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Mycovirus Containing *Aspergillus flavus* and Acute Lymphoblastic Leukemia: Carcinogenesis beyond Mycotoxin Production

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Abstract

Carcinogenic effects of *Aspergillus* spp. have been well established and generally attributed to a variety of mycotoxin productions, particularly aflatoxins. It is known that most carcinogenic mycotoxins, with the exception of fumonisins, are genotoxic and mutagenic, causing chromosomal aberrations, micronuclei, DNA single-strand breaks, sister chromatid exchange, unscheduled DNA synthesis *etc*. Some *Aspergillus* spp. are infected with mycoviruses which can result in loss of aflatoxin production. The effects of mycovirus containing *Aspergillus* on human health have not been fully evaluated. Recent studies in patients with acute lymphoblastic leukemia, in full remission, have revealed the existence of antibody to the products of a certain *Aspergillus flavus* isolate which harbored an unknown mycovirus. Exposure of blood mononuclear cells from these patients, but not controls, to the products of this organism had reproduced cell surface phenotypes and genetic markers, characteristic of acute lymphoblastic leukemia. Carcinogenic effects of *Aspergillus* spp. may not always be mycotoxin related and this requires further investigation.

Keywords: Acute lymphoblastic leukemia, Mycovirus, Aspergillus, Cancer, Etiology, Leukemogenesis, Carcinogenesis, Virus, Mycotoxin

1. Introduction

With a worldwide distribution and a significant level of genetic diversity, fungi are of importance in both medical and agricultural fields and represent major health and commercial concerns. Medically, fungal organisms can be a part of the normal flora of humans and animals. However, these also have the potential to cause mild to severe life-threatening invasive infections or toxicities. The immune response to fungal agents is variable and complex, ranging from lack of recognition to severe inflammatory reactions resulting in significant morbidity and mortality [1–6].

There is a broad and diverse spectrum of human and animal diseases attributed

to fungi. Major effects of fungal agents in human health include, but are not limited to, organ-specific and systemic infections, especially in immunocompromised individuals, toxicity emanating from fungal products, carcinogenicity, mutagenicity, growth impairment and stimulation of allergic reactions. Common and usually

non-life-threatening infections caused by fungal agents affecting humans are well recognized and often localized on nails, skin, oral cavity, throat and vagina. Severe and fatal infections, however, can be caused by a variety of fungi including *Aspergillus, Blastomyces, Candida, Coccidioides, Cryptococcus, Histoplasma, Mucoromycetes, Pneumocystis, Talaromyces, etc*. Despite the significance of fungal infections an understanding of their pathophysiology has lagged behind other human pathogens. While the immune system of healthy individuals, in general, can effectively prevent some fungal infections, this is not the case in immunosuppressed patients [7, 8].

In addition to causing direct infections, the products of some fungal organisms can be toxic to animals and humans. Also, the mycobiome has been implicated in the pathogenesis of various types of cancers. An example is the link between *Malassezia spp.* and development of pancreatic ductal adenocarcinoma (PDA) [9]. Based on a reported murine experiment, fungal migration from the intestinal lumen to the pancreas initiates the pathogenesis of PDA by driving the complement cascade through the activation of mannose-binding lectin (MBL) [10]. Another example is the carcinogenic potential of *Candida spp*. Some findings indicate that *Candida albicans* is capable of promoting cancer by several mechanisms, including production of carcinogenic byproducts, inflammation, induction of T helper type 17 (Th17) cell response and molecular imitations [10–12]. As will be discussed later in this article, possible relationships between fungal agents and hematological malignancies have been explored.

In light of the above, here the well-established significance of mycotoxins in carcinogenesis is discussed and novel findings illustrating that mycovirus infections may also play a role in human diseases is highlighted. In particular, focus is placed on a mycovirus containing *Aspergillus flavus* and its effects on leukemogenesis.

2. Mycotoxins

The toxicity, mutagenic and carcinogenic effects of some fungi is often attributed to their production of mycotoxins. Mycotoxins are low molecular weight metabolites produced by yeasts and filamentous fungi. These metabolites are heterogeneous chemicals, toxic to vertebrates, including humans. Several mycotoxins also have toxicities to invertebrates, plants, and other microorganisms [13, 14].

