Original Research Article

MicroRNA-122 mimic/microRNA-221 inhibitor combination as a novel therapeutic tool against hepatocellular carcinoma

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ABSTRACT

Background: Therapeutic microRNAs (miRNAs) delivery holds a lot of promise for treating human malignancies. So, this study was carried out to examine the potential of miR-122 mimic and/or miR-221 inhibitor as an innovative therapeutic strategy for HCC in an animal model.

Methodology: Mice were categorized into five groups comprising: (1) a normal control group, (2) an HCC group subjected to diethylnitrosamine (DEN) injection for 12 weeks, (3) a miR-122 mimic-treated HCC group, (4) a miR-221 inhibitor-treated HCC group, and (5) a miR-122 mimic/miR-221 inhibitor-treated HCC group. After 16 weeks, all animals were sacrificed and underwent biochemical, miRNAs and genes expression, histopathological, and immunohistochemical examinations.

Results: The miR-122 mimic/miR-221 inhibitor combination dramatically reduced the levels of proinflammatory, liver cancer, angiogenesis, and cell proliferation markers when compared to either treatment alone. It also down-regulated the expression of cyclin D1, TGF-β, and β-catenin genes, which are involved in promoting cell cycle progression and cancer cell proliferation. Furthermore, it caused the resolution of nearly all the histological malignant features as well as the reduction of malignant cellular markers, including α-smooth muscle actin, arginase-1, and tropomyosin-1.

Conclusions: The co-treatment with miR-122 mimic and miR-221 inhibitor amplifies the benefits of either treatment on HCC through targeting the SENP1 and ARF4 genes, respectively. This combination can inhibit cancer cell proliferation and angiogenesis while inducing tumor apoptosis and necrosis. This study demonstrates the therapeutic potential of reversing a dysregulated miRNAs expression pattern in HCC. As a result, future research should concentrate on turning miRNA understanding into therapeutic applications.

1. Introduction

Nucleic acid-based therapies hold great promise for the treatment of human cancers since they are key regulators in tumor development and progression [1]. Therapeutic miRNAs delivery may also offer distinct technological advantages. First, the probability of off-target gene silencing is lower than that associated with the manufactured RNAi triggers as physiological gene expression networks have developed to adapt the effects of endogenous miRNAs on gene regulation [2]. Second, unlike short interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs), which target a single transcript, miRNAs in the form of mimics or inhibitors can control both the expression and repression of multiple genes. This prevents the emergence of resistant clones in conditions like cancer where a lot of simultaneous mutations would be necessary to counteract the effects of miRNA expression [3]. Third, it has been demonstrated that the exogenous expression of some shRNAs can competitively inhibit the miRNAs biosynthesis, resulting in harmful effects. This might be owing to the shRNA sequences’ inadequate processing and/or nuclear-cytoplasmic transport that have not evolved to be precisely handled by this pathway [4]. Therefore, as these widespread effects could occasionally have negative consequences on health, miRNAs-based therapeutics will need comprehensive pre-clinical evaluation.

Hepatocellular carcinoma (HCC) is a disease that is both...
pathologically and clinically heterogeneous. It is caused by the disruption of several intracellular and extracellular signaling pathways [5,6]. The initial stages of HCC development entail the disruption of a set of interdependent mechanisms that control the cell cycle, cell growth, and apoptosis. Later in the carcinogenesis process, cells may develop angiogenic, invasive, and metastatic characteristics as a result of neoplastic cell interactions with the surrounding milieu [7]. In advanced stages, traditional chemotherapy has been proven to be barely effective or even harmful [8]. As a result, new therapeutic alternatives must be developed [9]. Improvements in our understanding of the molecular etiology of HCC will inevitably lead to the identification of new targets for unconventional therapy. Not only is abnormal cell cycle control a crucial component of HCC maintenance, but it also seems to be a crucial early occurrence. In fact, liver cirrhosis also exhibits an elevated proliferation rate and proliferating cell fraction and correlates with a higher risk of developing HCC [10]. It has been shown that many miRNAs are implicated in cell cycle regulation. MiR-221 is one of these miRNAs, and it has been reported to target CDKN1B/p27/Kip1 and CDKN1C/p57/Kip2 [11]. It is one of the most consistently expressed miRNAs, and its overexpression was proven to promote HCC tumorigenesis by influencing the cell cycle or apoptosis [12]. Additionally, miR-122 is considered as a liver-specific miRNA that accounts for 70% of the total miRNAs population in the liver. Its levels were found to be reduced in HCC compared to normal liver, and its lower expression correlates with metastasis and poor prognosis [13].

