



## DNA Methylation Profile of Breast Cancer Cell via Hif-1 $\alpha$ Regulation of DNA Methyltransferase

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### ABSTRACT

Breast cancer is a type of cancer that originates in the cells of the breast. It is the most common type of cancer in women, although it can also occur in men. Hif-1 $\alpha$  (hypoxia-inducible factor 1 alpha) is a transcription factor that plays a central role in the response to low oxygen levels (hypoxia). DNA methylation is an important regulatory mechanism in the cell where in breast cancer cells, HIF-1 $\alpha$  has been found to play a role in the DNA methylation profile, potentially influencing the way that genes are expressed and contributing to the development and progression of the cancer. The area of studying the anticancer effect of GQDs is attracting growing attention because of its valuable properties. Especially due to its nano-sized sheets, it tends to infiltrate the cell nucleus and interfere with DNA function due to its ultra-small size. The results emphasize the validity of using GQDs as anticancer agent, with varied concentrations of GQDs inhibiting the development of human breast. To achieve this aim, we conducted the study on MCF-7 cell lines, and evaluate the cytotoxicity of different concentration of graphene quantum dots by MTT assay, RNA extracted from MCF-7 cell lines by Qiagen method and reverse transcriptase to cDNA to observe the expression of Hif-1 $\alpha$  and DNA Methyltransferase (DNMT) by Real time PCR (RT-PCR). The results showed that different concentration of GQDs do affect the regulation of Hif-1 $\alpha$  and DNMT and hence can stop the breast cancer pathway.

**Keywords:** Breast cancer MCF7, DNMT, HIF-1 $\alpha$ , DNA Methylation, DNA methyl transferase, Epigenetic, Graphene quantum dots (GQDs), nano medicine.

### 1. Introduction

Cancer is neither a uniform nor a contemporary disease. Depending on the tissue, there are about 200 different types of cancer in people. The Egyptian "Edwin Smith" and "George Ebers" papyri, which were written between 3000 BC and 1500 BC, contain reports of cancer (Faguet, 2015).

The most common tumour in the world and a serious public health issue is breast cancer. Breast cancer detection and screening have been significantly impacted by public attention, advancements in breast imaging, and breast cancer awareness. Women are more likely to die from breast cancer than any other disease, and it poses a serious threat to their lives. In the last two decades, there has been a notable advancement in our understanding of breast cancer, which has resulted in more potent treatments. After menopause, breast cancer is the leading cause of death for women, accounting for 23% of all cancer-related deaths (Akram *et al.*, 2017).

The second most frequent malignancy among women to cause death is breast cancer. Breast cancer has multiple phases and different cell types, and prevention is still a major problem worldwide.

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One of the best ways to stop the disease is by early identification of breast cancer. Because of early identification, the average 5-year survival rate for patients with breast cancer in several industrialised nations approaches 80%. Significant progress has been achieved in the understanding of breast cancer and the creation of medicines for its prevention over the past ten years. Numerous genes linked to breast cancer were found, and the identification of breast cancer stem cells provided pathways for tumor-resistant pathophysiology and cancer. Alternative medications are now available for chemotherapy prevention (Sun *et al.*, 2017).

A number of hereditary and environmental factors contribute to the development of breast cancer. Although numerous mutations in primary breast tumours have been found, it is challenging to correct these changes. On the other hand, the majority of epigenetic alterations are post-transcriptional, reversible processes that are not directed against specific gene sequences, and it is possible that inhibiting these mechanisms will help treat breast cancer (Billam *et al.*, 2009).

Oxygen is essential for life in all human cells and plays an important function in cellular metabolism (Patel and Sant, 2016). Indeed, hypoxic zones in all human tumors act as a microenvironment that promotes disease development, including breast cancer. Indeed, hypoxic areas can be seen in 25-40% of breast cancer tumors (Liu *et al.*, 2015).

