

Research Article

Extremely Low Frequency Magnetic Fields Increase the Expression of HIF-1 α in Prostate Cancer Cells

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Abstract

Background: Increased levels of hypoxia-inducible factor 1 α (HIF-1 α) are related to poor prognosis and treatment failure in prostate cancer. The effect of extremely low frequency magnetic fields (ELF-MF), generally emitted from electronic devices on HIF-1 α in cancer cells and, specifically, in prostate cancer is not well described. This study aimed to investigate the effects of ELF-MF on HIF-1 α protein expression and HIF-1 transcriptional activity in prostate cancer cells.

Methods: Prostate cancer cells were irradiated with ELF-MF by means of a frequency generator at 875 MHz with an

intensity of 0.07 mW/cm² at various time periods and with an exposure system housed in a tissue culture incubator. The expression of HIF-1 α protein and HIF-1 transcriptional activity at the different conditions were evaluated.

Results: HIF-1 α expression in different prostate cancer cells was higher after one hour of exposure to ELF-MF. All exposed cells also demonstrated a significant 50% increase in HIF-1 transcriptional activity as measured by an HRE-dependent reporter gene assay after ELF-MF irradiation. Cells exposed to ELF-MF exhibited 3-fold longer HIF-1 α half-life compared to non-exposed control cells, while its mRNA levels were not affected. Immunofluorescence

staining confirmed nuclear HIF-1 α accumulation following exposure to ELF-MF. Cell proliferation was increased following ELF-MF exposure and significantly reduced when HIF-1 α was silenced even after exposure to ELF-MF.

Conclusions: Exposure of prostate cancer cells to ELF-MF increases HIF-1 α protein stability and upregulates HIF-1 transcriptional activity. These findings provide new insights into the effects of ELF-MF on the hypoxic pathway which are important for guiding future implications.

Keywords: Prostate cancer; Cancer; HIF-1; Extremely low frequency magnetic fields

Abbreviations: HIF-1 - hypoxia-inducible factor 1; ELF-MF - extremely low frequency magnetic fields; CHX - cycloheximide

1. Introduction

Prostate cancer is the most common solid malignancy in men [1]. Advanced disease is characterized by high-grade cancer and distant metastases. Hypoxic and angiogenic pathways are well known to be major contributors to the development of metastases and poor prognosis [2, 3]. Hypoxia is common among advanced human tumors in need of uncontrolled angiogenesis, and it is often associated with metastatic dissemination. Hypoxia-inducible factor (HIF)-1 and -2 are responsible for the primary mechanism that mediates adaptive responses to hypoxia. Cancer-specific HIF activity, especially in regions of intratumoral hypoxia, has been shown to mediate angiogenesis, epithelial-mesenchymal transition, stem cell maintenance, metastasis, and resistance to radiation therapy and chemotherapy [4, 5]. HIF-1 is a heterodimeric transcription factor composed of a constitutively expressed HIF-1 β subunit and an oxygen-regulated HIF-1 α subunit. Under

normal oxygen conditions, HIF-1 α is ubiquitinated by the tumor-suppressor protein, Von Hippel-Lindau, and is targeted for proteasomal degradation. Under hypoxic conditions, HIF-1 α rapidly accumulates in the cytoplasm and translocates into the nucleus where it dimerizes with HIF-1 β to recruit co-activators and drive transcription of many genes critical for key aspects of cancer progression. Increased levels of HIF-1 α have been demonstrated in the majority of primary human cancers and their metastases, and were shown to be related to poor prognosis and treatment failure [6].

Extremely low frequency magnetic fields (ELF-MF) are usually defined as radio waves with frequencies ranging from 3 Hz to 3 kHz, which are emitted from electronic devices. They are classified as being possibly carcinogenic, although the effect is not well described or understood. It is known that ELF-MF cannot traverse membranes or cause DNA damage. Many other mechanisms of actions have been studied among various diseases [7], however and some of the hypotheses that were put forth to explain them involved correlations between ELF-MF exposure and regulation of cell proliferation [8]. To the best of our knowledge, a correlation between ELF-MF and HIF-1 α in general and, specifically, a correlation between ELF-MF and prostate cancer have not been published to date. This study aimed to determine whether exposure to ELF-MF changes the expression of HIF-1 α and its transcriptional activity in prostate cancer cells.

