MALAT-1/p53/miR-155/miR-146a ceRNA circuit tuned by methoxylated quercitin glycoside alters immunogenic and oncogenic profiles of breast cancer

Mustafa Abdel-Latif1,2 · Ahmed Riad1,2 · Raghda A. Soliman2 · Aisha M. Elkhoully2,3 · Heba Nafae2,3 · Mohamed Z. Gad3 · Amira Abdel Motaal4,5 · Rana A. Youness2,6

Received: 27 October 2021 / Accepted: 27 January 2022 / Published online: 7 February 2022
© The Author(s), under exclusive licence to Springer Science+Business Media, LLC, part of Springer Nature 2022

Abstract
Triple-Negative Breast Cancer (TNBC) is one of the most aggressive and hot BC subtypes. Our research group has recently shed the light on the utility of natural compounds as effective immunotherapeutic agents. The aim of this study is to investigate the role of a methoxylated quercetin glycoside (MQG) isolated from Cleome droserifolia in harnessing TNBC progression and tuning the tumor microenvironment and natural killer cells cytotoxicity. Results showed that MQG showed the highest potency (IC50 = 12 µM) in repressing cellular proliferation, colony-forming ability, migration, and invasion capacities. Mechanistically, MQG was found to modulate a circuit of competing endogenous RNAs where it was found to reduce the oncogenic MALAT-1 lncRNA and induce TP53 and its downstream miRNAs; miR-155 and miR-146a. Accordingly, this leads to alteration in several downstream signaling pathways such as nitric oxide synthesizing machinery, natural killer cells' cytotoxicity through inducing the expression of its activating ligands such as MICA/B, ULBP2, CD155, and ICAM-1 and trimming of the immune-suppressive cytokines such as TNF-α and IL-10. In conclusion, this study shows that MQG act as a compelling anti-cancer agent repressing TNBC hallmarks, activating immune cell recognition, and alleviating the immune-suppressive tumor microenvironment experienced by TNBC patients.

Keywords Cleave droserifolia · Breast cancer · miR-146a · miR-155 · MALAT-1 · Natural Killer cells · Tumor microenvironment

Introduction
By the end of 2020, the number of women diagnosed with breast cancer (BC) reached 7.8 millions worldwide, ranking it as the most prevalent malignancy among females [1]. One of the most influential factors in terms of BC survival rates...
is the molecular subtyping of tumors. Hormone receptor-positive (HR⁺) BC correlates to the most forgiving proges-
ses followed by the human epidermal growth factor receptor-2 positive (HER2⁺) BC subtype [2, 3]. Bleaker still is
the onset of triple-negative breast cancer (TNBC) which
is characterized by the lack of hormonal receptors as well
as HER2, which are powerful therapeutic targets within
the context of the former two BC subtypes [4–6]. It is this
challenge that has prompted immunotherapy as the method
of choice when dealing with TNBC, whereby a variety of
immune checkpoint blockers are in the late stages of clinical
trials [7]. These agents do, however, harbor with them
several serious and potentially fatal side effects [8–10]. In
light of this, our research group has previously demonstrated
that natural compounds could be utilized in an immuno-
therapeutic capacity, possibly providing a means to avoid
the side effects of synthetic chemotherapeutic and immu-
notherapeutic agents [11–16]. Quercetin-3′-methoxy-3-O-
(4′-acetylrhamnoside)-7-O-α-rhamnoside (a methoxylated
quercetin glycoside (MQG) has been previously isolated
from our group from the Egyptian medicinal shrub Cleome
droserifolia [17, 18]. MQG showed highly inducing effects
on the tumor suppressor triad TP53, miR-15a, and miR-16
expression levels with potent antitumor activity in liver can-
cer cell lines [18]. However, its impact on TNBC progres-
sion has never been investigated.

One of the most well-defined aspects of cancer is the
myriad of epigenetic events associated with it [19]. A recent
layer of complexity has been added to the epigenetic circuit
tuning BC progression which is the association of long non-
coding RNAs (lncRNAs) in the regulation of BC hallmarks
and downstream targets [20–23]. MALAT-1 is an oncogenic
lncRNA that has been validated to modulate BC progress-
ion [24]. However, its role in tuning the immunological
profile has rarely been investigated. The dysfunction of the
TP53 gene, in particular, brings about the dysregulation
of a series of modulatory microRNAs (miRNAs) which in
turn shepherd the mutant cell towards an immortal malig-
nant phenotype [5, 22, 25–28]. Two of the most particularly
influential players in this scenario are miR-155-5p and miR-
146a-5p. MiR-155-5p and miR-146a-5p are well-known for
their roles as an immunostimulant, or pro-inflammatory
miRNAs [29–31] and hence are relevant in eliciting a
proper immune surveillance response against the malignant
transformation process. Within the tumor immune micro-
environment (TIME), miR-155-5p and miR-146a-5p were
found to alter cytotoxic T cells activity. However, their role
in altering the recognition of BC cells towards the innate
arm of the immune system and its impact on the cytokine
storm at the TIME is yet to be investigated. Therefore, this
study aims to unravel the impact of MQG on the oncologi-
cal or the immunological profiles of BC cells and to further
investigate the underlying molecular mechanism underneath
such alterations.

Materials and methods

Cell culture and treatment

Different cancer cell lines such as TNBC cell lines, MDA-
MB-231, HR⁺ BC cell lines, MCF-7, and HCC cell lines
Hep-G2 and Huh7 cells were obtained from ATCC and
Vacsera, Egypt. Adherent cells were cultured in DMEM (Lonza, Switzerland) media as previously described [18, 32,
33]. The respective compounds (1–3) were isolated from C.droserifolia as previously described in our group [18] and
as shown in Fig. 1. Stock solutions were prepared as 250 µM
stock solutions in 0.1% DMSO in culture media. Effective
concentrations ranging from 1 µM to 200 µM, depending on
the experimental setup, were prepared, and used to treat dif-
dferent cancer cells seeded in 96-well or 24-well plates. Cells
were treated for 24–96 h in normal growth conditions (37 °C
in a 5% CO₂ atmosphere). 5-Fluorouracil was used as a posi-
tive control in this study. In all experiments, cells used as a
control were labeled as vehicle control and were exposed to
0.1% DMSO in culture media as previously described [11,
14, 34, 35].

