAS, respectively. An increase in the number of chromosomes per cell with the normal and mutated H-RAS genes was also observed, as well as, the presence of apoptotic bodies.* **Conclusions:** *There are no H-RAS gene mutations in the 12 and 13 codons of exon 1 in MOLT-4 cells. The pSV2neo-Ras^{wt} and pSV2neo-Ras^{mut} plasmids contain normal and mutated versions of H-RAS, respectively. The normal and mutated H-RAS genes induce genomic instability and apoptosis in MOLT-4 cells. Plasmids pSV2neo-Ras^{wt} and pSV2neo-Ras^{mut} contain normal and mutated versions of the H-RAS gene. Normal and mutated H-RAS genes induce genomic instability and apoptosis in MOLT-4 cells.*

Keywords: *H-RAS. MOLT-4. Genomic instability.*

Introduction

Genome stability is very important to maintain cellular homeostasis and ensure genetic continuity during cell proliferation[1].

The DNA molecule is subject to constant changes that can cause stable alterations (mutations), thus modifying the genetic information[2]. Changes can arise spontaneously during the basic processes of cellular metabolism, being due to errors in repair, replication, or recombination[3]. Alterations in DNA can also be induced by exogenous physical, chemical, or biological agents, frequently present in our environment[4]. Genomic instability can be understood as the spontaneous mutation rate in cells. It has been established that genomic instability is an important characteristic of processes such as cancer or cellular aging[5]. However, in normal cells, despite the large amount of damage that occurs in their DNA, the spontaneous mutation rate is low, because basic cellular processes such as replication, repair, and cell cycle control function correctly[6]. Therefore, the genomic instability associated with the presence of a high rate of alterations in the genome of the cells indicates spontaneous damage and the cell's capacity to repair this,

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due to the saturation of the repair systems (produced by a high rate of DNA damage) or by genetic factors (alterations in the genes involved in the stability of the genome, which guarantee the fidelity of DNA synthesis and/or its adequate repair)[7]. To date, genes that induce genomic instability have been described, such as the H-RAS gene, which is located in band 15.5 of the short arm of chromosome 11[8]. It has been described that the mutated H-RAS gene has been considered a dominant oncogene, whose presence is sufficient for the development of the tumor phenotype. Among the most frequent point mutations in H-RAS occurring at codons 12, 13, or 61[9]. The introduction of normal and mutated H-RAS genes into HeLa cells caused alterations in cell morphology (micronucleus formation), in cell cycle phases, as well as genomic instability[10].

Therefore, in this work, we observed that the transfection of the normal and mutated H-RAS genes in a T-type acute lymphoblastic leukemia (ALL) (MOLT-4) cell line causes genomic instability and apoptosis of the cells.

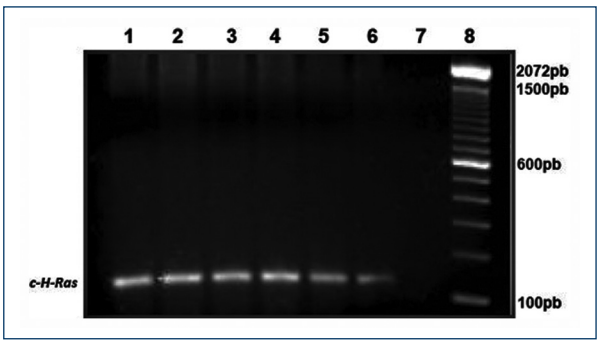

Figure 1. Expression of the H-RAS gene in MOLT-4 cells. Two percent agarose gel electrophoresis and ethidium bromide staining. Lanes **1-6:** 123 bp fragment of the H-RAS gene, **7:** negative control, **8:** weight marker.

Material and methods

Type of study

Prospective, experimental study

Obtaining bacterial clones

The DH5-alpha strains, which contain the plasmids pSV2neo, pSV2neo-Ras^{wt}, and pSV2neo-Ras^{mut}, were seeded in the solid culture medium Luria Bertani (LB) and the plates were incubated for 48 h at 37°C. Subsequently, under sterile conditions, a single colony was taken per plate and 10 mL of liquid LB medium was inoculated. Finally, the concentrates were centrifuged at 5500 ×g for 45 min at 4°C to obtain the bacterial buttons.

