STUDY OF BIOLOGICAL EPIGENETIC PATTERNS IN RESPONSE TO CHEMOTHERAPY TREATMENT OF MULTIFORM GLIOBLASTOMA

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ABSTRACT

Multiform glioblastoma (MGB) is an aggressive form of brain cancer which presents a low survival rate owing to its resistance against chemotherapy and radiotherapy treatments. Tumor apparition and progression are associated with dysfunction of DNA repair mechanisms. According to the state of the genetic material, there can be guessed the presence of expressed proteins which will lead to specific cellular responses. For the performance of this experiment, it has been studied in the first line the pattern of methylation of *methyl guanine methyl transferase* gen (MGMT). That pattern is clinically associated with an improvement response for chemotherapy treatment of glioblastoma. This is so because there have been studies of genomic DNA previously extracted from commercial cells of MGB, which were: LN18 & LN229. Those commercial cells were analyzed in the aim of knowing the methylation state of their gene MGMT. Thus, amplification by PCR was done by using specific primers of the gen MGMT. Subsequently, the resulting PCR samples were loaded into an agarose gel and electrophoresis was performed. Our results demonstrate that MGMT is expressed in the cell line LN229, whilst the cell line LN18 was not. Therefore, the commercial cell line LN18 is aimed to be sensitive to tumor whereas LN229 is to be resistant. Hence it is known that clinical patients of glioblastoma who express the gen MGMT will be resistant to chemotherapy.

Key words: DNA, protein, MGMT gen, methylation, electrophoresis, genetic primer

RESUMEN

El glioblastoma multiforme (GBM) es una forma agresiva de cáncer cerebral que presenta una baja tasa de supervivencia debido a su resistencia ante tratamientos de quimioterapia y radioterapia. La aparición y progresión tumoral están asociadas a la disfunción de los mecanismos de reparación del ADN. De acuerdo con el estado del material genético, se puede deducir la presencia de proteínas expresadas, que darán lugar a respuestas celulares específicas. Para la realización de este experimento, se estudió principalmente el patrón de metilación del gen *metil guanina metil transferasa (*MGMT). Dicho patrón está asociado clínicamente con una respuesta de mejoría al tratamiento quimioterápico de glioblastoma. Es por ello que se han realizado estudios de ADN genómico previamente extraído de líneas celulares de GBM, las cuales fueron: LN18 y LN229. Dichas líneas celulares fueron analizadas con el fin de conocer el estado de metilación de su gen MGMT. De este modo, se realizó una amplificación por PCR usando cebadores específicos para dicho gen. Posteriormente, se cargaron las muestras resultantes de la PCR en un gel de agarosa y se realizó electroforesis. Nuestros resultados demuestran que MGMT se expresa en la línea celular LN229, pero no en la línea LN18. Por

tanto, la línea celular LN18 será sensible al tumor y la línea LN229, resistente. De esta manera, se demuestra que los pacientes de GBM que expresen el gen MGMT serán resistentes a quimioterapia.

Palabras Clave: ADN, proteína, gen MGMT, metilación, electroforesis, cebador

1. INTRODUCTION

Every day it is becoming more common to hear the word cancer, which makes sense if we consider that it is nowadays the leading cause of death globally, accounting for around 10 million deaths in 2020. However, the increase in cases of this disease poses a challenge for public health, with an increase of 30,000 cases registered in Spain alone during the last 6 years according to the Spanish Association Against Cancer. In fact, the European Commission has forecasted that by 2040 there will be a 32.2% increase in the number of cases in the countries belonging to the European Union compared to the number of cases in 2020 (Dyba et al., 2021).

Brain tumors are a variant of this disease, which is more common in children under the age of 18. Every year in Spain, about 1,500 new cases of cancer are registered in children under the age of 15, which between 15% and 20% correspond to CNS tumors (Alvaro and Taboada, 2020).

Multiform glioblastoma is an aggressive form of brain cancer, which is particularly aggressive and has a low survival rate mainly due to the tumor's resistance to chemotherapy, which, along with radiation therapy, is the main treatment use d after surgery (Robert and Wastie, 2008). The appearance and development of this tumor is associated with the dysfunction of DNA repair mechanisms, changes in epigenetic patterns, and the deregulation of gene expression. It has been described that DNA repair mechanisms contribute to GBM chemotherapy resistance, which implies that genes involved in DNA repair can be used as predictive biomarkers of GBM treatment response (Mansouri *et al*., 2019).

The main medicine used in the treatment of GBM is Temozolomide (TMZ), which is an alkylating agent. TMZ induces DNA lesions such as 06-methylguanine, which can be repaired by O6-methylguanine-DNA methyltransferase (MGMT).

The aim of the study is to analyze the MGMT methylation pattern with respect to GBM cell lines and the relationship between methylation status and response to TMZ.

2. THEORIC BACKGROUNDS

2.1. Nucleic acid molecules

Genetics is the branch of biology whose aim relies in the study of genes and mechanisms that regulate the transmission of hereditary information characters. These studies let us discover the way that specific specie is related, and what do they biologically have in common.