Although technological advancements show that using miRNAs or their antagonim as treatments is both feasible and secure, additional research is still required to progress this field into the clinical setting [14]. Accordingly, the current study was conducted to examine, for the first time, the potential of combining a tumor suppressor miRNA mimic and an oncogenic miRNA inhibitor as a novel therapeutic strategy for HCC. So, miR-122 mimic and miR-221 antagonist were administered to an animal model of HCC, both individually and in combination, to explore whether the combined therapy would be more effective than a single miRNA restoration approach.

2. Methodology

2.1. Cytotoxic effect of miRNAs on normal murine cells

Isolated bone marrow-derived mesenchymal stem cells from mice were cultured in a 96-well cell culture plate, at a seeding density of 5 × 10^3 cells/well, in a growth medium Dulbecco’s Modified Eagle’s Medium (DMEM) (Biovest, USA, Catalog No.: L0066-500) until reaching full confluence. MiR-122 mimic and miR-221 inhibitor, alone and in combination, were added to each well at concentrations of 100, 50, 25, 12.5, 6.25, and 3.12 nM. After 24 h of incubation at 5% CO₂, crystal violet (Sigma-Aldrich, USA, CAS No: 548-62-9) staining was used to measure the cell viability percentage which is calculated by the equation: cell viability % = O.D. of the tested miRNA/O.D. of the control x 100 [15].

2.2. Experimental design

The protocol of this study was performed in accordance with the regulations of the Theodor Bilharz Research Institute (TBRI) Ethical Committee and the National Institutes of Health (NIH, 1996) recommendations for laboratory animals’ care and management. The animals used throughout this work were male Balb/C mice aged 8 weeks and were obtained from the animal house of TBRI. Prior to the initiation of treatment, mice were acclimated to the lab environment for 7 days. They were given proper care, kept at 12-h light/dark cycles, and provided with fresh tap water and a standard rodent diet.

In this experiment, mice were categorized into two main groups, including a normal control group (15 mice) that got saline injections and an HCC group (60 mice) that received intraperitoneal injections of diethylnitosamine (DEN) (Sigma-Aldrich, USA, CAS No: 55-18-5) at a rate of 50 mg/kg/week for 12 weeks [16]. The HCC group was subdivided into four groups (15 mice each): (1) a pathological control (HCC) group, (2) a miR-122 mimic-treated group that received intrahepatic injections of 100 nmol miR-122a mimic once/week for four weeks following DEN injection, (3) a miR-221 inhibitor-treated group that received intrahepatic injections of 100 nmol miR-221 inhibitor once/week for four weeks following DEN injection, and (4) a miR-122 mimic/miR-221 inhibitor-treated group that received intrahepatic injections of 100 nmol miR-122 mimic and 100 nmol miR-221 inhibitor once/week for four weeks following DEN injection. The injected miR-122 mimic and miR-221 inhibitor were obtained from Thermo Fischer Scientific, USA (Assay IDs: MC11012, MH10337, respectively). Their doses were chosen based on the maximum effective dose with the least cytotoxic effect on normal cells.

The mice were then put into anesthesia before being sacrificed via cervical dislocation. Their sera were collected and stored at −80 °C until being processed. Heparinized sera were dissected and kept in 10% formalin for histopathological and immunohistochemical examination. The remaining liver specimens were flash-frozen in liquid nitrogen and kept at −80 °C for subsequent biochemical and gene expression analyses.

2.3. Analysis of the biochemical parameters

The sera of mice were subjected to the measurement of alpha-fetoprotein (AFP), vascular endothelial growth factor (VEGF), tumor necrosis factor-α (TNF-α), and platelet-derived growth factor (PDGF) levels using enzyme-linked immunosorbent assay (ELISA) kits (MyBioSource, USA, Catalog Nos.: MBS730245, MBS704351, MBS825075, and MBS2503388).

