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RESEARCH PAPER

Fusaric acid decreases p53 expression by altering promoter methylation and m6A RNA methylation in human hepatocellular carcinoma (HepG2) cells

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ABSTRACT

Fusaric acid (FA) is a food-borne mycotoxin that mediates toxicity with limited information on its epigenetic properties. p53 is a tumour suppressor protein that regulates cell cycle arrest and apoptotic cell death. The expression of p53 is regulated transcriptionally by promoter methylation and post-transcriptionally by N-6-methyladenosine (m6A) RNA methylation. We investigated the effect of FA on p53 expression and its epigenetic regulation via promoter methylation and m6A RNA methylation in human hepatocellular carcinoma (HepG2) cells. HepG2 cells were treated with FA [0, 25, 50, 104, and 150 µg/ml; 24 h] and thereafter, DNA, RNA, and protein was isolated. Promoter methylation and expression of p53 was measured using qPCR and Western blot. RNA immuno-precipitation was used to determine m6A-*p53* levels. The expression of m6A methyltransferases (*METTL3* and *METTL14*), demethylases (*FTO* and *ALKBH5*), and readers (*YTHDF1-3* and *YTHDC2*) were measured using qPCR. FA induced *p53* promoter hypermethylation ($p < 0.0001$) and decreased *p53* expression ($p < 0.0001$). FA decreased m6A-*p53* levels ($p < 0.0001$) by decreasing *METTL3* ($p < 0.0001$) and *METTL14* ($p < 0.0001$); and suppressed expression of *YTHDF1* ($p < 0.0001$), *YTHDF3* ($p < 0.0001$), and *YTHDC2* ($p < 0.0001$) that ultimately reduced p53 translation ($p < 0.0001$). Taken together, the data shows that FA epigenetically decreased p53 expression by altering its promoter methylation and m6A RNA methylation in HepG2 cells. This study reveals a mechanism for p53 regulation by FA and provides insight into future therapeutic interventions.

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KEYWORDS
Fusaric acid; p53; promoter methylation; epitranscriptomics; m6A RNA methylation

Introduction

Fusaric acid (FA; 5-butylpicolinic acid) is a mycotoxin produced by the *Fusarium* species that parasitize agricultural foods and feeds and impacts on human and animal health. To date, little is known on the toxic and epigenetic effects of FA in humans and animals and the elucidation of cellular epigenetic mechanisms can lead to a better understanding of FA toxicity as well as assist in the development of preventative and therapeutic measures against FA toxicity. This is beneficial in underprivileged communities where the food supply and storage conditions are inadequate. Currently, the only epigenetic study on FA showed induction of DNA hypomethylation that led to genotoxicity and cytotoxicity in an *in vitro* model [1].

FA has diverse toxicological effects in plants [2–5] and animals [6–9]; it exhibits phytotoxicity by causing necrosis and wilt disease symptoms in various plants [5]. FA is also toxic to human and animal cells by inducing oxidative stress [10], mitochondrial dysfunction [11], DNA damage [12,13], and apoptotic cell death [10–12,14,15]. It has neurochemical effects in mice [16], rats [17], and pigs [18,19]; and reduced aggressive behaviour and motor activity [16]. Additionally, the toxicity of FA was associated with alterations in platelet function [20], delayed bone ossification [21], hypotension [7,22], and notochord malformation [8]. Synergism between FA and other *Fusarium*-produced mycotoxins such as deoxynivalenol (DON) [23], fumonisin B_1 (FB_1) [24], and 4,15-diacetoxyscirpenol (DAS) [25] have also been demonstrated.

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Supplemental data for this article can be accessed here

The tumour suppressor protein, p53 is a transcription factor that is activated in response to cellular stress [26]. The most common p53 activating stressors include oxidative stress, DNA damage, excessive oncogene activation, and hypoxia [26,27]. Once activated, p53 recruits core transcriptional machinery to its target promoters, enabling the transcription of genes, with cellular outcomes such as cell cycle arrest and apoptosis [28,29]. Dysregulation in p53 expression has been associated with several human diseases including neurodegenerative diseases [30,31] and cancer [32].

Although previous studies have indicated that p53 is regulated at the post-translational level by ubiquitination, phosphorylation, and acetylation [33–35], the expression of p53 is also regulated epigenetically at the transcriptional and post-transcriptional levels by promoter methylation and N-6-methyladenosine (m6A) RNA methylation.

Promoter methylation, methylation of CpG islands within the promoter regions of specific genes, is crucial in regulating gene transcription. The *p53* promoter region was sequenced and basal promoter activity was localized to an 85bp region (nucleotides 760–844) that is indispensable for its full promoter activity [36], and the *p53* promoter has putative binding sites for transcription factors. Alterations in *p53* promoter methylation have been linked with an array of *p53* mutations, loss in tumour suppressor function, and cancer progression [32]. Previously, it was shown that promoter hypermethylation of *p53* prevents binding of transcription factors and is associated with a reduction in *p53* expression, whereas promoter hypomethylation increases *p53* expression [37,38].

Post-transcriptional regulation of messenger RNA (mRNA) expression involves RNA-protein and RNA-RNA interactions [39]. M6A RNA methylation occurs in approximately 0.2–0.5% of adenines and is the most abundant post-transcriptional modification of mammalian mRNA [40,41]. M6A is commonly found in the coding region and 3 untranslated region (3 UTR) of mRNA and is involved in regulating cellular processes including mRNA translation [42,43], degradation [44], splicing [45], and cellular localization [46]. Dysregulation in the m6A methylation pattern has been associated with developmental abnormalities [46–48], obesity [49,50], type 2 diabetes [51], cancer [52–54], and other human diseases [55].

