Analysis of P53 Gene Mutations in Peripheral Blood Lymphocytes of Egyptian Spray Workers Exposed to Multiple Pesticides

Mohamed A. Noaishi [1], Samir H. Haggag [2] and Mostafa M.M. Afify[3]
[1]Department of Mammalian Toxicology, Central Agricultural Pesticides Lab (CAPL), Ministry of Agriculture, Egypt
[2]Department of Medicinal Chemistry, National Research Center (NRC) Egypt
[3]Department of Forensic Medicine and Clinical Toxicology, Faculty of Medicine, Bani Sweif University, Egypt
***Corresponding author: noaishi@gmail.com**
samirhih@yahoo.com

Abstract: The advanced research of the molecular genetic toxicology is focused on the fundamental molecular mechanisms involved the risk of mutations in genes related tumor. Mutations of the p53 tumor suppressor gene plays an important role in the development of common human malignancies. Previous reports revealed that the tumor suppressor gene p53 is considered to be the most frequently mutated gene in human tumors. The present study was designed to investigate the association among three factors, which focusing on occupational exposure to pesticides, aging and smoking habit and their effect on p53 gene mutation in fresh blood lymphocytes of workers occupationally exposed to a mixture of pesticides in outdoor fields in El-Fayoum governorate, Egypt. Because p53 gene mutation is associated with many factors not one factor effects on the result but may be many factors affect on this result. So we used a multiple Linear Regression statistical test to give the effect of each factor individual as well as the interaction among these factors and the results showed that there was a significant Linear Regression of p53 mutations with age and smoking factors with (P = 0.005) and (P = 0.002) respectively but there is no significant with pesticide exposure factor (P = 0.528). P53 genetic mutation occurs relatively with age after 40 years old and the workers in any occupation when they are smokers will be prone to P53 gene mutations than other environmental factors. In conclusion, PCR-"cold" SSCP is a rapid and sensitive method for identifying p53 genetic mutation and useful as biomarker but at least should be used with many other biomarker tests to give a clear picture about environmental genotoxicity.

[Mohamed A. Noaishi, Samir H. Haggag and Mostafa M.M. Afify. **Analysis of P53 Gene Mutations in Peripheral Blood Lymphocytes of Egyptian Spray Workers Exposed to Multiple Pesticides.** Journal of American Science 2011;7(3):538-543]. (ISSN: 1545-1003) . http://www.americanscience.org.

Key Words: Occupationally exposed, Pesticides, Age, Smoking, p53, SSCP

1. Introduction:

In recent years the pesticide problem has been focused of public interest. The reason is that these chemicals have a very important role in agriculture but in the same time the possible threat to human health has also been increased. Most of the pesticide sprayers in Egypt are not protected by safety measures when using the pesticides in the fields; therefore they are directly exposed to these compounds whose toxicity is ranged from moderate to hazardous. Many pesticides have been tested for mutagenicity by a variety of *in vitro* and *in vivo* assays, and mutagenic compounds have been found among all major categories of pesticides, including insecticides, fungicides and herbicides.

A limited number of field studies also have been done aiming to evaluate their genetic risk, at least at the molecular genetic level. In recent years, p53 tumor suppressor gene mutation has been found to be the most common genetic alternation in human cancers (Hollstein *et al.,* 1991). P53 protein appears to function in a cell cycle control as a negative regulator of cell division in the G1-S phase (Hartwell, 1992; Kastan *et al.,* 1991; Livingstone *et al.,* 1992).

The location and type of p53 gene mutation can reflect exposure of humans to certain types of carcinogenic agents according to (Wang *et al.,* 1996).Genetic mutations in genes controlling cell growth and proliferation, have been potential in discovering crucial biological alterations associated with long-term exposure to carcinogens; consequently, they may aid in leading to tumor formation. In this context, mutations of the tumor suppressor gene p53, which encodes a multi factorial transcription factor controlling cellular response to DNA damage (Levine, 1997) represent a potentially useful biomarker in the search for etiology, molecular mechanisms, and, hopefully, prevention of environmental cancers (Harris, *et al.,* 1996, Sidransky, *et al.,* 1996 and Hollstein *et al.,* 1997). So the present study was designed to investigate the association between three factors, which focusing on occupational exposure to pesticides, aging and smoking habit and their effect to induce mutation in the p53 gene. We supposed the mutations occurring at the molecular genetic level may be useful as biomarker of the DNA damage after exposure to pesticides. So we test the genetic damage in DNA of fresh blood lymphocytes for a population of workers occupationally exposed to a

mixture of pesticides in outdoor fields with addition two factors aging and smoking habit.

