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DNA methylation progress is involved in influenza a virus infection

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Abstract
The highly pathogenic strains of the influenza virus have been responsible for large epidemics and pandemic diseases characterized by severe pulmonary illness connected with high morbidity and mortality rate. Epigenetic reprogramming mechanisms including DNA methylation lead to host immune-related transcriptional programmers' perturbations by regulating chromatin structure and gene expression patterns. Therefore, here we investigated the further changes of DNA methylation in human lung epithelial cells in response to influenza A virus (IAV) infection and figured out how methylation might be activated upon IAV infection. By infecting A549 cells, we found that the expression of DNA methyltransferase 1(DNMT1) and methionine synthase (MTR) significantly increased according to MOIs of IAV infection 24 hours post infection. Interestingly, the relative gene expression of ten-eleven translocation 1 (TET1), 2, and 3, responsible for DNA demethylation activities, markedly reduced in infected cells reached the lowest expression in the cells infected with MOI of 2 of IAV. Furthermore, unlike interlukine-6 (IL-6), interferon beta (IFN-β) production increased upon IAV infection, while gradually decreasing by 6 hours post infection, indicated by ELISA assay of infected cells. These findings suggested the alteration in DNA methylation process in response to IAV infection proofed by the upregulation of DNMT1 and depletion of TET gene family. This alteration of DNA methylation may affect the cytokine production from infected cells and, subsequently, cellular immune response.

Keywords: Influenza A virus, DNA methyaltion, ten-eleven translocation, immune response

Introduction

Influenza A virus (IAV) belongs to the *Orthomyxoviridae* family, which has single-stranded negative RNA and is separated into eight segments coded for eleven different proteins (Gatherer, 2009) [9]. Influenza**,** A virus particle, is circular and usually has eighty to hundred-twenty nanometers. The filamentous forms is appeared in some IAV strains and are more common between some strains than others (Bouvier and Palese, 2008) [2]. The virion of IAV is a coated protein that gains its lipid bilayer from the host cell's membrane. Two main types of glycoproteins are encircled in the virion particle; almost 80% of these glycoproteins are hemagglutinin, which facilitates virus entry to the host cell (Rossman *et al*., 2010) [31]. The other 20% of the glycoprotein is neuraminidase, which facilitates virus release from the host cell (Bouvier and Palese, 2008; Khalil, 2012) [2, 17].

Once infected with IAV, transcriptional progress is launched in the host cell leading to stimulating host defense via regulating immune-related genes, programmed cell death, and cell cycle (Khalil, 2017) [18]. Epigenetics profile change in host genes is one of the most curtailed outcomes due to this progress following infection. Noteworthy, epigenetic modification mentions stable and heritable gene expression and cellular function alterations without changing the original DNA sequence. These alterations can cause chromosomal changes and modulate gene expression, cell division, and proliferation (Elawdan *et al*., 2022; Guirgis *et al*., 2023) [6, 10]. Modification in gene expression is due to methylation changes at the 5' of cytosine nucleotide that typically occur in CpG dinucleotide motifs and are stimulated by DNA methyltransferase (DNMT) (Jin *et al*., 2011) [16]. Methylation changes of CpG dinucleotides within the sequence of gene promoter can inhibit specific gene expression due to poor connection with transcription factors (Deaton and Bird, 2011) [4]. Subsequently, the alternation in methylation patterns affects specific protein expression profiles and is eventually implicated in various human diseases such as Alzheimer's and carcinogenicity

(Feinberg, 2007; Khalil *et al*., 2016) [8, 22]. Notably, ten-eleven translocation (TET) proteins family has been identified as dioxygenases that can convert 5-methylcytosine in DNA to 5- hydroxymethylcytosine, which plays an essential role in restoring and reducing the symmetrical methylation progress (Yang *et al*., 2013). However, methionine synthase (MS) encoded by the 5-methyltetrahydrofolate-homocysteine methyltransferase gene (MTR) is responsible for the renewal of methionine from homocysteine and implicated as a donor of the methyl group during DNA methylation activity (Banerjee and Matthews, 1990) [1]. Notably, epigenetic modifications refer to stable and heritable gene expression and cellular function changes without altering the original DNA sequence. These modifications can cause chromosomal changes and modulate gene expression, cell division, and proliferation (Mahmood and Rabbani, 2019) [26]. The promoter region's methylation changes of CpG dinucleotides inhibit certain gene expressions due to poor connection with transcription factors (Deaton and Bird, 2011) [4].