Cellular viability and proliferation experiments

3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
bromide (MTT) reagent was used in the cellular viability
experiments. BC cells (10,000) were seeded in 200 µL full
media in a 96-well plate. Forty-eight h post-transfection,
the media was replaced by a 20 µL working solution. After
6 h, the absorbance of the formed purple formazan crystals,
solubilized in 200 µL lysis buffer, was measured [18, 32, 33,
36]. For the cellular proliferation experiments, a bromo-
deoxyuridine (BrdU) incorporation assay was used. BC cells
were seeded into black 96-well plates at a cell density of
5 × 10⁴ cells/well. According to the Cell Proliferation ELISA
kit protocol (Roche Applied Science, Penzberg, Germany),
BC cells were incubated with BrdU for 4 h, then fixed for
30 min using Fix-Denate and finally incubated with Anti-
BrdU POD for 90 min [18, 32, 33, 36]. All experiments were
performed in triplicates and repeated three times or more.

Cellular migration and invasion

BC migration capacity was assessed using the wound-heal-
ing/scratch assay. Treated cells were left to grow to a conflu-
ency of 90–95%. Post-treatment, 3 scratches were performed
in each well using a 10-µL pipette tip. BC cells were washed using PBS and replenished with new low-serum media (1% FBS). After 24 h, the surface areas of the scratches were measured and wound closure was quantified with Zen2012 software [18, 32, 33, 36]. While for the invasion experiments, the modified Boyden chamber assay (BD Bioscience, Bedford, USA) was performed. In 24-well plates, BC cells were treated with MQG, and then 6 × 10^4 cells were re-suspended in 200 μL low-serum media (1% FBS) and were seeded in the upper well. Yet, the lower well contained high-serum media (20% FBS). Cells were washed from the upper surface using a cotton swab 8 h after seeding. Then the invaded cells were fixed and stained using 1% crystal violet (Sigma Chemical Co., California, USA) and counted under an inverted light microscope. All experiments were performed in triplicate and repeated three times or more [18, 32].

**Colony-forming assay**

For the colony-forming experiment, treated cells were harvested and seeded post-treatment in a 6-well plate at 800 cells/well. BC cells were incubated in full DMEM under normal conditions (37 °C and 5% CO₂) for 15 days. Colonies were fixed using 6% glutaraldehyde, stained by 0.05% crystal violet, and then counted [18, 32].

**Cell cycle analysis**

Expression vectors containing response elements for vital cell cycle proteins such as TP53 (pp53-TA-Luc), c-Myc (pMyc-TA-Luc), RB (pRB-TA-Luc), E2F (pE2F-TA-Luc; Clontech, France) were used. Similarly, BC cells were treated with a plus vector containing an unspecific binding site (Clontech, France). BC cells were seeded and transfected with the respective vectors using Superfect Transfection Reagent (Qiagen, Germany) according to the manufacturer’s protocol. After 24 h post-transfection of the plasmid DNA, cells were treated with MQG. After 72 h, BC cells were then lysed, and luciferase expression/luminescence measurement was quantified using Steady-GLO Luciferase Kit (Promega, Germany) according to the manufacturer’s instructions. Luminescence was plotted as % luciferase activity relative to cells transfected with the vector alone. Unspecific luminescence detected by the reagents and the empty plug vector (baseline luminescence) was subtracted from all values before plotting as previously illustrated [5, 32].

**Total RNA and miRNAs extraction**

Total RNA and miRNAs were isolated using a Biozol RNA extraction reagent. Extracted RNA was then quantified spectrophotometrically. RNA integrity was examined by 18 s rRNA bands detection on 1% agarose gel electrophoresis. RNA samples with 260/280 optical density > 2 were excluded [18, 32, 33, 36].

**Quantitative real-time PCR analysis**

Reverse transcription and relative expression quantification of MALAT-1, NOS2, NOS3, MICA, MICB, ULBP2, CD155, ICAM-1, TNF-α, IL-10, and β-actin mRNAs were performed. High-Capacity cDNA Reverse Transcription Kit (ABI, California, USA) was used according to the manufacturer’s instruction for the reverse transcription process. While for the extracted miRNAs TaqMan MicroRNA
Reverse Transcription Kit (ABI, California, USA) was used using specific primers for hsa-miR-155, hsa-miR-146a, and RNU6B. Concerning the relative expression analysis of all the targets and housekeeping genes used in this study, TaqMan Real-Time q-PCR-StepOne™ Systems (ABI, California, USA) was used. Relative expression calculations were performed using the $2^{-\Delta\Delta Ct}$ method. All PCR reactions were done in triplicates and repeated 3 times or more [18, 32, 33, 36–38].

**Quantification of NO production**

NO production was measured using Griess reagent assay (Promega, USA) according to the manufacturer protocol [5, 32]. Briefly, 50 µL of cells' supernatant were mixed with 50 µL sulphanilamide solution and incubated for 10 min. Then, another 50 µL of N-1-naphthyl ethylenediamine dihydrochloride (NED) solution is added and absorbance was measured at 540 nm using Wallac 1420 Victor 2 Multilabel Counter (Perkin Elmer, USA). Experiments were performed in triplicates and repeated 3 times or more [20, 32].

**NK cell isolation**

Peripheral blood mononuclear cells (PBMCs) were separated from peripheral blood of healthy donors upon their written informed consent using Ficoll–Hypaque centrifugation (Axis-Shield PoC AS, Norway). NK cells were then enriched by negative selection using a MACS NK cell isolation kit (Miltenyi Biotec, Cologne, Germany). Enriched NK cell populations were 96.9% CD56/CD3 and 0.8% CD3 positive, assessed by flow cytometry [3, 33, 36, 39].

**Lactate dehydrogenase (LDH) assay**

Treated BC cells were seeded in a 96-well plate at a cell density of 15,000 cells/well. After 2 h, primary NK cells were added to the target BC cells at a 5:1 effector to target ratio (E: T) and incubated for 8 h. Later, the lactate dehydrogenase (LDH) activity assay kit (MAK066-1K1-Sigma-Aldrich, St. Louis, MO, USA) was used to measure the in vitro NK cells cytotoxic potential following the manufacturer’s instructions. The lysis % was calculated according to the following equation: % cytotoxicity = (target maximum release – experimental release)/(target maximum release) × 100. The experiment was done in triplicate and repeated more than 3 times [16, 32, 33, 36–38].