2. Material and Methods

2.1 Subjects and blood samples

Prior to the study, a questionnaire was obtained from each subject to determine the lifestyle of each subject, age, smoking, smoking years, exposure to pesticides and exposure years. Seventy four subjects were selected for this study. They were lived in the same geographical area located in El-Fayoum governorate, Egypt. They were subjected to various mixtures of pesticides by inhalation, skin and eye contact. On the other hand, twelve healthy young individuals living in the same area and with no history of occupational exposure to pesticides or smoking habits were selected as a control group. The blood samples collected from each subject using heparinized vacutainers.

2.2 DNA extraction from fresh blood lymphocytes

Lymphocytes and other mononuclear cells were isolated from 2mL heparinized peripheral blood sample using a standard method (Histopaque-1077, Sigma). Genomic DNA was isolated from Lymphocytes using a standard method described by (Gustincich et al., 1991) with few modifications by authors. The suspended lymphocytes was added to 1.5ml microfuge tube containing 400µl lysis solution (25 mM Na_2EDTA, 2 % SDS) then pipetted up and down approximately 10 times to lyse the cells. 150µl Protein precipitation solution (10 M Ammonium Acetate) was added to cell lysised. The tubes were inverted gently no more than 3 times and place into an ice bath for 10 minutes. The tubes were centrifuged at 10000 rpm for 3 minutes. The precipitated proteins formed an irregular brown pellet. The supernatant (which containing the DNA)was pipette into a clean 1.5ml microfuge tube containing 600 µl 100% Isopropanol then mixed by inverting gently no more than 3 times. The tubes were centrifuged at 12,000 rpm for 6 minutes; the DNA will be visible as a small transparent pellet. The supernatant was poured off. A 300 µl of 70% ethanol was added and the tubes were inverted several times to wash the DNA pellet. The tubes were centrifuged at 12,000 rpm for 3 minutes. Carefully, the ethanol was pour off by draining each tube on clean absorbent paper and allowed them to air dry for 10-15 minutes. Finally, 50µl of TE buffer was added to each tube (this gave a concentration of 1µg/2µl). The solubilization of the genomic DNA pellet was facilitated by incubated for an hour at 55°C.

2.3. PCR amplification of the p53 gene

Most of the mutations reported in human p53 gene are clustered in exons 5-8, which are highly conserved among different species (Levine et al, 1991 and Hollstein, et al, 1991). The sequences of the primers and the annealing temperature are listed in Table (1). Oligonucleotide sequences of primers were cited from (Thongsuksaia et al., 2003). PCR reactions were performed in a 50µl volume containing 39.5?l sterile dd water, 5?l 10X optimized reaction buffer (DyNAzyme, Finnzymes), 2?l of 10mM dNTP Mix, 0.5?l of each sense and antisense primer(100 ?M stock), 2?l of DNA sample (0.5 ng/?l), 0.5?l of DyNAzyme Taq polymerase (2U/?l). Amplification was carried out in a DNA Thermal Cycler (PTC-100, MJ Research).

A hot-start PCR is used to reduce primer-dimer formation by added Taq DNA Polymerase after the reactions heated to 94°C for 5 min then run the PCR conditions, denaturation at 94°C for 1 min, annealing temperature (below 2 degree than melting temperature) for 1 min, and 72°C extension for 1 min. followed by 35 cycles. The final extension was conducted at 72 °C for 5 min. 10?l of the PCR product was added to 2?l of 6x loading dye then loaded onto a 1% agarose gel, stained with ethidium bromide, and photographed under UV light. This step was important to ensure the specific molecular weight of the p53 exons were obtained.

