

Detection of Aflatoxin Contamination through Biomarker Discovery in Human Intestinal Caco-2 Cells

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Abstract

Background and Objective: Aflatoxin includes four toxin types of B1, B2, G1 and G2. Due to the widespread contamination of food and agricultural products and the harmful effects of aflatoxin on the human body, investigating biological effects of aflatoxin and preventing its harmful effects on human health are important for the experts. The present study aimed to find a complementary method to modify standard detection methods of harmful levels of aflatoxin based on biomarker discovery.

Material and Methods: Data regarding changes in the gene expression profile of human intestinal Caco-2 cells were retrieved from the gene expression omnibus database, specifically concerning effects of aflatoxin B1 (AB1) on treated cells. Data were assessed via directed protein-protein interaction network analysis and gene ontology enrichment to identify the critical differentially expressed genes and the associated biochemical pathways.

Results and Conclusion: From the 934 differentially expressed genes, 623 were investigated through protein-protein interaction network analysis. Two directed protein-protein interaction networks were constructed from the significantly upregulated and downregulated differentially expressed genes. The COL4A6, IRF1, SLC2A2, CITED2, SULT2A1, RRAS and PCYT1B emerged as critical target genes affected by AB1. Various cellular functions, including cell proliferation, migration, apoptosis and metabolism, were identified as the key biochemical pathways that were affected. It was concluded that the levels of these critical target genes could be addressed as appropriate criteria for modifying standard methods of aflatoxin contamination detection.

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1. Introduction

Aspergillus flavus (AF) is a fungus with saprotrophic and pathogenic characteristics [1]. Wheat grains, legumes and tree nuts are best known for AF colonization [2]. The AF is used in production of fermented foods [3]. Aflatoxin is a dangerous mycotoxin in the food industry produced by AF [4]. Aflatoxin is classified as a Group 1 carcinogen [5] with four types of B1, B2, G1 and G2. The AF produces carcinogenic aflatoxins B1 and B2 [6]. More than 80% of

aflatoxin B1 (AB1) absorption occur in the proximal part of the gastrointestinal tract [GIT] via passive transport after ingestion [7]. The cytochrome P450 enzyme can metabolize AB1 in the liver [8]; However, AB1 metabolism to its toxic metabolites may occur in the digestive tract [9]. The intestinal epithelia serve as the first line of defense against contaminants, protecting against toxic components entering through tight junction proteins and are primary targets for mycotoxins such as AB1 [10].



The human Caco-2 intestinal cell line expresses typical intestinal nutrient transporters and is a widely used *in-vitro* model for drug delivery and metabolism studies [11]. Genome profile affected by AB1 as a biological system is investigated [12]. The AB1 increases lactate dehydrogenase (LDH) cytosolic enzyme levels, promotes dose-dependent accumulation of reactive oxygen species (ROS) and induces DNA damages *in vitro* [13]. The cytochrome P450 enzyme plays a significant role in bioactivation of AB1 to AB1-8,9-epoxide, indicating a high level of oxidative stress in intestinal cells *in vitro* [14]. There is a significant diversity in aflatoxin metabolism in various species [15]. Metabolism of AB1 in humans varies across regions and is affected by age, which affects individual resistance to AB1 as well [14]. Genome instability and cancer risk due to A-T mutations resulting in diminished DNA repair capability have been discussed by Engin et al. [16]. The AB1 exposure is associated with synergetic point mutations in the human P53 gene [17]. Diversity of metabolic pathways and various biomarkers involved in AB1 toxicity necessitate finding appropriate biomarkers for early detection of intestine cancers associated to AB1 exposure. Use of genomic data banks and network analysis software provides powerful tools to analyze data and identify hub genes associated with aflatoxin carcinogen characteristics [18]. Since effects of aflatoxin on gene profiles are not well-investigated in colon cancer, this study aimed to assess effects of AB1 on genes of Caco2 cells using information from valid data banks to carry out genes network analysis, introducing key biomarkers affected by AB1. This advancement holds significant implications for improving food safety protocols and early detection strategies of aflatoxin-associated health risks.

