Indian Journal of Experimental Biology
Vol. 62, July 2024, pp. 484-495
DOI: 10.56042/ijeb.v62i07.12272

Implication of HIF-1α signalling in human growth hormone (hGH) induced mammary carcinoma

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Received 31 January 2024; *revised 05 April 2024*

Mammary carcinoma is the leading cause of cancer related mortality in women globally. Human growth hormone (hGH) plays a vital role in the development of mammary glands by promoting cell proliferation. Hence, alteration in human growth hormone can contribute to mammary carcinoma. In the present study, we investigated the role of hGH in mammary carcinogenesis and metastasis. We used MTT assay for cell proliferation, and wound healing assay for cell invasion and migration, the cell cycle analysis and analysis of apoptosis. Luciferase reporter assay and Western blot analysis were performed to find out the role of HIF-1α signalling in association with hGH and also VEGF-A Western blot analysis was performed for angiogenesis regulating with hGH. Further, Western blot analysis was analysed for cadherin levels (E-cadherin and N-cadherin) and confocal microscopy and soft agar assay was performed to understand the role of hGH in metastasis through EMT. The results suggest that MCF-7 breast cancer cell lines transfected with hGH showed enhanced proliferation and cell cycle progression by decreasing apoptosis. This contributes to cancer cell progression via HIF-1α signalling pathway which also enhances the expression of VEGF-A contributing to angiogenesis. The EMT markers MMP-2 and N-cadherin expression also increased with decreased E-cadherin expression suggesting the role of hGH in metastasis. In conclusion, the present study establishes the role of hGH in breast cancer progression and suggests the possible signalling pathway involved is mediated by HIF-1α. These finding further helps in novel therapeutic interventions of mammary carcinoma. This present study reports that autocrine production of human growth hormone by human mammary cells alters cell morphology, proliferation and is responsible for enhanced migratory and invasive behaviour leading to breast cancer. Therefore, this study will further help in novel therapeutic interventions for the treatment of breast cancer, by utilizing knowledge of these signalling pathways as targets.

Keywords: Breast cancer, Hypoxia inducible factor-1 alpha (HIF-1α), Vascular endothelial growth factor-A (VEGF-A), Matrix metalloproteinases (MMPs)

Mammary glands are organs intended for nourishing offspring. Their growth and development are highly influenced and regulated by endocrine system through the action of hormones such as estrogen, progesterone, growth hormone, etc.[1]. The extensive modelling and remodelling of mammary glands during puberty determines their susceptibility to carcinogen induced tumours especially mammary epithelial carcinoma[2]. It is the leading cause of cancer related mortality in women worldwide. Among all the hormones, growth hormone is the major contributor of mammary cancer[1].

Growth hormone (GH) is an important regulator of mammary gland development by promoting cell proliferation[3]. Cell proliferation depends on progression through four distinct phases of the cell cycle (G0/G1, S, G2 and M), which is regulated by several cyclins (D, E and B) that act in complex with their cyclin partners. These were overexpression in cancer, particularly in estrogen receptor-negative cases, these cyclins drive tumor aggressiveness and worsen prognosis in breast cancer patients[4]. GH is a true cytokine whose local production and interaction with other genes such as NF-kB, cadherins directly contributes to tumor progression[5]. A crucial regulator of the metabolic reprogramming that occurs in hypoxic cancer cells is hypoxia-inducible factor 1 (HIF1-α)[6]. In addition to the GH, other proteins such as Hypoxia inducible factor 1 alpha (HIF1-α) also contribute to carcinoma[7]. HIF1-α is a transcriptional regulator of proteins that modulate transformation,

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survival, proliferation, invasion, angiogenesis, and metastasis. The HIF pathway is essential during physiological processes and is involved in cancer progression by regulating hundreds of genes[8-10]. This major regulator facilitates tumor growth by promoting TGF-β, angiogenesis via Vascular Endothelial Growth Factor (VEGF) and SDF-1 (stromal cell-derived factor), metabolism by GLUT-1 & GLUT-3 glycolytic enzymes regulation, cell apoptosis via p53 and cell cycle regulation[11]. Moreover, HIF-α contributes to cancer metastasis by altering cancer cell adhesion and motility through regulation of Epithelial-to-Mesenchymal Transition (EMT) by E-cadherin, and N-cadherin expression as well as migration[12] and invasion abilities through MMPs 2 (Matrix metalloproteinases)[13], COX (Cyclooxygenase) LOX (Lysyl oxidase) and CXCR4 (chemokine receptor type 4 (CXCR4).

Metastasis and recurrence are the primary cause of mortality in breast cancer. Hichem *et al.*[14] reported that the epithelial to mesenchymal and mesenchymal to epithelial transitions, mesenchymal epithelial transition factor receptor (EMT, MET) are the main causes of metastasis mechanism in breast cancer. The carcinoma cells shed their differentiated epithelial characteristics during EMT like cell-cell adhesion, polarity and lack of motility, and attain mesenchymal traits, including motility and invasiveness. However, the association of hGH and HIF-1α pathways in tumor progression is still unclear. Therefore, in the present study, we investigated the association of autocrine hGH and HIF-1α in mammary carcinoma along with E-cadherin and N-cadherin, the markers of EMT.

Materials and Methods

Cell lines and transfection

The human mammary carcinoma cell line MCF-7 was obtained from American type culture collection (ATCC). The cell line was cultured and maintained in DMEM medium (HIMEDIA) supplemented with 10% Foetal bovine serum (FBS) and Penicillin-Streptomycin cocktail (100 mg/L) at 37°C in a humidified 5% CO_2 incubator. For hGH gene transfection, MCF-7 cells were seeded into 6 well culture plates and kept for 24 h to reach 80% confluency. Spent medium was replaced with plain DMEM medium without antibiotics, and serum and were incubated with a mixture of Lipofectamine-2000 (200 μm/mL) and the plasmid vector containing the entire autocrine human growth hormone gene (pcDNA3-hGH), or an empty MUT vector (pcDNA3 vector, (200 μm/mL) (1:1 ratio of DNA and lipofectamine). Medium containing transfection mixture was then replaced with complete medium and incubated for 36 h after transfection and was designated as MCF-hGH and MUT-MCF-7, respectively. The pooled stable transfectant cells were selected in 800 μg/mL of G418 (Geneticin) over 21 days.