The very only tool of genetics is DNA (deoxyribonucleic acid) and RNA (ribonucleic acid). DNA is defined as the essential macromolecule for life. The importance of this compound lies in the fact that it is crucial for biological inheritance.

These DNA molecules are disposed in the cell core in a decondensed structure known as chromatin on as long the cell cycle. This chromatin is a substance formed by DNA associated to histones. As soon as the cell division commences, this chromatin condenses into chromosomes, preparing for the equitable distribution of DNA. Chromosomes are cylindrical structures of condensed chromatin of variable aspect, located in the nuclear plasm when the cell division phases take place.

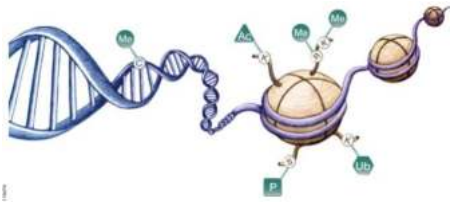

Figure 1. It is shown the structure of DNA condensation through the action of histones: DNA is shown being wrapped around histones.

DNA is biochemically defined as a nucleic acid. The unions among nucleotides form nucleic acids. Nucleotides are a biochemical structure formed by a nitrogenous base, phosphoric acid and carbohydrate as it is shown is figure 2.

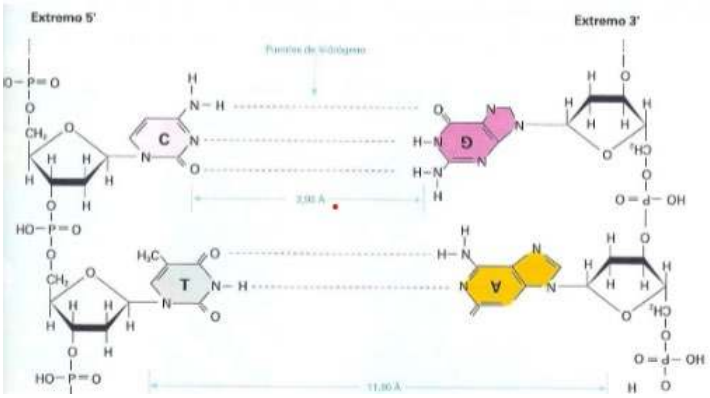

Figure 2: DNA structure shown.

Nitrogenous base could be either purine (Adenine, Guanine) or pyrimidine (Cytosine, Thymine and Uracil). This last base appears only in RNA molecules. The classification explained just above, depends whether the nitrogenous base belong from a nucleus of purine or pyrimidine either.

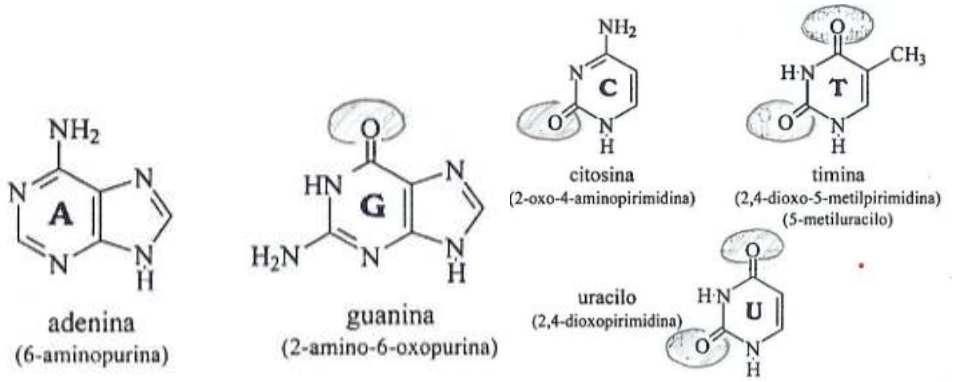

Figure 3: This image illustrates the five different nitrogenous bases which compose the internal structure of nitrogenous bases.

As before it has been explained, chromosomes are condensations of DNA, by the same token are unions of nucleotides. In addition, nucleotides form sequences called **genes.** A gen is a DNA fragment which contains the necessary genetic information capable of synthesizing a protein or a polypeptide chain. As a conclusion, chromosomes will have plenty of information for synthesizing proteins (Travers and Muskhelishvili, 2015).

Although all cells share the same genetic information, not all do they express the same proteins. The gene expression is highly regulated by different molecular mechanisms, one of them it is called epigenetic.

2.2. Epigenetics

Epigenetics is the branch of biology whose aim relies in the study of alterations among the genome (modifications that occur in DNA) without altering the sequences of nucleotides present in the gen taken. In relation of epigenetics with chromosomes, it is highly relevant to the fact of the implication of histones and other epigenetic marks in the condensation of chromatin.