2.4. Analysis of miRNAs expression

MiRNAs were extracted from the homogenized hepatic tissues using the miRNeasy Mini Kit (Qiagen, Germany, Catalog No.: 217,004) and reverse transcribed into cDNA using the TaqMan™ MicroRNA Reverse Transcription Kit (Thermo Fischer Scientific, USA, Catalog No.: 4366596) according to the manufacturers’ instructions. MiR-122 and miR-221 expression analyses were performed using the Taqman Universal Master Mix II No UNG (Thermo Fischer Scientific, USA, Catalog No.: 4440040) following the manufacturer’s protocol. The miRNA assays were ready-made and provided by Thermo Fischer Scientific, USA (Catalog No.: 4427975). The PCR reactions were run on a Real-Time PCR (StepOnePlus™ Applied Biosystems, USA) at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min. The comparative cycle threshold (CT) 2^-ΔΔCT approach was exploited to calculate the miRNAs relative expression (RQ) levels with the TaqMan® RNA60B assay used to normalize the data [17].

2.5. Analysis of genes expression

The hepatic specimens were homogenized, and total RNA was extracted from the homogenized tissues using the QIAamp RNA Blood Mini Kit (Qiagen, USA, Catalog No.: 52,304). Afterwards, the extracted RNA was reverse transcribed into cDNA using the ReverTaid First Strand cDNA Synthesis Kit (Thermo Fischer Scientific, USA, Catalog No.: K1622).

The expression of small ubiquitin-like modifier (SUMO)-specific protease 1 (SENP1), (ADP Ribosylation Factor 4) (ARF4), cyclin D1, transforming growth factor (TGF)-β, and β-catenin genes was analyzed using the TaqMan™ gene expression master mix (Thermo Fischer Scientific, USA, Catalog No.: 4369016) following the manufacturer’s protocol. All the gene assays were ready-made and obtained from Thermo Fischer Scientific, USA (Catalog No.: 4331182). The real-time PCR thermal cycling conditions included: incubation at 95 °C for 10 min and
2.6. Examination of hepatic tissues by hematoxylin and eosin

Parts of the hepatic specimens were fixed in 10% formalin for 24 h, rinsed in water for 2 h, dehydrated in escalating grades of alcohol, and cleared in xylene. Then, the samples were impregnated in pure soft paraffin for 2 h at 55 °C before being embedded in hard paraffin blocks. Microtome sections of 5 μm thickness were produced and stained with hematoxylin and eosin (H & E) [18]. Two pathologists, a screener, and a consultant quantified liver tissue sections blindly. Finally, the sections were examined by a light microscope (Scope A1, Axios, Zeiss, Germany), and photomicrographs were taken using a microscope camera (Axio-Cam, MRc5, Zeiss, Germany).

2.7. Examination of hepatic specimens by immunohistochemistry

The immunohistochemical (IHC) staining technique was carried out in two steps. After deparaffinizing and rehydrating the sections, the slides were heated in PT link (DAKO) for antigen retrieval. α-smooth muscle actin (α-SMA) (Anti-human mouse monoclonal antibodies Santa Cruz Biotecnology, USA, Catalog No.: sc-53142), arginase-1 (Arg-1) (Arg-1 polyclonal primary antibody, Chongqing Biospes Co., China, Catalog No.: BBP1013), and tropomyosin-1 (TPM-1) (anti-TPM1 polyclonal primary antibody, Chongqing Biospes Co., China, Catalog No.: YPA2402) antibodies were diluted according to the manufacturer’s instructions. IHC staining was done using DAKO Envision Flex + Detection Kit, High PH (Link) (Agilent, USA, code number: K8002). The reaction product was visualized with diamobenzidine as a chromogen and counterstained by hematoxylin. The percentage of positive cells was determined and analyzed statistically [19].

2.8. Statistical analysis

The results of this experiment were presented as mean ± standard deviation (SD). For the multivariate analysis, the one-way ANOVA was utilized, followed by Tukey’s post hoc test. SPSS version 25 for Windows (SPSS Inc., Chicago, USA) was used to examine the statistical differences, and p < 0.05 was designated as the statistically significant level.

3. Results

3.1. Cytotoxic effect of miRNAs on normal murine cells

Around 97.5%, 98.5%, and 97.3% of murine BM-MSCs cells remained viable when treated with miR-122 mimic, miR-221 inhibitor, and miR-122 mimic/miR-221 inhibitor, respectively. This indicated that the miR-122 mimic or miR-221 inhibitor, alone or in combination, had no harmful effect on normal living cells.