2. Materials and Methods

2.1 Culture

Human prostate cancer PC-3, LNCaP, and DU145 cells were maintained in RPMI 1640. Human embryonic kidney HEK293 cells were maintained in DMEM. The media were supplemented with 10% fetal calf serum and antibiotics. All

cells were cultured at 37°C in a humidified atmosphere and 5% CO₂ in air. For hypoxic exposure, the cells were placed in a sealed modular incubator chamber (Billups-Rothenberg, Del Mar, CA) flushed with 1% O₂, 5% CO₂, and 94% N₂ and then cultured at 37°C.

2.2 ELF-MF irradiation

The cells were irradiated inside a humidified incubator. Irradiation was by a frequency generator (TGR1040 signal generator; Thurlby Thandar Instruments, UK) at 875 MHz with an intensity of 0.07 mW/cm². The generator was set to the desired power and connected to the power amplifier, which was connected to a panel antenna (MA-CL 67-12) that was fixed in the incubator. Amplification was by an ERA-3SM device (Minicircuits, USA). The emitting antenna was placed in the center of a shelf in the incubator, and the walls of the incubator were covered to avoid reflection of the sound waves.

2.3 Protein extraction and Western blot

Whole cellular extracts were prepared and analyzed as previously described.²³ Protein concentration was determined with a bicinchoninic acid protein assay kit (Pierce Biotechnology, USA). Protein extracts were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies.

2.4 Antibodies, reagents and siRNA

The primary antibodies were mouse monoclonal anti-HIF-1 α (BD Biosciences, USA) and mouse monoclonal anti-tubulin. The secondary antibodies were conjugated with horseradish peroxidase (Jackson ImmunoResearch, USA) used for Western blotting. Immunofluorescence was by Alexa Flour 594 donkey anti-mouse (Invitrogene, USA) Cycloheximide (CHX), an inhibitor of protein synthesis, was purchased from Sigma-Aldrich. On-TARGET plus

human HIF-1 α siRNA-SMART pool and non-target SMART pool were obtained from Dharmacon.

2.5 Transient transfection

Subconfluent cells were transfected with 1 μ g DNA by means of the TransIT-X2 transfection reagent (Mirus Bio, USA) according to the manufacturer's protocol. The cells were transfected with siRNA by the Dharmafect transfection reagent (Dharmacon).

2.6 Luciferase luminescence assay

HIF-HRE-dependent luciferase activity was performed with the pBI-GL construct (pBI-GL V6L) containing six tandem copies of the VEGF hypoxia response element. The cells were grown in six-well plates and then transiently transfected in triplicate with 1 μ g DNA reporter plasmid. Forty eight hours after transfection, the cells were either exposed or unexposed to irradiation for one hour, and lysed and analyzed for luciferase luminescence assay six hours later.

2.7 RNA purification and quantitative real-time PCR

Total RNA was extracted from the cells with NucleoSpin RNA II kit (Macherey-Nagel, Germany) following the manufacturer's instructions. One μ g of total RNA was reverse transcribed into cDNA by means of a VersoTM cDNA kit (ABgene, Epsom, UK) with anchored oligo (dT) as the first-strand primer. Quantitative real-time PCR (qRT-PCR) analyses were performed to determine the expression of HIF-1 α mRNA. qRT-PCR reaction was performed in triplicates with a LightCycler FastStart DNA Master SYBR Green I (Roche Applied Science, Germany).

2.8 In vitro cell proliferation assay

The cells were transfected with 40 μ M non-target siRNA or siRNA to HIF-1 α . Forty-eight hours after transfection, they

were re-seeded on 96-well plates, 1000 cells/well, and exposed or unexposed to irradiation every day for one hour for the following 7 days. After the indicated times, cell proliferation was measured using a 3-bis-(2-methoxy-4-nitro-5 sulfanyl)-(2H)-tetrazolium-5-carboxanilide (XTT) kit (Biological Industries Ltd, Israel) following the manufacturer's instructions.

