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Increased Severity due to Aflatoxin in Viral Cause of Hepatocellular Carcinoma: A Case Control Study

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Abstract: The objective of our study was to assess the presence of aflatoxin in Hepatocellular Carcinoma patients and their severity. This case control study was conducted in the Asian Institute of Medical Sciences (AIMS) Hyderabad and Nuclear Institute of Medicine and Radiotherapy (NIMRA) Jamshoro for the period of 02 years from March 2018 to February 2020. The calculated sample size was 218 subjects, 109 patients were diagnosed with cases of HCC (76 male and 33 female), and 109 were included as control. Control was selected from the relatives of the patients who were Viral Hepatitis-Negative. Non-probability convenience sampling was used. Majority of the cases and controls were for 40 to 49 years of age. Serum α-fetoprotein (AFP) was significantly high in 80 (73.4%) cases who were culture positive with (p=0.013). There is no association between the AG ratio and mycological culture media-positive cases. A strong correlation is observed between Viral Hepatitis infection and the development of hepatocellular carcinoma (HCC) in Pakistan. The toxin produced by fungi has worse outcomes in patients with liver compromise especially hepatocellular carcinoma. The poor prognosis was observed in HCC and Liver Cirrhosis patients, especially in low-income countries where health services are poor. Therefore, preventive strategies need to be applied to control these toxins. Proper handling and cooking of common food and good hygiene, especially for high-risk patients, can help prevent and controlling the spread of these toxins.

Keywords: hepatocellular carcinoma, aflatoxins, hepatitis B, hepatitis C, Pakistan.

肝细胞癌病毒性病因中黄曲霉毒素的严重程度增加:一项病例对照研究

摘要：我们研究的目的是评估肝细胞癌患者中黄曲霉毒素的存在及其严重程度。本病例对照研究于2018年3月至2020年2月在亚洲医学科学研究所海得拉巴和核医学与放射治疗研究所詹肖罗进行，为期02年。计算的样本量为218名受试者，109名患者被诊断患有肝细胞癌（

76名男性和33名女性）和109名作为对照。选出来自病毒性肝炎阴性患者的亲属。使用非概率便利抽样。大多数病例和对照年龄在40至49岁之间。80例(73.4%)培养阳性的血清甲胎蛋白显着升高(p=0.013)。白蛋白/球蛋白和真菌培养基阳性病例之间没有关联。在巴基斯坦，病毒性肝炎感染与肝细胞癌的发展之间存在很强的相关性。真菌产生的毒素对肝功能的损害尤其是肝细胞癌患者的预后更差。肝细胞癌和肝硬化患者预后较差，尤其是在卫生服务较差的低收入国家。因此，需要采用预防策略来控制这些毒素。正确处理和烹饪普通食物以及良好的卫生习惯，特别是对于高危患者，可以帮助预防和控制这些毒素的传播.

关键词：肝细胞癌, 黄曲霉毒素, 乙型肝炎, 丙型肝炎, 巴基斯坦.

1. Introduction

The liver is a high energy requirement organ which plays a key role in the secretion and synthesis of endogenous compounds [1]. The liver is the first organ to detoxify these exogenous compounds that's why health is directly compromised due to harmful exogenous compounds and organisms such as viruses, fungi etc. These factors lead to acute and chronic liver disease. Hepatocellular carcinoma and Liver Cirrhosis are two important complications leading to death. Morbidity and Mortality are high due to Hepatocellular carcinoma (HCC) and liver cirrhosis around the worldwide, with a major proportion occurring in Asia and Africa [2, 3]. HCC is on the rise in these countries due to multiple factors, and the most common causes are Viral Hepatitis B and C in developing countries [3]. Alcohol and aflatoxins may act as co-factors and halt the disease process and recovery. The prevalence of Chronic Viral Hepatitis (CVH) varied from country to country, it is high in developing countries where basic health needs are poor. Poverty, illiteracy poor hygiene, etc, therefore it is essential to assess the etiological links, to minimize the development of HCC by controlling them [4]. Chronic hepatitis B and C virus infection are now considered a major risk factor for HCC in Asian countries [5]. Various studies have proven the link of the HBV and HCV with HCC development, which depends upon the geography and prevalence of the CVH [6].