**Statistical analysis**

Data are presented as mean ± standard error of the mean (SEM) for 3 different experiments. Non-parametric unpaired student-t-test was executed to compare between every two independent groups. One-way analysis of variance with post hoc analysis was adopted for multiple comparisons. P-value of < 0.05 was considered statistically significant, and the threshold of significance is denoted by *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Data were analyzed using GraphPad Prism 8.2.1 software as previously described [11].

**Results**

**Preferential selectivity of flavonol glycosides towards MDA-MB-231 TNBC cell lines**

The cytotoxic profiles of flavonol glycosides isolated from *C. droserifolia* (Compounds 1–3) were screened against different cancer cell lines. Flavonol glycosides showed a preferential selectivity towards the TNBC cell lines, MDA-MB-231 when compared to HR+ BC cells and HCC cell lines as shown in Table 1 and Fig. 2. Therefore, the other functional analysis experiments were performed on MDA-MB-231 TNBC cells. The IC50 values of the respective compounds were calculated using the corresponding dose–response curves of each compound in each cell line after 3 days of treatment.

**Impact of serial dilutions of flavonol glycosides isolated from *C. droserifolia* on MDA-MB-231 cellular viability and proliferation rates**

Serial dilutions of flavonol glycosides (1–10 µM) were prepared and applied to MDA-MB-231 cells for 72 h. Compounds (1–3) showed a concentration-dependent reduction in the cellular viability (Fig. 3a–c) and cellular proliferation rate (Fig. 3d–f). Compound 2 was the most potent compound (lowest IC50 as indicated in Table 1) and showed the most

| Table 1 | IC50 values of flavonol glycosides (Compounds 1–3) isolated from *C. droserifolia* against different cancer cell lines |
|---------|-------------------------------------------------|
| Flavonol glycoside | IC50 in Hep-G2 cells | IC50 in Huh-7 cells | IC50 in MCF-7 cells | IC50 in MDA-MB-231 cells |
| Compound 1 | 28 ± 1.63 µM | 50 ± 2.25 µM | 46.5 ± 1.05 µM | 21.7 ± 1.75 µM |
| Compound 2 | 18 ± 1.70 µM | 41 ± 3.68 µM | 38 ± 3.12 µM | 12 ± 2.41 µM |
| Compound 3 | 35 ± 3.27 µM | 60 ± 2.94 µM | 51.5 ± 1.85 µM | 30.3 ± 1.61 µM |
repressing effects on MDA-MB-231 cellular proliferation when compared to compounds 1 and 3.

Impact of flavonol glycosides isolated from *C. droserifolia* on MDA-MB-231 colony-forming ability, migration and invasion capacities

To investigate the long-term effects of serial dilutions of *C. droserifolia* flavonol glycosides (1–3) on MDA-MB-231 cell lines, an anchorage-independent growth assay was performed. In a similar pattern to cellular viability and proliferation assays, compounds (1–3) showed a concentration-dependent reduction in the clonogenic properties also with compound 2 (MQG) showing the most potent inhibitory impact as shown in (Fig. 4a–c). It was tempting to further investigate the impact of *C. droserifolia* flavonol glycosides, Compounds (1–3), on the migration and invasion capacities of MDA-MB-231 cells. Compounds (1–3) (IC<sub>50</sub> values) showed a significant attenuation of wound closure when compared to vehicle control cells. In a similar pattern to cellular viability and anchorage-independent growth assays, Compound 2 (MQG) showed the lowest % closure of the wound (26.36 ± 1.268) if compared to Compound 1 (48.49 ± 4.187) and Compound 3 (48.24 ± 3.645) (Fig. 4d).

Impact of methoxylated quercetin glycoside on cell cycle proteins

After validating the potential selective anticancer activity of MQG in halting the oncogenic profile of human BC cells
effectively and being non-toxic to human normal cells (Supplementary Fig. 1), our aim was extrapolated to unravel the mechanism by which MQG could harness BC progression. For that reason, vital cell cycle proteins were screened such as the tumor suppressor TP53, the oncogenic protein cMyc, and the RB/E2F complex proteins. The results showed that MQG led to a significant elevation in TP53 protein levels \( (P < 0.0001) \). However, it did not affect c-Myc and RB/E2F complex activity (Fig. 5a–d).

Impact of methoxylated quercetin glycoside on ncRNAs circuit around TP53

Then, it was interesting to further unravel the machinery downstream/upstream of the TP53. TP53 is a well-known upstream regulator for an array of miRNAs previously validated by our group [5]. Nonetheless, it was recently reported that MALAT-1 IncRNA acts as a direct upstream regulator for TP53 [40]. On the other hand, miR-155 and miR-146a are reported to act as downstream miRNAs to TP53 and have a dual role in tuning the oncological and immunological profiles. For that reason, screening of MALAT-1, miR-155, and miR-146a were performed in treated MDA-MB-231 cells by \( \text{IC}_{50} \) value (10 \( \mu \)M) of MQG. The results showed that the oncogenic MALAT-1 IncRNA has been significantly reduced \( (P < 0.001) \) while a significant increase of miR-155 \( (P = 0.0002) \) and miR-146a \( (P = 0.0026) \) levels were observed (Fig. 6); building up a novel axis MALAT-1/TP53/miR-155/miR-146a drawn downstream MQG in TNBC cells.