2.9 Immunofluorescence staining and microscope analysis

The cells were plated into six-well plates with 13-mm diameter cover glasses. After incubation and irradiation, the cells were fixed and permeabilized with cold Phemo buffer (0.068M PIPES, 0.025M HEPES, 0.015M EGTANa2, 0.003M MgCl2·6H2O, 10% DMSO, pH 6.8), 3.7% formaldehyde, 0.05% glutaraldehyde, and 0.5% Triton X-100 for 10 minutes. Briefly, blocking was done in 1% BSA/10% normal donkey serum/PBS and subsequently incubated with primary HIF-1 α antibody diluted 1:50 in primary antibody dilution buffer (Biomeda, USA) followed by incubation with Alexa Fluor 594 donkey anti-mouse diluted 1:200. The cells were then mounted with a fluorescence mounting medium (GBI labs, Israel), and fluorescence digital images were captured by an Olympus ix81-ZDC microscope. Image analysis was performed with ImageJ software. The cytosolic region of interest (ROI) was calculated by subtracting the nuclear ROI from whole-cell HIF-1 α staining of the ROI for each cell individually.

2.10 Statistical analysis

The experiments presented in the figures are representative of three or more independent repetitions. The data are expressed as means \pm SD. Student's t test was applied to compare differences between particular conditions. Group differences were assessed with a two-way ANOVA test. All

tests were two-tailed, and statistical significance was defined as a $P < 0.05$.

3. Results

3.1 ELF-MF increased HIF-1 α expression and its transcriptional activity

We tested HIF-1 α expression and its transcriptional activity following radiation in order to study whether HIF-1 is regulated by ELF-MF. ELF-MF exposure increased HIF-1 α protein levels in PC-3 (Figure 1A) and LNCaP (Figure 1B) cells by two- to three-fold. The maximum effect of this induction was seen immediately after the end of the radiation, and interestingly, this effect diminished over time. Similar results were also obtained from DU145 prostate cancer cells (not shown). HIF-1 α levels increased dramatically under hypoxic conditions, serving as a positive control. In addition, ELF-MF significantly increased HIF-1 transcriptional activity in these cells by 50-60%, as measured with a reporter plasmid expressing luciferase under the control of hypoxia-response elements (HRE) (Figure 2A and 2B). A similar increase in HRE-dependent luciferase activity was also seen in the non-cancerous HEK293 cells (Figure 2C).

3.2 ELF-MF regulates HIF-1 α at the posttranscriptional level

We determined HIF-1 α protein and mRNA following exposure to ELF-MF in order to characterize at which level it affects HIF-1 α expression. For this purpose, we measured the half-life of HIF-1 α after exposure to ELF-MF using the inhibitor of protein synthesis CHX. The HIF-1 α half-life in irradiated cells was prolonged by three-fold compared to the control cells (6 min vs. 2 min) (Figure 3A and 3B). These changes were not evident at the mRNA level as determined by real-time PCR (Figure 3C). These results indicated that ELF-MF increased HIF-1 α protein stability.

3.3 ELF-MF promoted HIF-1 α accumulation into the nucleus

PC-3 cells were exposed to ELF-MF and processed for immunofluorescence labeling with HIF-1 α antibody in order to test whether the increase in HIF-1 transcriptional activity following radiation was due to accumulation in the nucleus. Figure 4 demonstrates that the accumulation of nuclear HIF-1 α was significantly higher in the irradiated cells compared with the control cells.

3.4 ELF-MF influenced cell proliferation in a HIF-1 α -dependent manner

We applied an XTT proliferation assay in order to test whether ELF-MF could affect cell proliferation. ELF-MF caused a significant increase in cell proliferation in PC-3

and LNCaP during a period of 6 days in the irradiated cells compared to the control cells (PC-3 $P < 0.001$, LNCaP and $P < 0.05$, two-way ANOVA, Figure 5A and 5B). We applied siRNA to HIF-1 α in PC-3 cells in order to further explore the role of HIF-1 α in irradiation-induced changes in proliferation (Figure 5C). As shown in Figure 5D, ELF-MF failed to increase proliferation when HIF-1 α was silenced. In contrast, the proliferation was reduced, even after exposure to ELF-MF ($P < 0.05$, two-way ANOVA). In parallel, the irradiated cells expressing the non target siRNA demonstrated significantly higher proliferation compared to the control cells (data not shown). This phenomenon is most probably explained by the fact that the induction in cell proliferation by ELF-MF depends, at least partly, upon HIF-1 α .