In this work, we aimed to investigate the possible involvement of DNA methylation during IAV infection by using human lung epithelial cells A549 cells *in vitro*. Accordingly, A549 cells were seeded *in vitro* and infected with different MOI of IAV then the relative gene expression of DNA methyl transferase, MTR, and TET genes detected by quantitative real-time PCR (qRT-PCR).

Materials and Methods

Cell line

Lung epithelial cell line, A549 cell, were obtained from (VACSERA, Giza, Egypt) and regularly checked for any contamination with mycoplasma. A549 cells were cultured in RPMI media, containing 25 mM HEPS, 4 mM L-glutamine, and 10% of heat-inactivated bovine serum albumin (BSA) and incubated in CO2 incubator fixed at 37 °C and humidity of 95% (El-Fadl *et al*., 2021) [5].

Infection protocol

Influenza virus A/WSN/33 (IAV) was used to infect A549 cells. Accordingly, A549 cells were cultured overnight in 2ml of complete RPMI media with a density of $2x10^5$ cells per well using a 6-well culture plate. Cells were infected with different MOIs of IAV (0.5-2 MOI) for one hour at room temperature (RT). Finally, the infectious media was removed and infected cells were incubated in RPMI media for 24 hours (Khalil, 2012; Khalil *et al*., 2017b, 2019) [21, 19].

Detection of relative gene expression

Gene expression profile at RNA level was achieved in infected cells by using qRT-PCR. The cells were collected from the cell culture plates as previously described, and then the cells were precipitated in RNase and DNase-free Eppendorf tubes. Total RNA was isolated from precipitated cells by adding 500 µl TriZol (Invitrogen, USA), and 250µl chloroform, mixed carefully, and centrifuged at 10.000xg for 20 min at 4 °C. The supernatant was carefully transferred to clean tubes for RNA purification using an RNA purification kit (Invitrogen, USA). To synthesize the cDNA from the purified RNA, 5 unit of the reverse transcriptase M-MLV (Promega, USA) and 5µl from its buffer was added to 1µg of total RNA, in addition to 1µl oligo–dt primer, 1µl dNTPase, and unit of RNAase inhibitor (Promega, USA). This mixture was incubated for 3 hrs at 45 °C followed by 5 min at 75 °C to stop the reaction. The quantification analysis of the mRNA level of NP, NS1, DNMT1, MTR, TET1, TET2, and TET3 was monitored by using QuantiTect-SYBR-Green PCR Kit (Qiagen, USA) and the specific primers listed in table 1. The level of amplified GAPDH by the oligonucleotides, 5´-ggtatcgtggaaggactcatgac-3´ and 5´-atgccagtgagcttcccgttcag-3´, was used for normalization. The PCR reaction was prepared for each sample by using 10 µl SYBR green, 0.25 µl RNase inhibitor (25 U/µl), 0.2 µM of each primer, 2 µL of synthesized cDNA, and nuclease-free water up to a final volume of 25 µL. The following PCR conditions were used; 94 °C for 5 min, 35 cycles (94 °C for 35 sec, 62 °C for 20 sec, 72 °C for 35 sec) (Farghaly *et al*., 2018; Hamouda *et al*., 2021) [7, 14].

ELISA

ELISA assay was used to measure the released interleukins, Interferon beta (IFN-β) and IL-6 using human ELISA kits (Abcam, 278127) and (Abcam, 178013), respectively. A549 cells cultured in 96-well plates were overnight incubated. Then the cells were infected with IAV followed by an incubation period of (0, 6, 12, 24, 36, 48, and 72 hrs). At each time point, the cells were lysed using 1X cell lysis buffer (Invitrogen, USA). Then, 100 µl of the lysed cells were transferred into the ELISA plate reader and incubated for 2 hrs R.T. with 100 µl control solution and 50µl 1X biotinylated antibody. Then 100 µl of 1X streptavidin-HRP solution was added to each well of samples and incubated for 30 min in the dark. 100 µl of the chromogen TMB substrate solution was added to each well of samples and incubated for 15 min at R.T., away from the light. Finally, a 100 µl stop solution was added to each well of samples to stop the reaction. The absorbance of each well was measured at 450 nm (Khalil *et al*., 2017a; Mohamed *et al*., 2022) [20, 28].