Table (1) the sequences of the primers of p53 exons 5-8

Primers		Sequences		Fragments length (bp)	Annealing Temperature
Exon 5 Sense	5`	TCTTCCTACAGTACTCCCCT	3`	205	55 °C
Antisense	5`	AGCTGCTCACCATCGCTATC	3`		
Exon 6 Sense	5`	GATTGCTCTTAGGTCTGGCC	3`	136	57 °C
Antisense	5`	GCAAACCAGACCTCAGGCGG	3`		
Exon 7 Sense	5`	TTATCTCCTAGGTTGGCTCT	3`	130	59 °C
Antisense	5`	GCTCCTGACCTGGAGTCTTC	3`		
Exon 8 Sense	5`	TCCTGAGTAGTGGTAATCTA	3`	157	58 °C
Antisense	5`	GCTTGCTTACCTCGCTTAGT	3`		

2.4. Molecular analysis of P53 mutations

A rapid method for single-strand conformation polymorphism analysis of PCR products (SSCP) that allows using ethidium bromide staining was firstly described by (Hongyo et al.1993). This method is based on the PCR-amplified products can be separated into single strands by denaturation and electrophoresed on non-denaturating polyacrylamide gels. Under non-denaturating conditions, single-stranded DNA adopts a secondary structure that is dependent upon its sequence. Changes in the sequence (eg, point mutations) can cause a shift in the electrophoretic mobility of the analysed conformations compared with wild type DNA. In SSCP, detectable mutations include base substitutions, small insertions, deletions, and rearrangements (Peltonen, et al., 2007). Thus, the (PCR-SSCP) is a powerful approach for qualitative analysis of the DNA (Yap and McGee, 1992and Mohabeer et al., 1991). For SSCP analysis, 10?l of PCR product was mixed with 10?l gel loading dye (98% formamide, 10mM NaOH, 20mM EDTA, 0.05% bromophenolblue, 0.05% xylenecyanol) in 0.2ml ultra thin reaction vial. Denature the mix at 100°C, 7 min. in a heating block then putted in ice for 5min. The mixture was loaded on the 12% polyacrylamide gel (10 × 8× 0.1 cm3). The ratio of acrylamide/bisacrylamide was (37.5:1). Electrophoresis was carried out in 1.5x TBE buffer, initially at 200 V for two minutes, followed by 120 V for 4.5 hours, using the refrigerator as cooling system at 10°C. After the gel run was completed, the gel stained with 0.5mg/ml solution of ethidium bromide in 1 X TBE buffer for 15 minutes followed by washing in distilled water. The bands were visualized by using a 340 nm UV transilluminator and photographed.

3. Statistical Analysis

Statistical analyses were performed using the Multiple Linear Regression test. For all statistical tests, P-values < 0.05 were considered significant. Data were analyzed using SigmaPlot, Ver. 11 Statistics software. The obtained data by PCR and SSCP techniques were analyzed using Gel-Pro analyzer Ver. 3.1 software

3. Results

As shown in Fig. (1) The clear bands of PCR product (double-stranded DNA) show the specific molecular weight base pair (bp) of exons 5-8 of human p53 gene.

Mutations in exons 5–8 of the p53 gene were screened by cold SSCP analyses, and the mutation detects by monitoring the mobility shift of the normal two single strand bands of each exon which illustrated in Fig (2). Our results are summarized in Table(2), the results related to the age factor revealed that the high percentage of p53 gene mutation among workers with age ≥ 40 years, 22 cases (48.5%) and 1case (3.4%) among the workers with age < 40 years. The high significant multiple Linear Regression between the age and p53 mutation was high significant (p= 0.005). On the other hand, the negative control group consisting of young adult people with average age 18 years old did not show any mutation in the p53 gene.

The results related to exposure factor revealed that, the workers who occupationally exposed to pesticides more than 15 years were 15cases (51.7%) have mutations in p53 geneand8 cases (17.8%) for workers who exposed to less than 15 years and there is no significant regression between the mutation and the pesticide exposure factor (p= 0.528).

However, the results related to smoking factor investigate that 11 cases of workers who were smoking more than 20 years exhibited mutations with 84.6% than workers were smokers less than 20 years that showed only 7 cases with 28.0%. The smoking factor indicated high significant multiple Linear Regression (p= 0.002) but the non smoking workers revealed only 5cases of mutation (13.9%). Furthermore, according to our results the p53 gene mutation occurred only in exons 5 and 7, and we did not find any mutations either in exon 6 or 8.