Cellular responses to low oxygen tension are mediated primarily by the activating hypoxia-inducible factors (HIFs), which are comprised of a constitutively expressed component (HIF-1) and an oxygen-regulated subunit (HIF-1 $\alpha$  and HIF-1 $\beta$ ) (Keith *et al.*, 2012). HIF-1 overexpression has been associated with increased survival in a number of primary and metastatic human cancers. The connection between HIF-1 activity and patient survival in breast cancer has also been studied in clinical studies. Contradictory information has been found, though (Shamis *et al.*, 2021).

Hypoxia is often present in almost all solid tumors and is associated with the biological pathways that promote tumor growth. The deprivation of tumor oxygen has shown that tumor cells genetic modifications and adaptations can lead to survival and multiplication. These hypoxia-induced changes increase the sequential selection of functional hypoxia tumor cells and make the tumor more aggressive. As a result, the identification of hypoxia tumors can provide selection method for predicting the aggressiveness of specific tumors and can ultimately be used to prescribe treatment plans (Kim *et al.*, 2005).

(HIF-1) is a major transcription factor in the tissues of tumors and breast cancer, especially in triple-negative breast cancer. In breast cancer, HIF1 plays an important role in regulating the gene production that causes tumor development, angiogenesis, and tumor spreading (Bailey *et al.*, 2020).

In humans, one of the key epigenetic processes is DNA methylation, which is mediated by intergenic isoforms DNMT1, DNMT3A, and DNMT3B. Several studies have shown that DNMT isoforms are intricately and complexly regulated at the transcriptional, translational, and post-translational levels. The recent discovery of allosteric modulation of DNMT isoforms and being controlled by other associating chromatin altering proteins stresses functionality and structure and their role in the formation and progression of breast cancer. DNMT isoforms are controlled by a number of internal and extrinsic factors (Hegde & Joshi, 2021).

Carbon-based nanomaterials have gained a dominant place as drug delivery carriers. It is really intriguing to observe that a carbon nanostructure might also be utilized as a drug, in addition to its usual employment as a drug delivery carrier. Graphene quantum dots (GQDs) are presently in the media spotlight in this regard. GQDs are a recent addition to the list of carbon-based nanomaterials (Henna & Pramod, 2020).

GQDs have found use in a variety of fields, including nanomedicine. In compared to materials, quantum dots, GQDs have reduced toxicity and other specific features, making them ideal qualities for medical applications (Zhao *et al.*, 2020).

## 2. Material and Methods

### 2.1 Chemicals

All chemicals were Obtained from Egypt's Central Public Health Laboratories (CPHL). Primers were purchased from (Applied Biosystems), the RNA extraction kit from (Qiagen, Hilden, Germany), and the PCR kit HERA SYBER GREEN/ROX RT-qPCR from (Applied Biosystems) (Applied Biosystems, Foster City, California, USA). All work was done in Egypt's Central Public Health Laboratories (CPHL).

## 2.2 Graphene quantum dots

The graphene Quantum Dots were purchased from Sigma-Aldrich, Egypt.

## 2.3. Cell line and Cell culture

Human breast cancer cell line, MCF7, was obtained from central public health laboratories in Egypt (CPHL). The cells were cultivated in T75 tissue culture flasks in low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 µg/mL penicillin, 100 µg/mL streptomycin, 2 mM/L-glutamine and incubated in a 95% humidified incubator containing 5% CO<sub>2</sub> at 37°C. Now cells ready for treatment with Graphene quantum dots.