Currently, there are over 450 known mycotoxins, which along with their secondary metabolites, can produce varying degrees of toxicity ranging from mild gastrointestinal symptoms to cancer. A large number of common mycotoxins have been identified that are of major concern to human health, among which are aflatoxins, fumonisins, ochratoxins, patulin, zearalenone and nivalenol/deoxynivalenol. Some organisms can produce several different mycotoxins, and many different species may produce the same mycotoxins. Mycotoxin producing fungi are usually found in improperly saved edibles and agricultural commodities. They can enter and contaminate human and animal food supplies. Animals fed contaminated foods can pass aflatoxins through their eggs, milk, and meats, thus indirectly transmitting aflatoxins to humans [15, 16]. While toxicity in humans is often due to ingestion of large doses of mycotoxins, these can also permeate through the skin [17].

Many mycotoxins are cytotoxic and suppress the functions of lymphocytes, granulocytes, and monocytes. Exposure to some mycotoxins inhibits interferon gamma producing Th1 cells and results in decreased number of these cells. Mycotoxins may lead to T cell polarization toward the Th2 phenotype and is a risk factor for the development of allergies [18–23]. The principal function of Th1 cells is cell-mediated immunity and inflammation. In normal conditions, there is

a balance between Th1 and Th2 cells. A shift of such a balance results in various disorders. Th1 cells play an important role in the functions of immunity related cells such as macrophages, B cells, and cytotoxic $CD8^+$ T lymphocytes (CTLs). The latter stimulate cellular immune response, participate in the inhibition of the activation of macrophages and invigorate B cells to produce IgM and IgG1. For instance, it is found that T cells of children exposed to *Aspergillus* have significantly lower Th1 cytokines, including tumor necrosis factors (TNFs), interferon-γ, interleukin-2 and -10. These cytokines are involved in the development of CTLs and natural killer (NK) cells which are responsible for the cell-mediated immune response against viruses and detection and removal of tumor cells. Thus, exposure to fungal agents may significantly change cellular composition and cytokine production and immune function [24, 25].

Exposure to aflatoxins can lead to life threatening acute poisoning (aflatoxicosis) [26]. In turn, acute aflatoxicosis can result in acute hepatic necrosis often manifested by symptoms of liver failure [27]. This eventually may cause development of cirrhosis in the liver and hepatic carcinoma. Chronic low-level exposure to mycotoxins, particularly aflatoxins and especially aflatoxin B1, is known to be associated with increased risk of hepatic damage, liver and gallbladder cancer and impaired immune activity [27–29]. Several studies have documented liver and gallbladder toxicity and carcinogenicity related to mycotoxins. Other organs, including bones, kidneys, pancreas, bladder, viscera and central nervous system, can be subject to carcinogenesis [30].

A variety of mycotoxins have carcinogenic potential in animals and humans [16, 17, 26, 28, 31–35]. Certain mycotoxins, especially aflatoxins, produced by genetically diverse *Aspergillus* spp. including *A. fumigatus, A. parasiticus* and *A. flavus* can be genotoxic with damage to DNA, which is attributed to the development of cancer in animals and humans. The effects of aflatoxins B1, B2, G1 and G2 and their metabolites such as aflatoxins M1, M2a, P1, Q1, Q2a, R0, H1; B2a, M2; GM1, GM2a, parasiticol (B3) and GM2, produced by the *Aspergillus* spp., are well recognized [35].

The carcinogenesis of mycotoxins is reported to be due to the intercalation of aflatoxin metabolites into DNA which alkylate the bases through epoxide moiety. This can be as a result of the mutations in the *p53* gene or signaling apoptosis. The third base of codon 249 of the *p53* gene is reported to be more susceptible to aflatoxin-mediated mutations. For example, in hepatocellular carcinoma, upon exposure to aflatoxin, mutation of *p53* gene is fixed at codon 249 third base and take the form of G to T transversion [36, 37].