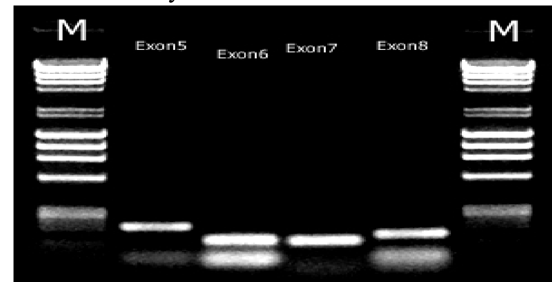

Fig. (1) PCR product shows molecular weight of exons 5-8 of human p53 gene (bp). (lane 1,6) DNA Marker a mix of Hind III digest and Hae III digest.

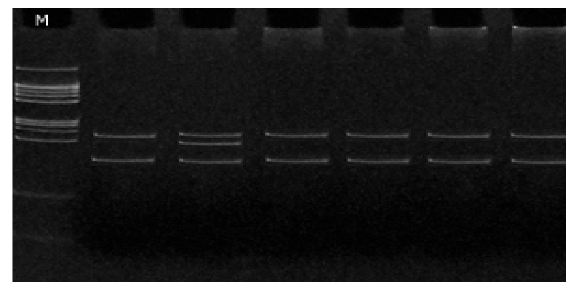

Fig. (2) SSCP analysis of P53 exon5. The band pattern reveals whether a mutation exists (lane 3) with heterozygous wt/mut in the same case; (lane 2) has no mutation and seems similar to (lanes 4, 5, and 6) which belong to control group; (lane 1) DNA marker. mix of Hind III digest and Hae III digest.

Table (2) the regressions between factors related to subjects and p53 Gene mutations(N=74)

Factors	No of subjects (%)	P53gene Mutations Positive (%)	P53gene Mutations Negative (%)	*P-values**
Age(y)				
<40	29 (39.2)	1 (3.4)	28 (96.5)	0.005
≥40	45 (60.8)	22 (48.9)	23 (51.1)	
Exposure duration				
< 15	45 (39.2)	8 (17.8)	37 (82.2)	0.528
≥15	29 (60.8)	15 (51.7)	14 (48.3)	
Non- Smoking	36 (48.6)	5 (13.9)	31 (86.1)	
Smoking				
< 20	25 (33.8)	7 (28.0)	18 (72.0)	0.002
≥20	13 (17.6)	11 (84.6)	2 (15.4)	

4. Discussion:

The incidence of P53 mutation varied in different population, which may be partly a result of the inclusion of people from different geographic areas and differences in the technique used to analyze the mutations (Hiseh et al., 2001). In the present study, we studied the probability of three factors to induce mutations in the p53gene as a biomarker of genotoxicity. These factors were focusing on occupational exposure to the pesticides, aging and the smoking habits. The statistical correlation test between pairs of variables was not performed because the investigation of our study (p53 gene mutation) is associated with many factors not one factor effects on the result but may be many factors affect on this result. So a Simple or multiple Linear Regression is the most commonly statistical test used in this case. By this test, we can discuss each factor as individual and then concluded the effect of all the factors together.

The age factor

Our results indicated the increase of percentage in p53 mutation by age older than 40 years. These results is appeared to be in agreement with (Tiwawechac et al., 2010) from 2.01 to 2.63 fold by age >40 to >50 years in the patient have Nasopharyngeal carcinoma (NPC) in Thailand. The study also revealed the polymorphism of gene p53 carriers with age of >40 years. our results are also in agreement with (Rugge et al., 2000)who found The rate of p53 gene mutationsin gastric cancers patients < 40 years are low versus (Hsieh et al., 2000) found the increasing in frequency of p53 gene mutations in intestinal-type tumors in elderly populations. Also studies related to age and p53 mutations seem to suggest the same idea(Pollack, et al., 2001) studied the relationship between frequency of p53 gene mutation exons 5-8 and age in childhood malignant gliomas and reported that children <3 years of age the frequency of mutations (2 of 17 tumors, 11.8%) and between 3 and 10 years old the mutations were 12 of 27 tumors (44.4%) and12mutations of 33 tumors (36.4%)from children between 10 and 18 years old at diagnosis. Therefore the age factor may be apparent has an essential role in the p53 gene mutation.