## 2.4. MTT Assays

To evaluate the cell viability and the cytotoxicity was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were seeded in 96-well plates in DMEM supplemented with 10% fetal bovine serum, and 1% antibiotic antimycotic mixture. After 24 h of cell preparation, the growth medium was aspirated from each well and the cells washed with 1X phosphate buffered saline (PBS). Different concentrations of Graphene Quantum dots were two fold serially diluted in DMEM then added to cultured cells in 96-well plate in triplicate and incubated for 24 h post treatment to determine the cytotoxic concentration 50 (CC50). The medium was then removed and the monolayer of cells washed with 1X PBS three times before adding MTT solution (20 µL/well of 5 mg/ml stock solution) and incubated at 37 °C for 4 h till formulation of formazan crystals. Crystals were dissolved using a volume of 200 µL of acidified isopropanol and the absorbance measured at λ<sub>max</sub> 540 nm using an ELISA microplate reader. Finally, the percentage of cytotoxicity compared to the untreated cells was determined. The CC50 of Graphene Quantum dots was determined from a linear exponential equation.

$$\% \text{ Cytotoxicity} = \frac{\text{Absorbance of cell without treatment} - \text{Absorbance of cell with treatment}}{\text{Absorbance of cell without treatment}} \times 100$$

## 2.5. Real-Time RT PCR Analysis

The total RNA was then extracted from cells using Qiagen extraction kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). Subsequently, 500 ng of the purified RNA were used to synthesize the complementary DNA (cDNA) with random hexamer primers (Thermo Scientific) and (HERA SYBR® green RT-qPCR kit) Reverse Transcriptase (Thermo Scientific) according to the manufacturer's protocol. The quantitative real-time PCR (qRT-PCR) reaction mixture (25 µl) comprises the following: 0.5 µl of cDNA template, 12.5 µl of Maxima SYBR green PCR master mix (Thermo Scientific) and 1 µl of each primer (100 µM forward and reverse primers). Reactions were run in triplicate on Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Foster City, California, USA). The cycling conditions were as follows: 2 min at 50 °C, 2 min at 95 °C, cDNA were amplified by 45 cycles of PCR, with each cycle consisting of 30 s at 94 °C, 30 s at 52 °C, and 30 s at 72 °C.

The primer sequences were as follows:

Primer	Sequence
DNMT Forward	5'- ACTATGGGCCTGGAGCTGTA-3'
DNMT Reverse	5'- AGGGTCACATCCAACCTCTGC-3'
HIF-1α Forward	5'- TGCAACATGGAAGGTATTGC-3'
HIF-1α Reverse	5'- GCTTGAGTTTCAACCCAGACA-3'

Ct values were normalized to the values of the control β-actin housekeeping transcripts and log fold change were calculated according to the equation of  $2^{-\Delta\Delta Ct}$

## 3. Results

In this study the effect of Graphene quantum dots was investigated on (MCF7) cell line as models of human Breast cancer cell line. B. Actin was used as housekeeping gene (control) , (HIF-1α) , (DNMT).

### Cytotoxicity of Graphene quantum dots against MCF-7 Cell Lines Using MTT assay

“Cytotoxicity assays are often used to measure damage to cellular membranes, cell viability, cell death, or cell growth. Creative Biolabs has investigated a number of assays for your flexible selection to best match my results. To assess the cytotoxic effect of two distinct concentrations of Graphene quantum dots against human Breast cancer cells (MCF-7), the cells were cultured with varied concentrations (0.5 percent to 1 percent) of Graphene quantum dots. The MTT test was used to assess cell viability after 24 hours of incubation. The cytotoxicity assay results are provided in (fig .1).”

“The ability of Graphene quantum dots inhibit MCF -7 cell lines growth was investigated by cytotoxicity assay after the MCF-7cell lines were treated with various concentrations of Graphene quantum dots (0.5% to 1%). Figure 1 shows the concentrations and time-dependent effect of Graphene quantum dots on the viability of MCF-7 cell lines for 24 and 48 h were treated with various concentrations of Graphene quantum dots (0.5% & 1%). In the viability of MCF-7cell lines for 24 and 48 h. All concentrations were able to inhibit the proliferation of the cancer cells MCF-7) and viability shown (fig .1)”.