In one report, using a mammalian cell line, the mutagenicity of various mycotoxins and the efficiency of mutagenic mycotoxins in producing DNA single strand breaks and chromosome aberrations were investigated. These experiments revealed that aflatoxin B1, mycophenolic acid, patulin, penicillic acid, and sterigmatocystin induce 8-azaguanine-resistant mutations. At higher concentrations, aflatoxin B1, mycophenolic acid, and sterigmatocystin were found to have minimal effects on single-stranded DNA. In contrast, treatment with patulin and penicillic acid at higher concentrations had resulted in severe breaks. Chaetoglobosin B, fusarenon X, luteoskyrin, and ochratoxin A had not induced 8-azaguanine-resistant mutations [38].

Overall, the mutagenicity of mycotoxins varies significantly and depends on their efficiency in causing DNA single-strand breaks, resulting in chromosomal aberrations. Adults are believed to have a higher tolerance to mycotoxins but exposure of children, while controversial and not uniformly accepted, can lead to delayed development and stunted growth [16, 31–33].

In addition to laboratory-based experiments, reports regarding isolation of mycotoxin producing strains of fungi, including that of *A. flavus*, from the

residences of leukemia patients are available [39–42]. In many reports, except for recent publications, fungal carcinogenesis is attributed to mycotoxins and their immunosuppressive effects. One report describes examination of sera from 36 cancer patients against an aflatoxin producing *A. flavus* which was isolated from the home of a patient with leukemia. A modified microimmunodiffiusion technique was used for this immunological evaluation. This study had found that 30% of cancer patients, 15 of whom had leukemia or lymphoid malignancy, and 6% of controls had shown a precipitation band indicating positive results [39]. Another published article reports four leukemic patients, from three families, in a residence where a mycotoxin producing fungus was isolated. The leukemogenesis was attributed to the immune depressive effects of mycotoxins [41]. In a house where a husband and wife had developed acute myelomonocytic and undifferentiated leukemia, respectively, fungal surveyance of the residence had been performed. Three fungal isolates were found, an extract of which had shown a depressive effect on a phytohemagglutinin skin test in guinea pigs as compared to negative findings using extracts isolated from a control residence [40]. As described below, a significant amount of data regarding the correlation of a mycovirus containing *A. flavus*, isolated from the home of a patient with acute lymphoblastic leukemia, has been recently published.

3. Viruses and human cancer

A vast amount of data on several viruses and their possible association with cancer development has been published [43–52]. While not the focus of this article, a brief review of the subject reveals the importance of the study of viral agents and their relation to occurrence of malignant disorders.

Both DNA and RNA viruses are capable of causing cancer in humans. Some of the known DNA viruses that are capable of causing human cancers are Epstein-Barr (EB) virus, human papilloma virus, hepatitis B virus, and human herpes virus 8. The relationship of EB virus to the development of Burkitt's lymphoma and nasopharyngeal carcinoma is well established [53–59]. Likewise, the relation of human papilloma virus and the development of cervical cancer and retention of HPV viral oncoproteins E6 and E7 for their continued expression and proliferation has been demonstrated [60–63]. Human T lymphotropic virus type 1, human immunodeficiency virus (HIV) and hepatitis C viruse are some of the RNA viruses that contribute to human cancers. It appears that viruses have diverse biological pathways to malignant disorders. The presence of viral gene products in cancer and precancerous cells are known. Despite the well-known carcinogenic role of viruses, little data regarding any possible health effects of mycoviruses alone, or in conjunction with their host, are available. This area needs to be further explored.

4. Mycoviruses

Viruses that infect fungi, also known as mycoviruses (*myco* = 'fungus' in Greek), are widespread geographically and are expected to infect all fungal taxa, from early divergent lineages to the most well-studied ascomycetes (sac fungi) and basidiomycetes (mushrooms). Mycovirus infection is persistent but does not result in disease or death of the host fungus, and often does not lead to obvious alterations in its phenotype under controlled laboratory conditions; therefore, mycovirology is an underappreciated and understudied field, similar to all non-disease associated virology [64].