Statistical analysis

All histograms and charts were prepared in Microsoft Excel. Delta-Delta Ct analysis was used in the quantification analysis of mRNA delivered from qRT-PCR assay based on the following formula: (1) delta-Ct = gene's Ct- GAPDH's Ct, (2) (delta–delta Ct) = experiment's delta Ct –control's delta Ct, (3) Quantified fold-change = ($2^{-\text{delta-delta ct}}$) (Rao *et al*., 2013; Khalil H *et al*., 2017). The student's two-tailed t-test was used for calculating. P-value ≤ 0.05 was considered statistically significant.

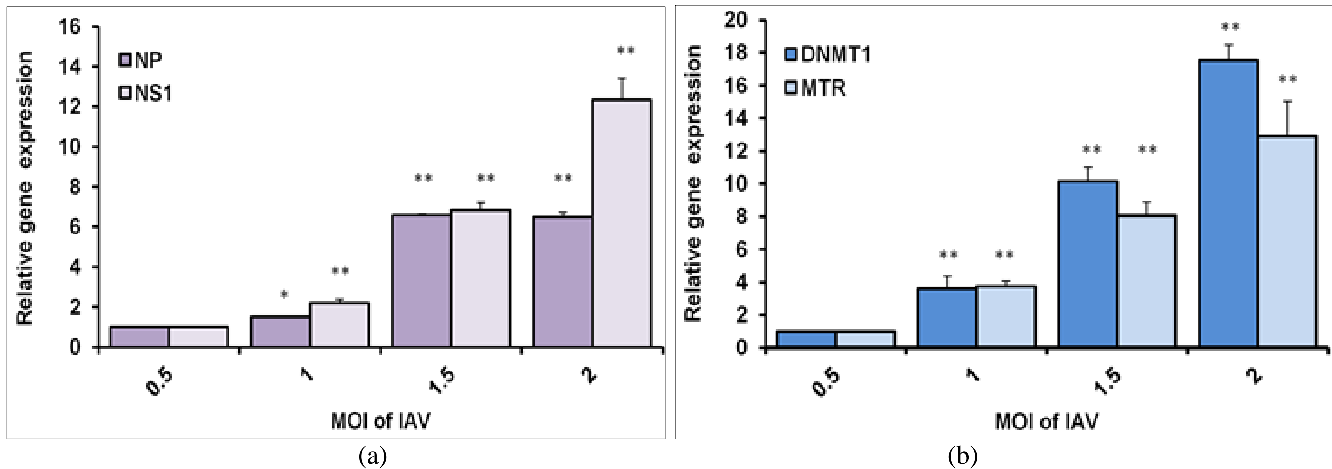
Table 1: Oligonucleotides sequences used for quantification analysis of mRNA of the indicated genes using qRT-PCR

Description	Primer sequences 5'-3'
NP-sense	ATATTGAGAGGGTCGGTTGC
NP-antisense	CCATCCACACCAGTTGACTC
NS1-sense	ATGGATCCAAACACTGTGTC
NS1-antisense	TACAGAGGCCATGGTCATTT
DNMT1-sense	AGGAATGTGTGAAGGAGAAATTG
DNMT1-antisense	CTTGAACGCTTAGCCTCTCCATC
MTR- sense	AGAAGAGGATTATGGTGCTGGATG
MTR- antisense	TCTTAATTCCTGTCTGGAGAGTT
TET1- sense	ACTCCCTGAGGTCTGTCCTGGGA
TET1- antisense	GGATCGAGACATAGCTACAGAGT
TET2- sense	ATGGAACAGGACAGAACCACCCAT
TET2- antisense	ATGGAGCCCAGAGAGAGATGGTTCA
TET3- sense	CGGACGCCTTCATTGCTGCTGCTT
TET3- antisense	TAGGTGCTGGGGCAGAACCACAGT

Results

IAV infecion modultes DNA methylation activity via regulating gene expression of DNMT1 and MTR

Total RNA and complementary cDNA have been performed from infected cells with IAV. Interestingly, the relative gene expression of NP and NS1 strongly up-regulated in cells infected with different MOI of IAV (Figure 1A). To recognize whether the differential methylation status is associated with IAV infection, we measured the expression profile of DNMT1 and MTR in A549 cells upon IAV infection (MOI = 0.5, 1, 1.5, 2) by qRT-PCR. Notably, the expression of DNMT1 and MTR genes showed drastic increases through all different MOIs, subsequently significantly increasing these genes' expression levels (Figure 1 B). These data suggest the correlation between IAV infection and alteration in gene expression of epigenetic-related factors such as DNMT and MTR in infected cells.