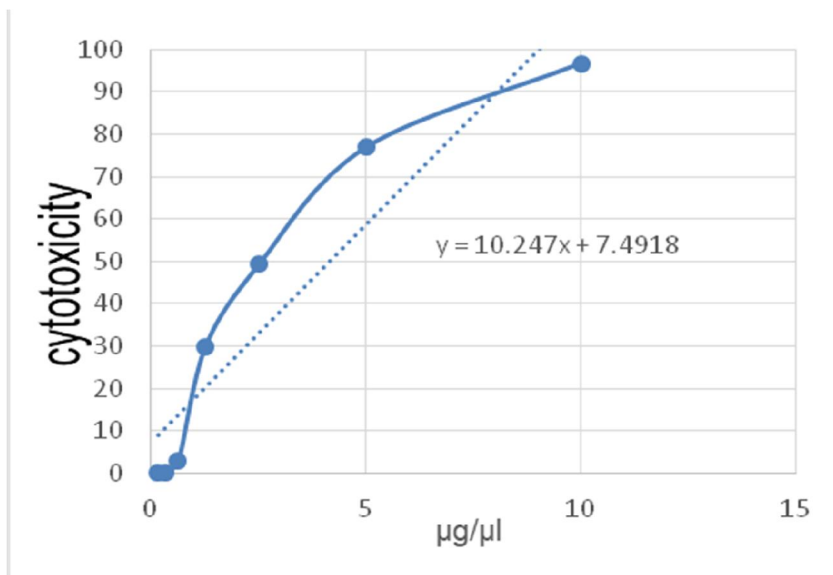


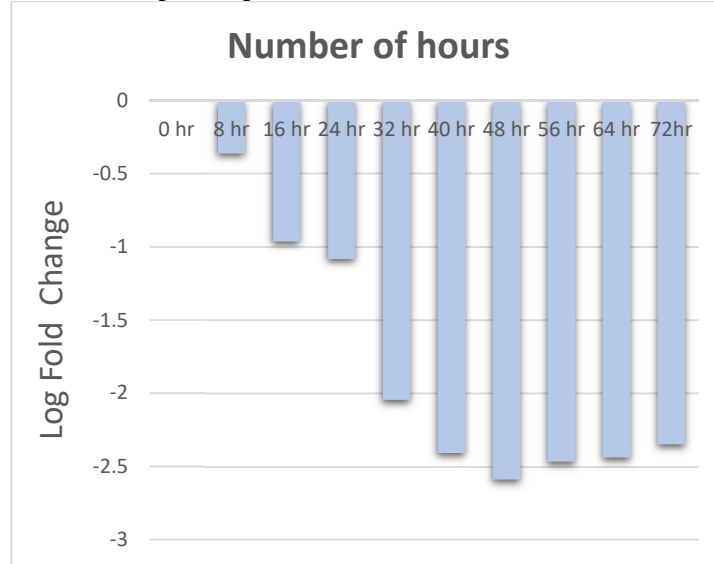
Fig.1: TC50=4.3 µg/µl

The cytotoxicity of the Graphene quantum dots extract was evaluated in MCF-7 cells using MTT assay. Graphene quantum dots was almost not toxic for studied cells up to a dose of 4.2 Or 4.3 µg/ml for Graphene quantum dots. The toxic effect of tested Graphene quantum dots was dose dependent. The result showed that the cytotoxic concentration 50 (CC50) value of Graphene quantum dots was 4.2 OR 4.3 µg. Therefore, for further studies we selected the safe concentrations of 1 -0.5 µg/ml for subsequent cellular signal studies.

### Evaluation of DNA methyl transferase and Hypoxia inducible factor gene expression after treatment with different concentration of Graphene quantum dots.