Mycoviruses are currently classified in 22 taxa (21 families and one genus) by the International Committee on Taxonomy of Viruses (ICTV; https://talk.ictvonline.org/) (**Figure 1**). Some of these taxa exclusively accommodate viruses infecting fungi, such as the families *Hypoviridae* and *Polymycoviridae*. Other taxa also accommodate viruses infecting protozoa, plants, insects and mammals, such as the families *Botourmiaviridae*, *Chrysoviridae*, *Partitiviridae*, *Reoviridae* and *Totiviridae*. Members of the DNA-containing *Genomoviridae* family have been discovered in sequencing data from a variety of samples, including plant and insect tissue, animal blood, serum and feces, human blood, plasma, cerebrospinal fluid, cervical biopsies, and feces, and sewage [65]. Mycoviruses may be closely related to viruses pathogenic for humans. For instance, family *Mymonaviridae* belongs to the order *Mononegavirales*, together with viruses that cause Ebola, measles, mumps, rabies and respiratory diseases. Families *Metaviridae* and *Pseudoviridae* belong to order *Ortervirales*, together with human immunodeficiency virus (HIV), cause of acquired immunodeficiency syndrome (AIDS), and other retroviruses.

Classification of exemplar mycoviruses known to infect *Aspergillus* spp is shown in **Figure 2**.

Almost all known mycoviruses have double stranded (ds) RNA genomes or single stranded (ss) RNA genomes, either positive sense or negative sense, with one family of mycoviruses having circular ssDNA genomes. Virions are often proteinaceous in nature, composed of virus capsid proteins and their structure may range from spherical, to bacilliform in the case of barnaviruses, to filamentous in the case of flexiviruses and mymonaviruses. The absence of true virions is also common: narnaviruses and mitoviruses exist as naked RNA molecules respectively in the cytoplasm and mitochondria, hypoviruses are encapsulated in host derived lipid vesicles, polymycoviruses are non-conventionally encapsidated by a viral protein [66, 67]. Mycoviruses move intracellularly within the infected fungus and spread in mycelia during cell division and growth. Almost all known mycoviruses lack an extracellular phase in their replication cycle; they are transmitted vertically during asexual and/or sexual spore production and horizontally between fungal strains following cell fusion. The absence of an extracellular phase explains the general lack of lipid envelopes in virions.

Early reports focused on the mycovirus-mediated alterations on fungal phenotype, including morphology, pigmentation, asexual and sexual sporulation, and growth. Production of viral toxins conferring a competitive advantage to the fungal host [68], clearly illustrate that virus infection can be beneficial to the host and viruses are undeserving of their name, derived from the Latin word for 'poison' or 'venom'. These killer yeast systems have been primarily studied in the eukaryotic model organism *Saccharomyces cerevisiae* [69], extensively used in biotechnological applications such as baking, brewing and winemaking. However, interest in mycoviruses stems mainly from their effects on the interaction between their host fungus and the plant, insect or mammalian/human host of the fungus.

An increasing number of studies clearly illustrate the importance of mycoviruses in host-microbe interactions. The discovery of 'transmissible hypovirulence', i.e., mycovirus-mediated decrease in fungal pathogenicity represents a major advance in the field and the first mycovirus-based biological control application to combat chestnut blight caused by the plant pathogen *Cryphonectria parasitica* [70, 71]. The opposite phenomenon called hypervirulence, i.e., mycovirus-mediated increase in fungal pathogenicity, has also been noted. For instance, two variants of *Aspergillus fumigatus* polymycovirus 1 (AfuPmV-1), the first virus demonstrated to be infectious as dsRNA [66], respectively cause hypovirulence in an immunosuppressed mouse infection model [72] and hypervirulence in the greater wax moth *G. mellonella* infection model [73]. Additionally, AfuPmV-1 renders its fungal host more