Epigenetics' activity is supported by the performances of biomarkers. Biomarkers are biological molecules located in blood, other liquids or tissues within the body. Its presence is a sign of normal or abnormal processes, in response to an illness. Biomarkers are used forward detections of the response of an illness against any treatment. Genes are used as biomarkers.

The process of methylation consists in the presence of a group methyl in the cytosine base of DNA. Methylation suppresses the genetic transcription of the specific gene on which it performs, therefore those genes or proteins methylated will not be expressed neither in that gene, nor in the following genes transcribed afterwards. Substances and environmental factors may modify this methylation pattern in DNA and then deregulate the gene expression (Esteller, 2007).

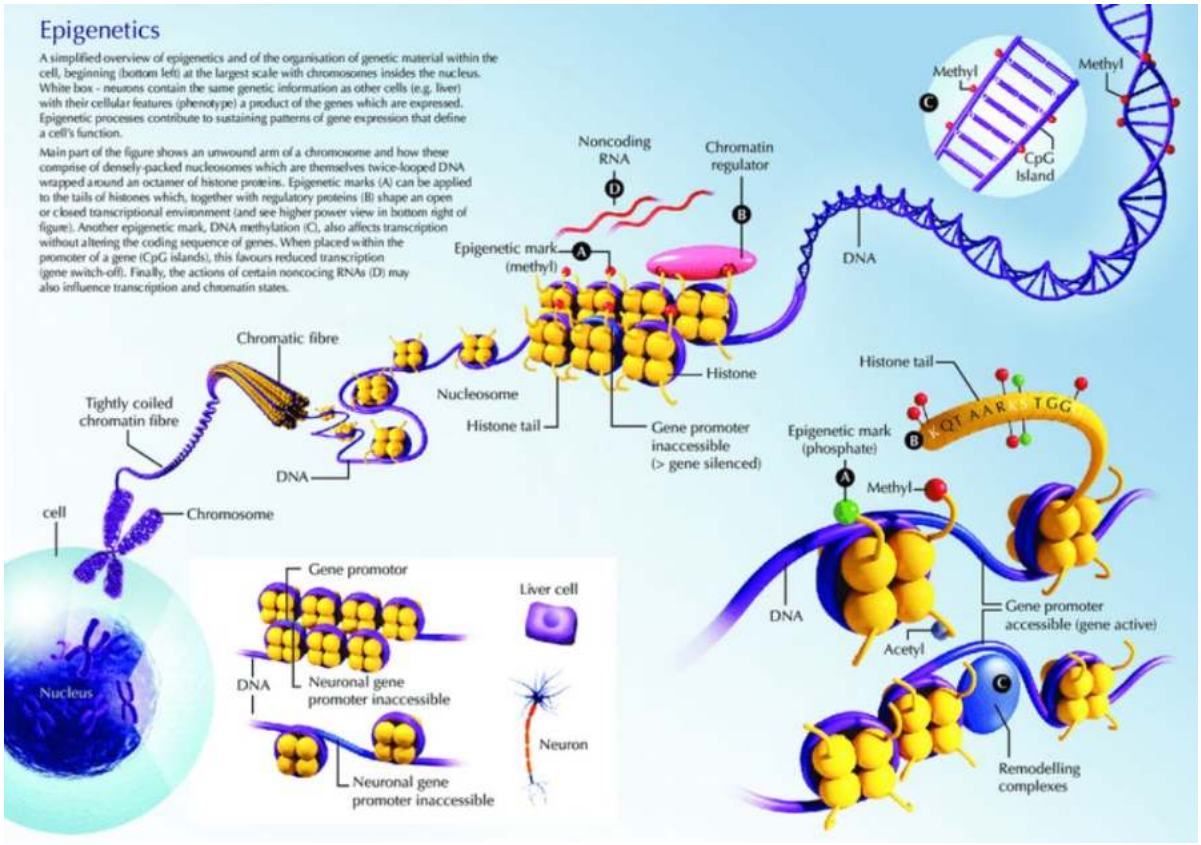

Figure 4: Brief synthesis of epigenetic activity shown in this picture.

2.3. qMSP

Quantitative Methylation of Specific Polymerase is a protocol aimed to analyze the grade of methylation of a molecule. Applied to the specific experimental procedure of this project, For describing the experimental procedure, on DNA samples of patients suffering from Glioblastoma, there will be performed a transformation of sodic bisulfite as an epigenetic mark.

Methylation of Cytosine is an epigenetic mark which reveals the state of methylation of DNA: Sodic Bisulfite transforms non-methylated cytosine into Uracil (owing to their chemical similarity), whereas methylated cytosine will remain equal. This reaction allows differencing methylated DNA from not methylated (Herman *et al*., 1996).

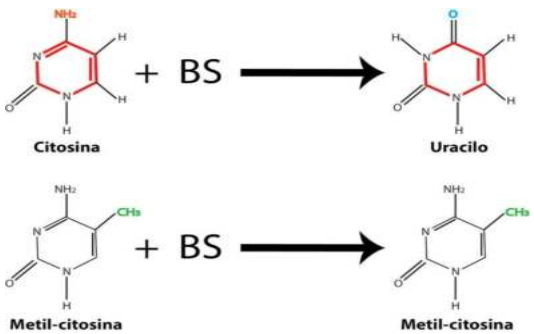

Figure 5: Biochemical reaction of *1. Cytosine; 2. Methylcytosine* in combination with bisulfite.