3.2. The biochemical parameters

The DEN-induced HCC group of mice showed a substantial rise in the liver cancer marker; AFP, angiogenesis marker; VEGF, pro-inflammatory marker; TNF-α, and cell proliferation marker; PDGF when compared to the control group (p < 0.001). The levels of these markers were significantly declined in mice that received either miR-122 or miR-221 or a combination of both in comparison to the HCC group. Mice given both the miR-122 mimic and miR-221 inhibitor had lower levels of AFP, VEGF, and PDGF than the miR-122 mimic-treated group (p < 0.001, p < 0.05, and p < 0.001, respectively), as well as diminished levels of AFP, VEGF, TNF-α, and PDGF when compared to the miR-221 inhibitor-treated group (p < 0.001). VEGF, TNF-α, and PDGF were considerably reduced in the miR-122 mimic-injected mice compared to the miR-221 inhibitor-treated mice (p < 0.001) (Fig. 1).

So, the administration of miR-122 mimic/miR-221 antagonist combination was the most effective technique for lowering the biochemical markers of liver cancer, angiogenesis, pro-inflammation, and cell proliferation, followed by miR-122 mimic injection.

3.3. Analysis of miRNAs expression

The miR-122 expression was found to be significantly lower in the HCC and miR-221 inhibitor-treated groups of mice when compared to the control group (p < 0.001). This down-regulation was reversed after the injection of mice with the miR-122 mimic either alone or in combination with the miR-221 antagonist (Fig. 2A).

On the other hand, the miR-221 expression was significantly greater in the HCC and miR-122 mimic-treated mice than in the normal group (p < 0.001), and when mice were given a miR-221 inhibitor, the miR-221 levels were restored and became significantly indifferent from the control group (Fig. 2B).

3.4. Analysis of genes expression

The SENP1 gene, a direct target of miR-122, was significantly up-regulated in the hepatic specimens of the HCC and miR-221 inhibitor-treated mice in comparison to the control group (p < 0.001). The SENP1 gene expression was markedly down-regulated following miR-122 mimic injection, reaching a value that was significantly lower than that in the HCC and miR-221 inhibitor-treated groups (p < 0.001 and p < 0.01, respectively) (Fig. 3A).

The HCC and the miR-122 mimic-injected mice had significantly higher levels of the ARF4 gene, which is a direct target of the miR-221, than the control group (p < 0.01). These levels, however, were significantly reduced in the mice that received intrahepatic injections of miR-221 inhibitor, either alone or in combination, when compared to the HCC (p < 0.01 and p < 0.001, respectively) and miR-122 mimic-treated mice (p < 0.01 and p < 0.001, respectively) (Fig. 3B).

The hepatic tissues of HCC mice had a significantly up-regulated cyclin D1, TGF-β, and β-catenin genes in comparison to the control group (p < 0.001). The expression of these genes was markedly declined in the HCC mice treated with miR-122 mimic and/or miR-221 inhibitor when compared to the HCC group (p < 0.001). The TGF-β gene expression was significantly lower in the miR-122 mimic-treated group than in the miR-221 inhibitor-treated group, while the expression of both the TGF-β and β-catenin were down-regulated in the co-treated group in comparison to the groups that received either miR-122 mimic or miR-221 inhibitor injections (p < 0.001) (Fig. 4).

Thus, co-administration of miR-122 mimic and miR-221 inhibitor was the best approach for down-regulating the expression of cyclin D1, TGF-β, and β-catenin genes which are involved in the regulation of the cell cycle, induction of epithelial-mesenchymal transition (EMT), and cell proliferation, respectively.