M6A is catalyzed by the methyltransferase complex which consists of methyltransferase-like 3 (METTL3), methyltransferase-like 14 (METTL14), and Wilm's tumour 1-associated protein (WTAP) [56,57]. METTL3 is catalytically active and regulates m6A levels by binding to s-adenosyl methionine and catalyzing the transfer of a methyl group to the N-6 position of specific adenines on the target mRNA, METTL14 functions to maintain structure and substrate recognition by interacting with and stabilizing METTL3, whereas WTAP is catalytically inactive and facilitates RNA binding and m6A deposition by coordinating nuclear localization of the METTL3-METTL14 complex [57]. The m6A demethylases, fat mass and obesity-associated protein (FTO) and ALKB homolog 5 (ALKBH5), are Fe^{2+} and alpha-ketoglutarate-dependent and function by oxidizing N-methyl groups of m6A to a hydroxymethyl group [39,58].

Chemical modifications of RNA transcripts alter the charge, base-pairing, secondary structure, and RNA-protein interactions, thereby, regulating gene expression by modulating RNA processing, localization, translation, and decay [42,44–46]. Similarly, m6A also affects RNA processing by recruiting specific reader proteins. The m6A readers such as the YT521-B homology domain containing proteins 1 and 2 (YTHDC1 and YTHDC2) and the YT521-B homology domain family proteins 1, 2, and 3 (YTHDF1, YTHDF2, and YTHDF3) specifically recognize m6A modified RNAs and regulate the expression and function of specific mRNAs and proteins. YTHDF1, YTHDF3, and YTHDC2 regulate mRNA translation [42,43], YTHDF2 regulates mRNA degradation [44], and YTHDC1 regulates mRNA splicing and cellular localization [45,46].

Previously, we determined that FA (104 μg/ml) post-translationally activates p53 in response to DNA damage in human hepatocellular carcinoma (HepG2) cells [12]; however, the mechanism underlying the effect of FA on p53 expression and its epigenetic regulation is not well understood. This study aimed to determine the effect

of FA on p53 expression and its epigenetic regulation at the transcriptional and post-transcriptional levels by promoter methylation and m6A RNA methylation in HepG2 cells. Results from this study provide insight into a possible mechanism of FA mediated regulation of p53 at the epigenetic level that may serve as an alternative mechanism for FA toxicity.

Results

Fusaric acid decreased p53 expression in HepG2 cells

The tumour suppressor protein, p53 is activated during cellular stress and functions in cell cycle control and apoptosis [27]. Previously, it was shown that FA activates p53 via phosphorylation and acetylation in HepG2 cells [12]; however, its effect on p53 mRNA and protein expression is not well understood. We determined the effect of FA on p53 mRNA and protein expression in HepG2 cells using qPCR and Western blot, respectively. FA significantly decreased p53 mRNA ($p < 0.0001$; Figure 1a) and protein ($p < 0.0001$; Figure 1b) expression in HepG2 cells compared to the control.

Fusaric acid altered p53 promoter methylation in HepG2 cells

The promoter methylation of genes is essential in regulating transcriptional activity and gene expression. Previously, *p53* promoter hypomethylation was shown to increase *p53* expression [59,60], whereas *p53* promoter hypermethylation was shown to decrease *p53* expression [37,38]. We determined if the decrease in *p53* mRNA expression observed in the FA-treated HepG2 cells was a result of alterations in *p53* promoter methylation. FA significantly increased *p53* promoter methylation in the 25, 104, and 150 µg/ml FA treatments; however, the promoter methylation of *p53* was significantly decreased by the 50 µg/ml FA in HepG2 cells ($p < 0.0001$; Figure 2).