Total cell count

The control MCF-7, MUT-MCF-7 and hGH-MCF-7 cell lines were seeded at a density of 2×10^3 cells/well in six-well culture plates as monolayers. The cells were grown in DMEM supplemented medium with 10% fatal bovine serum, 1 mM glutamine and 1% penicillin and streptomycin solution (HIMEDIA Labs) in 5% (v/v) CO_2 enriched air at 37°C in incubator. After 48 h the cells were washed with PBS buffer and trypsinized. The number of cells were determined by haemocytometer.

Cell viability assay (MTT)

Transfected cell lines (hGH-MCF, MUT-MCF-7) and control MCF-7 cell lines, were seeded in triplicates in 96-well plate at a density of 5×10^3 cells per well and cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT reagent (4 mg/mL solution diluted in PBS) was added to all the culture wells at a final concentration of 0.4 mg/mL, the cells were further incubated for 4 h at 37°C by adding 200 μL/well dimethyl sulfoxide (DMSO) the reaction was terminated and absorbance was read at 570 nm in a Varioskan™ LUX multimode micro plate reader (Thermo Fisher Scientific). The cell viability is proportional to the intensity of purple colour product produced in the wells. Wells containing complete media alone were used as blank, and the average values were subtracted from the average values of all clones. The results were analysed using Student's t-test. Data points represent the mean ± SD and $P < 0.05$ was considered significant.

Cell migration assay (Wound-healing assay)

The hGH- MCF-7, MUT-MCF-7 and control MCF-7 cell lines (5×10^4), were seeded in a six-well plate in triplicates and allowed to grow to full confluency. Cells were washed with PBS and left in serum free media for 5 h. A scratch was made and complete media was added. Images were captured at 200X magnification, using Invitrogen EVOS Digital Colour Fluorescence Microscope at 0, 24, 48 and 72 h. The results were analysed using Image J software, 2.02 versions.

Cell cycle analysis

After reaching 80-90% of confluency, the transfected hGH-MCF-7, MUT-MCF-7 and MCF-7 control cells were seeded at approximately 2×10^5 per well in a six-well plate and incubated for 24 h. Later, cells were washed with phosphate buffer saline (PBS) and harvested by trypsinization followed by centrifugation. Then the cell pellet was washed with PBS, followed by fixation in ice-cold ethanol (70%), and stored at 4°C for 18 h. Before measurement of fluorescence by flow cytometry, cells were washed with PBS and stained with nuclear stain propidium iodide (PI) (10 µg/mL) supplemented with 20 µg/mL RNase A followed by incubation at 37°C for 30 min. Subsequently cells were incubated at 4°C until further analysis. Then the cell cycle progression analysis was performed by Flow sight, Amnis Corporation, USA, inspire V.2 software.

Western blot analysis

Protein extraction and western blot analysis were performed as per the protocols described earlier. Briefly, Control MCF-7, MUT-MCF-7 and hGH-MCF-7 cell lines were lysed with hot sample buffer containing 1% sodium dodecyl sulfate (SDS) and then centrifuged for 15 min at 37°C. Protein extracts were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (Hybond C-Extra; Amersham Pharmacia, Aylesbury, UK). After blocking, membranes were incubated for 1 h with primary antimouse monoclonal antibodies obtained from Cloud-Clone Corp. (CCC, USA). The membranes were then washed and incubated with horseradish peroxidase–conjugated goat anti-mouse secondary antibody (1:10,000; Cloud-Clone Corp. (CCC, USA). The Blots were then stripped and re-probed using anti-actin antibody (Sigma Aldrich, St. Louis, MO, USA). Protein bands were detected by cell signalling technology using phototype horseradish peroxidase western blot detection system.

Apoptosis by flow cytometry

Asynchronously growing MCF-7 clones and mutants (hGH-MCF-7 and MUT-MCF-7) along with control MCF-7 cell lines were treated for 20 min with hypotonic buffer (1 mg/mL sodium citrate and 0.3% Igepal). The gentle pipetting to detach nuclei was done for a min, followed by its collection for green fluorescence acquisition. From each clone approximately 1×10^5 cells were plated and serum starved for 24 h. Briefly, cells were incubated with fluorescein isothiocyanate (FITC) -annexin V and propidium iodide (PI) (BD Biosciences) in binding buffer for 15 min in dark. Stained cells were allowed for flow cytometry analysis using Flow sight, Amnis Corporation, USA (The excitation wavelength of 380 nm, and for emission wavelength of 525 nm was used). All the samples were then acquired using flow cytometer (Guava 8 HT Easy Cyte; Millipore, Billerica, MA, USA) at around 50,000 events and flow rate of 0.59 mL/s. The obtained data were analysed using inspire V.2 software. All the experiments were performed in triplicates. After that, cells were prepared to see the impeding effect of hGH on apoptosis in MCF-7 cell lines using DAPI staining under confocal microscope.

Luciferase reporter assay

The hGH and MUT-hGH vectors were transfected in MCF-7 cell lines (2×10^5 cells/well in 6-well plate) together with luciferase reporter plasmids (2 µg/well) and an internal control (IC) pGL4.32 (luc2P/HRE/Hygro) vector (Promega, Madison, WI, USA; 2µg/well) expressing the Renilla luciferase. Transfections were performed using FuGENE 6 (Roche Applied Science, Indianapolis, IN, USA). The centrifugation was performed for 2 min at 14,000 g at 4°C following a brief freeze-thawing cycle and the insoluble debris was removed. Then 20 µL of supernatant was processed immediately in all the aliquots for sequential quantification of both Renilla luciferase activity (dual luciferase assay system) and firefly. Further, for normalization of transfection efficiency, the activity of the co-transfected Renilla reporter plasmid was used. All the reporter assays were performed in triplicates.

Confocal microscopy

The hGH-MCF-7, MUT-MCF-7 and Control MCF-7 cell lines were adhered on glass cover slips followed by fixation with 4% paraformaldehyde for 20 min. Then fixed cells were washed with phosphate-buffered saline (PBS), and permeabilized in 0.1% of triton X-100/PBS. After blocking in PBS having 1% bovine serum albumin (BSA) for 1 h at room temperature, the cells were incubated with primary antibody i.e., E-cadherin overnight at 4°C followed by incubation with Alexa 488-FITC for 1 h in a moist chamber at room temperature. The cover slips were mounted on glass slides with DAPI Fluoromount G (Southern Biotech, Birmingham, AL, USA) followed by PBS washing. Finally, images were acquired with Confocal Laser Scanning microscope (Leica SPE Biosystem).