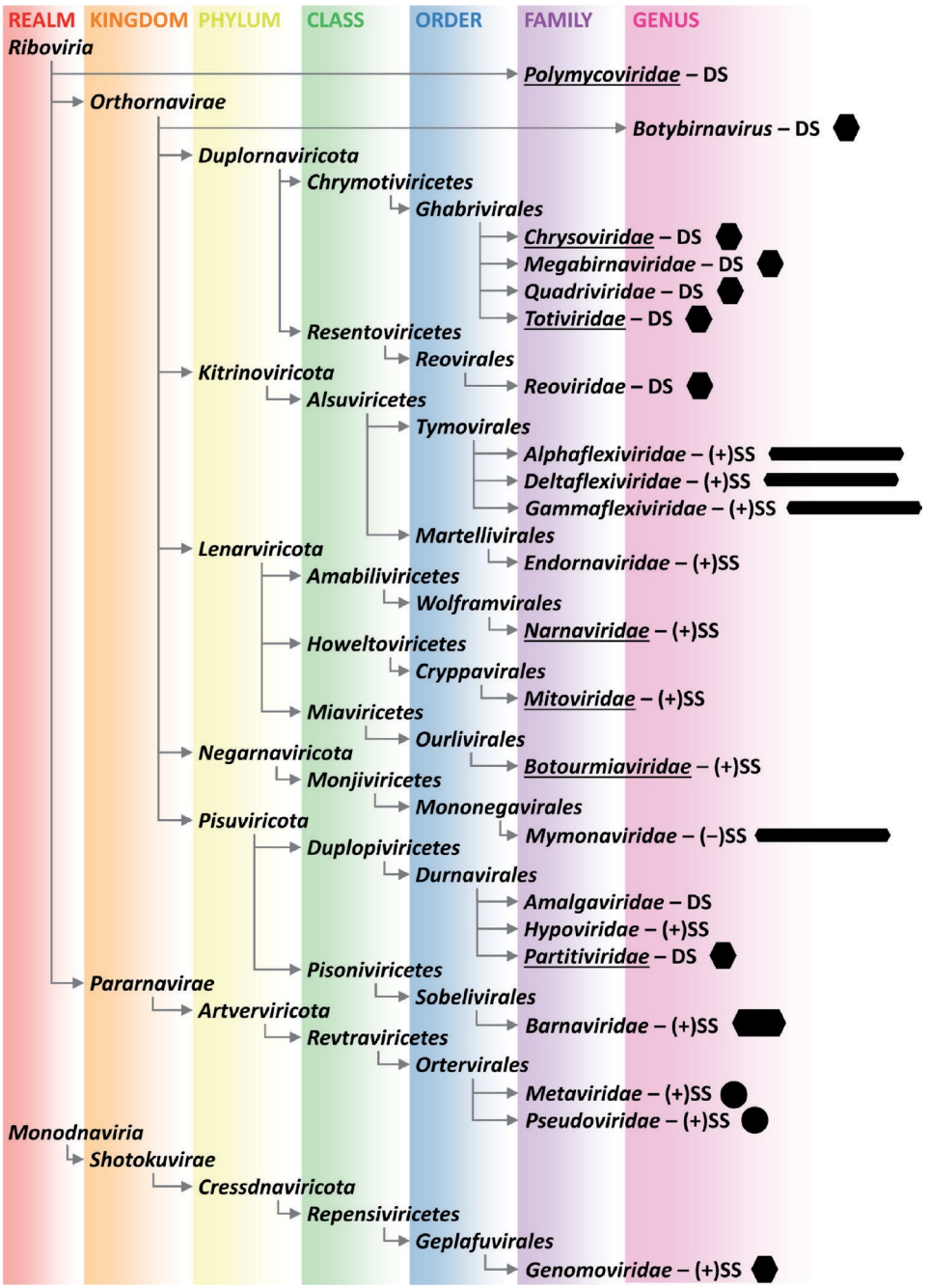

Figure 1.
Current classification of mycoviruses according to the International Committee on Taxonomy of Viruses. The realms Riboviria *and* Monodnaviria *accommodate viruses with respectively RNA and DNA genomes. Underlying family names accommodate mycoviruses known to infect* Aspergillus *spp. Next to family/genus names, (+)SS, (−)SS and DS indicate respectively, positive-sense single-stranded, negative-sense single-stranded and double-stranded genomes; hexagons indicate the presence of true virions, either isometric, bacilliform of filamentous.*

sensitive to the bacterium *Pseudomonas aeruginosa* [74]. Furthermore, partitivirus infection of *Talaromyces marneffei* leads to hypervirulence in a BALB/c mouse model [75]. Mycoviruses dsRNA genomes or replication intermediates are recognized by Toll-like receptor 3 (TLR-3) [76] and may induce an interferon immune response