Impact of methoxylated quercetin glycoside on NO machinery system

Furthermore, nitric oxide (NO) has been validated as an important cytokine at the TIME and at the same time has an indisputable role in altering the oncological profile of BC cells. Interestingly, the NO synthesizing enzymes (NOS2 and NOS3) are validated targets for miR-155 and miR-146a. Therefore, to draw the full axis downstream MQG in TNBC cells, the impact of MQG on the NO machinery system was probed. Interestingly, MQG resulted in marked repression of NOS2 \( (P = 0.0030) \), NOS3 \( (P = 0.0005) \) mRNA levels, and consequently a marked reduction in the NO produced from MDA-MB-231 \( (P = 0.0020) \) (Fig. 7).
Impact of methoxylated quercetin glycoside on NK cells cytotoxicity and tumor microenvironment

After validating the alteration of MALAT-1/TP53/miR-155/miR-146a machinery upon MQG treatment for MDA-MB-231 cell lines, we turned our attention towards miR-155 and miR-146a’s immunostimulant roles in the context of BC. It was essential to investigate the impact of MQG on miR-155 and miR-146a immune-related validated targets such as NK cells activating ligands (MICA/B, ULBP2, CD155, ICAM-1), known to be markedly downregulated in MDA-MB-231 cells, and vital immune-inhibitory cytokines (TNF-α, IL-10) known to have a dominant role in potentiating the immune-suppressive microenvironment in BC patients. MQG resulted in a marked induction of MICA (eight-folds, \( P = 0.0158 \)), MICB (tenfolds, \( P < 0.0001 \)), ULBP2 (threefolds, \( P < 0.0001 \)), CD155 (twofolds, \( P = 0.0038 \)), ICAM-1 (threefolds, \( P = 0.0188 \)) and significant reduction of immune-suppressive cytokines TNF-α (\( P = 0.0228 \)) and IL-10 (\( P = 0.0006 \)). Collectively, this resulted in induction in primary NK cells cytotoxicity (\( P = 0.0001 \)) (Fig. 8a–h).

Discussion

The current study sheds the light on a novel crosstalk between ncRNAs building up a novel ceRNAs circuit and their respective preys (targets) in TNBC cells. MQG was found to have the lowest IC\(_{50}\) in repressing the proliferation and viability of MDA-MB-231 TNBC cell lines. This could be directly linked to MQG chemical structure. It was reported that the methoxy and acetyl substitution are responsible for increasing the biological activity of substituted compounds compared to its respective congers [41, 42]. TNBC patients are the least fortunate if compared to other BC subtypes. The lack of therapeutic targets renders its patients especially needful of additional treatment options. Hence, TNBC patients comprised the chief scope of our research. Mechanistically, it was important to unravel the mechanism by which MQG acts as a potent selective anticancer agent against MDA-MB-231 cells. TP53 is a vital tumor suppressor protein and the most fundamental orchestrator of apoptosis and cell cycle arrest [43]. TP53 is markedly downregulated in TNBC patients and cell lines [3, 44, 45]. For that reason, TP53 was our primary target to unravel MQG molecular mechanism in TNCB cell lines. Consistent
with our previous study [18], the results showed that TP53 transcript and protein levels are induced by MQG in MDA-MB-231 cells. In the current study, we further assayed the protein levels of the alternative apoptotic genes E2F, RB, and c-MYC, all of which showed no increase upon treatment with MQG. Taken together, these results suggest that

**Fig. 5** Impact of Compound 2 (MQG) on vital cell cycle regulators. MDA-MB-231 cells treated with compound 2 (MQG) resulted in a significant induction of TP53 luciferase activity. However, it showed non-significant effects on A E2F, B c-Myc and C RB luciferase activity. Data are presented as mean ± SEM of three independent experiments; ***P < 0.001, **P < 0.01 compared with mock cells. miRNA microRNA, SEM standard error of the mean, siRNA small interfering RNA.

**Fig. 6** Impact of Compound 2 (MQG) on the ceRNA circuit MALAT-1/miR-155/miR-146a in TNBC cell line, MDA-MB-231. MALAT-1, miR-155 and miR-146a expression levels were assessed in MDA-MB-231 cells using qRT-PCR and normalized to RNU6B (for miRNAs) and β-actin (for MALAT-1) as internal controls. MDA-MB-231 cells were treated with 10 µM of Compound 2 (MQG) which resulted in a significant repression of A MALAT-1 with a marked elevation of B miR-155, C miR-146a simultaneously when compared to vehicle control cells. Student t test was performed. Data are presented as mean ± SEM of three independent experiments; ***P < 0.001, **P < 0.01, *P < 0.05 compared with control group.
as ceRNA molecules with MALAT-1 and at the same time, they are drawn downstream TP53 in several cellular contexts [29, 46]. Yet, such a novel axis has never been investigated in TNBC cells. For this reason, we extended our view towards the variety of implications p53 induction would entail. The results showed repression of MALAT-1 concomitant with marked repression with miR-155 and miR-146a in MQG treated TNBC cells; Thus building up a novel ceRNA axis orchestrated by TP53 drawn downstream MQG in MDA-MB-231 cells. The clear increase in the expression levels of these two miRNAs witnessed in our study likely supports the hypothesis of an induction mechanism facilitated by the initial modulation of the MALAT-1/p53 axis. This goes in line with previous studies highlighting the potent effect of quercetin derivatives on modulating the expression

Fig. 7 Impact of Compound 2 (MQG) on the NO machinery in TNBC cell line, MDA-MB-231. NOS2 and NOS3 expression levels were assessed in MDA-MB-231 cells using qRT-PCR and normalized to β-actin as internal controls. MDA-MB-231 cells were treated with 10 µM of Compound 2 (MQG) which resulted in a marked repression of A NOS2 and B NOS3 mRNA levels when compared to vehicle control cells. Moreover, Compound 2 (MQG) resulted in a significant reduction in C NO production in the supernatant of MDA-MB-231. Student t test was performed. Data are presented as mean ± SEM of three independent experiments; ***P < 0.001, **P < 0.01, *P < 0.05 compared with control group

Fig. 8 Impact of Compound 2 (MQG) on NK cells cytotoxicity and tumor microenvironment. MICA, MICB, ULBP2, ICAM-1, CD155, TNF-α and IL-10 expression levels were assessed in MDA-MB-231 cells using qRT-PCR and normalized to β-actin as an internal control. MDA-MB-231 cells were treated with 10 µM of Compound 2 (MQG) resulted in a marked increase in A MICA, B MICB, C ULBP2, D CD155, E ICAM-1 simultaneously with a marked repression of F TNF-α and G IL-10 transcript levels when compared to vehicle control cells. Moreover, Compound 2 (MQG) resulted in a significant induction of H primary NK cells cytolytic activity. Student t test was performed. Data are presented as mean ± SEM of three independent experiments; ***P < 0.001, **P < 0.01, *P < 0.05 compared with control group
of ncRNAs such as H19 lncRNA, miR-486-5p, miR-548a, miR-20a [14, 16, 34, 35].