Many studies have proved the correlation between alcohol consumption and the development of HCC [7, 8]. The exposure of Aflatoxins in the presence of hepatitis viruses has proved a synergistic effect and worsened the prognosis of HCC [9]. Pakistan is situated in a hot and humid environment that favors aflatoxins producing fungi and enhances food contamination with this hepatotoxic, but very limited research has been done to assess its effect on HCC in the presence of CVH [10].

Chronic HBV and HCV infections in Pakistan are increasing with twelve million active cases with a steep rise of 150000 cases each year. With the passage of time and availability of widespread HBV vaccination, the trend of prevalence has shifted from HBV to HCV [11]. It has been well documented that HBV plays a role in the development of HCC [12]. In India and other Asian nations, more than a quarter of all chronic liver diseases are caused by HBV mutations [13]. As the HCV is more prevalent in Pakistan in addition to higher aflatoxin contamination, a more systematic research is needed to assess the etiology of HCC prevalent in Pakistan [14].

1.1. Aflatoxin

Aflatoxin is a myotoxin produced by Aspergillus flavus and Aspergillus parasiticus, which are widespread and common in nature. These are found in common food stuff such as corn, rice, and oilseeds dried fruits, which are improperly stored in hot weather or unsanitary conditions. It is also found in meat eggs and milk. Nearly 4.5 billion people are at a risk of chronic exposure to Aflatoxin-contaminated food, especially in developing countries.

1.2. Estimation of the Population Risk of Aflatoxin-Induced Liver Cancer

The pattern of distribution is also interrelated with socioeconomic status and gender that is why found to be more common in low-income countries, due to improper handling of food, ineffective food regulations, and poor sanitation. As a consequence, individuals exposed to this toxin through contaminated food grains and animal products may develop both acute hepatotoxicity and HCC [15]. Aflatoxins are associated with toxicity and carcinogenicity in humans leading to acute aflatoxicosis, which results in death, whereas chronic aflatoxicosis results in more prolonged pathologic changes, like cancer and immunosuppression. The primary target organ is the liver, and its damage has been documented in poultry, and nonhuman primates following ingestion of aflatoxin B1. The risk of liver cancer in individuals exposed to chronic hepatitis virus infection increases up to 30 times greater due to Aflatoxin compared to

those exposed to aflatoxin alone. Two important risk factors for HCC are aflatoxin and Viral hepatitis, more prevalent in poor countries and the rural population worldwide. Studies have also shown that the age and sex of the infected person and genetic characteristics of the virus may play an important role in increasing the risk of aflatoxin-induced HCC [16].

The results of previous studies highlight the critical role of Aflatoxins uptake in the generation of toxicity and downstream carcinogenic effects in HCC, thereby providing a new avenue for the medical treatment and prevention of aflatoxin-induced liver cancer [17]. According to the data of the International Agency for Research on Cancer, Aflatoxin B1, which is a Group 1 carcinogen for HCC, is the fifth most common cause cancer in males and seventh in females. Severity and prognosis of HCC remain poor when Viral Hepatitis and Aflatoxin both carrying a poor prognosis in a high-incidence population with death within a year of diagnosis. Exposure of Aflatoxins increases susceptibility in the development of HCC [18].

Pakistan is also recognized as a country of the world where hepatitis C virus (HCV) is endemic. Recent large national surveys suggest an overall HCV prevalence of 4.8% and that of HBV as 2.5%. There are however, communities where the sero-prevalence of HCV can be as high as 23%. No wonder that chronic liver disease is the fifth most common reason for morbidity and mortality in the country and Pakistan has been perhaps accurately called a "cirrhotic state". Hence, majority of such patients are at risk of developing HCC [19].

2. Materials and Methods

2.1. Study Population

The subjects in this study were identified on clinical and laboratory evidence and diagnosed cases of Hepatocellular Carcinoma Asian Institute of Medical Sciences (AIMS) Hyderabad and Nuclear Institute of Medicine and Radiotherapy (NIMRA) Jamshoro, Pakistan. The patients were admitted in hospital or coming for follow-up for treating chronic HBV or HCV infection-related HCC for the period of 02 years from March 2018 to February 2020.