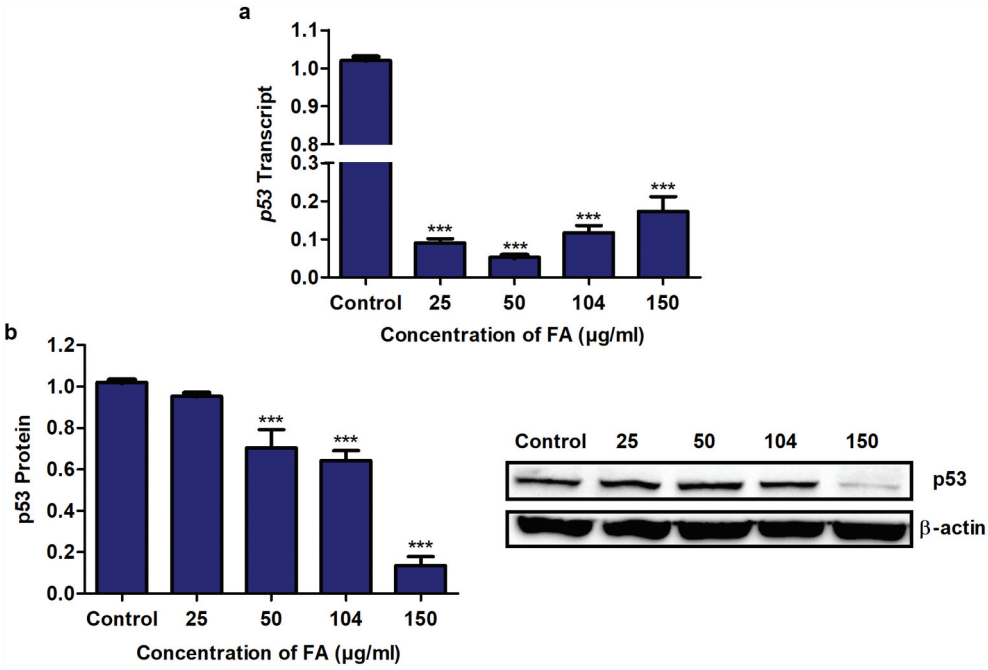

Figure 1. The effect of FA on p53 expression in HepG2 cells. (a) RNA isolated from control and FA-treated HepG2 cells were reverse transcribed into cDNA and analyzed for *p53* expression using qPCR. FA decreased the mRNA expression of *p53* in HepG2 cells. (b) Protein expression of p53 was determined using Western blot. FA decreased the protein expression of p53 in HepG2 cells. Results are represented as mean fold-change ± SD (n = 3). Statistical significance was determined by one-way ANOVA with the Bonferroni multiple comparisons test (***$p < 0.0001$).

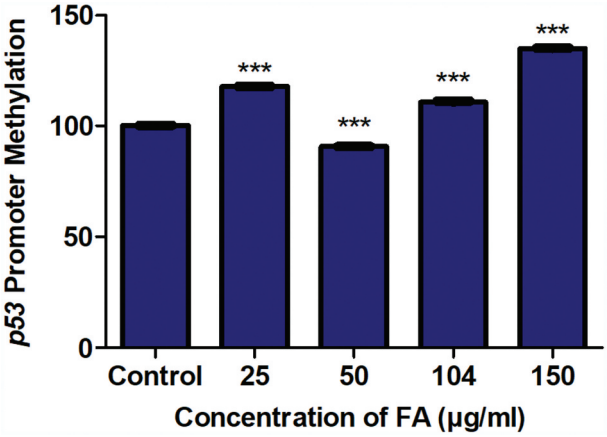

Figure 2. The effect of FA on the promoter methylation of *p53* in HepG2 cells. DNA isolated from control and FA-treated HepG2 cells were assayed for *p53* promoter methylation using the OneStep qMethyl Kit. FA altered *p53* promoter methylation in HepG2 cells. Results are represented as mean fold-change ± SD (n = 3). Statistical significance was determined by one-way ANOVA with the Bonferroni multiple comparisons test (***$p < 0.0001$).

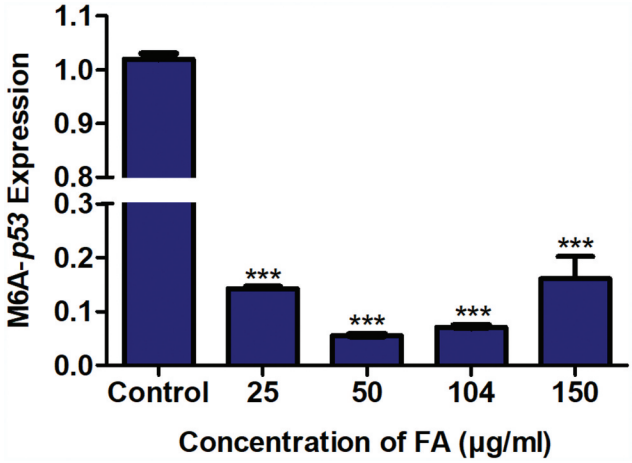

Figure 3. The effect of FA on m6A-*p53* levels in HepG2 cells. RNA immuno-precipitation using m6A antibody and quantification of *p53* mRNA levels in control and FA-treated HepG2 cells. FA decreased m6A-*p53* levels in HepG2 cells. Results are represented as mean fold-change ± SD (n = 3). Statistical significance was determined by one-way ANOVA with the Bonferroni multiple comparisons test (***$p < 0.0001$).

Fusaric acid decreased m6A-p53 levels in HepG2 cells

M6A, an abundant and dynamic post-transcriptional modification of mRNA, regulates mRNA degradation and translation [42–44]. Due to the FA-induced decrease in p53 expression at both the transcript and protein levels, we determined the effect of FA on m6A-*p53* levels in HepG2 cells using RNA immuno-precipitation. FA significantly decreased m6A-*p53* expression in HepG2 cells ($p < 0.0001$; Figure 3) compared to the control.

Fusaric acid decreased the expression of m6A methyltransferases and demethylases in HepG2 cells

The m6A methyltransferases, METTL3 and METTL14, and demethylases, FTO and ALKBH5, regulate m6A levels of RNA transcripts. Due to the FA-induced decrease in m6A-*p53* levels observed in the FA-treated HepG2 cells, we determined the effect of FA on the mRNA expression of *METTL3, METTL14, FTO*, and *ALKBH5*. FA significantly decreased the expression of *METTL3* ($p < 0.0001$; Figure 4a), *METTL14* ($p < 0.0001$; Figure 4b), *FTO* ($p < 0.0001$; Figure 4c), and *ALKBH5* ($p < 0.0001$; Figure 4d) in HepG2 cells compared to the control. This suggests that FA may decrease m6A-*p53* levels by modulating the expression of the m6A methyltransferases in HepG2 cells.