This state of methylation is afterwards observed by the electrophoresis made in relation with the PCR amplification of that gen. PCR is a laboratory method used so as to obtain numerous copies of a particular fragment of DNA from a sample that contains small quantities of DNA. With the Polymerase Chain Reaction (PCR), that DNA fragment is amplified (Liloglou and Nikolaidis, 2013).

2.4. Electrophoresis

Subsequently, this amplification result from PCR is observed in the electrophoresis. Electrophoresis is a technique used forward to separate molecules according to their mobility (speed) within an electronic field. Nucleic acids already have a negative electric charge, which will lead them to the positive pole (Slater *et al*., 2002).

The activity of electrophoresis frequently contributes to the detection of presence of DNA in a sample. This molecular separation may be performed on the hydrated surface of a solid supporting device, within a porous matrix or in dissolution. Nucleic acids, equally as proteins, they both are molecules. The greater mass the molecule has, the more difficult it will be for it to pass the gel pores as it is shown in figure 6.

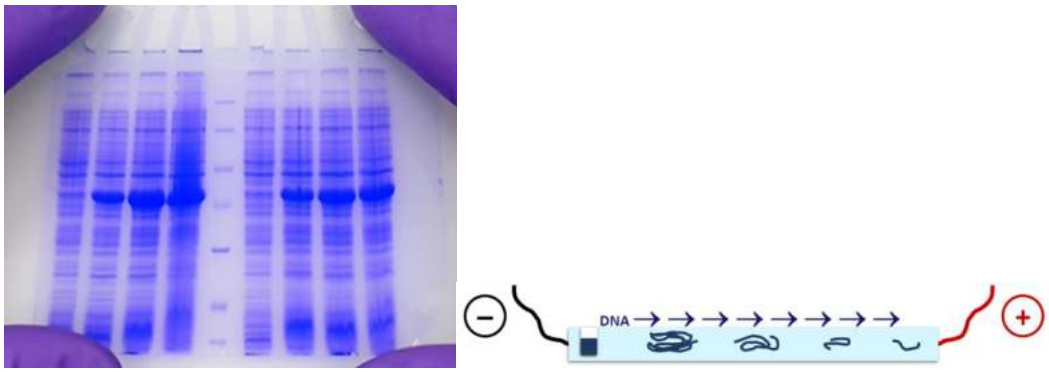

Figure 6: Revelation of results in electrophoresis techniques by agarose base. Each blue line is related to a protein. The bigger molecular weight the protein is, the bigger the blue mark will be when being revealed.

The commonly used variant forward to the analysis of mixes of proteins or nucleic acids, used as a gel support, is usually constituted by agarose or polyacrylamide. In order to perform this specific project, there will be used agarose gel, in a concentration 0.7g/100ml of dissolution. The procedure of charging the samples in the gel and the application of the electric current, will be explained in the methods section.

In this electrophoresis method, not only DNA samples as a result of PCR will be examined, but also a control base of actin. Positive and negative concurrent control treatments are necessary in scientific assay to validate the results obtained. Actin is a filamentous protein of the cellular protoplasm, which is responsible for conferring its form to the cell and then is constitutively synthetized. Applied to those methylated MGMT genes, electrophoresis should reveal the following results:

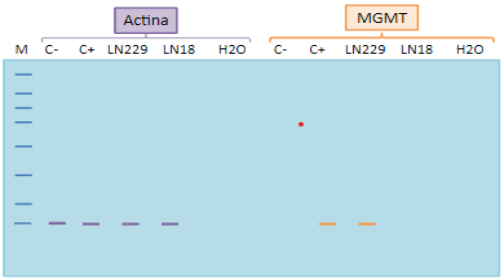

Figure 7: Revelation of results in electrophoresis by agarose base so as to confirm whether samples are polluted (show DNA in non-living substances such as water) or not. It is shown the study of each variable twice: 1) Study through actin primers: shows whether DNA is present or not. 2) Study through MGMT primers: shows whether MGMT gene is present or not.

2.5. Glioblastoma brain tumor and chemical treatment

During this project, epigenetics mechanisms have been applied to the genetic information of cancer cells from patients suffering from *multiform glioblastoma*. *Multiform glioblastoma* is a type of aggressive cancer generated within the brain or spinal cord. It is the most malignant astrocytoma. There are several types of *gliomas*, common brain tumors that affect glial cells. Gliomas are categorized according to their cellular skills, localization and severity. One group of gliomas is "astrocytoma". This brain tumor states a close relation forward to astrocytes (Purves *et al*., 2001).