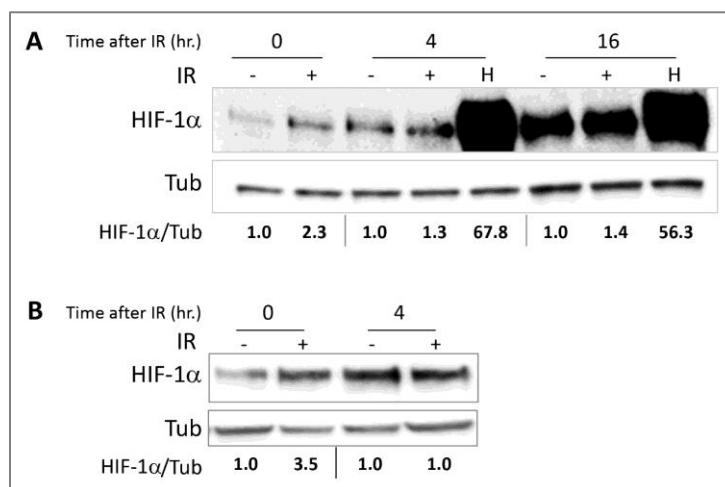


Figure 1: The ELF-MF irradiation increases HIF-1 α expression. (A) PC-3 and (B) LNCaP cells were irradiated (IR) for 1 hour. After irradiation, the cells were lysed immediately (0) or after the indicated times (4 and 16 hours). The cells that had been subjected to no treatment (-) or to hypoxia (H) for the same duration served as controls. Whole cell extracts were analyzed by SDS-PAGE and immunoblotted with antibodies to HIF-1 α and tubulin. Densitometric quantification of HIF-1 α levels that normalized to tubulin is denoted under the lanes.

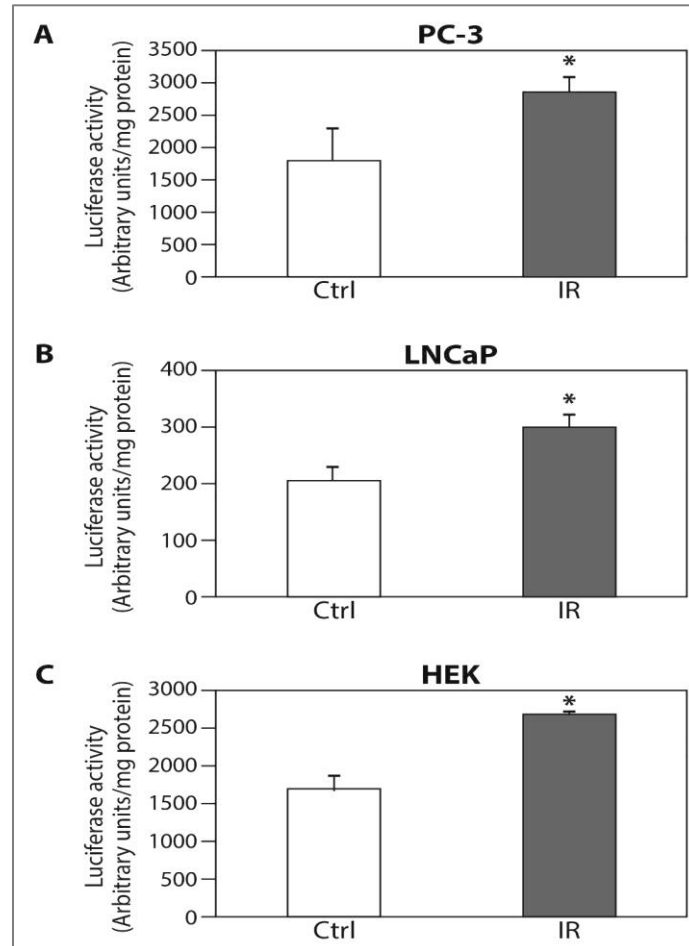


Figure 2: The ELF-MF irradiation increases HIF-1 α transcriptional activity. (A) PC-3; (B) LNCaP and (C) HEK cells were transiently transfected with expression vector encoding pBI-GL V6L expressing luciferase under the control of HRE. After 48 hours of transfection, the cells were exposed (IR) or unexposed (Ctrl) to irradiation for 6 hours, and then lysed and analyzed for luciferase luminescence assay. Relative luciferase activity, units/mg protein at each assay point. Columns show means (n = 3); bars show SD; * $P < 0.05$ compared to control.