Further downstream still is the observation of NOS2 and NOS3 downregulation validated target genes of miRNA-155 and miRNA-146a simultaneously [47, 48]. Oft dysregulated in cancer, the NO machinery (i.e. NOS2/NOS3) is highly implicated in driving tumor migration, angiogenesis, and mutation through radical-based DNA damage [49]. Indeed, high NOS activity is a significant predictor for poor prognosis among TNBC patients [50, 51]. Our results about the general mitigation of NO machinery upon MQG treatment thus demonstrate an additional facet of its antitumor activity.

The emergence of immunotherapy during the past decade brought about an entirely new line of thinking in the landscape of cancer therapy prompting a widespread consensus of the immune system as the future of the oncological field. With this in mind, we chose to furthermore investigate the MALAT-1/p53/miR-155/miR-146a axis’ significant role in modulating the innate immune recognition. Accordingly, our study showed significant increases in expression of the NK cell ligands MICA/B, ULBP2, CD155, and ICAM-1 as downstream targets for the ceRNA molecules MALAT-1/miR-155/miR-146a [52–54]. These ligands, in a normal context, act as signals for NK cell cytotoxicity during cellular stress. As a result, one mechanism of carcinogenic evolution is the shedding of these ligands, which are found to be especially downregulated in TNBC patients [55]. Their induction in MDA-MB-231 upon treatment with MQG and subsequent increase in NK-mediated cytotoxicity strongly alludes to a counteraction of this immune evasion mechanism. MQG administration also exhibited a significant downregulation of IL-10, an inhibitory cytokine to NK, CD4+, and dendritic cells among others, and a reportedly negative prognostic factor in BC [56, 57]. In doing so, a tentative but promising window is opened towards an entirely new addition to the immunotherapeutic arsenal shifting the focus from the currently dominant adaptive immunity-based agents for which resistance is growing and intense side effects are reported [9, 58–60]. MQG’s derivation from natural sources moreover represents a toxicologically safe and potentially much cheaper alternative to synthetic immunotherapeutic agents.

Curiously, we observed a concomitant downregulation of TNF-α upon MQG administration, despite reports of a positive correlation between miR-155 and the cytokine [61, 62]. High TNF-α production contributes to an inflamed, immunosuppressive TIME and has been reported to mitigate TNBC progression in vitro when knocked out [63]. Although somewhat counterintuitive, the downregulation of TNF-α witnessed in our study hence signifies another favorable modulation to the TIME imposed by MQG. It must be considered that, in inducing p53, the expression of several miRNAs apart from the ones we have tested is overwhelmingly likely to have been affected. The suppression of TNF-α, therefore, could be the result of a differential miRNAs expression such as miR-140-5p/miR-181a-5p [64, 65].

In conclusion, the current study probes a detailed molecular mechanism detailing the impact of MQG on the oncological and immunological profiles of TNBC cells. Furthermore, this study sheds the light on a novel selective multifaceted anticancer immunotherapeutic nutraceutical that holds great potential for TNBC patients that renders it as a potential candidate for clinical trials. This study also demonstrates a novel ceRNA circuit MALAT-1/miR-155/miR-146a orchestrated by p53 in TNBC cells and thus possesses potent effectiveness against TNBC progression and aggressiveness and immune-suppressive nature as summarized in Fig. 9.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11010-022-04378-4.

Author contributions MA-L and RAY contributed to most of the practical work and wrote the manuscript. AR, RS, AE-K, HN contributed to the practical work. MZG, AM co-supervised the work. RAY conceived the original idea, designed the experimental setup, supervised the work. All authors discussed the results and contributed to the final manuscript.

Funding This study was not supported by any funding agency.
Data availability  Data are available from the corresponding author based on a reasonable request.

Declarations

Conflict of interest All authors declare no conflict of interest.

Ethical approval This study complies with all Ethical Standards. The current study does not include any human participants or animals so informed consents are not applicable.