2.2. Sample Selection

Sample size was calculated according to the prevalence of Hepatocellular carcinoma in Pakistan, which was 8.9% using 95% confidence interval, 5% margin of error [20]. The calculated sample size was 218 subjects, 109 patients were diagnosed with cases of HCC, and 109 were included as control. Control was selected from the relatives of the patients who were Viral Hepatitis-Negative. Non-probability convenience sampling was used.

2.3. Inclusion Criteria

- Diagnosed cases of HCC due to Viral Hepatitis as cases and controls who were viral hepatitis negative.
- Age above 18 years.
- Both male and female

2.4. Exclusion Criteria

- Patients who were HCC other than Viral Hepatitis.
- Age above 18 years.
- Subjects not willing to participate in the study.

2.5. Data Collection Procedure

The case of HCC who were under treatment in the Asian Institute of Medical Sciences (AIMS) Hyderabad and Nuclear Institute of Medicine and Radiotherapy (NIMRA), Jamshoro, were included in this study who fulfilled the inclusion criteria. After taking written consent the information was gathered on the pre-designed questionnaire. The diagnostic work-up included HBV vaccine, transfusion, alcohol misuse (more than 80 g/day for 5 years), prolonged contraceptive use, and chemical exposure. A positive HBV or HCV test or a history of cirrhosis in the patient's family were also taken into consideration during the diagnosis. A thorough physical examination, all biochemical reports and Radiological evidences for the diagnosis of HCC were examined to confirm the diagnosis.

Serum Alpha Feto-Protein was also seen in these patients. A previous history of dietary information was collected with the assistance of an expert nutritionist. On the basis of protocols provided in various studies, the maximum contamination level of AFB1 was estimated for each food item based on the basis of reports issued by Pakistan. The presence of hepatitis was tested using Organon Teknika's 3rd generation EIA technology, which found HBsAg and anti-HCV antibodies.

2.6. Mycological Study

Mycological studies were conducted to determine the Mycoflora present in blood samples through the Agar plate (Potato Dextrose Agar) procedure [21], see fig. 1 and 2.

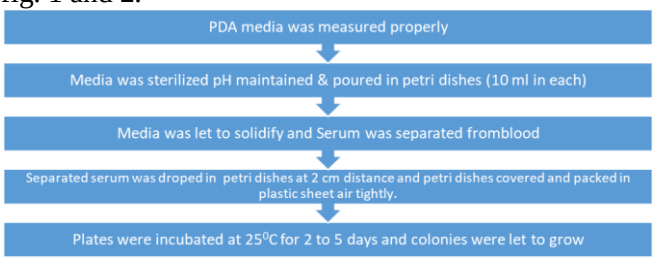

Fig. 1 The flowchart

The relative isolation frequency (Fq.) of all mycotoxin and aflatoxin-producing fungi (Aspergillus parasiticus and Aspargillus flavus) was calculated as noted by [22].

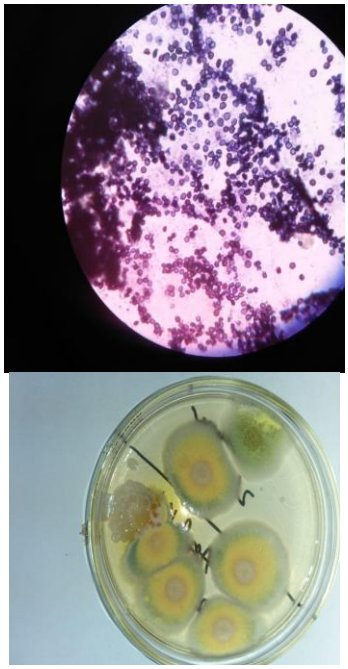

Fig. 2 Photo image of microflora present in the blood sample

2.7. Data Analysis Procedure

The data were analyzed in version 25.0 of the Statistical Package for Social Sciences (SPSS) and Microsoft Excel. For categorical variables, frequency and percentage were calculated. The data were formulated through Graphs and Charts.

