RESEARCH ARTICLE

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Low Frequency of Aflatoxin Induced *TP53* Gene Codon 249 Mutation in Hepatocellular Carcinoma from Egyptian Patients Living in the Nile Delta Region

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Abstract

Objective: Study the frequency of codon 7 (c.747 G>T, *p. R249S*) mutation associated with Aflatoxin B1 (AFB1) exposure in Egyptian patients with hepatocellular carcinoma (HCC). **Methods:** We utilized restriction fragment polymorphism and direct sequencing to assess codon 7 mutations in 104 hepatocellular carcinomas. The expression of *TP53* protein in the tumors were assessed in 44 tumors by a monoclonal rabbit antibody. **Results:** We identified a single 1/104 (1%) with c.747 G>T, *p. R249S* variant. 28/44 (63.6%) tumors showed no or occasional (less than < 5%) nuclear staining; 9/44 (20.4%) showed mild to moderate (5-49%) and 7/44 (15.9%) showed strong ≥ 50% staining. **Conclusion:** We observed much lower frequency of TP53 gene than previously published results suggesting geographical alterations in AFB1 exposure in Egypt.

Keywords: Hepatocellular carcinoma-*TP53*- Aflatoxin

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Introduction

HCC is the 5th and 7th most common cancer for men and women, respectively, but is the 3rd most common cause of cancer related death worldwide (Bosetti et al., 2014). HCC is diverse in etiology but unlike many cancers, its risk factors are well characterized and largely preventable. Nearly 75% of all liver cancer is preceded by persistent hepatitis B or C infection (IARC, 1994). In addition to being male, other strong risk factors include alcohol (Bagnardi et al., 2001) and tobacco(Lee et al., 2009) use, aflatoxin poisoning (Liu et al., 2012) and non-alcoholic steatohepatitis (NASH) secondary to obesity and type 2 diabetes mellitus (Baffy et al., 2012).

According to the first national population-based cancer registry conducted between 2008-2011 in Egypt, liver cancer was the most common with an overall prevalence of 23.8% of all cancers (Ibrahim et al., 2014). The calculated age specific rate (ASR) was 61.8/100,000 in men and 24.4/100,000 in women. In men liver cancer is currently the most common (33.6%) of all cancers while in women it is the second most common (13.5%) (Ibrahim et al., 2014). Although the exact subtypes of liver cancers were not reported in that registry our local experience at the National Liver Institute, Menoufia University indicates that most of these tumors are primary Hepatocellular Carcinomas (HCC).

Several studies have suggested that Aflatoxins B1 (AFB1) exposure could play a role in HCC in Egyptian patients. This was based on the observation of high level of serum AFB1, serum albumin adducts and a specific somatic mutation at codon 249 of the *TP53* tumor suppressor gene (p.R249S) (El-Kafrawy et al., 2005; Zekri et al., 2006; El-Din et al., 2010). This specific *TP53* mutation has been strongly linked to AFB1 exposure in the patients (Bressac et al., 1991). In the following study we investigated the possibility that the high frequency of HCC in Lower Egypt is linked to higher frequency of exposure to AFB1. The codon 249 mutation of the *TP53* in the tumors was used as a marker for such exposure. Our results indicate the rarity of *TP53* p. 249 suggesting the lack of significant contribution of AFB1 to HCC development in the delta region of Egypt.

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Materials and Methods

Samples

A total of 104 hepatocellular carcinomas were studied including 88 males and 16 females. All the samples were from the archive of the Pathology Department, National Liver Institute, Menoufia University. Samples were collected retrospectively from 2001 till 2016. Sample collection was in accordance with Institutional Review Board approved protocol (005/2008 and 051/2012). Most of the samples were surgical resections. Tissue obtained from only four needle biopsies were included. The average age of the patients was 55.1 years (range 35-70 years).

DNA extraction and Codon 249 of TP53 mutation screening

DNA was extracted from tumor tissues and non-tumor liver tissue, using DNeasy tissue kit (Qiagen, Valencia, CA). All tumors were tested for the variant by restriction fragment length polymorphism. In addition, thirty six tumors were also tested by direct sequencing. About 20-50 ng of the purified DNA was used as template for amplification of exon 7 of the *TP53* using the following primers (forward 5'CTTGGGCCTGTGTTATCTCC' 3, reverse: 5' TGGAAGAAATCGGTAAGAGGTG'3) with final primers' concentration of 0.4 µM. The PCR was carried out in an Applied Biosystem 9700 thermal cycler using the Qiagen HotStarTaq polymerase according to the following conditions a 10 min HotStarTaq polymerase activation at 95°C, followed by a 40 cycles of denaturation (95°C, 30 sec), primer annealing (56°C, 60 sec) and extension (72°C, 30 sec), followed by a final 10 min extension at 72°C. The size of the final undigested PCR fragment was 228 bp.