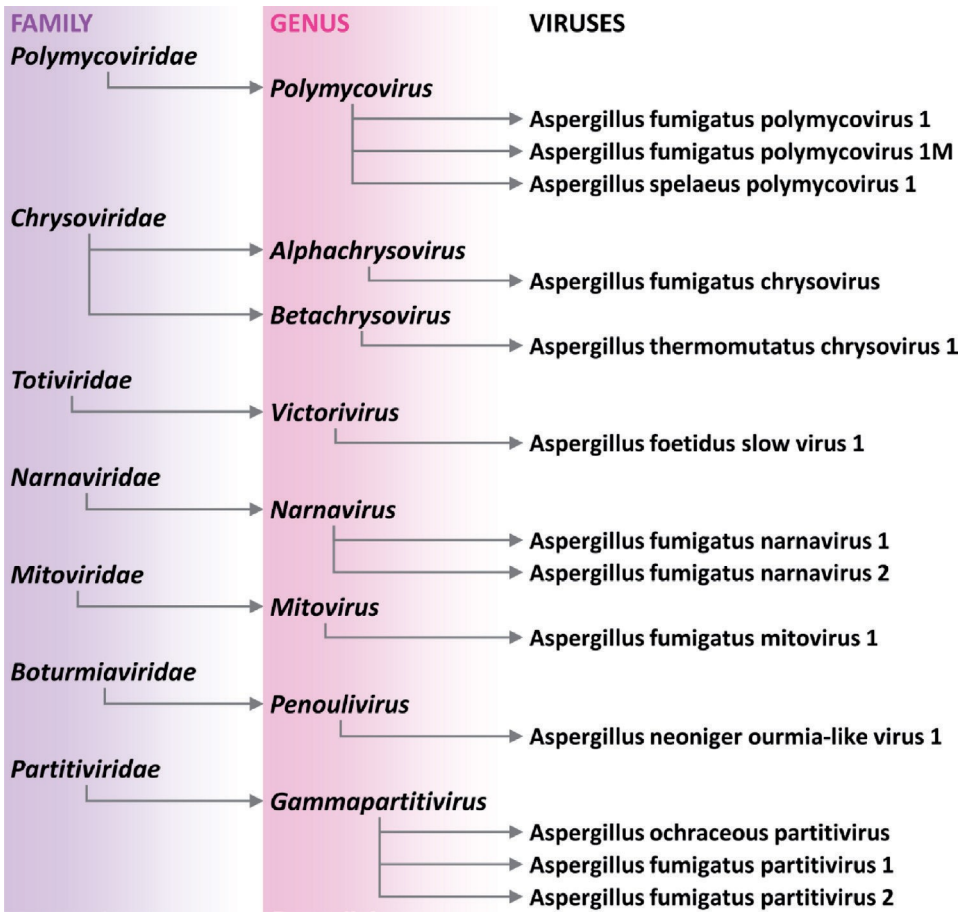

Figure 2.
Classification of exemplar mycoviruses known to infect Aspergillus *spp. Not all known mycoviruses found in* Aspergillus *spp. are officially assigned to recognized taxa. The phenotypes and effects of the majority of these mycoviruses on their* Aspergillus *host is unknown.*

in a TLR-3 dependent or independent manner, as illustrated with totivirus infected *Malassezia* [77, 78]. A link between azole resistance and mycovirus infection has been noted in *Penicillium digitatum* [79]. Finally, mycovirus infection is known to be responsible for modulation of fungal toxins and this phenomenon has been studied mainly in *Aspergillus* spp [80]. Carcinogenic aflatoxin production may be repressed by the presence of a mycovirus in *A. flavus* [81–84], while ochratoxin A is enhanced by the presence of a partitivirus in *A. ochraceus* [85].