(a) (b)

Fig 1: Relative gene expression of IAVand epigenetic related factors

Fold chnages in gene expression of viral NP and NS1 in infected A549 cells (24 hours post-infection). (B) Quantification analysis of DNMT1 and MTR in A549 cells that were infected with IAV (MOIs = 0.5, 1, 1.5, 2) by using the qRT-PCR assay. Error bars reveal to the standard deviation (SD) of two replicates. Student 2-tailed t-test was used for statistical analysis of Ct values of different groups. (*) indicates p-values ≤ 0.05, and (**) indicates the p ≤ 0.01.

IAV disturbs the expression profile of TET gene family in infected cells

Our analysis showed that the expression level of TET1, TET2, and TET3 in A549 cells infected with IAV (MOI = 0.5, 1, 1.5, 2) was significantly down regulation using qRT-PCR (Figure 3A, B, C). Our results strongly suggest that IAV infection reduces the expression profile of the TET gene family, which subsequently increases the hypermethylation activity of host genes following IAV infection.

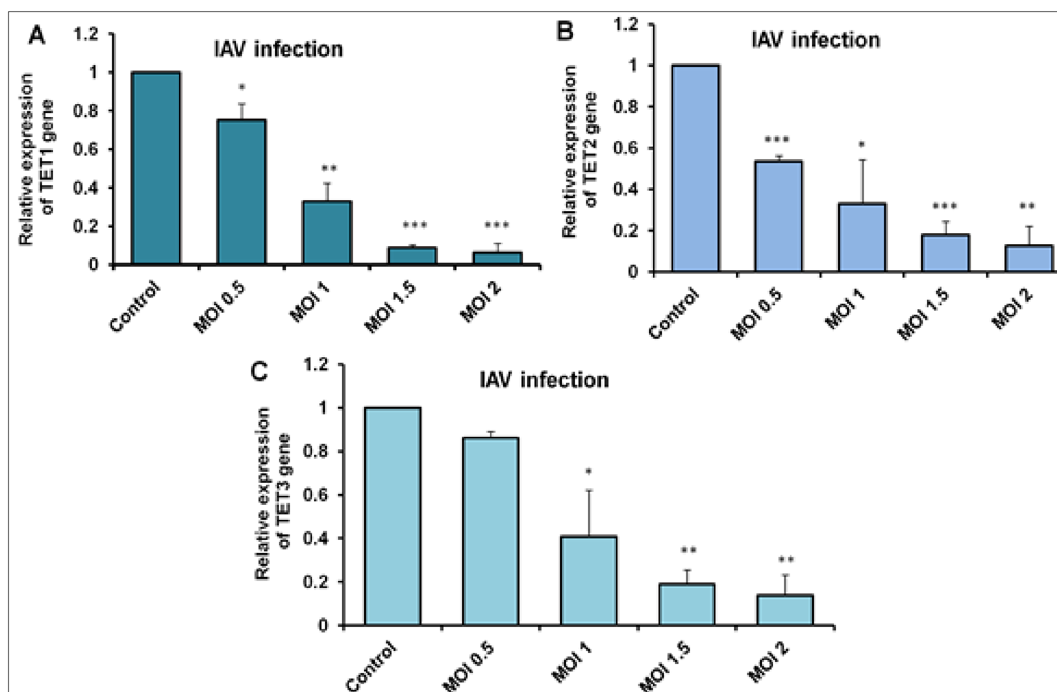


Fig 2: The relative gene expression of TET gene family upon IAV infection.

