Mutation Analysis of K-*ras* Gene in Peripheral Blood Lymphocytes of Egyptian Workers Occupationally Exposed to Multiple Pesticides

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Abstract: Proto-oncogenes are cellular genes that are expressed during normal growth and developmental processes. Mutated versions of normal proto-oncogenes have been implicated in the development of human neoplasia. The present study is designed to investigate the association among three factors, occupational exposure to pesticides, aging and smoking habit and their effects to induce k-*ras* gene mutation in lymphocytes of Egyptian workers occupationally exposed to a mixture of pesticides in outdoor fields in El-Fayoum governorate. The k-*ras* gene mutation can occur by many factors and not one factor induces the mutation. So we used a multiple linear regression statistical test to estimate the interaction among these factors. We have found k-*ras* mutations occurred in 9 of 45 workers ≥40 years while in workers <40 years the mutation was 1 of 29 and the workers who occupationally exposed to pesticides more than 15 years showed 9 cases of 29 (31.0%) and between workers exposed to less than 15 years showed 1 case of 45 (2.2%). In addition the results investigated the workers who were smoking more than 20 years exhibited mutations in 7 cases of 13 (53.8%) than workers were smoking less than 20 years who showed only 3 cases of 25 (12.0%). There is no significant multiple linear regression of both age and pesticide exposure factors (P= 0.766 and P = 0.232, respectively) but there is a significant association between k-*ras* mutation and smoking factor (P < 0.043). More addition a highly significant multiple linear regressions are found between k-*ras* mutation and smoking years factor (P < 0.001). In conclusion the interaction between tobacco smoking and other factors like aging and occupational exposure to pesticides may play together the main role of k-*ras* gene mutation and the sequential studies in the various animal models may be useful to give advanced information how these environmental mutagens affect on these genes.

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1. Introduction

Humans are unavoidably exposed to pesticides because their use in agriculture has been increasing steadily all over the world. Several epidemiological studies demonstrated that occupational exposure to some pesticides may be related to several kinds of cancer, including leukemia **(Blair and Zham, 1995)**, bladder cancer **(Webster *et al.*, 2002)** and pancreatic cancer **(Clary and Ritz, 2003)**.

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 **(Mansour, 2004)**.

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 molecular genetic level. Recent studies suggest that the activation of protooncogenes by genetic alterations may play a role in leading a cell to neoplastic development. These genetic alterations include gross chromosomal rearrangements, amplification of genes, and point mutations.

Previous evidence investigated that tumors development, at least in part, is the result of the abnormal activation of a small set of cellular genes. These genes, termed proto-oncogenes, were originally discovered as transforming genes by certain type of viruses called retroviruses (**Bishop, 1985**). Subsequent studies have established that these proto-oncogenes can also be activated as oncogenes by mechanisms independent of retroviruses **(Weinberg, 1985)**. Mechanisms for the conversion of proto-oncogenes to activated oncogenes include point mutations, gene amplification, chromosomal rearrangements, and promoter insertion **(Stowers *et al.*, 1987)**.

The most common activated oncogenes in human cancers are ras oncogenes. This oncogene

family contains three closely related cellular genes: K-*ras*-2, H-*ras*-1, and N-*ras* **(Bos, 1988)**. The K-*ras* gene codes for a protein that plays an important role in the epidermal growth factor receptor (EGFR-receptor). This protein regulates other proteins, downstream in the signaling pathway of dividing cell and proliferation. Mutant k-*ras* protein leads to unregulated in cell proliferation that may contribute to progression in tumor **(Salomon *et al.*, 1995).**

The k-ras genes are almost exclusively activated by single base-pair substitutions and this activation occurs usually via a point mutation at the 12th, 13th, or 61st codons in human tumors or tumor cell lines **(Barbacid, 1990)**. Studies in a variety of animal model systems have shown that specific activation of a protooncogene by point mutation can be caused by chemical or physical insult **(Stanley, 1995)**.