Fusaric acid decreased the expression of m6A readers in HepG2 cells

M6A plays a major role in RNA processing by recruiting specific readers which recognize m6A modified mRNAs and regulate the expression of the target mRNA and protein [55]. The m6A readers, YTHDF1, YTHDF3, and YTHDC2 have been shown to regulate mRNA translation/protein expression [42], whereas YTHDF2 was shown to regulate mRNA expression [44]. Due to the decrease in p53 mRNA and protein expression observed in the FA-treated HepG2 cells as well as the FA-induced decrease in m6A-*p53* levels, we determined the effect of FA on the mRNA expression of *YTHDF1, YTHDF2, YTHDF3*, and *YTHDC2*. FA significantly decreased the expression of *YTHDF1* ($p < 0.0001$; Figure 5a), *YTHDF2* ($p < 0.0001$; Figure 5b), *YTHDF3* ($p < 0.0001$; Figure 5c), and *YTHDC2* ($p < 0.0001$; Figure 5d) in HepG2 cells compared to the control.

Discussion

Exposure to mycotoxins causes adverse effects in humans and animals. FA is a common food-borne

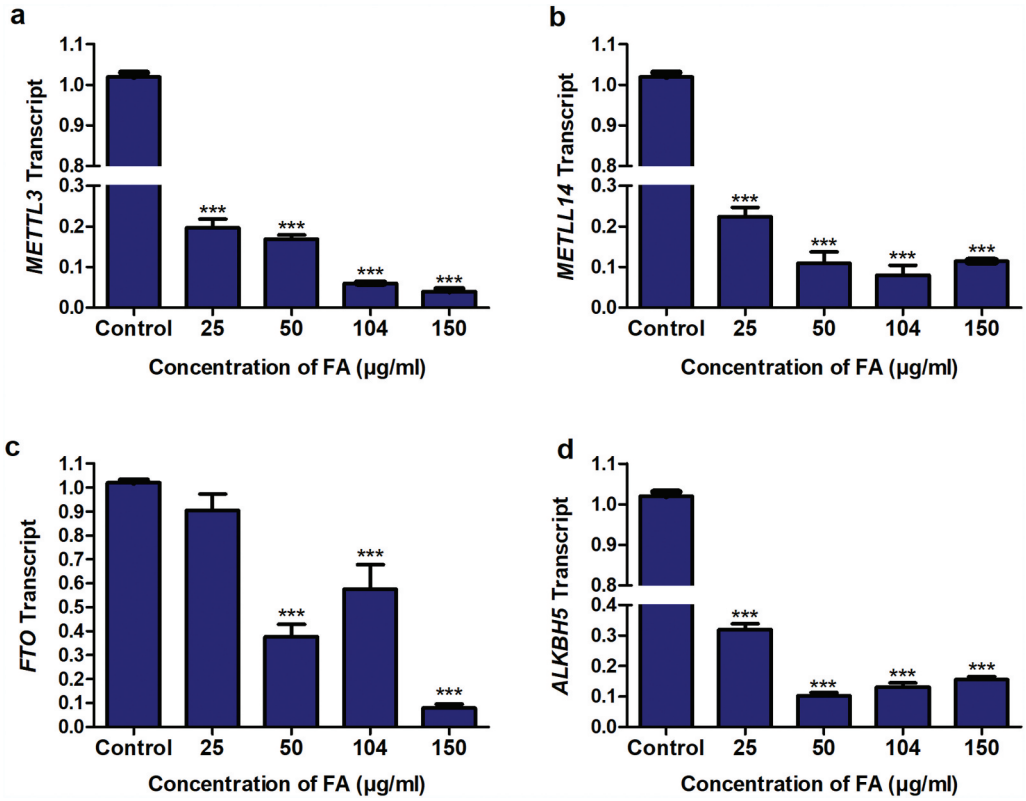

Figure 4. The effect of FA on the expression of m6A methyltransferases and demethylases in HepG2 cells. RNA isolated from control and FA-treated HepG2 cells were reverse transcribed into cDNA and analyzed for *METTL3, METTL14, FTO*, and *ALKBH5* expression using qPCR. FA decreased the mRNA expression of *METTL3* (a), *METTL14* (b), *FTO* (c), and *ALKBH5* (d) in HepG2 cells. Results are represented as mean fold-change ± SD (n = 3). Statistical significance was determined by one-way ANOVA with the Bonferroni multiple comparisons test (***$p < 0.0001$).

mycotoxin and chelator of divalent cations that alters cellular pathways in plants and animals [3,5,7,8,61]; however, its epigenetic mechanisms of toxicity are unclear. Recently, FA was shown to induce global DNA hypomethylation as an epigenetic mechanism of genotoxicity and cytotoxicity in liver cells [1]. Similarly, FB_1 (a common mycotoxin found in corn) caused chromatin instability and liver tumourigenesis by inducing global DNA hypomethylation and histone demethylation [62]. Zearalenone (a mycoestrogen) also reduced cell viability and caused apoptotic cell death by inducing global DNA hypomethylation [63] and histone demethylation [64]. Despite several studies indicating the genotoxic and cytotoxic effects of mycotoxins, no studies have been conducted on mycotoxins and its effect on the epigenetic regulation of p53 expression at the transcriptional and post-transcriptional level.

Previously, FA was shown to inhibit cell proliferation and induce apoptosis in HepG2 cells by post-translational modifications of p53 [12]; however, the effect of FA on p53 expression and its epigenetic regulation is not well understood. In addition to post-translational regulation of p53 protein stability and activity, the expression of p53 is also regulated at the transcriptional and post-transcriptional level by promoter methylation and m6A RNA methylation. In this study, we provide evidence for an epigenetic mechanism of FA-induced changes in p53 expression at both the transcript and protein levels by altering *p53* promoter methylation and m6A RNA methylation in HepG2 cells.