Colony formation assay/Soft agar assay

To determine the independent behaviour of transfected cells in metastasis, soft agar assay was performed. Here, 0.6% of low melting agarose was prepared in serum free medium and poured into 6 well plate and incubated at RT in Laminar Air Flow for 1hr until solidification. Trypsinized transfected cells were suspended in 0.3% Agarose (DMEM+FBS media) and were plated carefully to avoid multiple layer formation and covered with 1 mL complete supplemented media. On alternate days a 200 µL of complete media was added to the wells and at the end of the 10th day, cell colonies were observed. Pictures were captured under bright field microscope at 200X magnification. Colony formation was observed in 5 different fields. The number of colonies and mean colony was counted. The experiment was performed in duplicates.

Results

Effect of Autocrine hGH on breast cancer cell line of Cell viability assay

The growth and development of cancers are greatly contributed by enhanced cell proliferation and/or a decrease in apoptosis. To establish the role of autocrine hGH in modulation of mammary carcinoma cell behaviour, MCF-7 cell lines were stably transfected using empty vector and expression plasmid encoding the hGH (200 µg/mL) gene and un-transfected MCF-7 cell lines were used as a control. The MTT assay results after 24, 48 and 72 h showed increased growth ability of autocrine hGH transfected cells in both serum and serum free culture media (Fig. 1). The hGH-MCF-7 cell line showed higher viability/growth, in both serums contain and without serum media Loss of cell viability observed in control cells after 72 h under serum conditions can be attributed to the absence of hGH transfection. Without hGH, the cells lack the necessary stimulus for proliferation, compounded by the depletion of growth factors over time. Conversely, cells transfected with hGH exhibit sustained viability even without serum, demonstrating the potent growth-promoting influence of hGH. This highlights the critical role of hGH in promoting cell proliferation, irrespective of serum availability. (*Note: Serum growth factor may influence the proliferation of the cells*, *for the influence of human growth hormone it required both serum and without serum experiment need to done*, *so that the performance was shown with and without serum and growth hormone treated cells and hGH regulates high proliferation*).

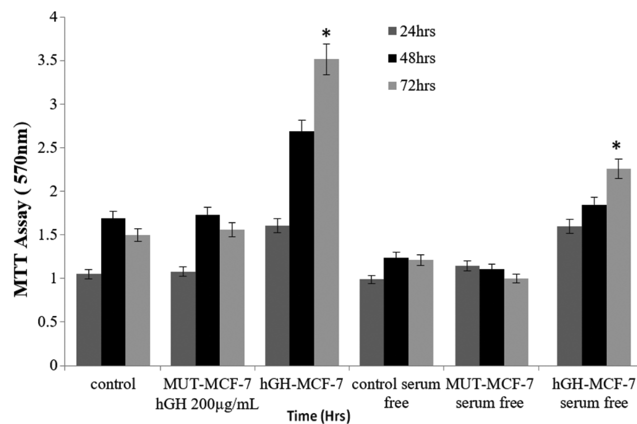

Fig. 1 — Cell proliferation assay showing the cell growth/viability of MCF-7 cell lines. The hGH-MCF-7 cell lines in complete and serum free media showed highest viability than control and MUT-MCF-7 cell lines after 72 h of transient transfection. [Each column represents the mean ± SD of OD at 570 nm. *P <0.05 is considered as significant]

Autocrine human growth hormone (hGH) induced proliferation in mammary cancer (wound healing assay)

The results did not show any morphological changes it shows invasion of cells in both the mutant and hGH transfected MCF-7 cell lines (Fig. 2A). However, wound healing assay showed significant changes in the rate of proliferation (invasion) in hGH expressed MCF-7 cell lines compared to others indicating that the proliferation was induced by growth hormone.

Analysis of apoptosis in control, MUT-MCF-7 and hGH-MCF-7 breast cancer cell lines using Flowcytometry

The investigation was aimed at whether the observed GH-induced growth in MCF-7 cell lines was due to reduced apoptosis. In the present study, apoptosis (early and late) was noticed to be 7.79% in hGH-MCF-7 cell lines, 13.58 % in control cell lines and 16.68% in MUT-MCF-7 cell lines which stained positively for annexin-V. A slight increase in the annexin V^+ PI^+ population was observed in control and MUT-MCF-7 cell lines, which indicate dead cells or late apoptosis. There was an increase in viable and non-apoptotic cell populations in MUT-MCF-7 cell lines compared to hGH-MCF-7 cell lines (30.26% and 8.81%, respectively) (Fig. 3). Further, the cell lines were stained with DAPI to confirm decreased apoptosis in hGH -MCF-7 cell lines (Fig. 4). Figure 5 shows the Western blot analysis of control, MUT-MCF-7 and hGH-MCF-7 breast cancer cells.

Cell cycle analysis of autocrine hG H transfected MCF-7 cell lines

Our study represents the cell number versus PI uptake of MUT-MCF-7 and hGH-MCF-7 cell lines.

hGH-MCF-7 cell lines displayed enhanced cell cycle progression in G1, S and G2 phases compared to MUT-MCF-7 and control cell lines. Similarly, it was noticed that expression of cell regulation of cyclin D, cyclin–E and cyclin-B (1:1000 Cloud-Clone Corp. (CCC, USA) was increased in hGH-MCF-7 cell lines **(**Fig. 6 A & B**)**. The results demonstrated that increasing trend in the percentage of DNA content in