2. Materials and Methods

2.1. Data collection

In this study, GSE75934 was investigated using toxicity, aflatoxin B1 and human intestinal keywords in gene expression omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=gse75934>). The gene expression profiles of five sets of cells; control and treated cells with PATULIN, ASCLADIOL, AB1, VERSICOLORIN A and STRIGMATOCYSTIN. Gene expression profiles of the treated cells with AB1 toxin and control cells were selected for analysis. Total RNA from the studied cells was extracted using EXTRACT-ALL® reagent (Eurobio, Les Ulis, France) following the manufacturer's manual. As described in the recorded data, for each sample, cyanine-3 (Cy3) labeled cRNA was prepared from 200 ng of total RNA using the One-Color Quick Amp Labeling kit (Agilent, USA) according to the manufacturer's instructions, followed by

Agencourt RNAClean XP (Agencourt Bioscience Corporation, Beverly, Massachusetts). Dye incorporation and cRNA yield were checked with the Biospec nano spectrophotometer (Shimadzu). More details of experiments are present in the following address.

(<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM1970394>).

2.2. Pre-assessment analysis

Human intestinal Caco-2 cells were exposed *in vitro* to 1 μ M of AB1 for 24 h. Then, RNA was extracted and compared with that of the control cells (cells in presence of DMSO). Details of the methods were described in a published document by Gauthier et al. [19]. The GEO2R analysis was carried out with default options to find significant differentially expressed genes (DEGs). The GEO2R program was used to assess comparability of the samples via box plot visualization and volcano plot presentation of the dysregulated genes. Significant DEGs were highlighted based on adjusted *p*-value. Samples matching was carried out and visualized using box plot analysis and GEO2R program. Presence of significant DEGs was assessed using volcano plot. Significant DEGs were addressed based on adjusted *p*-values less than 0.05. Repeated and uncharacterized DEGs were excluded from further analysis.

2.3. Gene ontology enrichment and directed protein-protein interaction network analysis

Significant up and downregulated DEGs were enriched separately to find the associated biochemical pathways using ClueGO plugin of Cytoscape software v.3.7.2. Biochemical pathways were extracted from the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. Biological terms were identified based on the kappa score value. Significant DEGs were assessed through directed protein-protein interaction (PPI) network analysis to identify critical genes. Queried genes were connected via activation, inhibition and expression links using CluePedia application of Cytoscape software. The common DEGs identified through pathway analysis and directed PPI network analysis were selected as the critical targeted genes affected by AB1.

2.4. Statistical analysis

Comparability of samples was verified via median-centered samples in box plot analysis. Significant DEGs were identified based on adjusted *p*-values less than 0.05. The statistical options included enrichment/depletion, two-sided hypergeometric test, Bonferroni step down and *p*-value correction. Network specificities greater than the medium threshold were addressed when investigating associated biochemical pathways.



3. Results and Discussion

3.1. Pre-assessment analysis

Gene expression profiles of the treated cells exposed to AB1 and the control cells were statistically matched (Figure 1). The compared profiles were median-centered and comparable. As shown in Figure 2, volcano plot

visualization of the analysis showed several significant up and down-regulated DEGs. Totally, 934 significant DEGs, distinguishing treated cells from the controls, were identified. Data were cleaned and 623 DEGs, including 294 upregulated and 329 downregulated DEGs, were selected for further investigations.

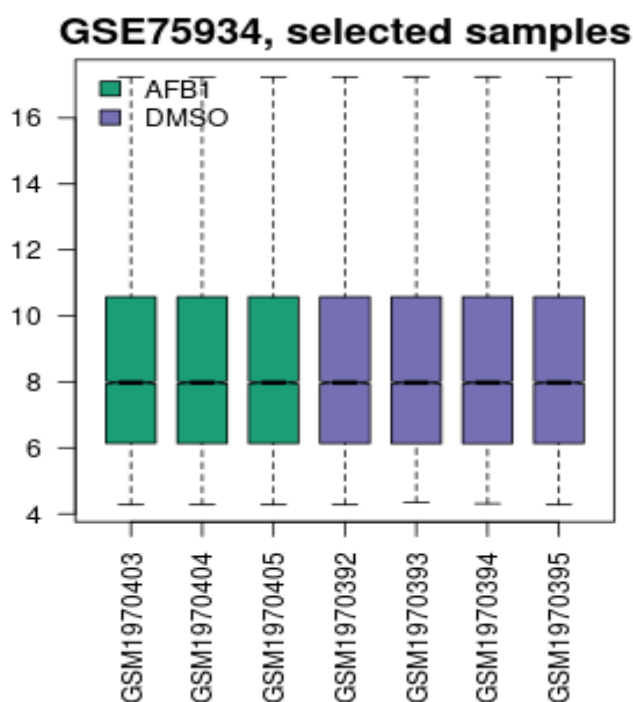


Figure 1. Box plot presentation of the studied gene expression profiles of the treated human intestinal Caco-2 cells with 1 micromole of aflatoxin B1 versus the control cells.