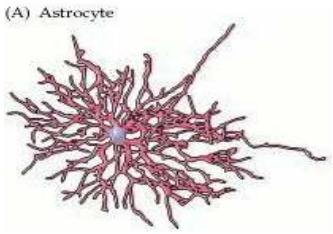

Figure 8. Astrocyte, Glial cell

"Astrocyte" is a term known to describe star shaped glial cells which have a great connection with blood vessels, carrying substances among blood and neurons, providing them support and nutrition. There are furthermore way more glial cells such as microglial cells, Schwann cells, oligodendrocytes, etc.

Glioblastoma may appear at any age, being adults the most affected beings to this cancer. It provokes headache, nausea, vomit and convulsions, being its unique treatment the combination between radiotherapy and chemotherapy (this last process provided by "**Temozolomide**"), previous to surgical removal of the tumor.

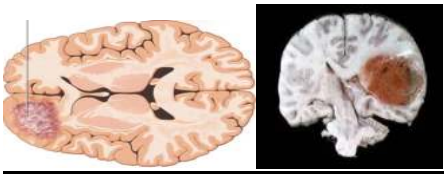

Figure 9. Transverse and sagittal dissections of the brain. Glioblastoma tumor is shown

Temozolomide is a medicine aimed at treating some sorts of brain tumors owing to its capacity of inhibiting the reproduction of tumor cells by causing their death. Nevertheless, not always is it effective. This chemotherapy treatment performs damages in DNA, modifying the base Guanine. This fact leads the cancer cell to death. Whether the cellular death is caused, *Glioblastoma* will be sensitive to chemotherapy.

MGMT protein is capable of repairing those damaged guanines from the DNA fragment treated with *Temozolomide*. Hence, when those MGMT proteins are expressed in tumor cells, they will not die and Glioblastoma cells become resistant to the treatment. Yet, MGMT is not expressed in all patients with Glioblastoma (Mansouri *et al*., 2019).

For this reason, this project consists in the analysis of epigenetic pattern of specific genes for reparation of DNA, specifically the pattern of methylation of MGMT gene. Indeed, methylation of MGMT gene has been associated with a better response forward chemotherapy treatment of patients with Glioblastoma. Hence, methylated MGMT genes will not be capable of repair those guanines destroyed by Temozolomide chemotherapy, and a result, cancer will be sensitive to medication, whilst MGMT not methylated will result to be resistant to Temozolomide.

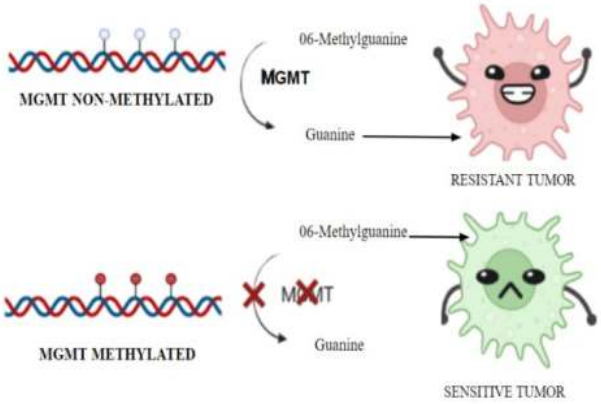

Figure 10. Effect of MGMT methylation status on glioblastoma cells.

3. HYPOTHESIS AND OBJECTIVES

Multiform Glioblastoma is an aggressive form of brain tumor, clinically treated with mechanisms of radio and chemotherapy, in addition to surgery as first line treatments. Being this project closely focused on the field of the epigenetic study forward the response of the tumor to chemotherapy, there will be used as a main variable the gene which is capable of reversing the effect of the treatment throughout the reparation of the harms caused within DNA by itself, that is to say Temozolomide.

This is so because the main hypothesis extracted previously to experimentation, is the fact that the cancer Multiform Glioblastoma is sensitive to its corresponding chemotherapy treatment as long as the gene MGMT is not present in the patient's genome. In turn, whilst the gene MGMT is expressed, harms caused in the genetic material within the cancer cells will be reversed, and by the same token the cancer will be resistant to treatment.

Thus, the following objectives suitable to this project are derivate, which are consistent to the previous hypothesis:

- To study biomarkers which may predict the responses to chemotherapy in MGB
- To analyze the epigenetic patterns of specific genes of reparation of DNA
- To observe the methylation grade of specific epigenetic marks so as to know the expression of those proteins which reverse the tumoral treatment.

4. PLANNING OF THE SESSION

Four sessions were carried out as follow:

Session 1 *16/02/22* (4hours): Institutional presentation of IMIBIC and visit to the facilities of the "GC22 Epigenetics" group of IMIBIC. Presentation of laboratory standards, project objectives and introduction to epigenetics and its importance in tumor processes.

Session 2 *16/03/22* (4hours): Learn the fundamentals of the analysis of epigenetic patterns and the most used methodologies. Analysis of epigenetic marks by modification with bisulfite and amplification by specific PCR of the methylation state.

Session 3 *30/03/22* (4hours): Observation of the results by electrophoresis in agarose gels and analysis of results.