Obtaining the plasmids

Bacterial boutons were resuspended in 4 mL of buffer P1 (with RNase A). The lysates were centrifuged for 15 min at 8000 × g and the supernatants were poured into the QIAFILTER cartridge (QIAGEN). The plasmid DNA was eluted and stored at –20°C until analysis. Then, 250 ng of DNA was taken from each plasmid and sequenced by the automated method (Sanger deoxy).

Plasmid digestion

In 1.5 mL Eppendorf tubes, 5 μg of plasmid DNA, bovine serum albumin (BSA), and 3U of the restriction enzyme EcoR1 were added. It was mixed gently and the tubes were incubated at 37°C for 3 h. Subsequently, the enzyme was inactivated by heat at 65°C for 15 min, and 0.12 μL of the digested and linearized plasmids were loaded on a 0.8% agarose gel for visualization. The rest of the samples were stored at -20°C for subsequent studies. The plasmids were purified and sequenced by an automated method.

Cell culture

The T-type ALL cell line (MOLT-4) was seeded at a density of 5 × 10^5 cells per dish in 5 mL of RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin antibiotics (100 u/mL) and streptomycin (100 μg/mL). At the beginning of each experiment, cell viability was determined using trypan blue dye (1%) and the number of cells by counting with the Neubauer chamber.

DNA extraction

MOLT-4 cells (5 × 10^6) were centrifuged at 200 ×g for 5 min and the RPMI 1640 medium was decanted. DNA was isolated using DNAzol, quantified, and stored at 4°C.

Polymerase chain reaction (PCR)

The H-RAS gene was amplified by PCR with the following conditions: 1 cycle (94°C 5 min), 35 cycles (94°C, 52°C, and 72°C 1 min), and 1 cycle (72°C 10 min). Sense: 5´-ATGACGGAATATAAGCTGC-3´, Antisense: 3´-ATATCTCCACTCGGACCGC-5´.