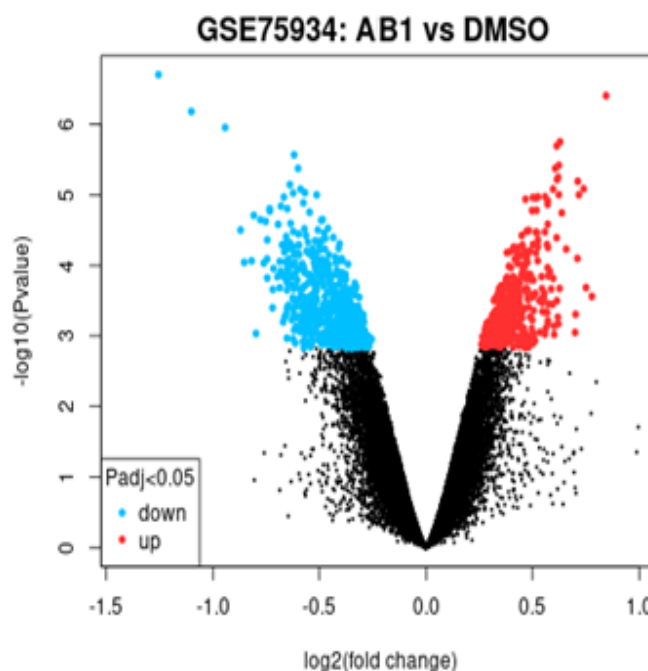


Figure 2. Volcano plot visualization of the studied gene expression profiles of the treated human intestinal Caco-2 cells with 1 micromole of aflatoxin B1 versus the control cells.

3.2. Gene ontology enrichment

Results of the gene ontology assessment are shown in Figures 3 and 4. As shown in Figure 3, 15 groups of biochemical pathways were associated to 43 upregulated DEGs. Network of the associated DEGs and biochemical pathways included eight subnetworks. The five down-regulated biochemical pathways were present together with ten associated genes (Figure 4). The sum of the 43 upregulated genes and the ten downregulated DEGs connected to the biochemical pathways were addressed as critical targeted genes by AB1 toxin. Significantly, analysis of Figures 3 and 4 indicated that the upregulation of genes was the dominant response to aflatoxin B1 exposure, highlighting a significant shift in gene expression profiles towards increased transcription in various biochemical pathways. As demonstrated in Figure 3, most biological terms were involved in cellular metabolism. Since downregulation was not a prominent process, no

significant subnetwork was shown in Figure 4. Results of the directed PPI network are present in Figure 5. All 623 significant DEGs were included in the constructively directed PPI network. Totally, 569 DEGs were recognized using Cytoscape software. The PPI network, including 492 isolated genes, 16 paired DEGs, six triple subnetworks (18 nodes), one tetrad component, two subnetworks of seven DEGs (14 nodes) and a major connected component consisting of 25 nodes was created. Subnetworks, including three or fewer nodes, were not included in further analysis. The COL4A6, IRF1, SLC2A2, CITED2, SULT2A1, RRAS and PCYT1B were identified as common DEGs between the pathway analysis and the directed PPI network analysis (see Table 1). It is noteworthy that the log (fold change) values for all the identified common genes were positive, indicating that these genes were upregulated in response to aflatoxin B1 exposure (Table 1).