Our results indicate that FA significantly decreased p53 mRNA (Figure 1a) and protein (Figure 1b) expression by inducing *p53* promoter hypermethylation (Figure 2) and decreasing m6A-*p53* expression levels (Figure 3) in HepG2 cells.

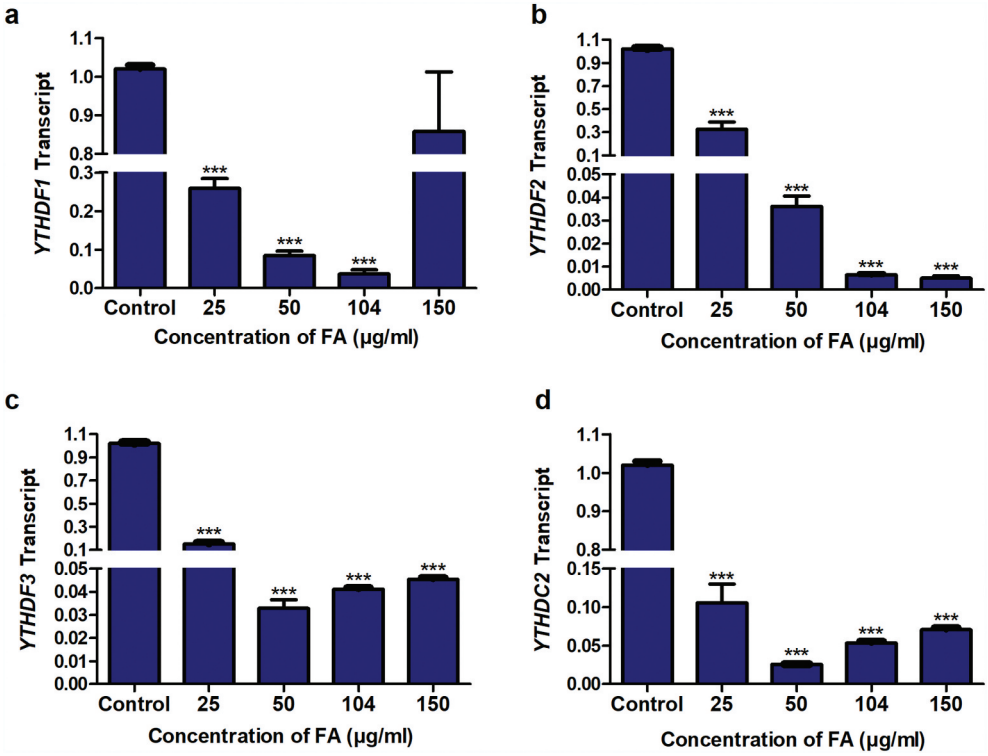

Figure 5. The effect of FA on the expression of m6A readers in HepG2 cells. RNA isolated from control and FA-treated HepG2 cells were reverse transcribed into cDNA and analyzed for the expression of *YTHDF1, YTHDF2, YTHDF3*, and *YTHDC2* by qPCR. FA decreased the expression of *YTHDF1* (a), *YTHDF2* (b), *YTHDF3* (c), and *YTHDC2* (d) in HepG2 cells. Results are represented as mean fold-change ± SD (n = 3). Statistical significance was determined by one-way ANOVA with the Bonferroni multiple comparisons test (***$p < 0.0001$).

This is in agreement with previous studies in which *p53* promoter hypermethylation was associated with a decrease in *p53* transcript levels [37,38] and *p53* promoter hypomethylation was associated with an increase in *p53* expression levels [59,60]. The data is also consistent with our previous findings in which FA decreased p53 protein expression in HepG2 cells [12]. Although it is clear that FA decreased p53 expression at the mRNA and protein levels, the decrease in p53 protein expression was dose-dependent, whereas the mRNA expression displayed a stepwise increase (albeit still lower than the control) at 104 and 150 µg/ml FA. This implied possible regulation by a post-transcriptional modification and may be the result of the FA-induced decrease in m6A levels on *p53* RNA transcripts.

As an epitranscriptomic marker, m6A is the most abundant post-transcriptional modification of mRNA that promotes translation efficiency and mRNA degradation by recruiting specific m6A-dependent readers [43,65,66]. Previously, aberrant regulation of m6A RNA transcripts were shown to affect many biological processes including circadian rhythm and lipid metabolism [67], adipogenesis [49], cell differentiation [41], and embryonic stem cell renewal [47]. Additionally, modulation of m6A RNAs were associated with various cancers such as acute myeloid leukaemia [68,69], breast cancer [70], liver cancer [71], and lung cancer [66].

Dietary factors have also been shown to affect RNA m6A levels [72,73], and studies on *p53* and m6A have indicated that m6A located within the coding region (codon 273) of *p53* pre-mRNA leads to the translation of mutant (R273H) p53 that alters the p53 signalling pathway and contributes to tumour formation and progression [74]. Similarly, mutations in m6A regulatory genes were correlated with a reduction in wild-type p53 expression and the presence of *p53* mutations in patients with acute myeloid leukaemia [69]. A decrease in m6A levels was also associated with cytotoxicity via activation of p53 and apoptosis in arsenite-transformed human keratinocytes [75].