In previous work (**Noaishi *et al.*, 2011)** demonstrated that numbers of mutations have been found in P53 tumor suppressor gene in lymphocytes of Egyptian Pesticide workers but these mutations may have not actual effects because the another allele in the second chromosome stills has effect and play the role to break the uncontrolled cell division in the same cell. So this gene needs homozygosity in phenotype to be shown the lost of its function. Otherwise the Oncogenes family which is positively regulates the cell cycle and one mutation in this gene cause excess in cell proliferation (Fig. 1).

Therefore the present study is performed to complete the previous work which was on the p53 gene as tumor suppressor gene and this study on k-ras gene as oncogene. Also to investigate how the analysis of these genes may be useful as biomarker of the DNA damage after exposure to mutagens.

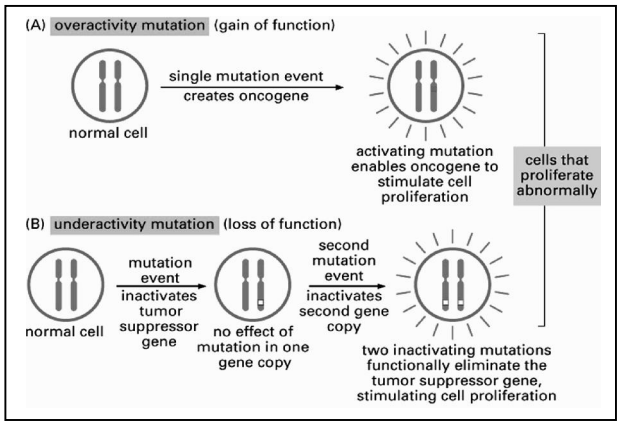

Fig. (1) The scheme shows in tumor suppressor gene the function is lost if both genes in two chromosomes become mutated but in oncogene one mutation in one chromosome is enough to stimulate the cell proliferation. Cited from Molecular Biology of the Cell, 4th edition

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 are 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. In addition a twelve healthy young individuals living in the same area and with no history of occupational exposure to pesticides or smoking habits were selected as control group. The blood samples collected from each subject using heparinised vacutainers.

2.2 DNA extraction from fresh blood lymphocytes

Lymphocytes and other mononuclear cells were isolated from 2mL heparinised peripheral blood sample using a standard method (Histopaque-1077, Sigma), and resuspended with 250 µl PBS. 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. 300µl 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 k-*ras* oncogene

The sequences of the specific primers and the annealing temperature are listed in Table (1).

Oligonucleotide sequence of primers was cited from **(Yamashita *et al.* 2001)**. PCR reactions were performed in a 50µl volume containing 39.5µl sterile dd water, 5µl 10X optimized reaction buffer (DyNAzyme, Finnzymes), 2µl of 10mM dNTP Mix, 0.5µl of each forward and reverse primer (100 µM stock), 2µl of DNA sample (0.5 ng/µl), 0.5µl of DyNAzyme Taq polymerase (2U/µl). 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 the PCR conditions run, 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. 10µl of the PCR product was added to 2µl 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 insure the specific molecular weight of the K-*ras* oncogene exons was obtained.

Table (1) the sequences of the K-*ras* gene primers

Primers		Sequences			Fragments length (bp)	Annealing Temperature
Exon 1	Forward	5`	GACTGAATATAACTTGTGG	3`	108	51 °C
	Reverse	5`	GCTATTGTTGGATCAATATTC	3`		
Exon 2	Forward	5`	GATTCCTACAGGAAGCAAGT	3`	185	43 °C
	Reverse	5`	TAATGGTGAATATCTTC	3`		

2.4. Molecular analysis of k-*ras* mutations

A rapid method for single-strand conformation polymorphism (SSCP) analysis of PCR products 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 the wild type DNA. Detectable mutations include base substitutions, small insertions, deletions, and rearrangements can monitor by SSCP technique (**Peltonen *et al.*, 2007**). Thus, the (PCR-SSCP) is a powerful approach for qualitative analysis of the DNA (**Yap and McGee, 1992** and **Mohabeer *et al.*, 1991).** For SSCP analysis, 10µl of PCR product was mixed with 10µl 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 1.5 x TBE buffers, 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.