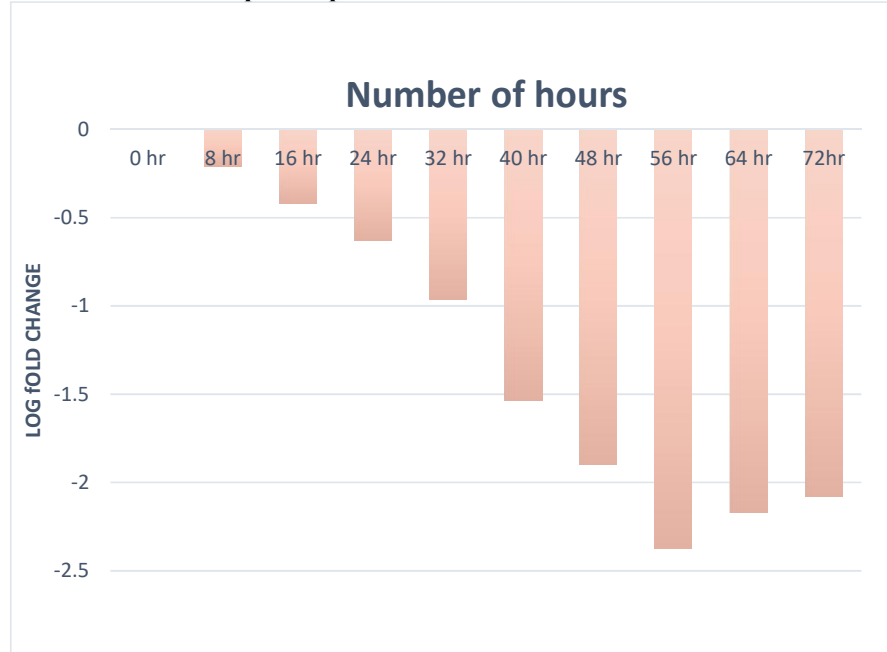
To determine the effects of Graphene quantum dots on DNMT and HIF-1α expression in Hepatocellular carcinoma, reverse-transcription PCR were performed following treatment with 1 -0.5 µg/ml Graphene quantum dots for different duration time (0 h, 8h, 16h, 24h, 32h, 40h, 48h, 56h, 64h and 72h). Gene expression of DNMT and HIF-1α was significantly down regulated (decreased) at 1 -0.5 µg/ml Graphene quantum dots treatment compared to untreated controls.

**Effect of concentration of Graphene quantum dots 1% on Cell line: MCF-7 on DNMT**



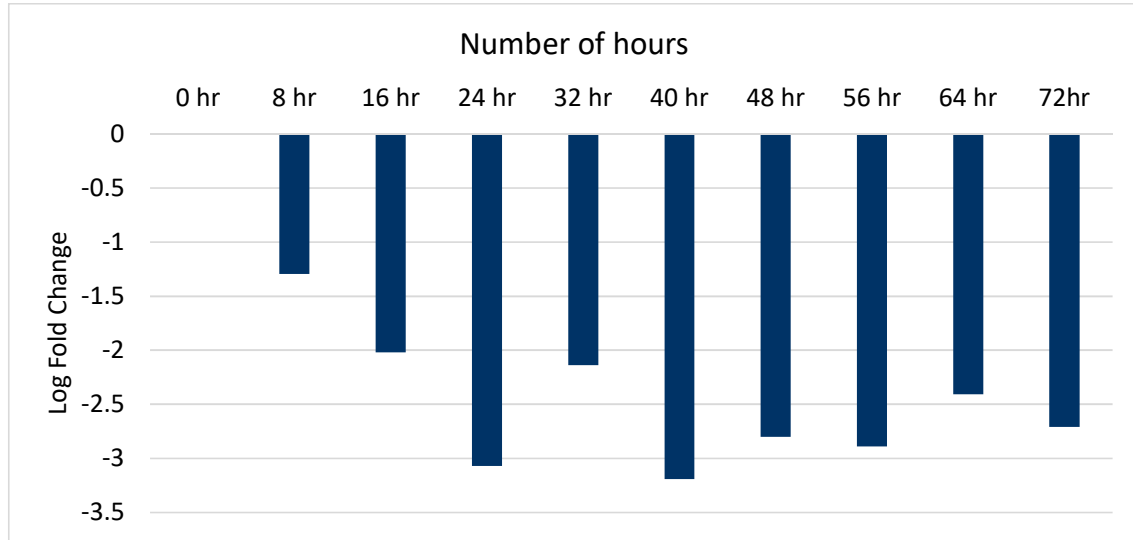
**Fig. 2:** The effect of concentration of Graphene quantum dots 1% on the gene expression of the DNA methyl transferase Gene in MCF-7 gene. The data represent a significant difference from control group across different exposure hours to Graphene quantum dots as when the time increase the down regulation of the gene DNA methyl transferase was increase.