Digestion of the PCR product was carried out using 10units of HaeIII restriction endonuclease (NeoEngland biotechnology,) which was added to a 9 µl aliquot of the PCR product. The enzyme cuts within a GG|CC sequence encompassing codon 249 (AGG). Digestion of wild-type DNA generates four bands of 24, 40, 66 and 92 base pairs, whereas mutant material, in which the restriction site has been altered, yields three bands of 24, 40 and 158 base pairs. The PCR product was visualized on 3% agarose gel stained with ethidium bromide. All analyses were repeated at least twice. Mutation was confirmed by forward and reverse sequencing utilizing the same primers by automated, dideoxy sequencing (sequencer AbiPrism 3100, PerkinElmer, Oak Brook, IL).

Immunohistochemistry

Tissue microarray (TMA) preparation was carried out at the Department of Pathology, National Liver Institute, Menoufia University. Tissue microarrays were prepared from paraffin embedded tissues as previously reported (Abdel-Rahman et al., 2006). At least two 2 mm representative cores from each tumor tissue were included. For tumors with heterogenous morphology up to six cores representing different morphological regions were included. Five different external control tissues were also studied (colon, lymph node, breast, spleen, gallbladder) to insure consistency between different experiments.

A monoclonal rabbit antibody (clone 318-6-11) was obtained from DAKO (Carpinteria, CA). Immunohistochemistry was carried out according to the manufacturers' suggested protocol. Briefly, heat induced target retrieval was carried out in a Tris/EDTA buffer pH 9 in a water bath at 95-99 ºC degree for 20min. After thermal treatment slides were allowed to cool for 20 minutes at room temperature then rinsed with phosphate buffer saline (PBS). After peroxidase blocking the slides were incubated with the primary antibody diluted in DAKO antibody diluent with background-reducing components at 1:50 concentration for 30min. DAKO EnVision System/HRP was utilized for detection of immunohistochemistry signals. Negative controls were carried out by incubating the tissue with the antibody diluent only. After counterstaining with hematoxyline and mounting, the slides were evaluated under a light microscope and assessed by a pathologist (MHA). At least 10 fields in each tumor sections were evaluated. Percentage of tumor cells showing nuclear staining was assessed.

Results

Mutation screening identified a heterozygous mutation in codon 249 in only one out of the 104 tumors tested. The mutation was confirmed by direct sequencing which showed a G to T transversion reportedly associated with AFB1 (c.747 G>T, *p. R249S*), Figure 1. Equal allele heights of the wild type and mutant alleles were observed.

We also tested the activation of *TP53* in HCC from in a subset of samples by immunostaining for the *TP53* protein on representative tissues from 44 tumors. Out of those 28/44 (63.6%) showed no or occasional (less than < 5%) nuclear staining; 9/44 (20.4%) showed mild to moderate (5-49%) and 7/44 (15.9%) showed strong ≥ 50% staining.

Discussion

The recent dramatic rise in the incidence of HCC in Egyptian patients from less than 1% of all cancers in the 70's to representing almost one third of all cancers 35 years later is extremely alarming. Understanding the

Table 1. Frequencies of *p.R249S* Mutation Detected in HCC from Egyptian Patients

Study location	Mutation Frequency	Techniques used	Reference
Cairo	8/41	Affymetrix/RFLP/Sequencing	El-Kafrawy et al. 2005
Cairo	1/25	SSCP/Sequencing	Zekri et al. 2006
Cairo	3/20	RFLP	El-Din et al.2010
Menoufia	1/104	RFLP/Sequencing	This study

Affymetrix, *TP53* Gene Chip, Affymetrix, SSCP, Single strand conformational polymorphism; RFLP, restriction fragment length polymorphism