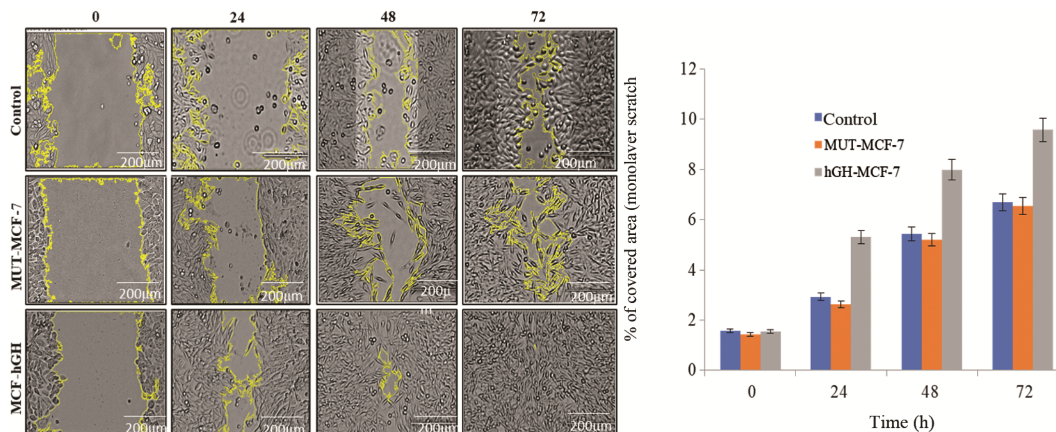

Fig. 2 — (A) Autocrine production of hGH in human mammary carcinoma cells results in increased cell motility and acquisition of an invasiveness. After transiently transfected with Control, MUT hGH and MCF-hGH, cells after 72 h created the scratch wound-healing assay, the wound areas were examined under 200X magnification. (B) Autocrine human growth hormone (hGH) signalling induced changes in cancer cell parameters. A) Representative micrographs of 2D cell cultures. B) Representative micrographs of wound healing assay. [Presented images are the best of biological triplicates]

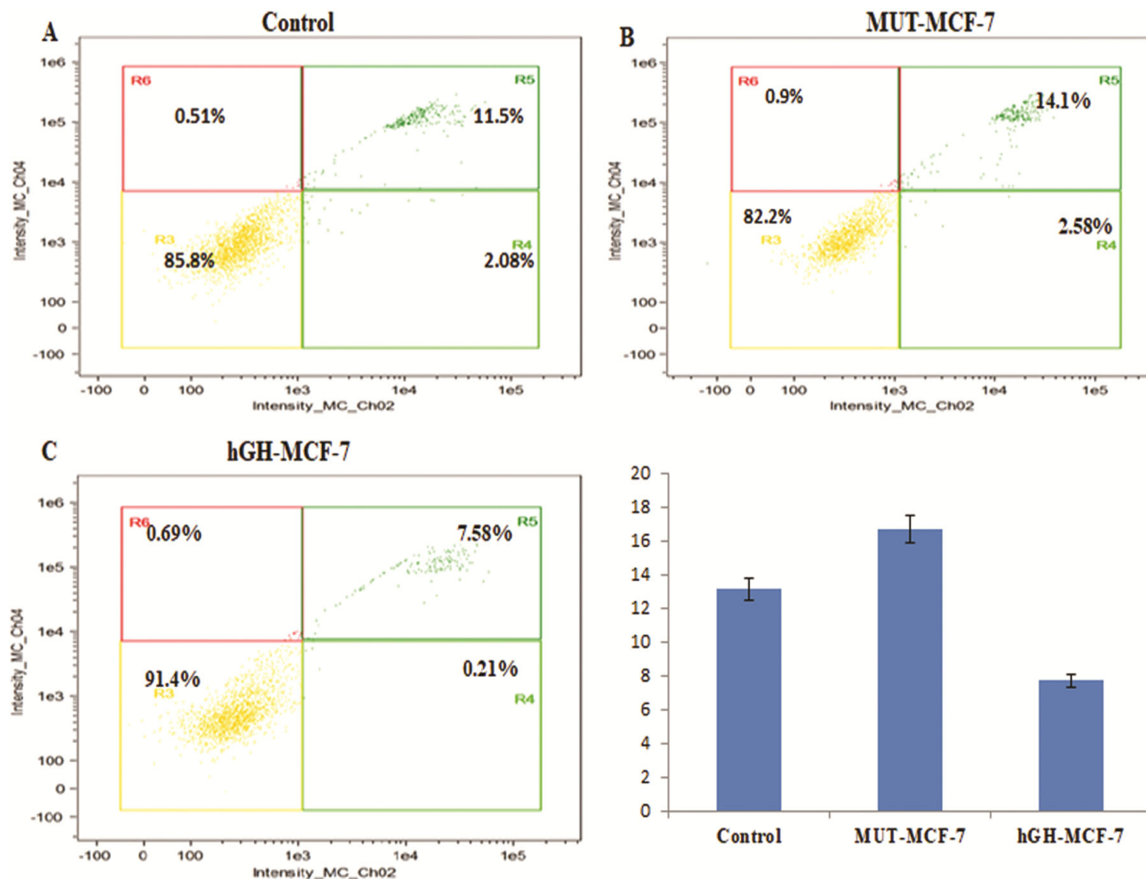

Fig. 3 — Apoptosis assay using Flow cytometric analysis for control, MUT-MCF-7 and hGH-MCF-7 breast cancer cell lines. [The percentage of events in each quadrant is shown and *P <0.05 is considered as statistical significance]

the S-phase and G2/M phase was clearly higher in hGH-MCF-7 transfected cell lines as compared to others.

Expression analysis of cyclins in human mammary carcinoma cells by western blot

On the other hand, in cell cycle and western blot analysis an increase in expression of protein levels of Cyclin D, Cyclin E and Cyclin B in hGH-MCF-7 cell lines was observed which is required for cell cycle progression (Fig. 6 C-F).

Luciferase reporter assay

The potential effects of autocrine hGH on HIF1-α in MCF-7 cell lines was investigated by co-transfection with hGH (hGH -MCF-7, HRE-hGH-MCF-7), MUT (MUT-MCF-7, HRE-MUT-MCF-7), HRE-MCF-7 cell lines (luc2P/HIF1-α/Hygro) and un-transfected MCF-7 cell lines as control were assayed for luciferase activity. An increased HIF1-α luciferase activity in hGH-MCF-7 cell lines was observed indicating elevated protein expression (Fig. 7).

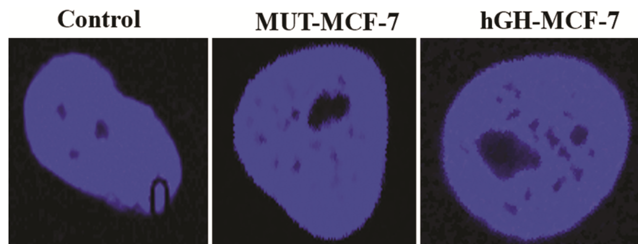

Fig. 4 — The data present cell invasion and nuclear morphology, characterized by nuclear condensation and increased blue fluorescence intensity of nuclei in Control, MUT-MCF-7, and hGH-MCF-7 cells, respectively.