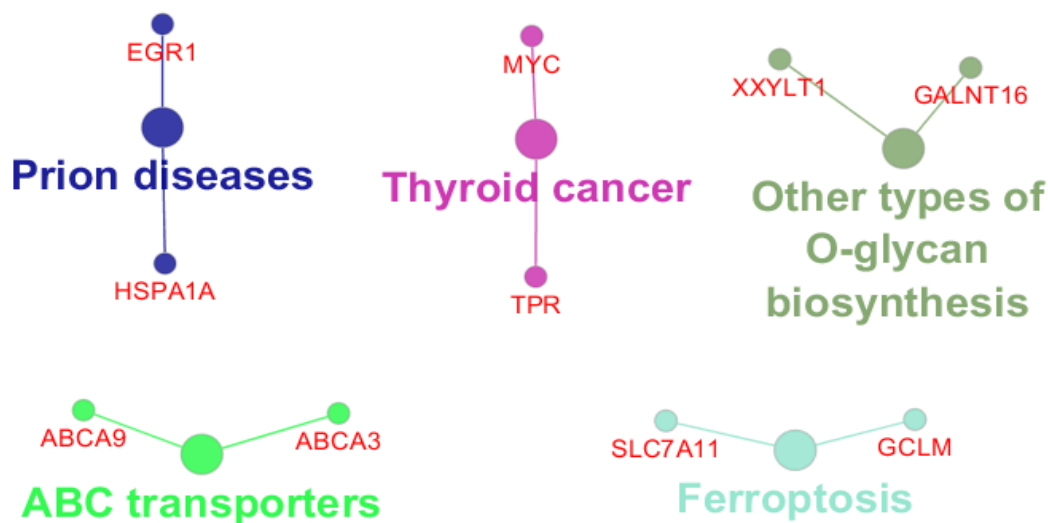


Figure 3. Biochemical pathways associated to upregulated differential expressed genes

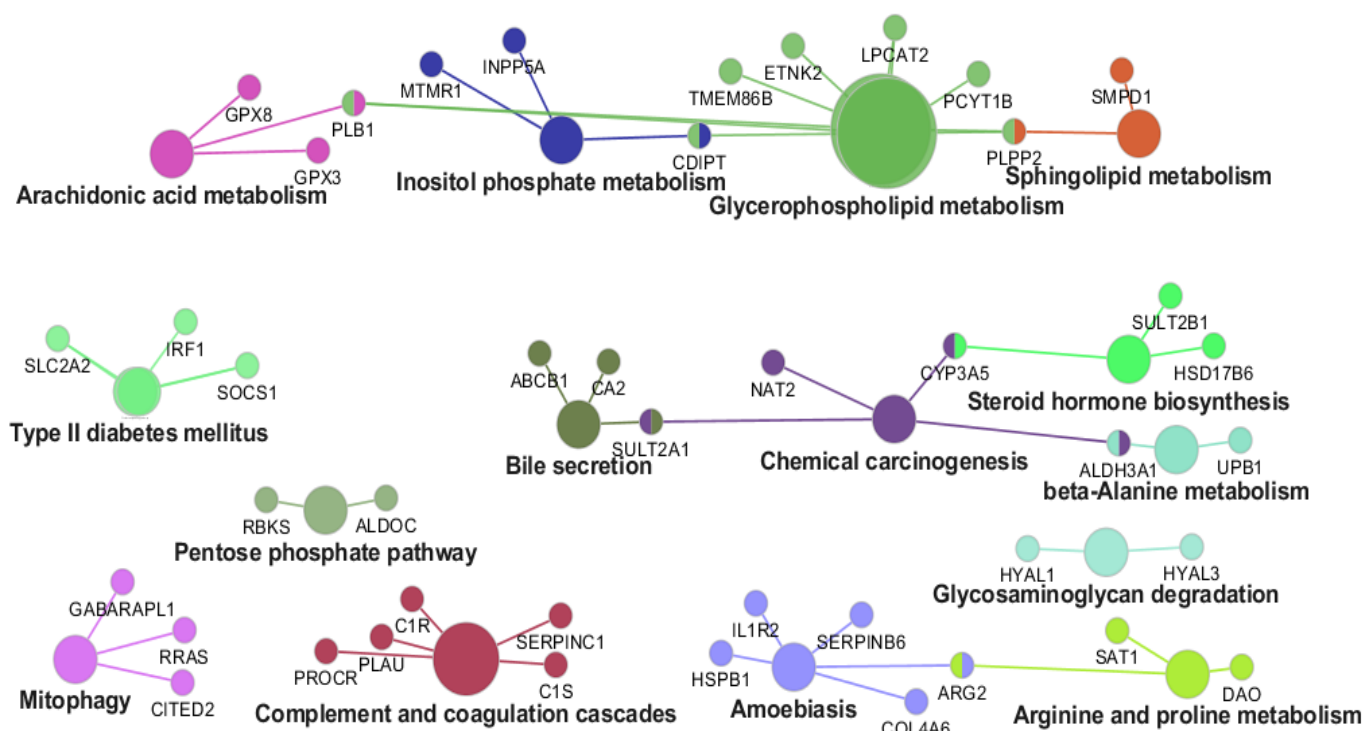


Figure 4. Biochemical pathways associated to downregulated differential expressed genes.