References

1. Siegel RL, Miller KD, Jemal A (2020) Cancer statistics, 2020. CA Cancer J Clin 70:7–30. https://doi.org/10.3322/caac.21590
2. O’Brien KM, Cole SR, Tse CK, Perou CM, Carey LA, Foulkes WD, Dressler LG, Geradts J, Millikan RC (2010) Intrinsic breast tumor subtypes, race, and long-term survival in the carolina breast cancer study. Clin Cancer Res 16:6100–6110. https://doi.org/10.1158/1078-0432.Ccr-10-1533
3. Youness RA, Gad AZ, Sanber K, Ahn YJ, Lee GJ, Khallaf E, Hafez HM, Motaal AA, Ahmed N, Gad MZ (2021) Targeting hydrogen sulphide signaling in breast cancer. J Adv Res 27:177–190. https://doi.org/10.1016/j.jare.2020.07.006
4. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, Lickley LA, Rawlinson E, Sun P, Nard SA (2007) Triple-negative breast cancer: clinical features and patterns of recurrence. Clin Cancer Res 13:4429–4434. https://doi.org/10.1158/1078-0432.Ccr-06-3045
5. Youness RA, Hafez HM, Khallaf E, Assal RA, Abdel Motaal A, Gad MZ (2019) The long noncoding RNA sONE represses triple-negative breast cancer aggressiveness through inducing the expression of miR-34a, miR-15a, miR-16, and let-7a. J Cell Physiol 234:20286–20297. https://doi.org/10.1002/jcp.28629
6. El-Layeh RA, Youness RA, Askary H, Abdelmotaal A, Assal RA (2019) 36PStructural diversity of the cardenolide calotropin renders it as a targeted therapy for harnessing TNBC progression through tuning nitric oxide (NO) levels. Ann Oncol 30(Suppl 1):i14. https://doi.org/10.1093/annonc/mdz026.007
7. Youness RA, Gad AZ, Sanber K, Ahn YJ, Lee GJ, Khallaf E, Hafez HM, Motaal AA, Ahmed N, Gad MZ (2021) Targeting hydrogen sulphide signaling in breast cancer. J Adv Res 27:177–190. https://doi.org/10.1016/j.jare.2020.07.006
8. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, Sawka CA, Lickley LA, Rawlinson E, Sun P, Nard SA (2007) Triple-negative breast cancer: clinical features and patterns of recurrence. Clin Cancer Res 13:4429–4434. https://doi.org/10.1158/1078-0432.Ccr-06-3045
9. Katz H, Alsharedi M (2017) Immunotherapy in triple-negative breast cancer. Med Oncol 35:13. https://doi.org/10.1007/s12032-017-1071-6
10. Beck K, Blansfield J, Tran K, Feldman A, Hughes M, Royal R, Kammula U, Topalian S, Sherry R, Kleiner D, Quezado M, S12032-017-1071-6
11. Ahad AR, Youness RA, Ibrahim M, Motaal AA, El-Askary HI, Assal RA, Gad MZ (2019) An acetylated derivative of vexitxin halts MDA-MB-231 cellular progression and improves its immunogenic profile through tuning miR-20a-MICA/B axis. Nat Prod Res. https://doi.org/10.1080/10786419.2019.1686372
12. El-Layeh RA, Youness RA, Askary H, Abdelmotaal A, Assal RA (2019) Structural diversity of the cardenolide calotropin renders it as a targeted therapy for harnessing TNBC progression through tuning nitric oxide (NO) levels. Ann Oncol 30(Suppl 1):i14. https://doi.org/10.1093/annonc/mdz026.007
13. Elkhouly A, Youness R, Abdelmotaal A, Gad M (2020) miR-486-5p and miR-17-5p: novel immunomodulatory non-coding RNAs drawn downstream 3′-O-acetylvinetin in triple negative breast cancer. Eur J Cancer 138:S70. https://doi.org/10.1016/S0959-8049(20)30715-2
14. Awad AR, Youness RA, Ibrahim M, Motaal AA, El-Askary HI, Assal RA, Gad MZ (2021) An acetylated derivative of vexitxin halts MDA-MB-231 cellular progression and improves its immunogenic profile through tuning miR-20a-MICA/B axis. Nat Prod Res 35:3126–3130. https://doi.org/10.1080/10786419.2019.1686372
15. Shaalan YM, Handoussa H, Youness RA, Assal RA, El-Khatib AH, Linscheid MW, El TayebiAbdelaziz HMAI (2018) Destabilizing the interplay between miR-1275 and IGF2BP5 by Tamarix arbutifolia and quercetin in hepatocellular carcinoma. Nat Prod Res 32:2217–2220. https://doi.org/10.1080/10786419.2017.1366478
16. Abdallah RM, Elkhouly AM, Soliman RA, El Meckawy N, El Sebai A, Motaal AA, El-Askary H, Youness RA, Assal RA (2021) Hindering The synchronization between Mir-486-5p and H19 Lncrna By hesperetin halts breast cancer aggressiveness through tuning ICAM-1. Anticancer Agents Med Chem. https://doi.org/10.2174/187152062166621041909365
17. Ezzat SM, Abdel Motaal A (2012) Isolation of new cytotoxic metabolites from Cleome droserifolia growing in Egypt. Z Nat 67:266–274
18. Youness RA, Assal RA, Ezzat SM, Gad MZ, Abdel Motaal A (2018) A methoxylated quercetin glycoside harnesses HCC tumor progression in a TP53/miR-15/miR-16 dependent manner. Nat Prod Res. https://doi.org/10.1080/10786419.2018.1509326
19. Sharma S, Kelly TK, Jones PA (2010) Epigenetics in cancer. Carcinogenesis 31:27–36. https://doi.org/10.1093/carcin/bgp220
20. Nafea H, Youness RA, Abou-Aisha K, Gad MZ (2021) LncRNA HEIH/miR-939-5p interplay modulates triple-negative breast cancer progression through NOS2-induced nitric oxide production. J Cell Physiol 236:5362–5372. https://doi.org/10.1002/jcp.30234
21. Youness RA, Gad MZ (2019) Long non-coding RNAs: Functional regulatory players in breast cancer. Noncoding RNA Res 4:36–44. https://doi.org/10.1016/j.ncrna.2019.01.003
22. ElKhouly AM, Youness RA, Gad MZ (2020) MicroRNA-486-5p and microRNA-486-3p: Multifaceted pleiotropic mediators in oncological and non-oncological conditions. Noncoding RNA Res 32:2217–2220. https://doi.org/10.1080/10786419.2017.1366478
23. Selem NA, Youness RA, Gad MZ (2021) What is beyond LncR- NAs in breast cancer: a special focus on colon cancer-associated Transcript-1 (CCAT-1). Noncoding RNA Res 6:174–186. https://doi.org/10.1016/j.ncrna.2021.11.001
24. Soliman R-A, Youness R-A, Manie T-M, Khallaf E, El-Shazly M, Abdelmohsen M, Handoussa H, Gad M-Z (2022) Uncoupling tumor necrosis factor-α and interleukin-10 at tumor immune microenvironment of breast cancer through miR-17-5p/ MALAT-1/H19 circuit. Biocell 46:769–783
25. Abd J, Rastgoo N, Li LH, Chen WM, Chang H (2017) Role of tumor suppressor p53 and micro-RNA interplay in multiple myeloma pathogenesis. J Hematol Oncol. https://doi.org/10.1186/s13045-017-0538-4
26. Hattori H, Janky R, Nettelfd W, Aerts S, Babu MM, Venkitaraman AR (2014) p53 shapes genome-wide and cell type-specific changes in microRNA expression during the human DNA damage response. Cell Cycle 13:2572–2586. https://doi.org/10.4161/15384101.2015.942209
27. Sandhu R, Rein J, D’Arcy M, Herschkowitz JI, Hoadley KA, Troester MA (2014) Overexpression of miR-146a in basal-like breast cancer cells confers enhanced tumorigenic potential in association with altered p53 status. Carcinogenesis 35:2567–2575. https://doi.org/10.1093/carcin/bgu175