2.8. Statistical Analysis

The results are presented as mean standard deviation (SD). Using Student's t-tests for continuous variables and the chi-squared test for non-continuous ones, the significance of the various variables was determined.

3. Results

A total of 218 subjects were included in our study according to the inclusion and exclusion criteria. 109 patients were diagnosed with cases of HCC and 109 were included as control, and almost all controls were relatives of the patients.

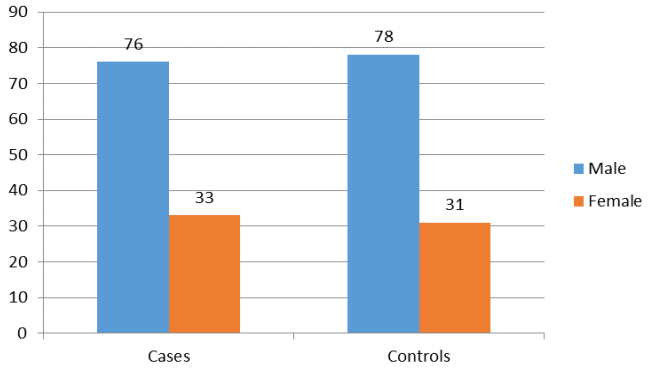

Fig. 3 Gender distribution of cases and control (cases = 109, controls = 109)

Fig. 3 shows the gender distribution of cases and control. There were 76 (69.72%) males and 33 (30.27%) females in cases. In controls, 78 (71.55%) were males and 31 (28.44%) were females.

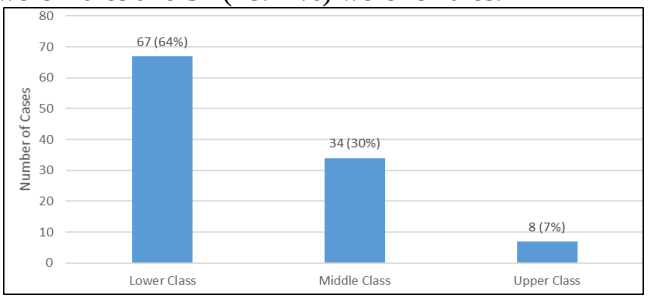

Fig. 4 Distribution of social class on hepatocellular carcinoma cases (n = 109)

The patients were mostly from the rural areas and belonging to low-income families. The above figure shows the relative incidence of the HCC in different social classes. A more appropriate comparison of social status and access to hygienic food was used to determine any difference between the different classes of the patients. As shown in fig. 4, 67 (64%) belonged to the lower class, 34 (30%) were from the middle class and the remaining 7 (6%) were from the upper class. A significant correlation was found between the different classes of the people ($p < 0.05$).

Table 1 Hematologic and biochemical profiles of hepatocellular carcinoma patients (n = 109)

Parameter	Mean Value	Standard Deviation
Hemoglobin	10.4 (g/dL)	± 1.9
Serum bilirubin	2.0 (mg/dL)	± 0.3
Alanine Transaminase (AST)	189.0 (IU/L)	± 35.0
Alanine Transaminase (ALT)	141.0 (IU/L)	± 42.0
Alkaline phosphatase	352.0 (IU/L)	± 17.0
Serum albumin	3.0 (g/dL)	± 0.2
a-Fetoprotein	2708.0 (ng/dL)	± 123.0

The results of the hematological and liver function tests were significantly deranged in all cases, as shown in table 1. Hemoglobin level was below normal with a mean value of 10.4 (g/dL) with SD ± 1.9. ALT was significantly high in all cases and the mean was 141.0 (IU/L) with an SD of ± 42.0. Elevated levels of α-fetoprotein with a mean value of 2708.0 (ng/dL) with an SD of ± 123.0, as shown in table 1. The serum level of α-fetoprotein (AFP) was high in almost all cases. The presence of cirrhosis was significantly less in patients with HCC patients who had no detectable evidence of viral infection ($P < 0.05$). The detailed history has shown that no one has any link to the alcohol consumption.