(A) TET1 in infected A549 cells with the indicated MOIs (MOI of 0.5, 1, 1.5, 2) using the qRT-PCR. (B) TET2 in infected A549 cells with the indicated MOIs (MOI of 0.5, 1, 1.5, 2) using the qRT-PCR. (C) TET3 in infected A549 cells with the indicated MOIs (MOI of 0.5, 1, 1.5, 2) using the qRT-PCR. (Error bars reveal the SD of 3 replicates. Student 2-tailed t-test was used for statistical analysis of Ct values in different groups. (*) means that P-values ≤ 0.05 and (**) means that the $p \leq 0.01$.

Cytokines secretion including IFN-β expression and interleukin 6 upon IAV infection.

ELIZA assay was used to detect the cytokine secretion including interferon beta (INF β) and interleukin 6 (IL6) from A549 cells infected with IAV in a time-dependent manner. Our result illustrated that the concentration of INF β from A549 cells with different MOI of influenza was significantly upregulated upon 6 hours post-infection following remarkable downregulated in a time-dependent manner (Figure 3 A). Moreover, the concentration of IL 6 produced from A549 cells infected with different MOI was significantly upregulated 24 hours post-infection and continuously increased till 48 hours post-infection (Figure 3 B).

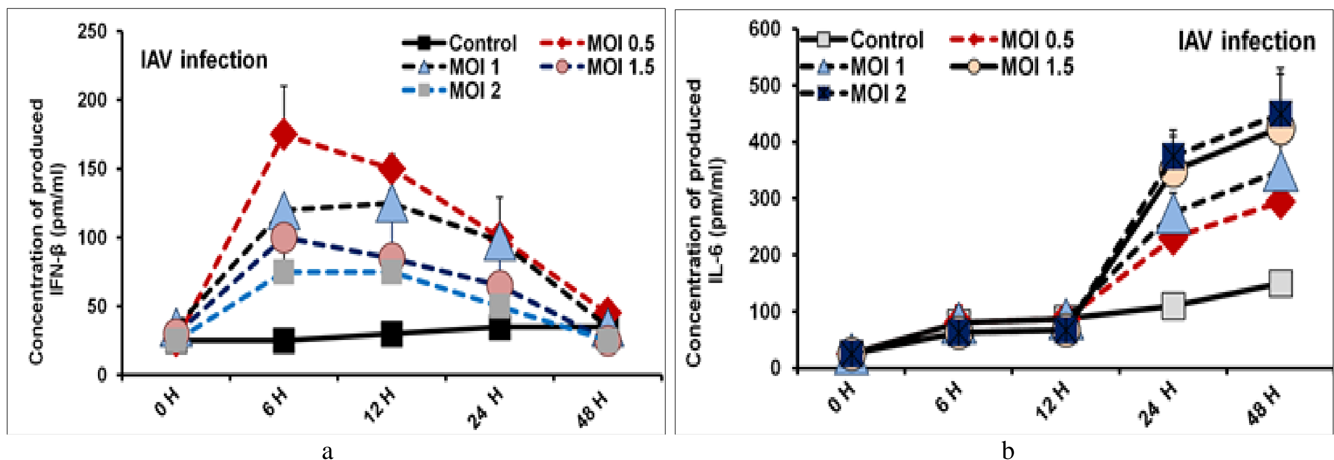


Fig 3: IFN-β expression and interleukin 6 secretions following IAV infection.

(A)The concentration of IFN-β expression produced in A549 cells infected with IAV infection at the indicated time point (MOI 0.5, 1.5). (B) The Concentration of secreted IL6 from infected cells with IAV by pm/ml. Error bars reveal the SD of 3 replicates. The data are represented by three independent experiments.

Discussion

A549 cells were subjected to different MOIs of IAV to investigate the relative expression of NS1 and NP to confirm the viral infection of the cells. Then, we determined the expression of DNMT1, MTR, TET1, TET2, and TET3 by using qRT-PCR. Further, we also measured the concentration of IFN-β and IL6 by ELIZA assay after IAV infection. Interestingly, the relative expression of NS1 and NP was up-regulation and subsequently significantly up-regulation of DNMT1 and MTR while significantly down regulation of TET1, TET2, and TET3. The concentration of IFN-β expression is up-regulation 6 hours after infection and then, gradually down-regulation till 48 hours while IL6 was upr-egulation promptly.