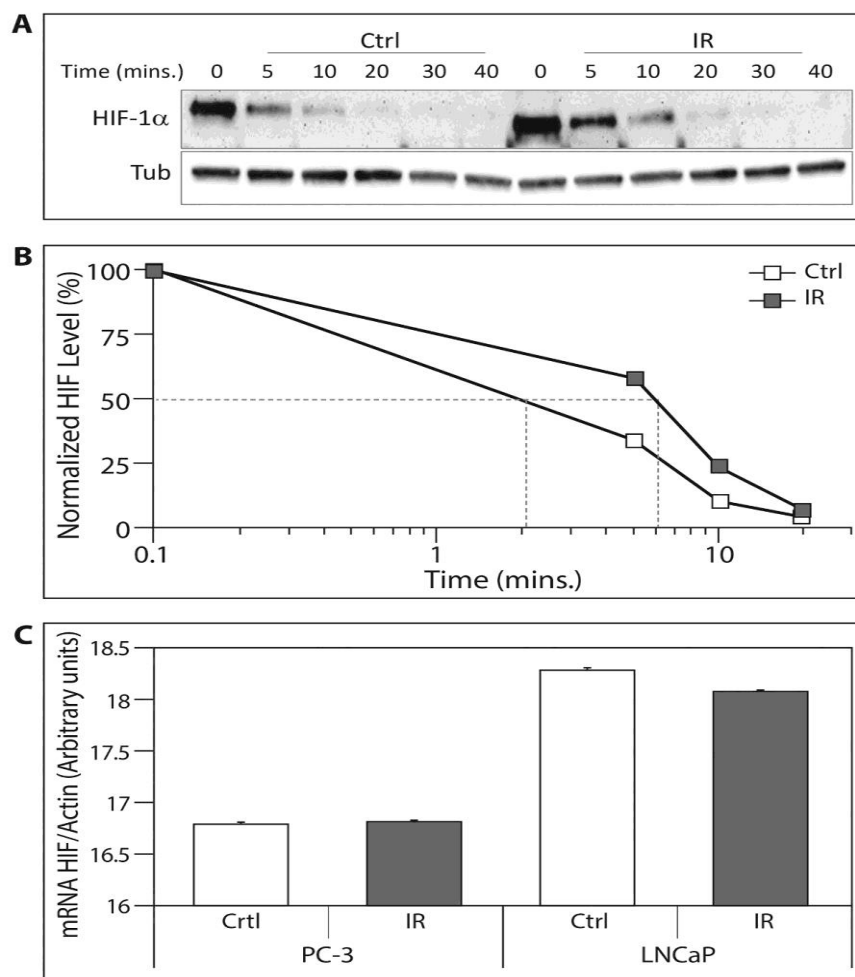


Figure 3: The ELF-MF irradiation upregulates HIF-1 α protein on the posttranscriptional level. (A) 10 μ M CHX was added to the PC-3 cell medium. Immediately afterwards, the cells were exposed (IR) or unexposed (Ctrl) to irradiation for 1 hour and lysed at the indicated times. Whole cell extracts were analyzed by SDS-PAGE and immunoblotted with antibodies to HIF-1 α and tubulin; (B) Chart displaying densitometric quantification of HIF-1 α levels in normalized to tubulin. The 50% decrease of HIF-1 α levels is delineated in grey lines. This is a representative experiment of three independent repetitions; (C) PC-3 and LNCaP cells were exposed (IR) or unexposed (Ctrl) to irradiation for 1 hour. RNA was isolated and reverse transcribed for HIF-1 α and actin mRNA real-time PCR.