Autocrine production of hGH in MCF-7 cells using western blot analysis

Increased HIF1-α luciferase activity was confirmed by western blot analysis where increased expression of HIF1-α in hGH-MCF-7 cell lines was observed compared to others (1:1000 Cloud-Clone

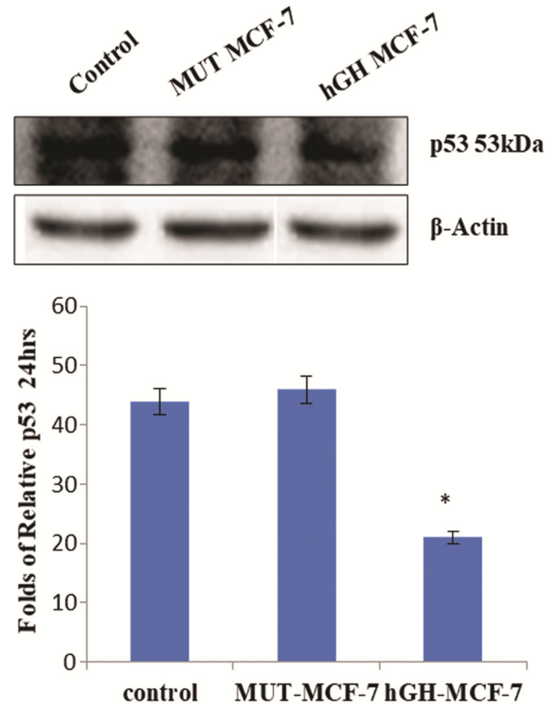

Fig. 5 — Autocrine production of hGH in human mammary carcinoma cells: Expression of p53 protein was increased when compared with MUT-MCF-7 and control cells, the protein bands detected in the immunoblots and histogram analyzed by Imaj J 2. [Data are mean +SD of three independent experiments. (*P<0.05 compared with controls)]

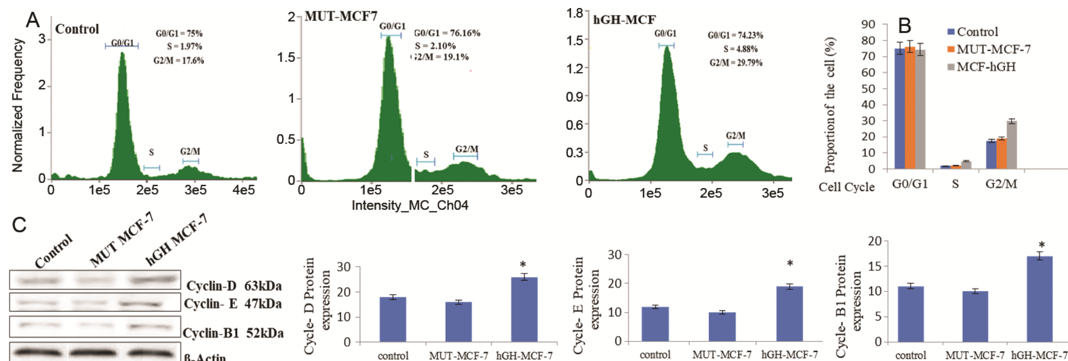

Fig. 6 — (A) After 24 h of serum starvation, the cells were transfected with hGH, (MUT) and control. The cells were harvested and processed for cell cycle analysis by flow cytometry. hGH-MCF-7 cells displayed enhanced cell cycle progression in G_0/G_1, S and G_2/M phase when compared to control and MUT-MCF-7. Fluorescence of the PI-stained cells was measured using Flow sight, Amnis Corporation, USA, inspire V.2 software. (B) Histograms were plotted by the MultiCycle (Flow sight , Amnis Corporation, USA, inspire V.2 software) program. provide the estimate of percentage of cells with fractional DNA content (cell cycle: G_0) and cells in G_1, S and G_2/M phases of the cycle. (C) Expression of cyclin D, cyclin E and cyclin B proteins was increased in hGH-MCF-7 cell lines compared to others. The protein bands detected in the immuno blots were analyzed by Image J 2 and histograms were generated. [Data represented as mean ± SD of three independent experiments. *P <0.05 is considered as statistically significant]

Corp. (CCC, USA). Western blot analysis also showed GH-dependent increase in VEGF-A expression (1:1000 Cloud-Clone Corp. (CCC, USA) indicating the role of HIF1-α in induction of VEGF-A expression in mammary carcinoma (Fig. 8). This data indicates that epithelial HIF1-α is an active contributor to angiogenesis, tumour progression and it demonstrates that the inhibition of HIF1-α has significant therapeutic impact even at later stages of mammary tumour progression.

Evaluation of epithelial mesenchymal transition (EMT) by Western blot analysis of N-cadherin and E-cadherin

The experiment was aimed at analysing the expression of HIF1-α targets i.e., MMP-2, E- and N-cadherin that induce epithelial mesenchymal transition (EMT) in metastasis. The results revealed that hGH induced EMT transition in MCF-7 cell lines is due to increased expression of MMP-2 and N-cadherin. Whereas, E-cadherin showed decreased expression by Western blot analysis (Fig. 9). Additionally, to confirm the decreased expression of E-cadherin by western blot analysis, confocal microscopy was used. The results showed decreased expression of E-cadherin in [illegible] MCF-7 cell lines when compared to control and MUT-MCF-7 cell lines. Precisely, immunofluorescence revealed a significant number of internalized E-cadherin particles in MUT-MCF-7 cell lines than hGH-MCF-7 cell lines (Fig. 10). It depicts E-cadherin expression using confocal laser scanning microscope in various cell lines.