**Effect of concentration of Graphene quantum dots 0.5% on Cell line: MCF-7 on DNMT**



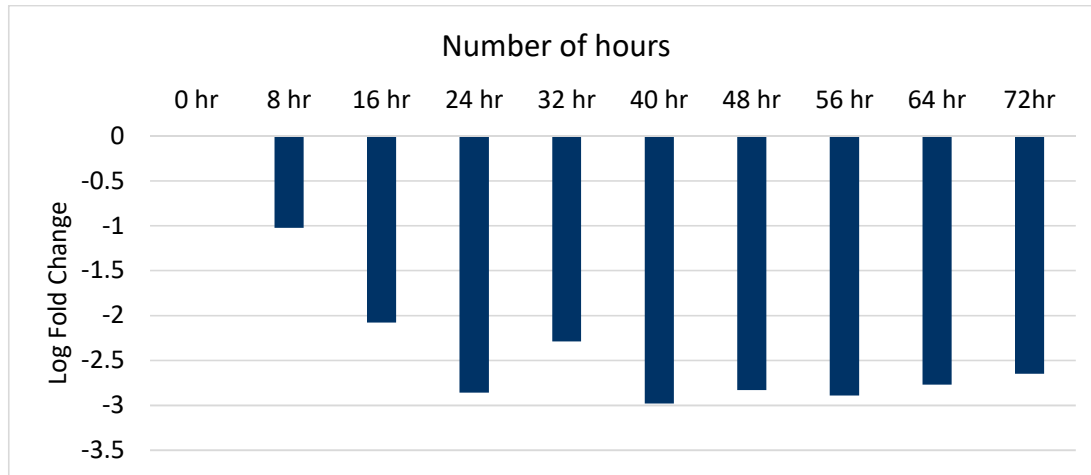
**Fig. 3:** The effect of concentration of Graphene quantum dots 0.5% on the gene expression of the DNA methyl transferase Gene in MCF-7 gene. The data represent a significant difference from control group across different exposure hours to Graphene quantum dots as when the time increase the down regulation of the gene DNA methyl transferase was increase.

**Effect of concentration of Graphene quantum dots 1% on HIF-1 $\alpha$  gene expression.**  
Cell line: MCF-7



**Fig. 4:** The effect of concentration of Graphene quantum dots 1% on the gene expression of the HIF-1 $\alpha$  Gene in MCF-7 gene. The data represent a significant difference from control group across different exposure hours to Graphene quantum dots as when the time increase the down regulation of the gene HIF-1 $\alpha$  was increase.

**Effect of concentration of Graphene quantum dots 0.5% on HIF-1 $\alpha$  gene expression**  
Cell line: MCF-7



**Fig. 5:** The effect of concentration of Graphene quantum dots 0.5% on the gene expression of the HIF-1 $\alpha$  Gene in MCF-7 gene. The data represent a significant difference from control group across different exposure hours to Graphene quantum dots as when the time increase the down regulation of the gene HIF-1 $\alpha$  was increase.