Session *4 20/04/22* (4hours): Counselling on the development of a scientific poster (structure, number of graphic elements, colors, letter).

5. MATERIALS AND METHODS

5.1. Cell lines

Throughout experimentation, there have been used samples of:

- **POSITIVE CONTROL OF METHYLATION:** DNA completely methylated
- **NEGATIVE CONTROL OF METHYLATION:** DNA completely non methylated
- **LN229:** Cell line of Glioblastoma
- **LN18:** Cell line of Glioblastoma

5.2. Sodium bisulfite treatment

PCR amplification of each DNA sample is performed for observing the methylation status in each one. Those MGMT genes non-methylated, in presence of bisulfite, contain uracil in their sequence of nucleotides instead of cytosine, as before it has been explicated; whereas the sequences of methylated MGMT conserve the chemical structure of cytosine. Therefore, those MGMT genes methylated will not concord with the PRIMER input for MGMT established for the PCR amplification, and DNA will not replicate (Liloglou and Nikolaidis, 2013).

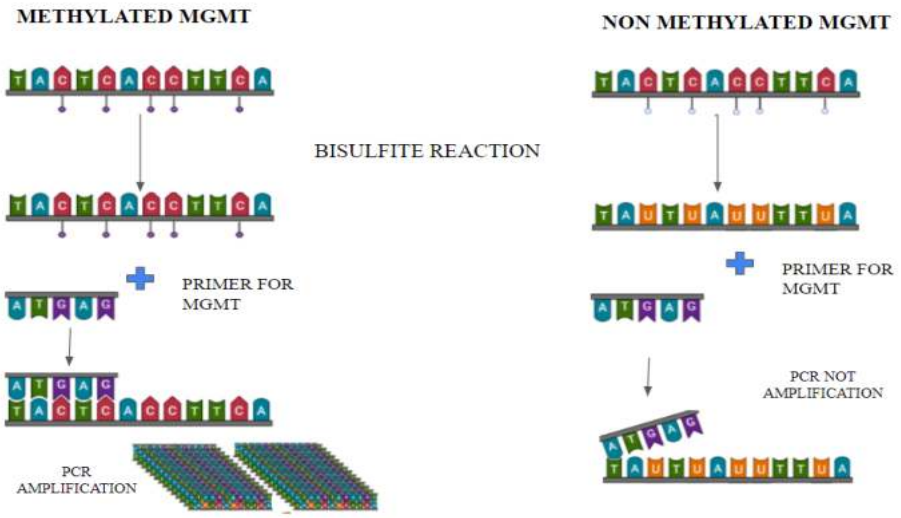

Figure 11: This picture graphic representation which show us how the DNA strand with methylated MGMT which contains uracil in its sequence of nucleotides concords with the DNA strand from the PRIMER, and the sequence with the non-methylated MGMT doesn't concord with the DNA strand from the PRIMER

5.3. PCR

In order to analyze the methylation state of all cellular lines, 2 groups of primers were performed: the 1º group contains actin primers (actin acts such as control, leading us to know if the DNA has been recuperated after the bisulfite treatment) leading us and the 2º MGMT primers.

Five PCR reactions were carried out, 4 of them according to the cellular lines, and another one with water for making sure DNA has not been contaminated during the process. One mix for each primer are prepared in an Eppendorf tube (1.5 ml) by adding these quantities:

Reactives	1 reaction	6 reactions
10x Mix Inmolase	1µL	6µL
dNTPs 2mM	1µL	6µL
$MgCl_2$ 50 mM	0.3µL	1.8µL
Primer Fw 5 µM	0.2µL	1.2µL
Primer Rv 5 µM	0.2µL	1.2µL
Inmolase	0.1µL	0.6µL
H_2O MQ	6.2µL	37.2µL
	9µL	

Table 1. It is shown all substances and quantities we need to performance the mix for PCR.

From this mix, 9µl are distributed in each PCR tube and 1 µ of bisulphite samples.
After this the tubes are put into a thermal cycler, programming:

- 95º 10 minutes
- 95º 30 seconds
- 60º 30 seconds
- 72º 30 seconds
- 72º 7 minutes
- 16º infinite

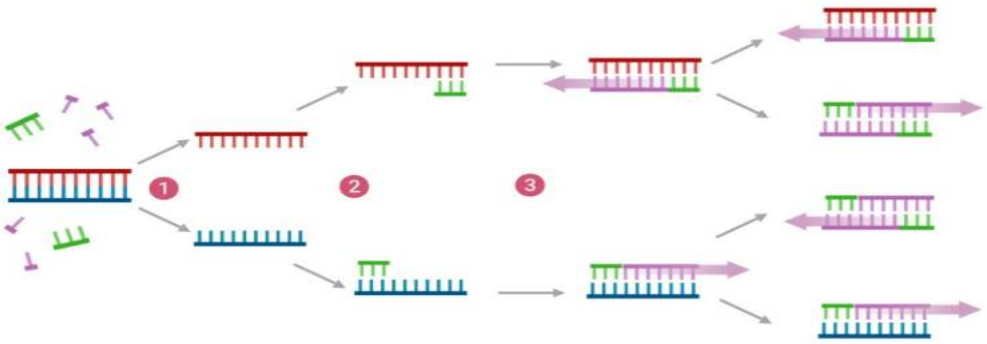

Figure 12: This picture shows how primers are attached and synthesizes the DNA strand to duplicate them.