In our study, we found that FA decreased m6A-*p53* levels in HepG2 cells (Figure 3). This occurred

despite the significant decrease in both m6A methyltransferases (*METTL3* (Figure 4a) and *METTL14* (Figure 4b)) and demethylases (*FTO* (Figure 4c) and *ALKBH5* (Figure 4d)) in the FA-treated HepG2 cells. The FA-induced decrease in *FTO* and *ALKBH5* suggests that it may not necessarily be responsible for the decrease in m6A-*p53* levels in the HepG2 cells, and that the decrease in m6A-*p53* levels is rather a consequence of the FA-induced decrease in *METTL3* and *METTL14*. This is in agreement with previous studies in which knockdown of *METTL3* and/or *METTL14* was associated with a substantial decrease in m6A mRNA levels [57], whereas overexpression of *METTL3* and/or *METTL14* was associated with an increase in m6A mRNA levels [67]. Additionally, knockdown of *METTL3* and *METTL14* was shown to downregulate the expression of several tumour suppressor genes including *CDKN2A, BRCA2*, and *TP53I11* [76], thereby, reducing cell proliferation, migration, and colony formation of cancer cells *in vitro* [71]. The depletion of *FTO* and *ALKBH5* was also found to reduce cell proliferation and invasiveness *in vitro* [66,77]. Interestingly, although FTO and ALKBH5 have been recognized as m6A demethylases, it was shown that FTO is highly co-expressed with the m6A methyltransferases *in vitro*, thereby, establishing a dynamic equilibrium between methylated and un-methylated RNA transcripts within the cells [78], and this may account for the positive correlation observed between *FTO* and *ALKBH5* and *METTL3* and *METTL14* expression in the FA-treated HepG2 cells. This simultaneous expression in both the m6A methyltransferases and demethylases has been observed in other studies [79,80]. However, further investigation is required to determine whether or not this simultaneous expression counteracts the effects of each other.

YTHDF1, YTHDF2, YTHDF3, and YTHDC2 specifically recognize m6A modified mRNAs and regulate mRNA degradation [44] and translation [42,43]. In HepG2 cells, the FA-induced decrease in m6A-*p53* levels led to a decrease in the expression of *YTHDF1* (Figure 5a), *YTHDF2* (Figure 5b), *YTHDF3* (Figure 5c), and *YTHDC2* (Figure 5d). YTHDF2 plays a major role in mRNA degradation; the carboxy-terminal domain of YTHDF2 selectively binds to m6A-containing mRNA, whereas the amino-terminal domain is responsible for the localization of the YTHDF2-mRNA complex to RNA decay sites such as processing bodies [44]. The decrease in *YTHDF2* expression, decrease in *p53* mRNA expression, and increase in *p53* promoter methylation observed in the FA-treated HepG2 cells suggests that FA may decrease *p53* mRNA expression via promoter hypermethylation and inhibition in *p53* transcription, and not YTHDF2-mediated degradation of *p53* mRNA.

YTHDF1, YTHDF3, and YTHDC2 function by interacting with translational machinery and actively promote protein synthesis to ensure effective protein production from dynamic transcripts that are marked by m6A [43]. Therefore, the FA-induced decrease in *YTHDF1, YTHDF3*, and *YTHDC2*, in addition to the FA-induced decrease in *p53* transcription, may be responsible for the decrease in p53 protein expression observed in the HepG2 cells. Similar results were observed in a previous study where a decrease in YTHDF3 reduced the protein expression of FOXO3 without affecting the expression of *FOXO3* mRNA [81]. These results are also in agreement with the study by Wang et al (2015) in which ribosome profiling on *METTL3* knockdown cells showed that YTHDF1 promotes translation efficiency in an m6A-dependent manner, and knockdown of *YTHDF1* reduced ribosome occupancy and translation efficiency of m6A targeted transcripts [43]. Similarly, YTHDF3 and YTHDC2 promote protein synthesis in synergy with YTHDF1 by interacting with ribosomal proteins and unwinding the 5 UTR of mRNA [82–84].

In conclusion, this study provides evidence for a possible mechanism of FA-induced changes in p53 expression at the epigenetic level. The results indicate that FA epigenetically decreases p53 expression at both the transcript and protein levels by increasing *p53* promoter methylation and decreasing m6A-*p53* methylation levels in HepG2 cells (Figure 6). The results further indicate that the decrease in m6A-*p53* expression levels was mediated by a decrease in the expression of *METTL3* and *METTL14*, and may have occurred independently of *FTO* and *ALKBH5*. Together, these results may serve as an alternative mechanism of FA-induced toxicity in the liver and are beneficial in poverty stricken areas