#### 4. Discussion

Breast cancer treatment is a significant burden for patients since it frequently comes with a variety of adverse effects. Women who get chemotherapy for breast cancer are more likely to be hospitalized as a result of these adverse effects. This would be reasonable if the treatment helped all patients. However, numerous breast cancer patients who are treated with hazardous cytostatic medicines may only have chemotherapeutic side effects rather than the desired tumor-shrinking impact. It is critical to

select the proper therapy as early as possible in order to kill the cancer cells avoid or postpone the emergence of drug resistance. Unfortunately, there are currently no viable procedures that are frequently used in the hospital to detect poor responders prior to treatment (Younis *et al.*, 2020).

HIF-1 $\alpha$  has been identified as a significant cancer therapeutic target. Many recent investigations have found a substantial association between high HIF-1 $\alpha$  levels and tumor spreading, angiogenesis, poor patient prognosis, and tumor resistance treatment. Hypoxia (low O<sub>2</sub> levels) was discovered to be a prevalent feature in many forms of solid tumors. Hypoxic tumor cells activate various survival pathways as a physiological adaptation to hypoxic stress, allowing them to carry out their critical biological functions in different ways than normal cells. Recent advancements in cancer biology have identified the HIF-1 $\alpha$  pathway as an important survival mechanism for which innovative cancer therapeutic techniques might be created. However, addressing the HIF-1 $\alpha$  pathway has proven difficult (Masoud & Li, 2015).

HIF-1 $\alpha$  in the activated form plays a critical role in tumor cell adaptation to oxygen fluctuations via increased expression of over 100 associated genes that regulate important biological processes necessary for tumor survival and growth. Genes involved in glucose metabolism, cell growth, migration, and angiogenesis are examples. In a rapidly developing tumor tissue, for example, HIF-1 $\alpha$  assists hypoxic tumor cells in shifting glucose production from the more productive oxidative phosphorylation route to the less effective glycolytic pathway in order to sustain energy output (the Warburg effect) (Warburg, 1956).

GQDs are currently focused on. GQDs are a relatively new addition to the family of carbon-based nanomaterials. They are currently being used to treat Parkinson's and Alzheimer's disorders. Furthermore, GQDs have antimicrobial and anti-diabetic properties. Furthermore, they are presently being intensively examined for medication delivery applications. They show promise for being used as a drug delivery to pass the blood-brain barrier. GQDs can also deliver drugs to specific tumors. Their bioimaging and biosensing applications are also being researched thoroughly (Henna & Pramod, 2020).

overall, the major portion of available research indicates that GQDs have minimal *in vivo* and *in vitro* toxicity and great biocompatibility, particularly when compared to their contemporaries graphene oxide (GO), carbon nanotubes, and traditional semiconductor quantum dots. GQDs are an attractive contender for bio-applications such as bio-imaging, bio-sensing, and biomedicine "e.g. medication administration" due to their low toxicity and great biocompatibility (Wang *et al.*, 2016).

In the present study, our results showing that the effect of the different concentrations of Graphene quantum dots on different genes on Breast Cancer cell line (MCF-7) for different duration time and gene expression was measured by real time polymers chain reaction (PCR), when the time increase the down regulation of the gene HIF-1 $\alpha$  was increase and also when the concentration of the Graphene quantum dots increase the down regulation of gene DNMT gene was highly increase. Graphene quantum dots shown great potential in drug delivery and biological imaging applications, in addition, Graphene quantum dots also shown promising effects against Breast Cancer. Finally, I hope that these findings will soon be translated to clinical trials, potentially improving Breast cancer patient outcomes and quality of life.