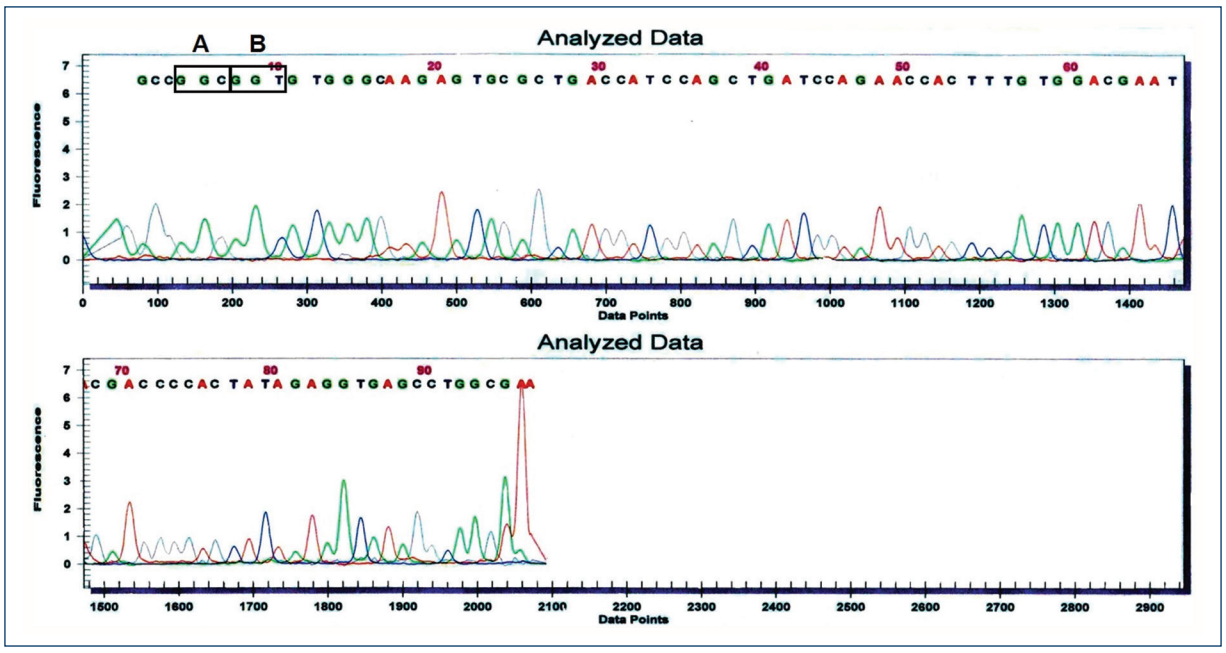

Figure 2. Sequence of the H-RAS gene in MOLT-4 cells. Region of codons 12 and 13 of exon 1 of the H-RAS gene. Note the triplet **A:** (GGC) and **B:** (GGT), which code for the amino acid glycine in normal DNA.

The amplified fragment was sequenced by an automated method. The internal control used was the endogenous beta-actin gene.

Transfection assay of normal and mutated H-RAS genes in MOLT-4

MOLT-4 cells were grown at a density of 10×10^6 per dish in 10 mL of supplemented RPMI 1640 medium. The cells were transfected by electroporation (960 F, 200 V) with the linearized DNA of the plasmids pSV-2neo-Ras^{wt} and pSV2neo-Ras^{mut}. In all cases, cells were transfected with the pSV2neo plasmid as a control. At 24 h after transfection, the cells were passed through Ficoll Hypaque to eliminate dead cells. Subsequently, the cells were washed twice with PBS and incubated for 24 h at 37°C and 5% CO_2. 600 μg/mL of the antibiotic G418 (INVITROGEN) was added to select resistant cells.

Determination of chromosome number in cells transfected with H-RAS

MOLT-4 cells (10×10^6) transfected and selected as previously described were grown for 96 h and then exposed to colcemid for 5 h and a triplicate count of 100 metaphases was performed.

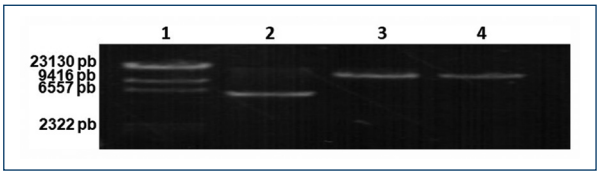

Figure 3. Plasmid DNA integrity. 0.8% agarose gel electrophoresis and ethidium bromide staining. Lane **1:** weight marker, **2:** pSV2neo, **3:** pSV2neo-Ras^{wt}, and **4:** pSV2neo-Ras^{mut}.

Determination of the number of apoptotic cells due to the effect of H-RAS

To analyze the number of apoptotic cells, they were washed twice with PBS, RNase (100 μg/mL) was added, and a mixture of acridine orange/propidium iodide (V/V) was added. It was incubated in the dark for 5 min and observed under a fluorescence microscope.

Statistic analysis

Analysis of variance was used to see if there were statistically significant differences between the pSV2neo-Ras^{wt} and pSV2neo-Ras^{mut} groups, with respect to the MOLT-4 and pSV2neo control groups. The p-value considered significant was < 0.05. Likewise, Dunnet's test was used to corroborate the differences between the groups.