Table 2 Association of mycological culture media positive in cases and controls with gender (cases = 109, controls = 109)

			Mycological Culture Media Cases				Mycological Culture Media Control			
			Positive	**Negative**	**Total**	**P-Value**	**Positive**	**Negative**	**Total**	**P-Value**
Gender	Female	Count	8	29	37	0.001	0	30	30	0.270
		Expected Count	23.8	13.2	37.0		1.1	28.9	30.0	
		% within Gender	21.6%	78.4%	100.0%		0.0%	100.0%	100.0%	
	Male	Count	62	10	72		4	75	79	
		Expected Count	46.2	25.8	72.0		2.9	76.1	79.0	
		% within Gender	86.1%	13.9%	100.0%		5.1%	94.9%	100.0%	
Total (Male and Female)		Count	70	39	109		4	105	109	
		Expected Count	70.0	39.0	109.0		4.0	105.0	109.0	
		% within Gender	64.2%	35.8%	100.0%		3.7%	96.3%	100.0%	

Mycological Culture Media was significantly associated with hepatocellular Carcinoma (table 2); it was found that the total number of positive cases both females and males were 70 (64.2%) and negative cases were 39 (35.8%). On the other hand control group had only 4 (3.7%) positive in Non HCC controls. These are results are highly significant in the study group with (p = 0.001) compared to controls with (p = 0.270).

Table 3 Association between mycological culture media and alpha-feto-protein in case and control groups (cases = 109, controls = 109)

			Alpha-Feto-Protein Cases							Alpha-Feto-Protein Control	
			200-400 ng/dl	**401-600 ng/dl**	**601-800 ng/dl**	**801-1000 ng/dl**	**> 1000 ng/dl**	**Total**	**P-Value**	**< 12 ng/dl**	**P-Value**
Mycological Culture Media	Positive	Count	0	0	0	5	75	80	0.013	4	0.292
		Expected Count	8.1	6.6	6.6	3.7	55.0	80.0		4.0	
		% of Total	0.0%	0.0%	0.0%	4.6%	68.8%	73.4%		3.7%	
	Negative	Count	11	9	9	0	0	29		105	
		Expected Count	2.9	2.4	2.4	1.3	20.0	29.0		105.0	
		% of Total	10.1%	8.3%	8.3%	0.0%	0.0%	26.6%		96.3%	
Total		Count	11	9	9	5	75	109		109	
		Expected Count	11.0	9.0	9.0	5.0	75.0	109.0		109.0	
		% of Total	10.1%	8.3%	8.3%	4.6%	68.8%	100.0%		100%	

Table 3 shows the association of mycological culture media in HCC cases who had high Alpha Feto Protein. From our study data, 73.4% cases were culture positive and the majority of these culture-positive cases (68.8%) had high alpha feto protein more than 1000ng/dl. There was a strong association between High alpha feot protein and mycological culture media positive with p-value of (0.013).

Table 4 Association between albumin globulin ratio (AG Ratio) and mycological culture media (cases = 109, controls = 109)

			Mycological Culture Media Cases				Mycological Culture Media Control			
			Positive	**Negative**	**Total**	**P-Value**	**Positive**	**Negative**	**Total**	**P-Value**
AG Ratio	0.1-0.9	Count	69	22	91	0.000	0	61	61	0.022
		Expected Count	58.4	32.6	91.0		2.2	58.8	61.0	
		% within AG Ratio	75.8%	24.2%	100.0%		0.0%	100.0%	100.0%	
	1.0-1.9	Count	1	17	18		4	44	48	
		Expected Count	11.6	6.4	18.0		1.8	46.2	48.0	
		% within AG Ratio	5.6%	94.4%	100.0%		8.3%	91.7%	100.0%	
Total		Count	70	39	109		4	105	109	
		Expected Count	70.0	39.0	109.0		4.0	105.0	109.0	
		% within AG Ratio	64.2%	35.8%	100.0%		3.7%	96.3%	100.0%	

As shown in the table 4, the albumin globulin ratio was measured in mycological culture media case and control groups and was observed an AG ratio from 0.1 to 0.9 in a total of 91 study subjects and ratio from 1.0 to 1.9 was observed in 18 study subjects. The AG ratio in the control group was also measured ranging 61 cases were having AG ratio from 0.1 to 0.9 and 48 were having a ration of 1.0 to 1.9. Both values for cases and control were statistically significant (p = 0.000 and p = 0.022 respectively) which shows that there was no association between the AG ratio and mycological culture media-positive cases (Table 4).