28. El Kilany Youness FHRA, Assal RA, Gad MZ (2021) miR-744/eNOS/NO axis: a novel target to halt triple negative breast cancer progression. Breast Dis 40:161–169. https://doi.org/10.3233/bd-200454

29. Farooni I, Antonentzi FR, Cardone J, Bonmassar E (2009) miR-155 gene: a typical multifunctional microRNA. BBA-Mol Basis Dis 1792:497–505. https://doi.org/10.1016/j.bbadis.2009.02.013

30. Wang H, Zhang Y, Wu X, Wang Y, Cui H, Li X, Zhang J, Tun N, Peng Y, Yu J (2018) Regulation of Human Natural Killer Cell IFN-γ Production by MicroRNA-146a via Targeting the NF-κB Signaling Pathway. Front Immunol 9:293. https://doi.org/10.3389/fimmu.2018.00293

31. Hargreaves BKV, Roberts SE, Derfalvi B, Boudreau JE (2020) Highly efficient serum-free manipulation of miRNA in human NK cells without loss of viability or phenotypic alterations is accomplished with TransIT-TKO. PLoS ONE 15(4):e0231664

32. Youness RA, Assal RA, Abdul Motaal A, Gad MZ (2018) A novel role of sONE/NOS3/NO signaling cascade in mediating hydrogen sulphide bilateral effects on triple negative breast cancer progression. Nitric Oxide 80:12–23. https://doi.org/10.1016/j.jnio.2017.08.004

33. Youness RA, Rahmoon MA, Assal RA, Gomaa AI, Hamza MT, Waked I, El Tayebi HM, Abdelaziz AI (2016) Contradicting interplay between insulin-like growth factor-1 and miR-486-5p in primary NK cells and hematoma cell lines with a contemporary inhibitory impact on HCC tumor progression. Growth Factors 34:128–140. https://doi.org/10.1007/s00705-019-04232-x

34. Ahmed Youness R, Amr Assal R, Mohamed Ezzat S, Zakaria Gad M, Abdul Motaal A (2020) A methoxylated quer cetin glycoside synthesizes HCC tumor progression in a TP53/miR-15/miR-16 dependent manner. Nat Prod Res 34:1475–1480. https://doi.org/10.1080/14786419.2018.1509326

35. Youness RA, El-Tayebi HM, Assal RA, Hosny K, Esmat G, Abdelaziz AI (2016) MicroRNA-486-5p enhances hepatocellular carcinoma tumor suppression through repression of IGF-1R and its downstream mTOR, STAT3 and c-Myc. Oncol Lett 11:2567–2573. https://doi.org/10.3892/ol.2016.4914

36. Youssef SS, Abbas E, Youness RA, Elemeemy MN, Nasr AS, Seif S (2019) PNPLA3 and IL-28B signature for predicting susceptibility to chronic hepatitis C infection and fibrosis progression. Arch Physiol Biochem. https://doi.org/10.1080/13813455.2019.1694039

37. El Din Youness GSRA, Assal RA, Gad MZ (2020) microRNA-506-3p directly regulates rs10754339 (A/G) in the immune checkpoint protein B7–H4 in breast cancer. Microrna 9:346–353. https://doi.org/10.2174/221153660966662019152949

38. Rahmoon MA, Youness RA, Gomaa AI, Hamza MT, Waked I, El Tayebi HM, Abdelaziz AI (2017) MiR-615-5p depresses natural killer cells cytostrophy through repressing IGFR-1 in hepatocellular carcinoma patients. Growth Factors 35:76–87. https://doi.org/10.1080/08977194.2017.1354859

39. Zhang T, Wang H, Li Q, Fu J, Huang J, Zhao Y (2018) MALAT1 Activates the P53 signaling pathway by regulating MDM2 to promote ischemic stroke. Cell Physiol Biochem 50:2216–2228. https://doi.org/10.1159/000495083

40. Nour AM, Khalid SA, Kaiser M, Brun R, Abdalla WE, Schmidt TJ (2010) The antiproteolal activity of methylated flavonoids from Agaratum conyzoides L. J Ethnopharmacol 129:127–130. https://doi.org/10.1016/j.jp.tal.2010.02.015

41. Begum S, Wahab A, Siddiqui BS (2008) Antimycobacterial activity of flavonoids from Lantana camara Linn. Nat Prod Res 22:467–470. https://doi.org/10.1080/14786410600898714

42. Petitjean A, Achatz MI, Borresen-Dale AL, Hainaut P, Olivier M (2007) TP53 mutations in human cancers: functional selection and impact on cancer prognosis and outcomes. Oncogene 26:2157–2165. https://doi.org/10.1038/sj.onc.1213032

43. Horigome E, Fujieda M, Handa T, Katayama A, Ito M, Ichihara A, Tanaka D, Gombodorj N, Yoshiyama S, Yamane A, Yamada K, Horiguchi J, Shinozuka K, Oyama T, Nishiyama M, Rokudai S (2018) Mutant TP53 modulates metastasis of triple negative breast cancer through adenosine A2b receptor signaling. Oncotarget 9:34554–34566. https://doi.org/10.18632/oncotarget.26177

44. Li JP, Zhang XM, Zhang Z, Zheng LH, Jindal S, Liu YJ (2019) Association of p53 expression with poor prognosis in patients with triple-negative breast invasive ductal carcinoma. Medicine (Baltimore) 98:e15449. https://doi.org/10.1097/md.00000000000015449

45. Grespi F, Landré V, Molchadsky A, Di Daniele N, Mellerin G, Rotter V (2016) Differential regulated microRNA by wild type and mutant p53 in induced pluripotent stem cells. Cell Death Dis 7:e2567–e2567. https://doi.org/10.1038/cddis.2016.419

46. Dai R, Phillips RA, Zhang Y, Khan D, Crasta O, Ahmed SA (2008) Suppression of LPS-induced Interferon-γ and nitric oxide in splenic lymphocytes by select estrogen-regulated microRNAs: a novel mechanism of immune modulation. Blood 112:4591–4597. https://doi.org/10.1182/blood-2008-04-152488