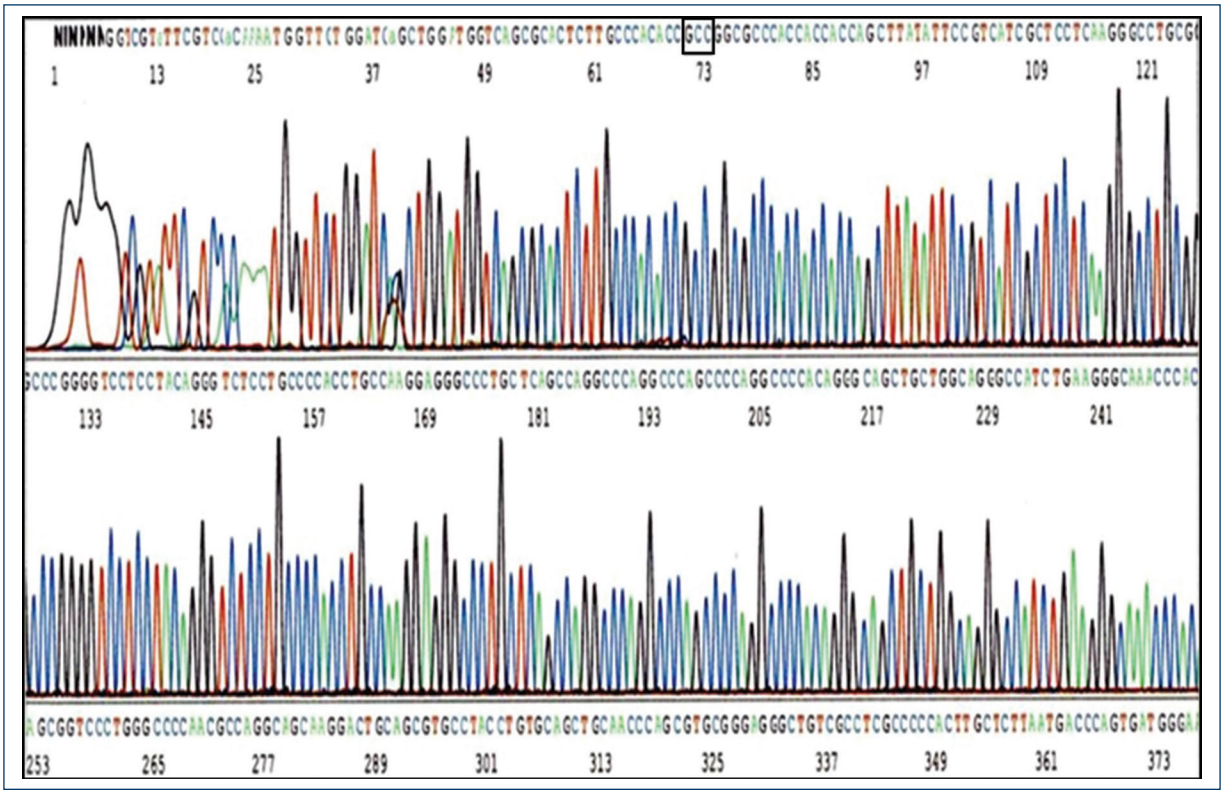

Figure 4. Sequences of the pSV2neo-Ras^{wt} plasmid. The sequence showed 100% homology with exon 1 of the H-RAS gene. Note in the upper box the codon (GCC) corresponding to normal DNA (glycine).

Results

Amplification of the H-RAS gene in the MOLT-4 cell line

One of the first objectives was to analyze whether the MOLT-4 cell line derived from ALL had any mutation in the H-RAS gene. Therefore, the gene was amplified by PCR, the fragment was 123 bp that covers the region of codons 12 and 13 of exon 1 of the gene. Fig. 1 shows electrophoresis in agarose gel stained with ethidium bromide, where the fragment is observed.

Determination of H-RAS gene mutations in MOLT-4 cells

Once the fragment was amplified, sequencing was performed to reveal the most frequent mutations of the H-RAS gene. The sequence was aligned in the Basic Local Alignment Search Tool (BLAST) showing 100% homology with codons 12 and 13 (GGC and GGT) that code for the amino acid glycine; no mutation of the H-RAS gene was found in MOLT-4 cells at the sites analyzed (A and B) (Fig. 2).