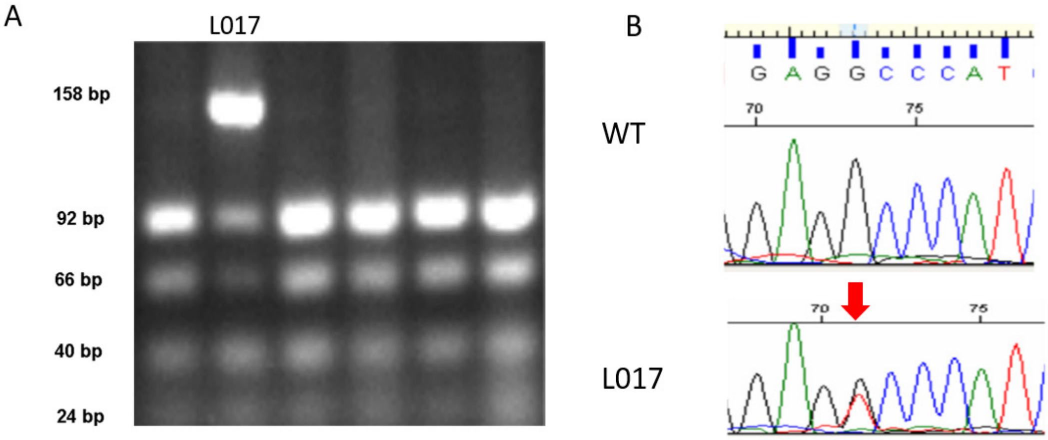

Figure 1. Restriction Fragment Length Polymorphism (RFLP) and Direct Sequencing Identifies the c.747 G>T, *p. R249S* Mutation in *TP53* Gene. A) Digestion of wild-type (all cases except L017) allele generates four bands of 24, 40, 66 and 92 base pairs, whereas mutant allele alters one restriction site leading to three bands of 24, 40 and 158 base pairs. B) direct sequencing of tumor tissue from case L017 shows a heterozygous G>T transversion with minor allele frequency close to 50%.

etiological factors contributing to such increase is very important to address this major health problem. Several etiological factors have been suggested to contribute to HCC risk in Egyptian patients. Viral hepatitis is by far the most significant risk factor but other environmental factors in particular pesticides and exposure to AFB1 have been suggested.

Several approaches have been utilized to assess the contribution of AFB1 to HCC risk in Egyptians. This includes assessing of serum concentration of albumin adduct and *TP53 p. R249S* mutation. The highest frequency of *TP53 p. R249S* mutation was reported by El-Kafrawy et al., (2005) where they identified 10/41 subjects with mutations in codon 249 including 8 (19.5%) with the p.R249S mutation, one with the p.R249T and one with the p.R249G mutation, Table 1. In two additional small studies the frequency of the p.R249S mutation was 1/25 (4%) and 3/20 (15%), Table 1 (Zekri et al., 2006; El-Din et al., 2010). All three studies were from centers located in Cairo (El-Kafrawy et al., 2005; Zekri et al., 2006; El-Din et al., 2010). The significant lower frequency of the p.R249S mutation detected in our study is not related to the RFLP technique we used for the screening as two of the other publications also used the same technique. Also, direct sequencing of a subset of 36 samples didn't identify any additional mutations. In tumors caused by AFB1 exposure, the mutation is an early alteration in tumorigenesis so one would expect detection of the mutation in all tumor cells with minor allele frequency ~50% so the RFLP should have sufficient sensitivity to detect that. The geographical location (i.e. Cairo versus Menoufia) may be a factor though highly unlikely. Another potential factor is the time frame for sample collection; our samples were collected over a very long period of time from 2002 until 2016.

Large difference in geographical distribution of liver cancer was also observed in Egypt. The proportions and ASR of liver cancer were highest in Lower Egypt (Nile delta) (29.6% and 56.8/100,000), less in Middle Egypt (15.2% and 27.4/100,000), and least in Upper Egypt (8.2% and 13.1/100,000) (Ibrahim et al., 2014). The cause of such large difference in geographical distribution is not clear.

HCV is the major risk factor for HCC In Egypt (El-Zayadi et al., 2005). Egypt has one of the highest prevalence of HCV in the World with an estimated prevalence of 18.9% for HCV antibodies in the general population (Mohamed and Aoun, 2002). Although spatial variation in the prevalence of HCV has been reported, clusters of high prevalence was observed in areas of Upper and Middle Egypt such as BeniSuef, Minya, Faiyum as well as areas of Lower Egypt as Dakahlia, Menoufia and Kafr El-Sheikh. While clusters of low prevalence were reported in areas of Lower Egypt such as Cairo and Alexandria as well as area of Upper Egypt such as Luxor. Thus, spatial variation in HCV prevalence doesn't fully explain the large difference in the prevalence of HCC between Lower, Middle and Upper Egypt.

In conclusion, we observed a relatively low frequency of *TP53 p. R249S* mutation in HCC patients accrued from a single center located in the Nile Delta region in Egypt suggesting geographical variation in the AFB1 exposure in Egypt. The cause of such variation and strategies to lower it in high frequency region should be considered.

Author Contribution Statement

Asmaa Mosbeh: experimental work, preliminary analysis and wrote first draft of manuscript; Waleed Abdelmaguid: experimental work, preliminary analysis and assisted with the first draft of manuscript; Sameera Ezzat (deceased): study design, statistical analysis, reviewed and approved near final manuscript draft; Mervat Sultan: pathological analysis, reviewed and approved final manuscript; Mohamed S. Kohla: patients' accrual, participated in study design, reviewed and approved final manuscript; Mohamed H. Abdel Rahman study design and overall supervision, pathological analysis and editing final manuscript.

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Ethical approval

The research has been approved by the IRB of the National Liver Institute, Menoufia University Egypt. IRB protocols 005/2008 and 0051/2012.

Conflict of Interest

Authors report no conflict of interest.

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