The pesticide exposure factor

There is an association between occupational exposure to complex of pesticides and the genotoxicity which have been established in a number of studies in long ago (Sorsa *et al.,* 1990) and (Hagmar *et al.,* 1994). Also previous studies have shown that loss of P53 function leads to genomic instability (Livingstoneet al., 1992). Recently Loyant et al., 2005 investigated the P53 mutations in exons 2-11 by denaturing high performance liquid chromatography (DHPLC) and they explored the hypothesis that occupational exposures to pesticides and organic solvents could raise the frequency of P53 mutations in brain tumour cells. These mutations are present in approximately 30% of brain tumours. On the other hand our results indicated no significant between pesticide exposure factor and the incidence of mutation in p53 gene this result seems to in parallel with previous study(Pastor et al., 2001) reported that no statistically significant differences in the frequencies of cytogenetic damage were detected between exposed pesticide worker and control individuals in lymphocyte cultures but the multiple linear regression analysis indicated that the cytogenetic endpoints were inversely influenced by other factors which included also in the control group e.g (alcohol, tobacco, and coffee, etc.), and may be these factors together affect on the result not only one factor .

Our results showed high significant multiple Linear Regression between p53 gene mutations and

smoking factor than any other factors. These results are in agreement with several other studies (IARC, 1986: Hackshaw et al., 1997) reported that the smoking is the most important risk factor for lung cancer, but passive smoking increases the risk as well as. Also these results appear to be in agreement with the previous report (Bennett et al., 1999; Hainaut and Pfeifer, 2001; Pfeifer et al., 2002) they reported that Both qualitative and quantitative data link To P53 gene mutations at CpG sites in lung cancer with smoking and the incidence of P53 mutations is10–26% in non-smokers and about 60% in smokers with overrepresentation of G to T base pair shift mutations. Thus, the existing molecular epidemiology studies on different aspects of smoking-related lung cancer support theP53 gene as a target of carcinogens in cigarette smoke and a tobacco-specific P53 mutation spectrum at a population level. However, so far no studies exist on P53 mutations specifically in lung cancers putatively due to occupational exposure to passive smoking. Versus studies have been noted the passive smoking effect on P53 mutations by (Husgafvel-Pursiainen et al., 2000) showed that lung cancers of passive smokers have an increased risk for p53 mutations. In western Chinese's population, the high incidence of P53 mutation (42.56%) is associated with known risk factors, such as tobacco smoking and alcohol drinking (Ostwald. et al., 2000). Recently (TANG et al., 2010) investigated that the high incidence of P53 mutation has proven to be related to tobacco smoking in most oral squamous cell carcinoma patients. It seems that, P53 gene mutation frequency is increased in smoking, the effect may be strong enough to overcome the effect of other carcinogens. Furthermore, recent studies have shown that various kinds of carcinogens produced by smoked cigarettes as polycyclic aromatic hydrocarbon (PAH) might be responsible for different p53 gene mutations and p53 over-expression (Taghavi, et al., 2010). Therefore, it has been hypothesized that continuous exposure to specific carcinogenic components of tobacco smoke may cause mutations in some important cell cycle genes, such as p53, leading to over-expression and abnormal accumulation of the translated proteins. This specific mutation may result in the formation of a dysfunctional protein, which is sequestered and accumulated in the cell, leading to cancer development. This means that workers in any occupation when they are smokers or exposed to tobacco smoke will be prone to P53 gene mutations and at risk to cancer initiation through the inactivation of p53.

5. Conclusion

It can be concluded that PCR with cold-SSCP analysis is a rapid and sensitive method for identifying p53 gene mutation. Although p53 genetic mutation polymorphisms play an important role to explore the effects of gene-environment interaction, probably not a lone is useful as biomarker but at least should be used with many other biomarker tests to give a clear picture about environmental genotoxicity.

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1/12/2011