Statistical Analysis

Statistical analyses were performed by 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. (2) The bands of PCR product (double-stranded DNA) show the specific molecular weight base pair (bp) of exons 1 and 2 of human k-*ras* gene. The mutations observed only in exon 1 and exon 2 did not show any mutation.

Abnormal SSCP patterns showed more bands than those expected in the wild type that suggesting the coexistence of distinct heterozygous of phenotypes. This pattern is illustrated in Fig (3) and our results are summarized in Table (2); the results related to age factor revealed that the relative increase in the percentage of k-*ras* gene mutation among workers aged more than 40 years, 9 cases of 45 (20.0%) and one case of 29 (3.4%) among workers aged less than 40 years but we found no significant multiple Linear Regression between k-*ras* mutation and the age factor (P= 0.766). Furthermore the negative control group consisting of young adult people with average age 18 years old did not show any mutations in this gene. The results related to exposure factor revealed that, the workers who occupationally exposed to pesticides more than 15 years showed 9 cases of 29 (31.0%) and between the workers exposed to less than 15 years showed one case of 45 (2.2%). Also there is no significant

multiple linear regression between the mutation and the pesticide exposure factor (P=0.232). On the other hand the statistical analysis of the mutations related to smoking factor revealed a significant association between K-*ras* mutation and smoking factor (P < 0.043). More addition a highly significant correlation was found between the K-*ras* mutation and the smoking years factor (P < 0.001) and the results investigated that 7 cases of 13 (53.8%) who were smoking more than 20 years exhibited mutations than workers were smokers less than 20 years who showed only 3 cases of 25 (12.0%). Furthermore the non smoking workers 36 subjects (48.6%) did not show any mutation.

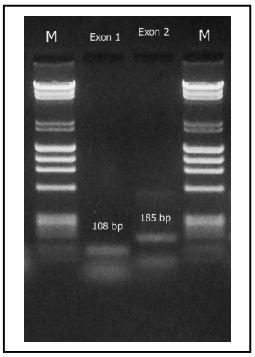

Fig. (2) PCR products show molecular weight of exon1 108 (bp), exon2 185 (bp) and (lane 1,4) DNA Marker (a mix of Hind III digest and Hae III digest).

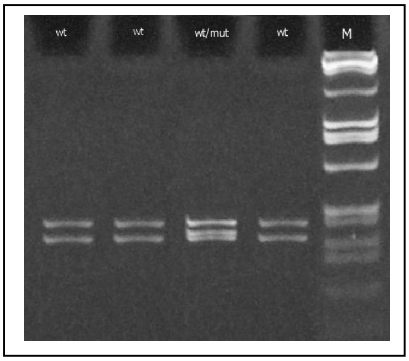

Fig. (3) SSCP analysis of k-*ras* exon1. The band pattern reveals a mutation exists in lane 3 with heterozygous wt/mut in the same case; lanes 1, 2, and 4 which belong to control group have no mutations and lane 5 DNA markers mix of Hind III digest and Hae III digest.

Table (2) the correlation between factors related to subjects and k-*ras* gene mutations

Factors	Total Subjects (n =74)		K-*ras* mutated (n= 10)		K-*ras* wild type (n= 64)		*P-values**
	No.	(%)	No.	(%)	No.	(%)	
Age(y)							0.766
<40	29	(39.2)	1	(3.4)	28	(96.6)	
≥40	45	(60.8)	9	(20.0)	36	(80.0)	
Exposure to pesticides(y)							0.232
< 15	45	(60.8)	1	(2.2)	44	(97.8)	
≥ 15	29	(39.2)	9	(31.0)	20	(68.9)	
Smoking(y)							0.001
< 20	25	(33.8)	3	(12.0)	22	(88.0)	
≥ 20	13	(17.6)	7	(53.8)	6	(46.2)	
Non-smoking	36	(48.6)	0	(00.0)	36	(100.0)	