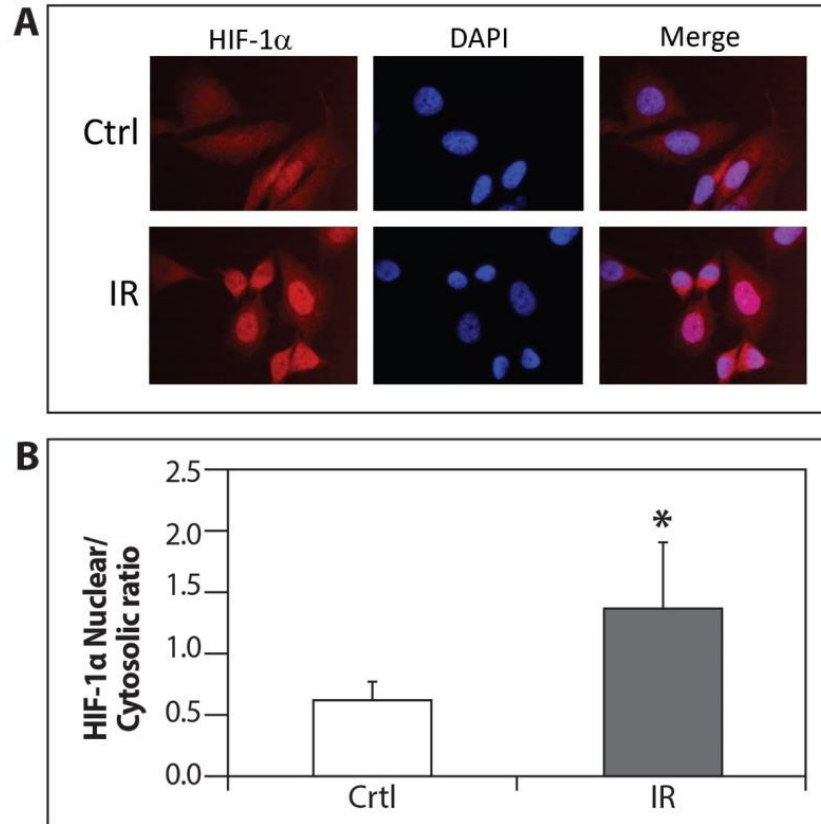


Figure 4: Accumulation of nuclear HIF-1α by ELF-MF irradiation. (A) PC-3 cells were seeded on cover glasses. After 24 hours, the cells were exposed (IR) or unexposed (Ctrl) to irradiation for 1 hour and processed for immunofluorescence labeling with HIF-1α antibody. Staining was analyzed by fluorescence microscopy (magnification x64); (B) The densitometric quantification of HIF-1α fluorescence signal was measured by Image J software. This is a representative experiment of three independent repetitions. A signal of all cells from five different fields was measured from each experiment. * $P < 0.05$ compared with control.