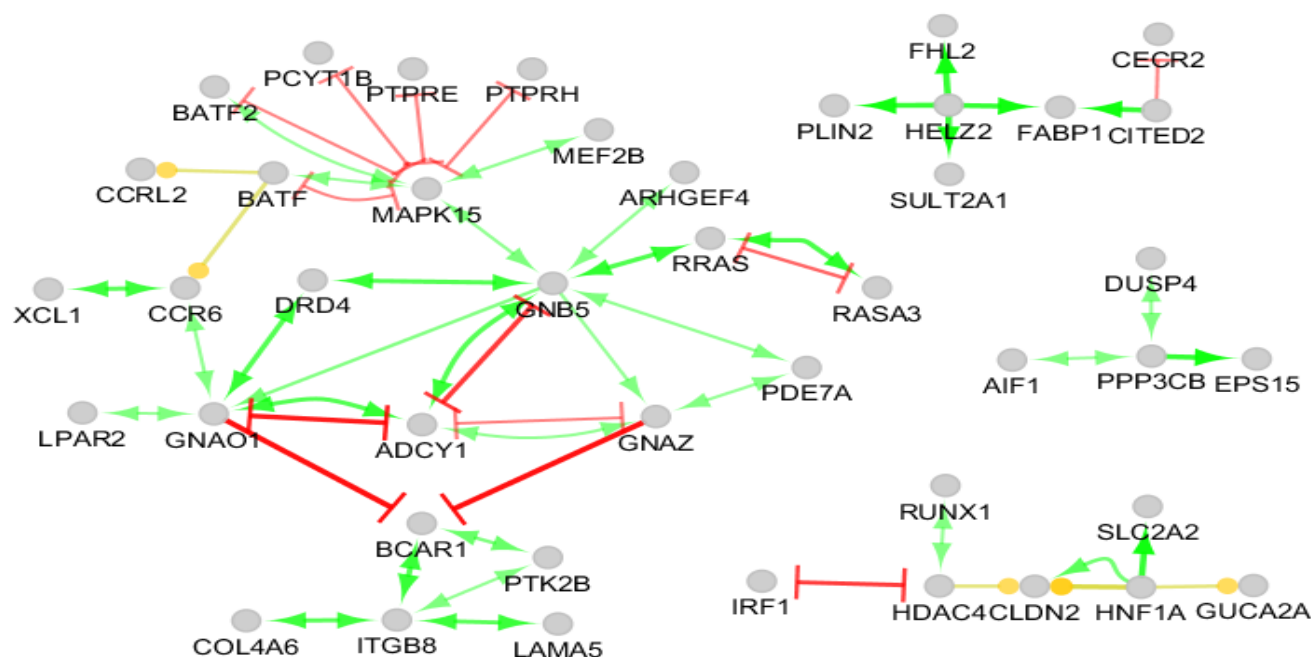


Figure 5. Directed protein-protein interaction network of recognized significant differential expressed genes. The subnetworks, including three or fewer nodes, were ignored. Green, red and yellow lines refer to activation, inhibition and expression, respectively. Round tips refer to positive relationships.

As demonstrated, seven genes including COL4A6, IRF1, SLC2A2, CITED2, SUL2A1, RRAS and PCYT1B, were highlighted in response to the presence of AB1 toxin in human intestinal Caco-2 cells. The following descriptions briefly review their physiological and pathological roles. Type IV collagen lattice network was accumulated from six chains coded by the COL4A1 to COL4A6 genes. These genes were major components of the basement membranes [20]. Investigation indicated that the COL4A6 gene was highly downregulated in prostate cancer and this down-regulation was accompanied by the cancer progression and metastasis [21]. Furthermore, it has been reported that COL4A6 is significantly downregulated in stomach adenocarcinoma tissues, compared to normal adjacent tissues [22]. As shown in Figure 5, COL4A6 was a part of the largest connected component of the directed PPI network. As shown in Figure 3, COL4A6 was directly associated to the biochemical pathway of amoebiasis,

which is a leading cause of death from parasitic illness globally [23].