Metastasis of MCF-7 cell lines by soft agar/ colony formation assay

To further analyse the effects of hGH transfected MCF-7 cell lines on the characteristics of metastatic outgrowth, a soft-agar colony assay was used in the current study (Fig. 11). When hGH transfected MCF-7 cell lines were used, the cells formed compact spherical colonies that grew larger in size and in number, when compared to the control and MUT-MCF-7 cell lines. It suggests that the colony

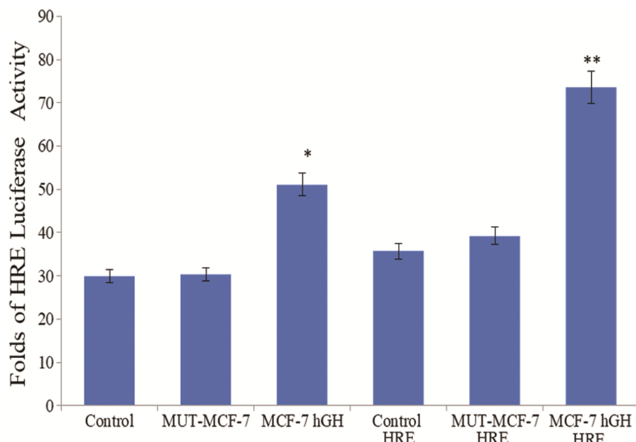

Fig. 7 — Control, MUT-MCF-7 and hGH-MCF-7 cell lines ($2{\times}10^5$ cells/well in six-well plate) were co-transfected with Luciferase reporter plasmids (3 µg/well) and an internal control, pGL4.32 (luc2P/ HIF1-α/Hygro) vector (Promega; 2 µg/well) expressing the Renilla luciferase. The hGH-MCF-7 HRE cell lines showed increased HIF1-α expression compared with control MUT-MCF-7 cell lines. [Each column represents the mean ± SD of luciferase fold induction, *P <0.01 is considered statistically significant]

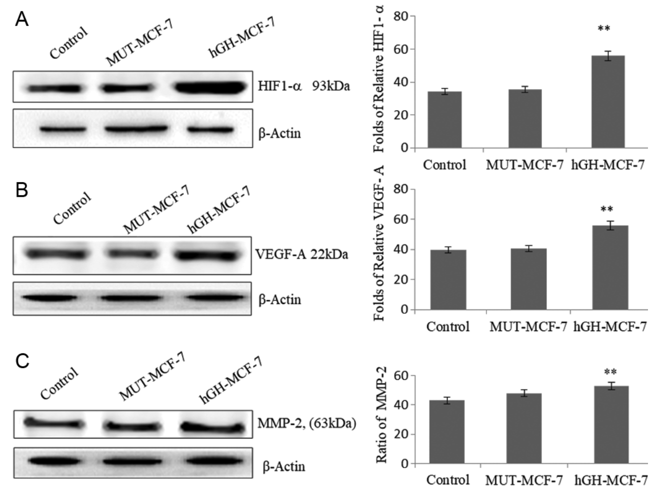

Fig. 8 — Protein levels of HIF1-α, VEGF-A and MMP-2 were increased in hGH-MCF-7 cell lines. The protein bands detected in the Western blot analysis were analyzed by Image J 2 and histograms were generated. [Data are represented as mean ± SD of three independent experiments, **P <0.01 is considered as statistically significant]

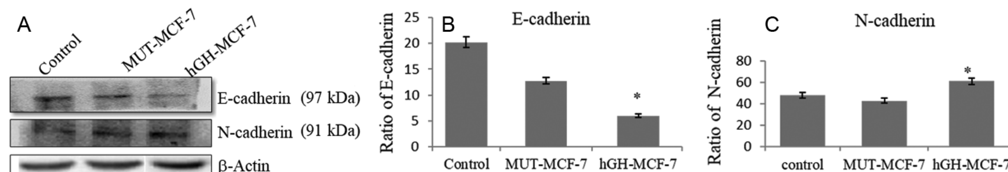

Fig. 9 — Growth hormone dependent regulations of epithelial markers. (A) Decreased levels of E-cadherin (97 kDa), Increased levels of N-cadherin (91 k Da); and (B) Histogram, right panel upper side (E-cad), histogram, right panel down side (N-cad). [All histogram analysis was by Image J 2. Software. Data expressed are mean ± SD of three independent experiments. *P <0.05, *P <0.01 are considered statistically significant]