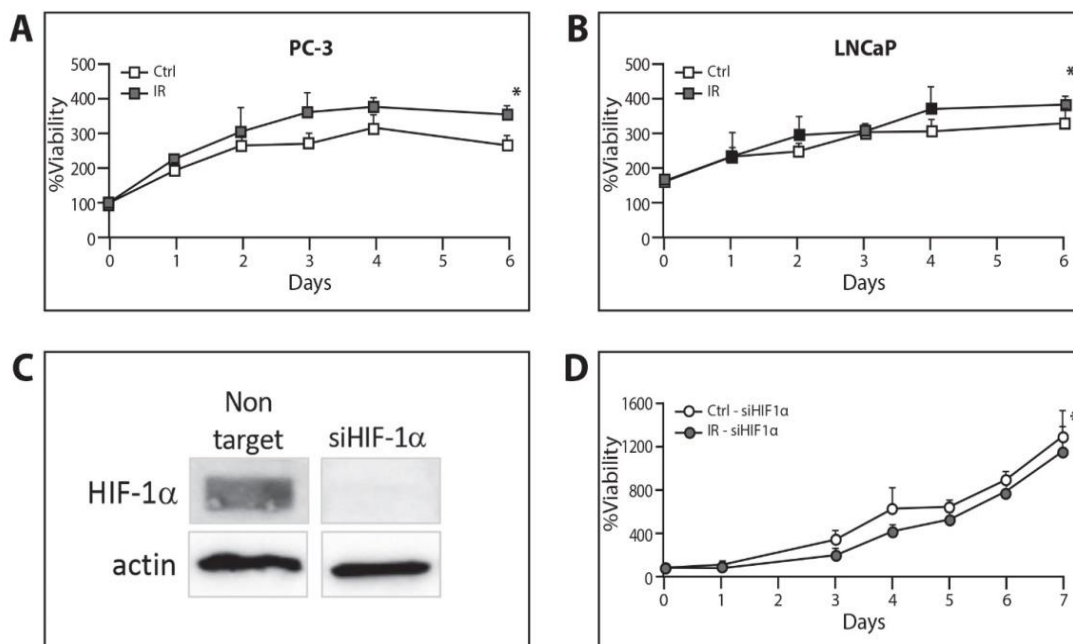


Figure 5: Increase of cell proliferation by ELF-MF irradiation in a HIF-1 α -dependent manner. (A) PC-3 and (B) LNCaP cells were seeded on 96-well plates, 2000 cells/well for PC-3 and 5000 cells/well for LNCaP. The cells were exposed or unexposed to irradiation every day for 1 hour for the following 6 days. The proliferation rate was measured with XTT assay and expressed as an increase in percentage of initial absorbance that was measured before irradiation (100%).* $P < 0.05$ compared with control; (C) PC-3 cells transfected with 40 μ M non-target siRNA or siRNA against HIF-1 α . The cells were lysed 72 hours after transfection and whole cell extracts were analyzed by SDS-PAGE and immunoblotted with antibodies to HIF-1 α and tubulin; (D) PC-3 cells transfected with 40 μ M siRNA against HIF-1 α . They were re-seeded on 96-well plates, 1000 cells/well, 48 hours after transfection and exposed or unexposed to irradiation every day for 1 hour for the following 7 days. The proliferation rate was measured with an XTT assay and expressed as an increase in percentage of initial absorbance that was measured before irradiation (100%).

4. Discussion

The relation between ELF-MF and cancer has been the object of scientific and public discussions for decades. The World Health Organization published recommendations and concluded by stating that there is no convincing scientific evidence that weak radiofrequency signals cause adverse health effects [9], although many studies raise further concerns regarding their effect on cancer development [10-12]. Although prostate cancer is a very common type of malignancy, evidence of its relationship

with ELF-MF is lacking [13]. This study, therefore, investigated the effects of ELF-MF on HIF-1 α protein expression and HIF-1 transcriptional activity in prostate cancer cells.

HIF-1 α is a key element in the hypoxic pathway, which is one of the most well-described and understood pathways important for cell survival and cancer progression [14]. Interestingly, there is a paucity of data on the correlation between HIF-1 α and ELF-MF. Only a single study

evaluated the effect of ELF-MF on neuron-like cells, and it demonstrated that ELF-MF exposure significantly counteracted hypoxia damage by reducing cell death and apoptosis and inhibited the activation of the HIF-1 α pathway [15].

Our results are in agreement with reports in the literature that different ELF-MF exposures can elicit various effects on various cells [16]. Prostate cancer cells that were exposed to ELF-MF in the current study showed immediate higher expression of HIF-1 α , although the effect was transient (Figure 1). The effects that were exhibited in our *in vitro* cannot be applied to the *in vivo* setting in which exposure is long-standing.

Another interesting finding is that exposure to ELF-MF prolonged the half-life of HIF-1 α (Figure 3A & B), leading to an increase in HIF-1 transcriptional activity (Figure 2) without affecting HIF-1 α mRNA (Figure 3C). Many prior studies showed that HIF-1 α mRNA levels were not affected by hypoxia or other interventions in both *in vitro* and *in vivo* models, whereas HIF-1 α protein levels were drastically increased. It is possible that HIF-1 α is regulated at the post-mRNA level [17-19]. We also demonstrated that prostate cancer cells exposed to ELF-MF showed greater levels of proliferation compared to cells control counterpart cells, but these cells did not show any increase in proliferation when HIF-1 α was silenced (Figure 5). The results may indicate on such undiscovered mechanisms where HIF-1 α is involved in the response to ELF-MF irradiation.

HIF-1 α phosphorylation is another level of regulation which promotes nuclear accumulation of HIF-1 α and leads to an increase of HIF-1 transcriptional activation [20-22]. Our findings showed that exposure to ELF-MF enhanced

the accumulation of nuclear HIF-1 α compared to the non-exposed cells (Figures 3 and 4). Further studies are warranted to explore the exact mechanism of how ELF-MF stabilizes HIF-1 α protein.

It must be borne in mind that exposure to ELF-MF at all strengths is too low to induce any physiological or pathological effects by themselves, and that our results cannot be regarded as evidence of the involvement of ELF-MF in human cancer. Conclusions from *in vitro* studies performed on cells cannot be applied to interpret epidemiological findings in human populations. Our findings that the exposure of prostate cancer cells to ELF-MF increases HIF-1 α activation suggest that ELF-MF might facilitate and promote dormant insignificant prostate tumors, and they call for further investigation of these issues in the clinical setting.

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Conflicts of Interest

The authors have no financial interest or benefit that has arisen from the direct applications of this research.

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