5.4. Electrophoresis

An agarose gel has been prepared with the aim of analyzing the products from the PCR, to determine the MGMT methylation state. For preparing this gel, 0.7 grams of agarose gel and electrophoresis´ tampon TAE1x (100mL) are deposited on a flask (250 mL).This mix is heated in a microwave, in order to melt the sucrose, after that it is cooled until 40-45º.
It was necessary to prepare the tray of electrophoresis, sealing the edges with scotch tape and putting the comb with wells on the tray and depositing the agarose. After letting sucrose cool until it has gelled, the comb is carefully removed. Finally, the tray has been submerged in the electrophoresis tray, which has the tampon 1X TAE.

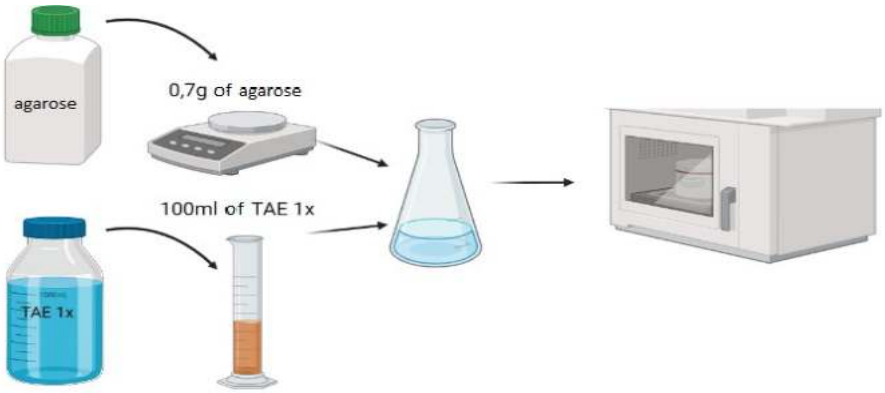

Figure 13: Scheme which shows the preparation of the agarose gel.

2µL of blusafe are added into the PCR tubes (it is an interleaving agent that let see the ADN with fluorescence), in addition another tube with a molecular weight marker is prepared (adding 4µL of marker, 2µL of Blusafe and 2µL of water) ,that contains DNA of different known sizes, which let us knowing the weight of the samples which we are amplifying.

Each sample is applied in a well, for that automatics micropipettes will be used the micropipette´s tip is introduced in the well without touching the walls and slowly the sample is included in the well. We connect the tray to a source of electricity, being careful with the positive and negative poles, and turn on the fountain to 120V. The approximated duration of this event is normally 40 minutes.

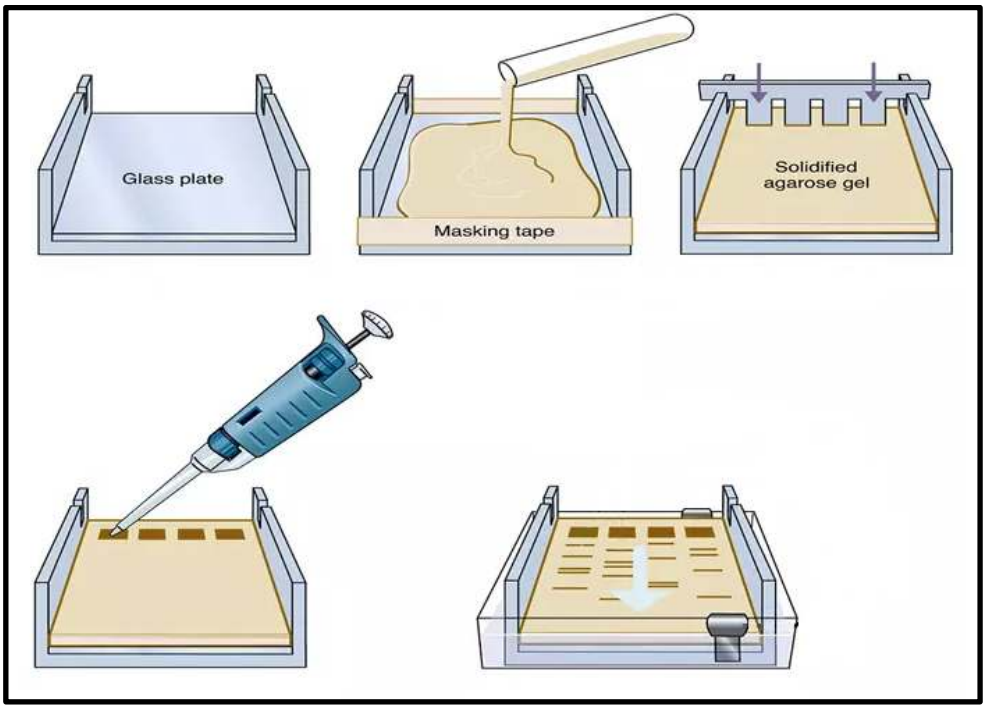

Figure 14: Steps followed with the aim of completing the electrophoresis.