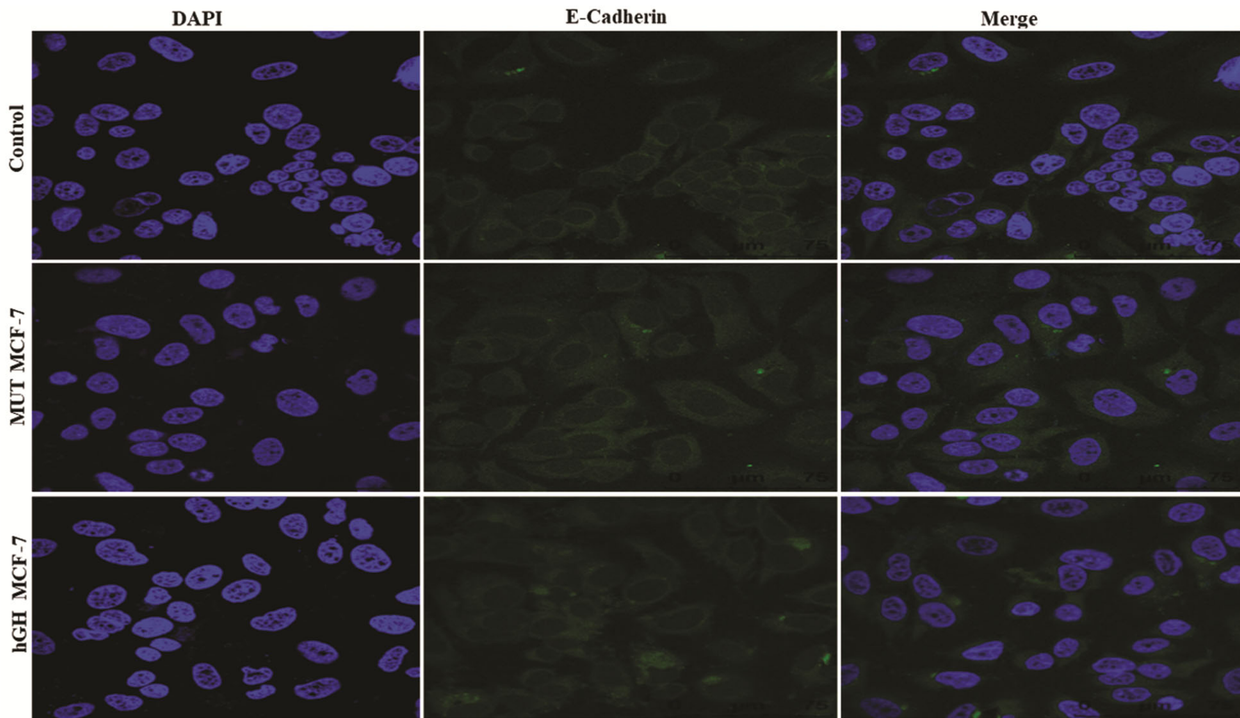

Fig. 10 — Left, middle and right panel shows micrographs of DAPI, E-cadherin and merge cells of control, hGH-MCF-7 and MUT-MCF-7 cell lines. [48 h after transfection, cells were stained for distribution of E-cadherin using FITC-conjugated secondary antibody (green) and counterstained with DAPI (blue), Scale bar, 10 µm]

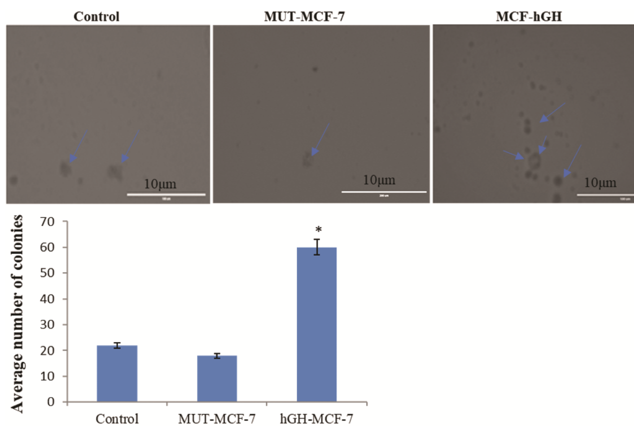

Fig. 11 — Autocrine hGH production by human mammary carcinoma cells results in enhanced anchorage-independent growth. [*P <0.05, is considered as statistically significant Scale bar, 10 µm]

outgrowth is prompted by the autocrine hGH in the MCF-7 cell lines.

Discussion

Various epidemiological studies have discovered that breast cancer is influenced by multiple factors including place of residence, ethnicity, environment, genetics, hormones, and radiation, reproductive history and socio-economic status[15]. Cancer progression is a multistage process involving the regulation of cell proliferation. The metastatic processes can be broadly split into three main types: invasion, intravasation and extravasation. The invasion is a process where loss of cell-to-cell adhesion allow malignant tumour cells to detach from the primary tumour mass and alter the cell-matrix to invade the surrounding stroma. This involves the secretion of substances to degrade the basement membrane and EMT also the expression or suppression of proteins involved in the control of motility and migration[16].

However, the role of endocrine system in development of mammary gland has been well established. Among the hormones, growth hormone (GH) is an important regulator of mammary gland development. On the other hand GH is considering being true cytokine whose local production contributes to tumor progression[1,17].

Therefore, in the present study the autocrine production of hGH by human mammary carcinoma cells and its role in the acquisition of a mesenchymal phenotype with increased migration and invasion through EMTs was observed. Firstly, the study aimed at the identification of the human growth hormone induced alterations in mammary gland contributing to mammary cancer progression. Hence the wound-healing assay revealed that autocrine hGH stimulate

cell migration with a more rapid closing of the wound than observed in MCF-7 control and MCF-MUT cell lines. Similar to our observations, two other studies have examined enhanced endothelial cell proliferation or increased total cell number after treatment with exogenous hGH. Moreover, increased invasiveness of certain mammary carcinoma cell lines has been correlated with our results. The present study also provided direct evidence that hGH- MCF-7 established the maximum proliferation followed by MUT-MCF-7 and MCF-7 cell lines. Growth factor exerts its function locally in an autocrine and/or paracrine fashion to initiate and progress the malignancy[18]. Likewise, transcriptional regulation of genes required for cell cycle progression as well as cell survival is involved in activation of cell signalling pathways that causes oncogenic transformation of mammary cells[19, 20, 21]. However, protection from apoptosis of mammary cells is rendered both by autocrine-hGH and also exogenously added hGH[22] .The autocrine hGH may possibly regulate genes required for cell survival, and it is demonstrated from a previous study that autocrine hGH increases transcription of the CHOP gene that results in survival of mammary carcinoma cells in a p38 MAP kinase-dependent manner[23]. Similarly, the current study revealed the autocrine hGH mediated modulation of mammary carcinoma cell cycle progression in breast cancer cell line. However, it still remains to determine how autocrine hGH influence the mammary carcinoma cell behaviour and whether it is mediated directly by autocrine hGH or indirectly via utilization of effector molecules, which in turn directly affect the cellular function.

The hGH-induced mitogenesis was shown to be associated with the recruitment of non-cycling resting phase (G_0) cells into the cell cycle and an increased rate of progression through first gap phase (G_1), with focused attention on the estrogenic regulation of molecules with a known role in the control of first gap phase (G_1) to synthesis (S) phase progression (G_1-S phase) [4, 24]. Hence, the present study was undertaken to evaluate the G_0/G_1, S and G_2/M cell phases. It is observed that hGH-MCF-7 cell lines displayed enhanced cell cycle progression in all the above cell phases when compared to the controls and MUT-MCF-7 cell lines. It was also demonstrated that the autocrine hGH induced cell cycle progression showed major changes in different cell cycle regulatory proteins i.e., Cyclin-D, E and B in hGH-MCF-7 cell lines after 48 h of transfection. The cyclins increased cell proliferation by decreased apoptotic cell death. Hence, the findings from our results are suggestive of the influence of autocrine hGH on mammary cancer. The implication of growth hormone levels in cancer can be decided by the ratio of apoptotic to anti-apoptotic proteins. It is known that members of the p53 i.e., Bcl-2 and HIF1-α[17,25,26] protein families are briefly involved in cytokine-signalling of cell survival[27]. However, identification of signalling pathway for growth hormone via activation of HIF1-α[6], still remains as a vital route in mediating the antiapoptotic effect of growth hormone, that prompted us for a brief analysis of this protein in mammary cancer. Additionally, NF-κB factors also promote cell survival in a number of cells and growth conditions but its role in mammary cancer remains undefined[5]. The above observations strongly suggest that locally formed GH is adequate to rescue hGH-MCF-7 cell lines from apoptosis. Therefore, the present investigation demonstrated the role of GH in rescuing hGH-MCF-7 cell lines from apoptosis using DAPI staining that predict differences in nuclear morphology of apoptotic and viable cells. Likewise, p53 tumour suppressor protein expression using western blot analysis also showed decrease in apoptotic and increase in viable cells. The findings are suggestive of hGH autocrine production in immortalized human mammary epithelial cells for an overall condensation or presence of fragmented chromatin forming the basis for this oncogenic transformation. Therefore, to understand the condensation or presence of fragmented chromatin by apoptosis, the nature of single cell after transfection of MUT-MCF-7 and hGH-MCF-7 in MCF-7 cell line was observed under the confocal microscopy, which showed enhanced proliferation and conferred protection from apoptosis.