3.5. Examination of hepatic tissues by hematoxylin and eosin

Mice injected with DEN developed all features of HCC with marked infiltration of the hepatic sinusoids and portal tracts by mononuclear inflammatory cells, with focal hepatocytic degeneration, scattered apoptotic figures, focal cholestasis, vascular and lymphatic proliferation, and patchy fibrosis. The HCC mice treated with miR-122 mimic showed focal liver cell dysplasia with inflammatory cellular reaction and liver cell degeneration and apoptosis. In the HCC mice that received miR-221 inhibitor, there was a low-grade liver cell dysplasia with less evident inflammation and cell degeneration and necrosis. However, the HCC mice co-treated with miR-122 mimic and miR-221 inhibitor almost restored the normal hepatic architecture with the absence of dysplasia and had milder inflammatory and degenerative liver alterations (Fig. 5).
3.6. Examination of hepatic specimens by immunohistochemistry

The induction of HCC led to an escalation in the expression of α-SMA, Arg-1, and TPM-1 compared to the normal control group (p < 0.01). The expression of TPM-1 was significantly reduced when HCC mice were treated with miR-122 mimic with a more pronounced decline in the
Fig. 4. The relative quantification (RQ) values of cyclin D1, TGF-β, and β-catenin genes in the examined groups. **Significant increase compared to the control group ($p < 0.001$); *significant increase compared to the control group ($p < 0.05$); $^*$significant increase compared to the control group ($p < 0.01$); $^\dagger$significant decrease compared to the control group ($p < 0.001$); $^\ddagger$significant decrease compared to the HCC group ($p < 0.001$); $^\S$significant decrease compared to the HCC, miR-122 mimic-treated, and miR-221 inhibitor-treated groups ($p < 0.001$); $^\c$significant decrease compared to the HCC and miR-221 inhibitor-treated groups ($p < 0.001$).

Fig. 5. Histopathological appearance of liver sections from: (a) normal control mice (x400), (b) HCC mice showing malignant hepatocytes (black arrow) and sinusoidal dilatation (red arrow) (x400), (c) miR-122 mimic-treated mice showed focal liver cell dysplasia with inflammatory cellular reaction and both liver cell degeneration and apoptosis (x200), (d) miR-221 inhibitor-treated mice showed less evident inflammation (red arrow) and cell degeneration and necrosis (black arrow) (x400), (e) miR-122 mimic/miR-221 inhibitor-treated showed mild focal inflammatory reaction (red arrow) and degenerative liver changes (black arrow) (x400).
miR-221 inhibitor-treated mice. The miR-122 mimic/miR-221 inhibitor combination lowered the expression of α-SMA when compared to the DEN- or miR-122 mimic-treated groups ($p < 0.05$). In addition, the co-treatment of mice with the miR-122 mimic and miR-221 inhibitor considerably decreased the expression of Arg-1 and TPM-1 in comparison to the DEN, miR-122 mimic, or miR-221 inhibitor therapy ($p < 0.05$) (Fig. 6 and Table 1). This indicates that combining the delivery of a miR-122 mimic and a miR-221 antagonist was the most efficient strategy for reducing the expression of hepatocellular markers of malignancy.

4. Discussion

The injection of a mixture of two synthetic miRNAs was examined in the current study for the first time as a potential therapeutic strategy for HCC in a mouse model. Together, the miR-122 mimic and miR-221 inhibitor considerably improved the benefits of either treatment on HCC by targeting the SENP1 and ARF4 genes, respectively. This combination markedly reduced the levels of pro-inflammatory, liver cancer, angiogenesis, and cell proliferation serological markers, including TNF-α, AFP, VEGF, and PDGF, respectively. It also down-regulated the expression of cyclin D1, TGF-β, and β-catenin genes which are involved in promoting the progression of the cell cycle, EMT, and the proliferation of liver cancer cells. Additionally, it caused the resolution of nearly all the histological malignant features induced by DEN injection and reduced the expression of the malignant cellular markers such as α-SMA, Arg-1, and TPM-1.

Hepatocellular carcinoma (HCC) is the 3rd leading cause of cancer death and the 5th most prevalent malignant disorder worldwide. It is frequently detected at an advanced stage when curative therapies are no longer effective [20]. The effectiveness of current therapeutic regimens

|                  | α-SMA (%) | Arg-1 (%) | TPM-1 (%) |
|------------------|-----------|-----------|-----------|
| Control          | 3.50 ± 1.38 | 9.33 ± 3.32 | 7.00 ± 2.64 |
| HCC              | 18.50 ± 7.0* | 89.17 ± 12.7* | 90.00 ± 10.08* |
| miR-122 mimic    | 13.33 ± 2.88* | 80.00 ± 10.05* | 62.34 ± 5.97** |
| miR-221 inhibitor| 15.61 ± 2.38* | 75.75 ± 11.83* | 34.38 ± 4.82** |
| miR-122 mimic/miR-221 inhibitor | 11.65 ± 1.47* | 25.47 ± 8.65** | 25.00 ± 5.48** |

Table 1

The percentages of positive cells stained with α-smooth muscle actin (α-SMA), arginase-1 (Arg-1), and tropomyosin 1 (TPM-1) antibodies.