Currently most mycovirus studies are focused on economically important phytopathogenic fungi, while scant data regarding fungi containing mycoviruses and human disorders are available. Since mycoviruses do exist in fungi, and humans are exposed to them, further research on these organisms may expand our knowledge of their possible role and effects of their interaction with humans.

5. Studies of mycovirus containing *Aspergillus flavus*

A report describing plasma of patients with acute lymphoblastic leukemia (ALL) having a positive reaction to an *A. flavus* isolate containing an unknown mycovirus is available [86]. Exposure of the peripheral blood mononuclear cells

(PBMCs) obtained from a group of ALL patients who were in a complete remission to the culture of this organism was reported to reproduce genetic and cell surface phenotypes, characteristic of active ALL [87]. Conversely, this was not observed in the control group of patients [87]. To describe these findings, which are patented in more details, in a series of experiments, a mycovirus infected *A. flavus* separated from the home of a patient with B-cell ALL was found to contain unknown mycovirus particles. These mycovirus particles were found within the body of the organism and culture supernatant. Chemical analysis of the isolated mycovirus containing *A. flavus* had revealed a lack of aflatoxin production [86]. The latter may be due to the influence of the unknown mycovirus which may have caused suppression of the production of aflatoxin as described previously [80–84]. Utilizing fast protein liquid chromatography (FPLC) for the analysis of the supernatant of the culture of this isolate, three separate peaks were identified. As noted above, in controlled experiments using plasma of patients with ALL in complete remission, with no evidence of the disease, using crude supernatant of the culture of the mycovirus containing *A. flavus* and enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies, plasma of patients with ALL had reacted positively. The plasma obtained from three separate groups of controls, including normal individuals, patients with sickle cell disease and individuals with various solid tumors, had been negative. In a separate study evaluating peaks obtained by fractionation using FPLC, of the three peaks which were found, peak 1 had the strongest positive effect [86]. The authors suggest that this technique can be used for screening for ALL or a test to identify patients who have had this disease [86].

As noted before, in a related publication, exposure of PBMCs obtained from ALL patients in complete remission, and long-term survivors of this disease, to the supernatant of the culture of the mycovirus containing *A. flavus* resulted in the re-development of the genetic and cell surface phenotypes, characteristic of ALL. The cell surface phenotypes examined were CD10/CD19, CD19/CD34 and CD34/CD117. The redevelopment of the ALL cell surface phenotypes was reported to be gradual, completed in 24 hours, and remained stable thereafter. Following exposure to the supernatant of the mycovirus containing *A. flavus*, alterations in gene expression were evaluated using microarray technique. Some of these alterations were reported to be upregulation of JAK1 (12.87-fold), JAK2 (1.5-fold), JAK3 (2.73-fold), IKZF1 (10.12-fold), MCL1 (59.37-fold), MYC (14.19-fold), HDAC1 (26.39-fold) and downregulation of PAX5 (3.05-fold). Following incubation, a significant and robust activation of transcription factor NF-κB p65 was reported by immunoblotting in ALL patients without any changes in the controls. The supernatant of the culture of *Mycocladus corymbifer*, which was used as a negative control, was reported to have no effects on PBMCs either from the ALL or control patients [87]. The above studies suggest a possible role for the mycovirus containing *A. flavus* in the process of leukemogenesis and opens a venue for vaccination and prevention of this disease.

6. Conclusion

It is apparent that fungal *spp.* are important in human and animal health. The mechanism of the effects of fungal agents in the development of human diseases appears to be multifaceted. Fungi are widespread in nature and inevitably, humans encounter these organisms. Many fungi contain mycoviruses. Although a significant amount of data regarding the carcinogenic effects of mycotoxins in the development of malignant disorders are available, possible pathogenicity and role of the mycoviruses in fungi, if any, in human and animal health, including malignant disorders, are not known. Recent reports describing *in vitro* effects of a mycovirus

containing *A. flavus* isolate in redeveloping characteristic ALL cell surface and genetic phenotypes in the PMBCs of acute lymphoblastic leukemia patients in complete remission is of interest. The existence of antibody to this organism in plasma of these patients is intriguing and further indicates its possible role in leukemogenesis. This area needs to be further investigated.

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