Markedly, IAV infection stimulates various internal signaling pathways that either have antiviral effects or are required to promote viral replication (Khalil, 2017) [18]. A well-known cellular antiviral response is the activation of type I interferon (IFN) signaling cascades (Levy *et al*., 2011) [25]. Consequently, an appreciated number of the so-named IFN-stimulated genes (ISGs) are expressed such as the Mx1 gene, which encodes for Mx1 protein, a member of the dynamin-like large guanosine triphosphatases (GTPases) (Haller *et al*., 2007, 2015) [12-13]. Importantly, the AP-1 associates with NF-κB and interferon regulatory factors-3, 7 (IRF-3, 7) resulted in stimulation of IFN production. Secretion of IFN-β and/or IFN-α, from infected cells, initiate an event that stimulates the expression of IFN-dependent genes such as Mx genes as an antiviral response. Several studies indicated that the viral NS1 protein is a crucial regulator of host gene expression that can inhibit mature mRNA processes and nucleocytoplasmic export of pre-mature mRNAs. In addition, viral NS1 protein has RNA-binding activity that can disturb the function of the antiviral protein; RNA-dependent enzymes (RIG-I), protein kinase R (PKR), NF-κB, IRF-3, and IRF-7 transcription factors (Sha *et al*., 2020; Ji *et al*., 2021; Cruz and Joseph, 2022) [32, 15, 33].

To date, two studies have reported epigenetic alteration during IAV infection. One study existed that the NS1 protein prevents cellular antiviral responses by acting as a histone mimic that disturbs transcriptional elongation (Marazzi *et al*., 2012) [22]. The other study revealed that IAV infection causes methylation changes within the promoter region of genes encoding to inflammatory proteins (Mukherjee *et al*., 2013) [29]. Another study found that interleukin 32 (IL-32) is up-regulated by aberrant DNA methylation modifications during IAV infection (Shi *et al*., 2019) [33]. Another evidence indicated that IAV infection inhibits DNMT3B expression, leading to cyclooxygenase 2 and lambda-1 interferon production (Shi *et al*., 2019) [33]. Overall, these studies indicated the potential role of viral NS1 on DNA methylation activity to facilitate viral replication. Likeminded, as shown in the present study, the infection of A549 cells with IAV showed increasing DNMT1 and MTR gene expression levels as crucial factor in DNA methylation. Interestingly our findings parallel showed a decreasing expression of TET gene family in dose-dependent manner of IAV infection. These data suggested the potential epigenetic modification of TET gene expression following IAV

infection. The role of the TET protein family in the regulation of hypermethylation progress has been reported via modifying methylcytosine and erasing DNA methylation (Elawdan *et al*., 2022) [6]. Three identified TET proteins namely TET1, TET2, and TET3 can catalyze the oxidation of 5mC to 5hmC, and 5fC, to 5-5caC (Tan and Shi, 2012) [34]. These 5 mC oxidation products immediately interact as intermediates in the conversion of 5mC to unmodified cytosines, as the first steps in the activation of the DNA demethylation pathway (Klungland and Robertson, 2017) [24].

Conclusion

In the current work we amid to confirm the involvement of epigenetic alteration in IAV infection by addressing the relative gene expression of epigenetic-related factors, including DNMT1, MTR, and TET gene family. Interestingly, by infecting A549 cells with different MOIs of IAV, the relative expression of DNMT1 was significantly up-regulated following infection and in a dose-dependent manner. The relative expression of the MTR and TET gene family was markedly reduced in response to IAV infection. Further, we figured out that the production of IFN-β is increased immediately upon IAV infection, while markedly decreased from 6 hours post infection and during replication cycle. Unlike IFN-β, IL-6 production from infected cells strongly increased from 12 hours post infection in a dose-dependent manner. These findings conform the implication of epigenetic alteration in IAV replication cycle and suggest the correlation between these alterations and cellular immune response following infection.

Consent for publication

All authors read the manuscript and approved the submission and publication.

Availability of data and materials

All data supporting these findings are available at any time upon request to the corresponding author.

Competing of interest

All authors declare that there are no conflicts of interest.

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Authors' contributions

Dina El Bery performed the experiments. Adel Guirgis and Samir El-Masry assisted in supervising and conceptualizing experiments. Hany Khalil designed the research plan, led the overall research, and provided and interpreted data organized. Dina El-Berry and Hany Khalil wrote the manuscript.

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