Integrity of plasmid DNA and H-RAS genes

The plasmids were isolated from the DH5-alpha strains, purified, and analyzed on an agarose gel (0.8%). Fig. 3 shows the integrity of the three plasmids pSV-2neo, pSV2neo-Ras^{wt}, and pSV2neo-Ras^{mut} after having been digested with the restriction enzyme EcoR1. The size of the plasmids is as follows: pSV2neo (5.7 Kb), pSV2neo-Ras^{wt}, and pSV2neo-Ras^{mut} (12.3 Kb).

Verification of the mutation in the plasmid DNA by sequencing

To ensure that plasmids pSV2neo-Ras^{wt} and pSV-2neo-Ras^{mut} contained the H-RAS genes in their normal and mutated versions, respectively, both plasmids were sequenced. Fig. 4 shows the sequences obtained from the wt and mut. When aligned in BLAST, the results showed 100% homology with the H-RAS gene. It can also be observed that in codon 12 of exon 1, there is the GCC triplet that codes for glycine (normal DNA) (H-Ras^{wt}) and codon 13 of the same exon contains the GAC triplet that codes for valine (mutated DNA) (H-Ras^{mut}) (Fig. 5).

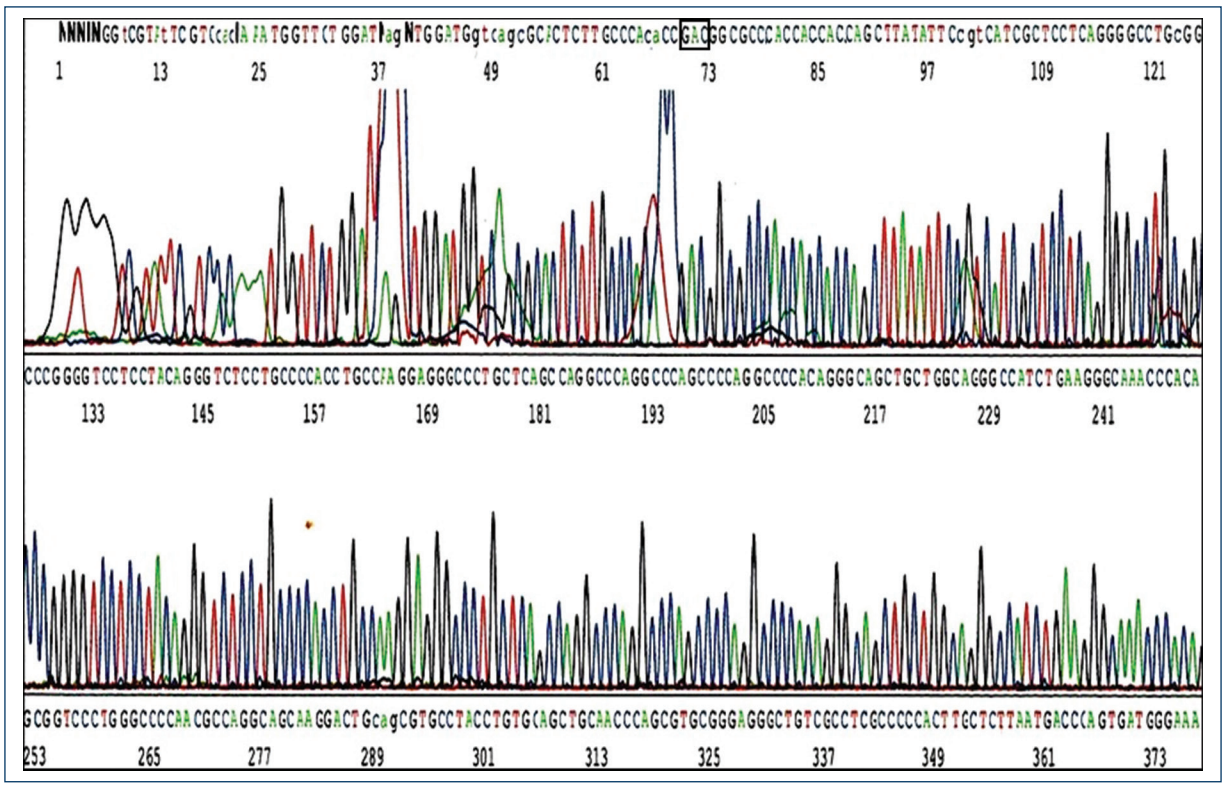

Figure 5. Sequences of the pSV2neo-Ras^{mut} plasmids. The sequence showed 100% homology with exon 1 of the H-RAS gene. They showed 100% homology with exon 1 of the H-RAS gene. Note in the upper box the triplet (GAC) (valine) of the mutated DNA.