## References

- Akram, M., M. Iqbal, M. Daniyal, and A.U. Khan, 2017. Awareness and current knowledge of breast cancer. *Biological Research*, 50(1). <https://doi.org/10.1186/s40659-017-0140-9>
- Bailey, C.M., Y. Liu, G. Peng, H. Zhang, M. He, D. Sun, and Y. Wang, 2020. Liposomal formulation of HIF-1 $\alpha$  inhibitor echinomycin eliminates established metastases of triple-negative breast cancer. *Nanomedicine: Nanotechnology, Biology and Medicine*, 29, 102278.
- Billam, M., M.D. Sobolewski, and N.E. Davidson, 2010. Effects of a novel DNA methyltransferase inhibitor zebularine on human breast cancer cells. *Breast Cancer Res Treat*, 120: 581–592 (). <https://doi.org/10.1007/s10549-009-0420-3>
- Faguet, G.B., 2014. A brief history of cancer: Age-old milestones underlying our current Knowledge Database. *International Journal of Cancer*, 136(9): 2022–2036. <https://doi.org/10.1002/ijc.29134>
- Hegde, M., and M.B. Joshi, 2021. Comprehensive analysis of regulation of DNA methyltransferase isoforms in human breast tumors. *Journal of Cancer Research and Clinical Oncology*, 147(4): 937-971.

- Henna, T.K., and K. Pramod, 2020. Graphene quantum dots redefine nanobiomedicine. *Materials Science and Engineering: C*, 110: 110651.
- Keith, B., R. Johnson, and M. Simon, 2012. HIF1 $\alpha$  and HIF2 $\alpha$ : sibling rivalry in hypoxic tumour growth and progression. *Nat. Rev. Cancer* 12: 9–22. <https://doi.org/10.1038/nrc3183>
- Kim, D., J. Cho, S. Lee, and Y. Lee, 2005. Cobalt chloride-induced estrogen receptor  $\alpha$  down-regulation involves hypoxia-inducible factor-1 $\alpha$  in MCF-7 human breast cancer cells. *Molecular Endocrinology*, 19(5):1191-1199.
- Liu, L., J.-Z. Liao, X.-X. He, and P.-Y. Li, 2017. The role of autophagy in hepatocellular carcinoma: Friend or foe. *Oncotarget*, 8(34): 57707–57722. <https://doi.org/10.18632/oncotarget.17202>
- Masoud, G.N., and W. Li, 2015. HIF-1 $\alpha$  pathway: role, regulation and intervention for cancer therapy. *Acta Pharmaceutica Sinica B*, 5(5): 378-389.
- Patel, A., and S. Sant, 2016. Hypoxic tumor microenvironment: Opportunities to develop targeted therapies. *Biotechnology Advances*, 34(5): 803–812. <https://doi.org/10.1016/j.biotechadv.2016.04.005>
- Shamis, S.A., D.C. McMillan, and J. Edwards, 2021. The relationship between hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) and patient survival in breast cancer: Systematic review and meta-analysis. *Critical Reviews in Oncology/Hematology*, 159: 103231.
- Sun, Y.-S., Z. Zhao, Z.N. Yang, F. Xu, H.J. Lu, Z.Y. Zhu, W. Shi, J. Jiang, P.P. Yao, and H.P. Zhu, 2017. Risk factors and preventions of breast cancer. *International Journal of Biological Sciences*, 13(11): 1387–1397. <https://doi.org/10.7150/ijbs.21635>
- Wang, S., I.S. Cole, and Q. Li, 2016. The toxicity of graphene quantum dots. *RSC Advances*, 6(92): 89867-89878.
- Warburg, O., 1956. On the origin of cancer cells. *Science*, 123(3191), 309-314.
- Younes, M., C. Ammoury, T. Haykal, L. Nasr, R. Sarkis, and S. Rizk, 2020. The selective anti-proliferative and pro-apoptotic effect of *A. cherimola* on MDA-MB-231 breast cancer cell line. *BMC Complementary Medicine and Therapies*, 20(1): 1-10.
- Zhao, C., X. Song, Y. Liu, Y. Fu, L. Ye, N. Wang, and J. Liu, 2020. Synthesis of graphene quantum dots and their applications in drug delivery. *Journal of Nanobiotechnology*, 18(1):1-32.