4. Discussion

The incidence of *ras* mutation varies and is greatly dependent on the tissue or cell type from. Although the k-*ras* mutation occurred in 75% to 95% of pancreatic carcinomas this percentage decreases to 50% in colon carcinomas **(Jebar *et al.*, 2005)**. Also k-ras gene mutation is associated with many factors not one factor affects on the result but may be many factors affect on this result. So a 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 k-*ras* mutation by age older than 40 years. Previous studies related to age and sex factors reported the low frequency k-*ras* mutations in the colon cancer of younger male patients than in older, and in women is to be higher than in men (**Breivik *et al.*, 1994)** but according to the statistical analysis of our results did not show significant effect (P=0.766) may be due to the effect of other factors. Several lines of evidence strongly suggest that no significant association between K-*ras* mutation status and age or sex factor **(Alguacil *et al.*, 2002)** who reported that there is no differences in the frequency of k-*ras* mutations according to age and in the previous work (**Robert *et al.*, 1990)** found there was no association between the K-*ras* point mutation and the age. Also **Park *et al.* (2004)** investigated the k-*ras* mutation in

colon cancer by use of oligonucleotide microarrays and they found no significant relationship between the K-*ras* mutation and sex or age. Therefore the age factor may be apparent has no essential role in the k-*ras* 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)**. In our study the result related to the pesticide exposure indicated the increase of k-*ras* mutation in workers exposed to pesticides ≥15 years 9(31%) versus the workers < 15 years exposure 1(2.2%) although the multiple linear regression analysis indicated that no statistically significant association between K-*ras* mutation and pesticide exposure factor because this endpoint influenced by other factors e.g (age, sex, and smoking, etc.), and may be these factors effect together on the result not only one factor .

This result seems to be in parallel with recently study **(Soliman *et al.*, 2007**) reported that the rates of K-*ras* and p53 mutation in exons 5–8 were higher in tumors of Egyptian pancreatic adenocarcinomas patients from the high-pollution region (61.5%) as compared with the low-pollution regions (34.2%) and they suggested that the environmental factors and pollution may explain these differences.

The smoking factor

Our results showed only high significant multiple Linear Regression between k-*ras* gene mutations and smoking factor versus the two previous factors. The results investigated there is a significant association between k-*ras* mutation and smoking factor ($P < 0.043$). More addition a highly significant multiple linear regressions were found between k-*ras* mutation and smoking years factor ($P < 0.001$). These results are in agreement with several previous studies (**IARC, 1986: Hackshaw *et al.*, 1997)** reported that the smoking is the most important risk factor for lung cancer, and passive smoking increases the risk as well as. **(Husgafvel *et al.*, 1993)** investigated a clear association between K-*ras* mutations and the heavy life-time smoking i.e.(≥50 years of cigarette smoking). Also the results appear to be in agreement with (**Ahrendt *et al.*, 2001**) who found the cigarette smoking is strongly associated with mutation of the K-*ras* gene in patients with primary adenocarcinoma of the lung.

In contrast to **(Miller *et al.*, 2007)** who reported that the presence of *K-ras* mutations was not related to smoking history when compared with never smokers, there was no significant difference in frequency of *K-ras* mutations for lung cancer patient with history of cigarette smoking and non-smokers. More over (**Gregory *et al.*, 2008**) mentioned based on their data the frequency of *KRAS* mutation was not associated with age, gender, or smoking history. The number of pack years of cigarette smoking did not predict an increased likelihood of *K-ras* mutations.

Recently, (**Gregory *et al.*, 2009**) evaluated the frequency of *K-ras* mutations in lung adeno-carcinomas from nearly 500 patients, and noted that *K-ras* transition mutations (G→A) were more common in patients who had never smoked cigarettes. In contrast, transversion mutations (G→T or G→C) were more common in former or current smokers. This information suggests that while some mutations in *K-ras* are associated with cigarette smoking, another type of *K-ras* mutations do occur also in never-smokers.

Therefore these findings support the hypothesis that smoking influences increase the risk of induction, acquisition and persistence of *K-ras* mutations and not the unique reason.

Conclusion

The interaction between tobacco smoking and other factors like aging and occupational exposure to pesticides may play together the main role of k-*ras* gene mutation and the sequential studies in the various animal models may be useful to give advanced information how these environmental factors affect on these genes.

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