Data are presented as mean ± standard deviation (SD).

*Significant increase than the control group ($p < 0.001$); **significant increase than the HCC group ($p < 0.05$); a significant decrease than the miR-122 mimic-treated group ($p < 0.05$); b significant decrease than the miR-221 inhibitor-treated group ($p < 0.05$).

Arg-1, and TPM-1. On the other side, the efficacy of either the miR-122 mimic or the miR-221 antagonist administration was much lower than that of their combination.

Hepatocellular carcinoma (HCC) is the 3rd leading cause of cancer death and the 5th most prevalent malignant disorder worldwide. It is frequently detected at an advanced stage when curative therapies are no longer effective [20]. The effectiveness of current therapeutic regimens
for this cancer type is further reduced by the highly active drug-metabolizing pathways and multidrug resistance transporter proteins in the malignant cells [21]. Alternative strategies are consequently required to get beyond these impediments to obtain effective therapy. Fortunately, these alternate strategies work well on the liver because both viral and non-viral genes, as well as small molecule delivery systems, may easily target the liver [22].

Multiple aberrantly expressed miRNAs were revealed to be implicated in hepatocarcinogenesis. For instance, miR-221 up-regulation has been shown to promote cell cycle progression, reduce apoptosis, favor angiogenesis, and confer cell migratory properties on HCC malignant cells [23]. One of the down-regulated miRNAs in liver cancer is the hept-specific miR-122, which makes up about 70% of the miRNAs population in the adult liver and has a critical role in regulating the metabolism of fatty acids and cholesterol. It has been demonstrated that the miR-122 expression levels rise in the mouse liver over the course of development, peaking immediately before birth. Therefore, the loss of miR-122 expression in HCC may signify either a reversal of differentiation or a block at a less differentiated state [10,24]. Accordingly, this study was designed as an attempt to restore the normal miRNA expression pattern of an oncogene; miR-221, and a tumor suppressor miRNA; miR-22, that had been disrupted during the process of hepatocarcinogenesis and to explore whether this therapeutic strategy would be more effective than a single miRNA restoration approach.

In the present study, it was detected that administration of the miR-122 mimic, either alone or in combination with the miR-221 antagonist, dramatically reduced the proliferation of malignant cells and significantly improved the hepatocyte architecture that has been distorted by the carcinogenesis process. In agreement with our findings, it has been found that overexpressing miR-122 in Hep3B cells and in vivo induces apoptosis, slows tumor growth, and suppresses proliferation [25]. In addition, transfection of miR-122 mimic into HepG2 cells induces cell-cycle arrest and makes these cells more susceptible to antitumor agents, including doxorubicin, cisplatin, vincristine, and sorafenib, by modulating the expression of multidrug resistance genes and the unfolded protein response [26–29]. Another study indicated that the hydrodynamic injection of miR-122 into miR-122 knockout mice has successfully impaired the hepatocarcinogenesis process and tumor progression, as reflected by reduced tumor occurrence and size [30]. The establishment of miR-122 knockout mice has proven the significance of miR-122 in liver cancer. These animals developed hepatic inflammation, fibrosis, and spontaneous tumors comparable to HCC, demonstrating for the first time the therapeutic potential of reversing a combination of dysregulated miRNAs expression patterns in HCC. As a result, future research should concentrate on turning miRNA understanding into therapeutic applications.

Research involving human participants and/or animals

All procedures performed in this study were in accordance with the ethical standards of the Institutional Research Committee and Review Board (FWA00010609) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Data availability statement

All data generated or analyzed during this study are included in this published article.

CRediT authorship contribution statement

Marwa Hassan: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Visualization, Writing – original draft, Writing – review & editing. Mohamed Elzallat: Conceptualization, Project administration, Data curation, Methodology, Visualization. Tarek Aboushousha: Data curation, Formal analysis, Methodology, Validation. Yasmine Elhusseiny: Data curation, Methodology, Validation.
Declaration of competing interest
The authors have no conflicts of interest to declare.

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