47. Xu C, Ren G, Cao G, Chen Q, Shou P, Zheng C, Du L, Han X, Jiang M, Yang Q, Lin L, Wang G, Yu P, Zhang X, Cao W, Brewer G, Wang Y, Shi Y (2013) miR-155 regulates immune modulatory properties of mesenchymal stem cells by targeting TAK1-binding protein 2. J Biol Chem 288:11074–11079. https://doi.org/10.1074.jbc.M111.414862

48. Jadeski LC, Chakraborty C, Lala PK (2002) Role of nitric oxide in tumour progression with special reference to a murine breast cancer model. Can J Physiol Pharmacol 80:125–135. https://doi.org/10.1139/y02-007

49. Walsh EM, Keane MM, Wink DA, Callagy G, Glynn SA (2016) Review of triple negative breast cancer and the impact of inducible nitric oxide synthase on tumor biology and patient outcomes. Crit Rev Oncog 21:333–351. https://doi.org/10.1615/CritRevOnc.2017021307

50. Garrido P, Shalaby A, Walsh EM, Keane N, Webber M, Keane MM, Sullivan FJ, Merri CJ, Callagy G, Ryan AE, Glynn SA (2017) Impact of inducible nitric oxide synthase (iNOS) expression on triple negative breast cancer outcome and activation of EGFR and ERK signaling pathways. Oncotarget 8:80568–80588. https://doi.org/10.18632/oncotarget.19631

51. Lu C, Huang X, Zhang X, Roensch K, Cao Q, Nakayama KI, Blazar BR, Zeng Y, Zhou X (2011) miR-221 and miR-155 regulate human dendritic cell development, apoptosis, and IL-12 production through targeting of p27kip1, KPC1, and SOCS-1. Blood 117:4293–4303. https://doi.org/10.1182/blood-2010-12-322503

52. Cerutti C, Soblecher-Martin P, Wu D, López-Ramirez MA, de Vries H, Sharrack B, Male DK, Romero IA (2016) MicroRNA-155 contributes to shear-resistant leukocyte adhesion to human brain endothelium in vitro. Fluids Barri CNS 13:8. https://doi.org/10.1186/s12987-016-0032-3

53. Abdel-Latif M, Afiﬁ A, Soliman R, Elkhouly A, Abdmotalal A, Youness RA (2019) 23P-A new quercetin glycoside enhances TNBC immunological profile through TP53/miR-155/MICA/
55. de Kruijf EM, Sajet A, van Nes JG, Putter H, Smit VT, Eagle RA, Jafferji I, Trowsdale J, Liefer GJ, van de Velde CJ, Kuppen PJ (2012) NKG2D ligand tumor expression and association with clinical outcome in early breast cancer patients: an observational study. BMC Cancer 12:24. https://doi.org/10.1186/1471-2407-12-24

56. Llanes-Fernández L, Alvarez-Goyanes RI, Arango-Prado MdC, Alcocer-González JM, Mojarrieta JC, Pérez XE, López MO, Odio SF, Camacho-Rodriguez R, Guerra-Yi ME, Madrid-Marina V, Tamez-Guerra R, Rodríguez-Padilla C (2006) Relationship between IL-10 and tumor markers in breast cancer patients. Breast 15:482–489. https://doi.org/10.1016/j.breast.2005.09.012

57. Chavey C, Bibeau F, Gourgou-Bourgade S, Burlinchon S, Boissière F, Laune D, Roques S, Lazennec G (2007) Oestrogen receptor negative breast cancers exhibit high cytokine content. Breast Cancer Res 9:R15. https://doi.org/10.1186/bcr1648

58. Jenkins RW, Barbie DA, Flaherty KT (2018) Mechanisms of resistance to immune checkpoint inhibitors. Br J Cancer 118:9–16. https://doi.org/10.1038/bjc.2017.434

59. O’Donnell JS, Long GV, Scolyer RA, Teng MWL, Smyth MJ (2017) Resistance to PD1/PDL1 checkpoint inhibition. Cancer Treat Rev 52:71–81. https://doi.org/10.1016/j.ctrv.2016.11.007

60. Downey SG, Klapper JA, Smith FO, Yang JC, Sherry RM, Royal RE, Kammula US, Hughes MS, Allen TE, Levy CL, Yellin M, Nichol G, White DE, Steinberg SM, Rosenberg SA (2007) Prognostic factors related to clinical response in patients with metastatic melanoma treated by CTL-associated antigen-4 blockade. Clin Cancer Res 13:6681–6688. https://doi.org/10.1158/1078-0432.Ccr-07-0187

61. Pedersen IM, Otero D, Kao E, Miletic AV, Hather C, Ralfkiaer E, Rickert RC, Gronbaek K, David M (2009) Onco-miR-155 targets SHIP1 to promote TNFα-dependent growth of B cell lymphomas. EMBO Mol Med 1:288–295. https://doi.org/10.1002/emmm.200900028

62. Bala S, Marcos M, Kody K, Csak T, Catalano D, Mandrekar P, Szabo G (2011) Up-regulation of microRNA-155 in macrophages contributes to increased tumor necrosis factor alpha (TNF alpha) production via increased mRNA half-life in alcoholic liver disease. J Biol Chem 286:1436–1444. https://doi.org/10.1074/jbc.M110.145870

63. Pileczki V, Braicu C, Gherman CD, Berindan-Neagoe I (2012) TNF-α gene knockout in triple negative breast cancer cell line induces apoptosis. Int J Mol Sci 14:411–420. https://doi.org/10.3390/ijms1410411

64. Zhu J, Wang F-L, Wang H-B, Dong N, Zhu X-M, Wu Y, Wang Y-T, Yao Y-M (2017) TNF-α mRNA is negatively regulated by microRNA-181a-5p in maturation of dendritic cells induced by high mobility group box-1 protein. Sci Rep 7:12239. https://doi.org/10.1038/s41598-017-12492-3

65. Zhu TT, Zhang WF, Yin YL, Liu YH, Song P, Xu J, Zhang MX, Li P (2019) MicroRNA-140-5p targeting tumor necrosis factor-α prevents pulmonary arterial hypertension. J Cell Physiol 234:9535–9550. https://doi.org/10.1002/jcp.27642

Publisher’s Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.