4. Discussion

Aflatoxin B1 (AFB1) is a category 1 carcinogen with an established role in the development of hepatocellular carcinoma (HCC) in high-risk areas and low-income countries ultimately accumulating in the

liver and possibly contributing to liver cancer. The main cause of Hepatocellular carcinoma in developing and low-income countries is Viral Hepatitis C now days with lower socioeconomic status. As a consequence Individuals are exposed to many toxins through contaminated food grains and animal products, most common of them is aflatoxin produced by fungi due to improper handling of food, ineffective food regulations, and poor sanitation.

Pakistan is one these countries where the prevalence of Viral Hepatitis C is high, leading to HCC developing more frequently in cirrhotic livers than non-cirrhotic livers. A similar pattern has been observed in regions where HCV infection is prevalent, such as the Far East and sub-Saharan Africa [21]. The cirrhosis of the liver has been found in up to 90% of patients with HCC in these regions. A cirrhotic patient's annual risk of developing hepatocellular carcinoma (HCC) ranges from 3% to 11% in Japan [22].

Aflatoxins in the diet have a clear-cut history of carcinogenesis, and act synergistically in the presence of HCV [23]. The dietary Aflatoxin intake was determined on the basis of reports from Pakistan. Except for a few patients with HCV infection who presented in the late fifth decade, the majority of our HCC patients presented in the late fourth or early fifth decade. This is the first report from areas with a high prevalence of HCV show that hepatocellular carcinoma (HCC). In our study, approximately four-fifths of the patients were in their forties with a mean age of 49.5±11.5 years, it is in accordance with this study as well.

Aflatoxins are categorized as the main carcinogens causing liver cancer, Exposure to chronic low levels of aflatoxin-contaminated food can lead to immune suppression and nutritional consequences that greatly contributed to the increase in hepatocellular carcinoma (HCC). In our study, blood sample mycological culture was positive in 70 (64.2%) cases of HCC and was found negative in 39 (35.8%) HCC cases. In a study by Farag RM, fifty (50) bold samples were collected, and it was found that aflatoxins were present in 38 (76%) cases of HCC and were detectable serum level of AFB1, while the remaining 12 patients were AFB1 negative and used as control participants in that study [24]. This is in accordance with our study as well.

Alfa feto-protein is a reliable tumor marker for HCC, and in our study data, it was found that alfa feto-protein was significantly high in almost all cases of HCC, even 73.4% cases had > 1000 ng/dl as noted. And alfa feto-protein level above 10 ng/dl was only in 3.7% non HCC controls. In a study by Jasirvan COM, et al in Indonesia concluded that alfa feto-protein levels above 10 ng/ml were seen in 82.6% (109 of 132) HCC patients and 29% (57 of 198) non-HCC patients [24]. This is in accordance with our study as well.

It was reported in Japan, where the high prevalence of HCV-related hepatocellular carcinoma (HCC), disease is more likely to present at a later age and with more severe cirrhosis than HBV HCC. The role of dual infection in the pathogenesis of HCC has not yet been established, but has been proved that dual infections speed up the progression of chronic hepatitis to cirrhosis and to hepatocellular carcinoma (HCC) in high-endemic areas [25].

5. Conclusion

Aflatoxin produced by Aspergillus flavus and Aspergillus parasiticus is wide spread found in common food stuff such as corn, rice, and oilseeds dried fruits, which are improperly stored in hot weather or unsanitary conditions. The toxin produced by these fungi have worse outcomes in patients with liver compromise especially hepatocellular carcinoma. The poor prognosis was observed in HCC and Liver Cirrhosis patients, especially in low-income countries where health services are poor. Therefore, preventive strategies are applied to control these toxins to enter the body. Proper handling and cooking of common food and good hygiene, especially for high-risk patients, can help prevent and controlling the spread of these toxins.

Ethical Approval

The institutional ethics committee of the Institute of Biochemistry, University of Sindh Jamshoro, Pakistan approved this study Ref. No. IOB/401/2017, Dated: 07-08-2017.

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