5.5. Software

In order to visualize the gel and obtain the results from the electrophoresis, we used the program Nano drop, which shows us if the DNA is methylated or not. In this program it is necessary to put the gel on a trans illuminator and selecting the EtBr/ultraviolet filter to see it, we have to put the exposure to 400ms too, but it can increase depending on the gel´s appearance. After that the image of the gel was saved for work with it.

In addition, Google Drive was used to performance all documents which were required in it was also used to work on line all together.

Figure 15: It is shown the results of the samples after being studied.

5.6. Experimental design

In the aim of knowing whether MGMT gene is expressed having received chemotherapy, the following study was performed. From Genomic DNA, which had before been extracted from specific cell line of MGB, it was performed a treatment with bisulfite as an epigenetic mark so as to know the state of methylation of DNA. Afterwards, it will be performed by an amplification by PCR of this genetic material. Those MGMT genes non methylated will not be replicated whilst methylated ones, will.

Finally, using Nano Drop to reveal the results, the conclusions of all our investigation could be evaluated and discussed.

6. RESULTS

For results, it is observed the electrophoresis that we carried out in which different cell lines using actin protein as concurrent control (It is shown in figure 16).

Actin is a protein that is used to detect DNA since it is expressed constitutively Therefore, it is observed how bands have appeared in C-, C+, LN229 and LN18, which are the cell lines studied and in which there is presence of DNA.

However, it is observed that in water there is no band, since this substance is an organic macromolecule in which there is no presence of DNA, and this shows that the PCR has been run correctly and there are no failures and then, our results are validated.

MGMT is a gene that has the function of repairing the genome by eliminating the alkyl group, from its use we will observe which cell lines will be resistant or sensitive to chemotherapy.

Primers C+ and C- allow to distinguish methylated and non-methylated DNA respectively, so they are suitable for use in this study.

LN229 DNA shows amplification since methylated cytosine is reproduced, so no MGMT protein is expected and it can be known that this cell line is sensitive to Temozolomide and therefore, that the tumor will not be resistant to chemotherapy. Instead, LN18 DNA does not show amplification as it reproduces the non-methylated cytosine, and therefore MGMT protein is expected, so it can be known that it is a cell line resistant to Temozolomide and chemotherapy. Again, it is observed that in the water no band appears, since as we have said before, there is no presence of DNA.

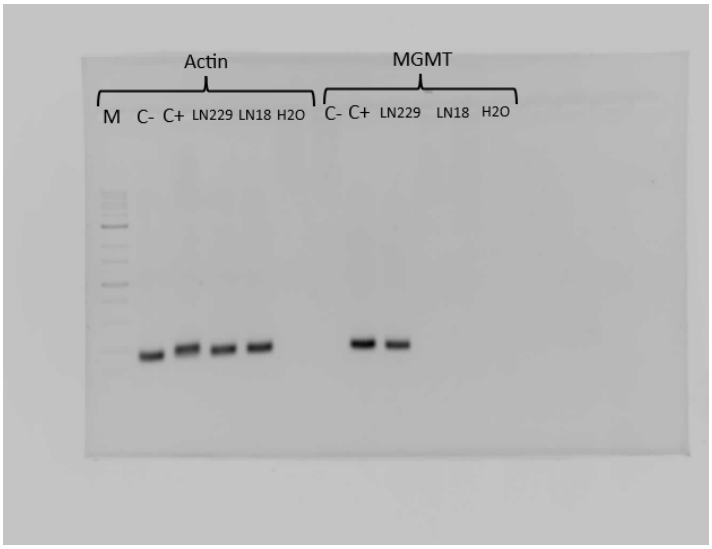

Figure 16: It shows the results of the samples.

7. CONCLUSION

Thanks to the research we did, we were able to draw several conclusions:

- There is no amplification in either of the samples which do not contain DNA.
- DNA modified with sodium bisulfite is in optime conditions. All DNA samples show amplification in qMSP when Actin primers were used.
- According to MGMT, primers allow to distinguish DNA methylated and DNA not methylated.
- LN229 DNA shows amplification for MGMT. Consequently, presence of protein MGMT is not expected, that's why that cellular line is sensitive to temozolomide.
- LN18 DNA does not show amplification for MGMT gen. LN18 does not have MGMT DNA methylated and MGMT protein is not expected, so tumor cells will present resistance to temozolomide as a result.

8. ACKNOWLEDGEMENTS

Having reached this part of the project, it is important to recognize those ones who have helped the investigation body, whether in and outside the working phase.

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