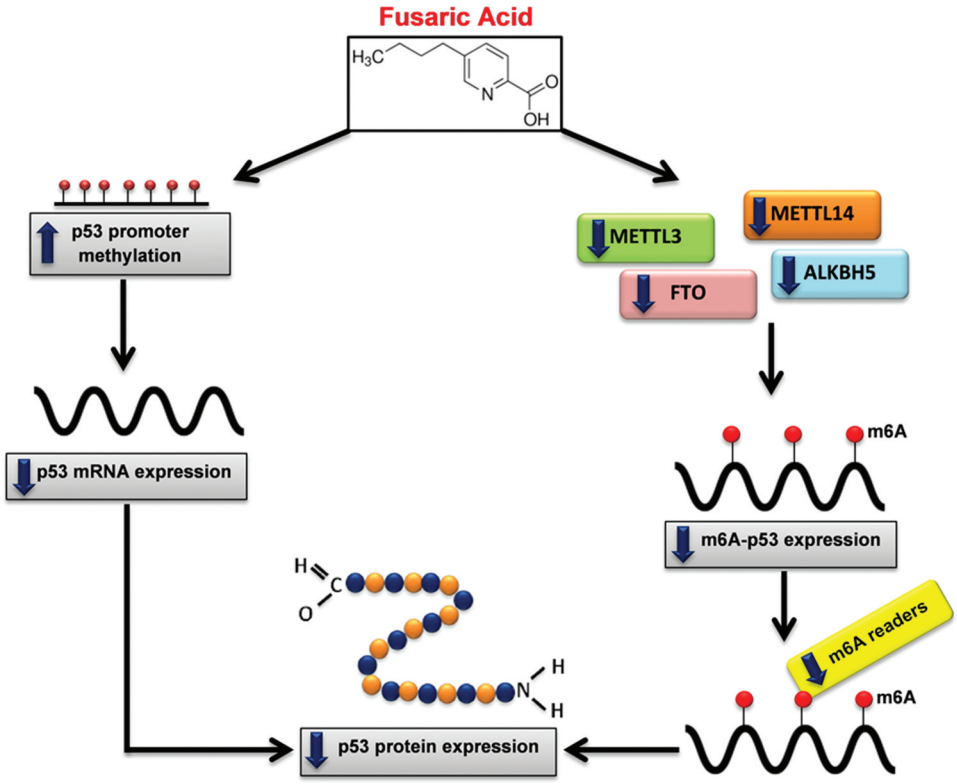

Figure 6. Proposed mechanism of FA-induced decrease in p53 expression. FA decreases p53 expression at both the transcript and protein levels by inducing *p53* promoter hypermethylation and decreasing m6A-*p53* methylation levels. FA decreased m6A-*p53* levels by decreasing *METTL3* and *METTL14*; and suppressed expression of the m6A readers, *YTHDF1*, *YTHDF3*, and *YTHDC2*, which led to the decrease in p53 translation/protein expression. Furthermore, the decrease in p53 protein expression may be a consequence of the FA-induced decrease in *p53* mRNA expression.

where there are high levels of FA contamination. This study provides insight for future studies on FA and p53 in an *in vivo* model as well as assessing the ability of FA to induce p53 mutant proteins by altering m6A levels.

Materials and methods

Materials

FA (*Gibberella fujikuroi*, F6513) was purchased from Sigma-Aldrich. The HepG2 cell line (HB-8065) was purchased from the American Type Culture Collection (ATCC). Cell culture reagents were purchased from Lonza Biotechnology. Western blot reagents were purchased from Bio-Rad. All other reagents were purchased from Merck.

Cell culture and treatment

HepG2 cells (1.5 X 10^6, passage 3) were cultured (37°C, 5% CO_2) to 90% confluency in 25 cm^3 cell culture flasks containing complete culture media (CCM; Eagle's Minimum Essentials Medium (EMEM) supplemented with 10% foetal calf serum, 1% penicillin-streptomycin fungizone, and 1% L-glutamine). A stock solution of FA (1 mg/ml) in 0.1 M phosphate buffered saline (PBS) was prepared and the cells were incubated (37°C, 5% CO_2, 24 h) with a range of FA concentrations (25, 50, 104, and 150 µg/ml) [1]. An untreated control (CCM only) was also prepared. The viability of the cells was assessed using the trypan blue cell exclusion method. All results were verified by performing two independent experiments in triplicate.

RNA isolation and quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from control and FA-treated HepG2 cells using Qiazol Reagent (Qiagen, 79,306), as previously described [1]. The RNA was quantified using the Nanodrop2000 spectrophotometer (Thermo-Fisher Scientific), standardized to

1,000 ng/µl, and reverse transcribed into complementary DNA (cDNA) using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo-Fisher Scientific, K1652). Thereafter, the mRNA expression of *p53, METTL3, METTL14, FTO, ALKBH5, YTHDF1, YTHDF2, YTHDF3*, and *YTHDC2* was determined using the PowerUp™ SYBR™ Green Master Mix (Thermo-Fisher Scientific, A25742) and the CFX96 Real Time PCR System (Bio-Rad) with the following cycling conditions: initial denaturation (95°C, 8 min), followed by 40 cycles of denaturation (95°C, 15 s), annealing (Supplementary Table S1, 40 s), and extension (72°C, 30 s). Primer sequences and annealing temperatures are listed in Supplementary Table S1. *GAPDH* was used as the internal control to normalize mRNA expression. The comparative threshold cycle (Ct) method was used to determine relative changes in expression [85].