Determination of the number of chromosomes in MOLT-4 cells transfected with the plasmids

MOLT-4 cells transfected with the pSV2neo vector showed no significant differences from untransfected MOLT-4 cells (average = 52; range = 10-130) (average = 49; range = 12-136), respectively. However, cells transfected with the normal or mutated plasmid exhibited a significant increase in chromosome number: average = 86, (40-151) and average = 77, (31-180), compared to the pSV2neo vector p = 0.05. These results indicate that the transfection of the H-Ras genes produces genomic instability due to alteration at the chromosomal and molecular level (Fig. 6).

Determination of the number of apoptotic MOLT-4 cells by transfection of the H-RAS gene

When evaluating cell death by means of the incorporation of acridine orange/propidium iodide and visualization in the fluorescence microscope, the cells transfected with the normal and mutated H-RAS plasmids presented an increase in the number of apoptotic cells compared to the cells untransfected (Fig. 7).

Discussion

The participation of N-RAS and K-RAS oncogenes in acute myeloblastic leukemias and myelodysplastic syndromes has been described. However, H-RAS gene mutations appear to have not been explored in other leukemia models. We looked for the most frequent mutations of the H-RAS gene in the region of codons 12 and 13 of exon 1 in the MOLT-4 cell line[11]; however, we did not find the mutation at those sites. Although it should be noted that other mutations in sites not yet explored in the complete DNA sequence of MOLT-4 cells are not ruled out. On the other hand, we know that mitotic death is a form of death that occurs during mitosis (cytokinesis), as a result of damaged DNA or due to the formation of the defective spindle linked to alterations in the mechanisms of the cell cycle checkpoints and occurs when apoptosis cannot be

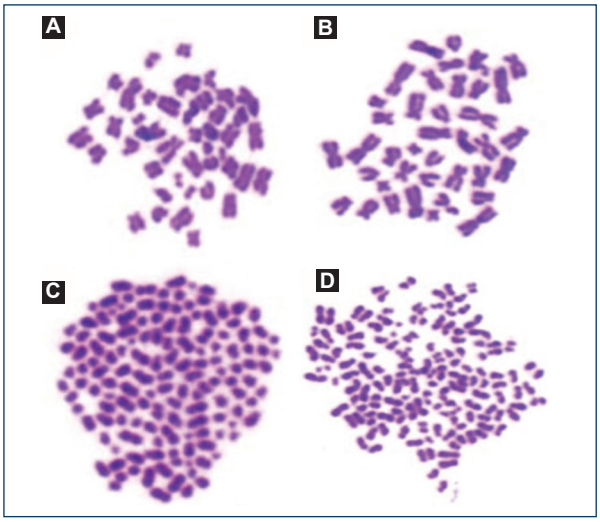

Figure 6. Chromosomes from untreated and H-RAS transfected cells. Cells were grown for 96 h and then exposed to colcemid for 5 h. Cytogenetic analysis was performed by counting 100 metaphases. Wright stain, magnification ×60. **A:** untreated, **B:** pSV2neo, **C:** pSV2neo-Ras^{wt}, and **D:** pSV2neo-Ras^{mut}.