Interferon regulatory factor-1 (IRF1) is a transcription factor involved in the regulation of several genes associated to various pathological and physiological processes such as tumor immune surveillance, viral infection, development of the immune system and pro-inflammatory injuries. The critical role of IRF1 in connecting the adaptive and native immune systems has been reported in the literature [24]. Growth inhibition of various human cell types due to IRF1 overexpression is attributed to its regulatory effects on targeted genes that downregulate cell growth and proliferation. Investigations indicate that the upregulation of IRF1 significantly suppresses (15-fold) levels of the survival protein. This is similar to cancer therapy in breast cancer. Similar to P53, IRF1 acts as a tumor suppressor and regulates DNA damage-induced apoptosis [25].

Table 1. Common differential expressed genes between the pathway analysis and directed protein-protein interaction network analysis

R	Gene	Description	Log (fold change)
1	COL4A6	Collagen, type IV, alpha 6	0.316
2	IRF1	Interferon regulatory factor 1	0.326
3	SLC2A2	Solute carrier family 2 (facilitated glucose transporter), member 2	0.459
4	CITED2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	0.422
5	SULT2A1	Sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone (DHEA)-preferring, member 1	0.466
6	RRAS	Related RAS viral (r-ras) oncogene homolog	0.411
7	PCYT1B	Phosphate cytidylyltransferase 1, choline, beta	0.376



Solute carrier family 2 (facilitated glucose transporter), member 2 (SLC2A2) is another upregulated gene in response to the presence of AB1 toxin in the treated cells. The SLC2A2, which is known as glucose transporter type 2 (GLUT2), is complicated with glucose transport into cells. This function of SLC2A2 is linked to its role in the overexpression associated with tumor invasiveness and development [26]. Chai et al. investigation demonstrated that the overexpression of SLC2 family members was associated to poor survival outcomes in papillary thyroid carcinoma [27]. The transcription co-factor Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 2 (CITED2) is another highlighted gene in the current study. Technically, CITED2 plays significant roles in various cellular processes, including apoptosis, proliferation, migration, differentiation and autophagy as addressed in the literature [28]. As demonstrated in Figure 3, CITED2 was connected to the mitophagy biochemical pathway.

Sulfotransferase family cytosolic 2A dehydroepiandrosterone (DHEA)-preferring member 1 (SULT2A1) is associated to the bile secretion pathway (Figure 3). Prominent role of the sulfotransferase family in regulating hormones and excreting xenobiotics is verified by the researchers [29]. It has been reported that SULT2A1 plays a role in initiation and progression of hepatocellular carcinoma [30]. Related RAS viral (r-ras) oncogene homolog (RRAS) such as CITED2 is linked to the mitophagy biochemical pathway. The R-RAS subfamily is closely associated to RAS proteins. Studies have shown that RRAS proteins are involved in regulating various cellular functions, including cell migration, adhesion and morphological changes [31]. Downregulation of RRAS has been documented in progression and pathogenesis of gliomas [32]. The last highlighted gene was phosphate cytidyltransferase 1 choline beta (PCYT1B), which is associated with the glycerophospholipid biochemical pathway. This gene includes a mutual inhibition relationship with MAPK15 (Figure 5). The PCYT1B is involved in the Kennedy pathway [33]. Stored lipids in cells form lipid droplets, including a neutral lipid core and a peripheral phospholipid monolayer, are primarily composed of phosphatidylcholine. Balances between the neutral lipid core and the outer layer result from the activity of the Kennedy pathway [34]. The AB1 is known as a potent contaminant and a harmful type of aflatoxin. In fact, AB1 is associated with immune system suppression, characteristics of hepatocarcinogenesis, and mutagenic and teratogenic effects. Additionally, AB1 exposure significantly affects metabolic processes, highlighting comprehensive biochemical disruptions caused by this mycotoxin (Figure 3).

To minimize aflatoxin contamination and increase food safety, modification in farming practices and use of resistant crop varieties are recommended [35]. Due to the widespread contamination of food and agricultural products by mycotoxins, many people face health risks associated with the consumption of aflatoxin-contaminated foods [36]. Level of the expressed biomarkers such as the recommended genes (COL4A6, IRF1, SLC2A2, CITED2, SULT2A1, RRAS and PCYT1B) is an appropriate criterion with aflatoxin measurement for introducing a novel standard method to detect practical harmful levels of aflatoxin contamination. The present findings suggest that the expression levels of these targeted genes can serve as reliable biomarkers for assessing aflatoxin contamination, potentially complementing existing detection methods. Regarding the current risks posed by aflatoxin contamination in food and agricultural products, integration of the biomarker-based criteria into standard detection protocols can enhance food safety measures and hence public health consequences. Future studies should focus on validating these biomarkers in clinical settings and investigating further interplays between the aflatoxin exposure and metabolic pathways to develop effective moderation strategies.