Protein isolation and Western blot

The protein expression of p53 was determined using Western blot. Briefly, crude protein was isolated from control and FA-treated HepG2 cells using cytobuster reagent (200 µl; Novagen, 71,009) supplemented with protease and phosphatase inhibitors (Roche; 05892791001 and 04906837001, respectively). The bicinchoninic acid (BCA) assay was used to quantify the proteins and the samples were subsequently standardized to 1 mg/ml. The samples were then boiled (100°C, 5 min) in a 1:1 dilution with 1X Laemmli buffer [dH_2O, 0.5 M Tris-HCl (pH 6.8), glycerol, 10% SDS, 5% β-mercaptoethanol, 1% bromophenol blue], separated in sodium dodecyl sulphate polyacrylamide gels (10% resolving gel, 4% stacking gel; 1 h, 150 V), and transferred onto nitrocellulose membranes using the Bio-Rad Trans-Blot® Turbo Transfer System (20 V, 30 min). The membranes were then blocked in 5% non-fat dry milk (NFDM) in Tris buffered saline with 0.05% Tween 20 [TTBS; 150 mM NaCl, 3 mM KCl, 25 mM Tris, 0.05% Tween 20, dH2O, pH 7.5; 1 h, RT] and probed overnight (4°C) with primary antibody [p53 (1:500; Santa Cruz, sc-6243)]. Membranes were rinsed five times in TTBS (10 min, RT) and incubated with a horse-radish peroxidase (HRP)-conjugated secondary antibody [goat anti-rabbit (1:5,000; Cell Signalling Technology, #7074 S); 1 h, RT]. Membranes were rinsed five times in TTBS (10 min, RT). Immunoblots were visualized using the Clarity™ Western ECL Substrate Kit (Bio-Rad, #170-5060) and the images were captured using the ChemiDoc™ XRS+ Molecular Imaging System (Bio-Rad). Following detection, membranes were quenched in hydrogen peroxide (5%, 37°C, 30 min) and probed with the housekeeping protein, anti-β-actin (1:5,000, 30 min, RT; Sigma-Aldrich, A3854) to normalize protein expression. Protein expression was determined using the Image Lab Software version 5.1 (Bio-Rad) and the results were represented as a fold-change in band density (RBD) relative to the control.

Promoter methylation of p53

Genomic DNA was extracted from control and FA-treated HepG2 cells using the Quick-g-DNA MiniPrep Kit (Zymo Research, D3007) and purified using the DNA Clean and Concentrator™-5 Kit (Zymo Research, D4003), as per manufacturer's instructions. DNA concentration was determined using the Nanodrop2000 spectrophotometer and standardized to 4 ng/µl. The purity of the DNA was assessed using the A260/A280 absorbance ratio. The promoter methylation of *p53* was determined using the OneStep qMethyl Kit (Zymo Research, 5310) in which 20 ng DNA was subject to a test and reference reaction containing specific primers. Primer sequences and annealing temperatures are listed in Supplementary Table S1. Cycling conditions were as follows: digestion by methyl sensitive restriction enzymes (37°C, 2 h), initial denaturation (95°C, 10 min), followed by 45 cycles of denaturation (95°C, 30 s), annealing (Supplementary Table S1, 60 s), extension (72°C, 60 s), final extension (72°C, 60 s), and a hold at 4°C. The percentage methylation was calculated using the supplied formula (Supplementary Information) and represented as a fold-change relative to the control.

RNA immuno-precipitation

Quantification of m6A-*p53* levels were conducted using RNA immuno-precipitation. Briefly, control and FA-treated HepG2 cells were incubated in nuclear isolation buffer [500 µl; 1.28 M sucrose,

40 mM Tris-HCl (pH 7.5), 20 mM magnesium chloride, 4% Triton X-100; 4°C, 20 min] and centrifuged (2,500xg, 4°C, 15 min). Nuclear pellets were re-suspended in RNA immunoprecipitation buffer [1 ml; 150 mM potassium chloride, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, 0.5 mM DTT, 0.5% IGEPAL, 100 U/ml SUPERase IN™ RNase Inhibitor (Thermo-Fisher Scientific, AM2694), protease inhibitors (Roche, 05892791001), phosphatase inhibitors (Roche, 04906837001)] and the chromatin was mechanically sheared using a needle (20 gauge/20 strokes). Thereafter, the nuclear membrane and debris were pelleted by centrifugation (13,000xg, 4°C, 10 min). The supernatant containing RNA was separated into two fractions of 500 µl each. Qiazol Reagent (500 µl) was added to one fraction and stored at −80°C for reference RNA isolation. The second fraction was incubated with m6A antibody [1:100; Abcam, ab208577] overnight at 4°C and the antigen-antibody complex was precipitated using protein A beads [20 µl 50% bead slurry (Cell Signalling Technology, #9863), 4°C, 3 h]. Thereafter, the immuno-precipitates were recovered by centrifugation (2,500xg, 4°C, 60 s), washed three times in RNA immuno-precipitation buffer, followed by re-suspension in Qiazol Reagent (500 µl). RNA was isolated from both the reference and m6A-precipitated samples, as previously described [1]. The RNA was quantified using the Nanodrop2000 spectrophotometer, standardized to 400 ng/µl, and reverse transcribed into cDNA using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo-Fisher Scientific, K1652). The expression of *p53* was then determined using qPCR as mentioned above. The expression of *p53* in the m6A-precipitated sample was normalized against the expression of *p53* in the reference sample in order to determine the ratio of m6A methylated *p53* relative to the total *p53* expressed. Primer sequences and annealing temperatures are listed in Supplementary Table S1.

Statistical analysis

GraphPad Prism version 5.0 (GraphPad Prism Software Inc.) was used to perform all statistical analyses. The D'Agostino and Pearson tests were used to determine normality. All data was analyzed using the one-way analysis of variance (ANOVA), followed by the Bonferroni multiple comparisons test. Results were represented as a mean fold-change ± standard deviation (SD) (n = 3). Statistical significance was considered at $p < 0.05$.

Author contributions

TG and AC conceptualized and designed the study. TG conducted all laboratory experiments, analyzed the data, and wrote the manuscript. SN and AC revised the manuscript. All authors have read the manuscript prior to submission.

Disclosure statement

The authors report no conflict of interest.

Data availability

All datasets generated in this study are available in Supplementary Information and from the corresponding author on reasonable request.

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