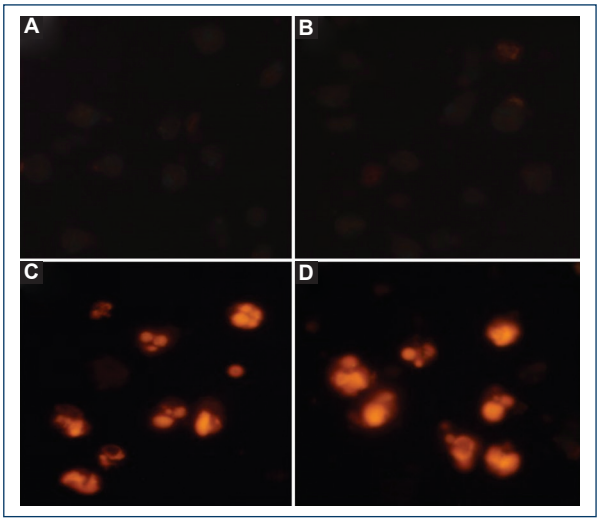

Figure 7. Transfection of the H-RAS gene induces apoptosis in MOLT-4 cells. The cells were transfected with: **A:** untreated, **B:** pSV2neo, **C:** pSV2neo-Ras^{wt}, and **D:** pSV2neo-Ras^{mut}. They were subsequently stained with acridine orange-propidium iodide and photographed (×40 magnification). **C** and **D:** note the formation of apoptotic bodies.

triggered due to alterations present in apoptotic proteins such as BCL2 or P53. Curiously, MOLT-4 cells present mutations in the P53 protein, which increases the possibility that the cells have died by another different mechanism such as mitotic death[12-14].

We demonstrated that the transfection of the plasmids with the normal and mutated versions of the H-RAS gene caused an increase in the number of chromosomes, producing genomic instability and apoptosis. On the other hand, there have been controversies regarding considering the H-RAS gene as a therapeutic target due to the large number of effectors it has; However, the possibility of using chemical inhibitors to block the most important signaling pathways in which RAS participates, such as PI3K or the RAF protein, among others, is not ruled out[15-18]. Although gene therapy stopped being used for a long time due to the consequences of the use of retroviruses, it has currently been resumed throughout the world, at least in animal models and as a therapeutic alternative for cancer in the not-too-distant future[19,20]. For now, we can say that the introduction of the normal and mutated H-RAS gene induces important changes in the leukemogenesis of MOLT-4 cells, a model derived from one of the most frequent leukemias in our country such as ALL. There are two main categories of genomic instability: chromosomal and molecular. Chromosomal instability refers to a persistently high rate of chromosome missegregation causing changes in the number of chromosomes, such as gain or loss of chromosomes[21-23]. Chromosomal instability is mainly caused by failures in the mitotic apparatus that in turn cause alterations in the mitotic process[24]. Furthermore, recent studies in other cell lines have shown that mitotic errors along with the extended mitotic arrest that is often associated with them can also lead to DNA damage, chromosome rearrangements, and fragmentations[25,26]. ALL is the most common leukemia in our environment and its prognosis remains unfavorable[27]. At present, only treatments based on chemotherapeutics are available that have effects on the cell cycle, causing the arrest of mitosis in metaphase, with dispersion and disorganization of the chromosomal material; as well as interfering with the cell's microtubules. In addition to this, transplants of hematopoietic progenitor cells are performed (graft versus tumor effect), and some phase 1 studies with signaling pathway inhibitors[28]. It is necessary to identify the role of various oncogenes such as H-RAS on leukemogenesis so that in the near future, they can be considered therapeutic targets.

Conclusion

Our work group demonstrated that there are no H-RAS gene mutations in the region of codons 12 and

13 of exon 1 in MOLT-4 cells. The Plasmids pSV-2neo-Ras^{wt} and pSV2neo-Ras^{mut} both contain normal versions and mutated of the H-RAS gene respectively. By transfecting the two types of Plasmids induce genomic instability and apoptosis in MOLT-4 cells.

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Conflicts of interest

The authors declare that no conflicts of interest.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Use of artificial intelligence for generating text. The authors declare that they have not used any type of generative artificial intelligence for the writing of this manuscript nor for the creation of images, graphics, tables, or their corresponding captions.

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