4. Conclusion

In conclusion, exposure to AB1 significantly alters numerous cellular functions, including cell proliferation, migration and apoptosis as well as various metabolic processes in human intestinal Caco-2 cells. The COL4A6, IRF1, SLC2A2, CITED2, SULT2A1, RRAS and PCYT1B were identified as the critical targeted genes for AB1. Due to the significant effects of aflatoxin contamination on food and agricultural products, it has been concluded that the levels of these critical targeted genes in response to aflatoxin can be addressed as an appropriate criterion for modifying standard methods of aflatoxin contamination assessments. Several trends are anticipated in the field of aflatoxin detection and management. First, integration of multiple-omics approaches such as genomics, proteomics and metabolomics can provide a further comprehensive understanding of the aflatoxin biological effects and enhance biomarker discovery. Second, use of machine learning algorithms to analyze complex datasets may facilitate identification of novel biomarkers and improve accuracy of detection methods. Third, educating farmers and food manufacturers on aflatoxin prevention strategies and diminishing techniques is critical in decreasing risks of aflatoxin exposure in the food supply chain. These not only include promises for improving food safety but also for enhancing overall understanding of mycotoxin-linked health effects. However, it is difficult to introduce the real safe levels of contamination.



5. Ethical Code:

This study was approved via
IR.SBMU.RETECH.REC.1403.423 ethical code.

6. Acknowledgements

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7. Author contributions:

Vahid Mansouri contributed to the conception and design of the study and literature review. All authors participated equally in study administration and writing of the primary draft of the manuscripts, providing critical revision and editing. All authors approved the final version of the manuscript.

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تشخیص آلودگی آفاتوکسین از طریق کشف نشانگر زیستی در سلول های Caco-2 روده انسان

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چکیده

سابقه و هدف: آفاتوکسین شامل چهار نوع سم B1، B2، G1 و G2 است. با توجه به آلودگی گسترده مواد غذایی و محصولات کشاورزی و اثرات مضر آفاتوکسین بر بدن انسان، بررسی اثرات بیولوژیکی آفاتوکسین و پیشگیری از اثرات مضر آن بر سلامت انسان مورد توجه متخصصین قرار می گیرد. مطالعه حاضر با هدف یافتن روشی تکمیلی برای اصلاح روش های استاندارد تشخیص سطوح مضر آفاتوکسین بر اساس کشف نشانگرهای زیستی انجام شد.

مواد و روش ها: داده های مربوط به تغییرات در پروفایل بیان ژن سلول های Caco-2 روده انسان از پایگاه داده بیان ژن omnibus، به ویژه در مورد اثرات آفاتوکسین (B1 (AB1) بر سلول های تیمار شده، بازیابی شد. داده ها از طریق آنالیز شبکه برهمکنش پروتئین-پروتئین جهت دار و غنی سازی هستی شناسی ژن برای شناسایی ژن های که بیان افتراقی داشته اند و مسیرهای بیوشیمیایی مرتبط ارزیابی شدند.

یافته ها و نتیجه گیری: از ۹۳۴ ژن بیان شده گوناگون، ۶۲۳ ژن از طریق تجزیه و تحلیل شبکه برهمکنش پروتئین-پروتئین مورد بررسی قرار گرفتند. دو شبکه برهمکنش پروتئین-پروتئین جهت دار از ژن های بیان شده-ای، که که افزایش و کاهش بیان قابل توجهی داشتند، ساخته شد. ژن های SLC2A2، IRF1، COL4A6، PCYT1B و RRAS، SULT2A1، CITED2 به عنوان ژن های هدف مهم متأثر از AB1 مشخص شدند. عملکردهای گوناگون سلولی، از جمله تکثیر سلولی، مهاجرت، آپوپتوز و متابولیسم، به عنوان مسیرهای بیوشیمیایی کلیدی، که تحت تأثیر قرار گرفتند، شناسایی شدند. نتیجه گیری شد که میزان بیان ژن های هدف مهم را می توان به عنوان معیارهای مناسب برای اصلاح روش های استاندارد تشخیص آلودگی آفاتوکسین در نظر گرفت.

تعارض منافع: نویسندگان اعلام می کنند که هیچ نوع تعارض منافع مرتبط با انتشار این مقاله ندارند.

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