The epithelial HIF1-α is an active contributor to VEGF-A that promotes angiogenesis in breast cancer. The activation of HIF1-α in ER+ (MCF-7 and T47-D) and ER- (MDA-MB-231) breast cancer cell lines is estrogen-independent[28]. In this study, we have compared the constitutive activation of HIF1-α, VEGF-A and their properties with other hormone-dependent growth factors[29] by relying on autocrine hGH invasive and metastatic growth properties of breast cancer cell lines using reporter assay and western blot analysis. It was shown that potential effects of HIF1-α regulates in cancer, not only

MMP-2, MMP-9 but also VEGF-A[30] and even other metastatic markers. In this study, we investigated the role of autocrine hGH on Luciferase activity of HIF1-α in transfected MCF-7 cell lines. The activity of HIF1-α was found to be increased in hGH-MCF-7 cells but not in MUT-MCF-7 and control cells. Furthermore, we investigated the protein expression study by western blot analysis of HIF1-α, and it also revealed that GH-dependent increase of HIF1-α, expression. Furthermore, we focused on the association of HIF1-α and VEGF-A activity in hGH MCF-7 cell lines[31]. Through western blot analysis, it was determined that autocrine human growth hormone (hGH) exerted potential effects on the activity of vascular endothelial growth factor-A (VEGF-A) and hypoxia-inducible factor 1-alpha (HIF1-α). A consistent upregulation in the expression levels of VEGF-A and HIF1-α was observed in comparison to control and MUT-MCF-7 cell lines. Notably, the GH-dependent increase in VEGF-A expression was found to be mediated via HIF1-α. Consequently, HIF1-α is implicated in angiogenesis, apoptosis prevention, and sheds light on the mechanisms underlying the invasive and metastatic growth induced by hGH in breast cancers. Moreover, emerging evidence suggests that matrix metalloproteinases (MMPs) may facilitate processes associated with epithelial-to-mesenchymal transition (EMT), thereby enhancing tumor cell invasion and metastatic potential[32]. These specific proteins can interrupt cell adhesion by processing the components of cell-cell and cell ECM contacts and interfere with the function of other full-length E-cadherin molecules. E-cadherin is a transmembrane glycoprotein involved in ca^+ dependant intracellular adhesion[33], and is specifically associated with epithelial cell to cell adhesion. E-cadherin processed by MMP contributes to the initiation of EMT, detachment of cancer cells and their transfer in to the stroma, which allows stationary epithelial cells to become motile[34]. In this study, signalling pathway for GH via activation of HIF1-α, revealed increased MMP-2 and N-cadherin expression by western blot analysis. However, decrease in E-cadherin expression is due to its down regulation in the mesenchymal state[35].

In normal conditions, epithelial cells are typically anchored firmly to the basement membrane. However, during epithelial-to-mesenchymal transition (EMT), these cells lose their attachment to the basement membrane, facilitating migration and invasion of tumor cells. A well-established hallmark of tumorigenesis is the reduction in E-cadherin junctions. In this study, we demonstrated that the decreased expression of E-cadherin is attributed to proteolytic degradation mediated by MMP-2, which was observed in hGH-transfected MCF-7 cells but not in MUT-MCF-7 or control cells. E-cadherin molecules function as Ca2+-dependent transmembrane adhesion proteins involved in homophilic interactions, whereas N-cadherin plays a role in ductal mammary branching[36]. In further investigations, we examined the reduced expression of E-cadherin using confocal microscopy. This approach allowed for a detailed visualization and analysis of cellular localization and abundance of E-cadherin, providing additional insights into its regulation in response to hGH stimulation.

Moreover, our study assessed the anchorage-independent growth of cells in soft agar, a recognized indicator of metastatic potential in cancer. This assay enables the assessment of cellular ability to grow and proliferate in a three-dimensional environment without reliance on a solid substrate for attachment. The findings from this assay provided independent confirmation of the metastatic potential associated with hGH stimulation, thereby corroborating our observations from other experimental assays.

Conclusion

Crucially, we elucidate a putative signaling cascade initiated by hGH in mammary carcinogenesis, wherein the activation of hypoxia-inducible factor 1-alpha (HIF1-α) emerges as a pivotal mediator mediating its anti-apoptotic effects. Furthermore, the observed GH-dependent elevation in HIF1-α and vascular endothelial growth factor-A (VEGF-A) expression within MCF-7 cell lines implicates hGH and HIF1-α in fostering cancer progression through angiogenic pathways. Additionally, our study unveils hGH's involvement in metastatic dissemination, attributed to its promotion of epithelial-to-mesenchymal transition (EMT) via upregulated expression of matrix metalloproteinase-2 (MMP-2) and N-cadherin, coupled with downregulation of E-cadherin expression. Overall, these findings contribute crucial insights into the mechanistic underpinnings of mammary cancer progression and highlight potential therapeutic avenues targeting these signalling pathways. Our study lays the groundwork

for the development of novel interventions aimed at mitigating the deleterious effects of hGH in mammary cancer, thereby advancing the prospects for improved clinical management of this disease.

Acknowledgement

Authors thank Prof. Surya Satya Narayana Singh, Department of Biochemistry, Osmania University, Hyderabad, India for providing plasmid vector in this research work (SAP). This study was supported by the National fellowship Programme (NF-UGC New Delhi).

Conflict of interest

Authors declare no competing interests.

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