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RESEARCH ARTICLE

Genetic aberrations of the K-ras proto-oncogene in bladder cancer in relation to pesticide exposure

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Abstract

In Egypt, bladder cancer is one of the most popular cancers, accounting for 31% of all cancer cases. It ranks first in males about 16.2% of male cancer. The incidence in rural areas among males is near 32 per 100,000. The exact etiology of bladder cancer is still unknown; K-ras gene is known as a critical DNA target for chemical carcinogens such as pesticide. Some occupational hazard exposure is thought to be directly genotoxic, while others might enhance the mutagenicity and carcinogenicity of directly acting genotoxic agents. Analysis of the relationship between pesticide exposure and mutation in the K-ras gene in human bladder cancer. One hundred patients were diagnosed with bladder cancer and two hundred controls attended the outpatient clinic; after taking consent and filling a questionnaire for age, sex, occupation and pesticide exposure, surgically resected specimens were collected and the samples were used to determine the k-ras mutation. Blood samples were taken to analyze the level of acetylcholinesterase enzyme and level of P_{53}. The present study indicated that pesticide exposure may play a great role in malignant transformation of the bladder cells through mutation in the K-ras gene; there was a significant correlation between the acetylcholinesterase enzyme level and k-ras mutation ($p < 0.001$). The results revealed that the level of P_{53} was significantly high in comparison with the control group ($p < 0.001$). These findings give an alarm to decrease the amount of pesticides used in our area; also, p_{53} may be used as an indicator to bladder cancer.

The original publication of this paper contains a mistake.

Line 6 in the abstract, line should read "One hundred patients were diagnosed with bladder cancer and two hundred controls attended the outpatient clinic;"

2nd paragraph of the Methods section, the correct line should read "This study included 100 cases newly diagnosed with histopathologically proven bladder cancer and 200 controls who attended the outpatient clinic."

The original version of this article was revised.

Highlights:
- Pesticide exposure is one of the most dangerous poisons in many countries.
- One of the most common causes for occurrence of bladder cancer is pesticide exposure.
- Relationship between exposure to pesticide and bladder cancer is k-ras mutation.
- K-ras gene mutation was found to be related to bladder cancer occurrence and severity.

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Keywords Bladder cancer · Acetylcholinesterase · K-ras · Mutation

Background

Bladder cancer is a great health problem all over the world. It ranks the ninth worldwide in the incidence of cancer (Public health impact of schistosomiasis 1993). It represents 16.2% of male cancer and considered the first cancer among males (Khaled 2005). The incidence in males of rural areas in Egypt is about 32 per 100,000 (Amal and EL Sebai 1983).

The main etiology of bladder cancer is still obscure. Many risk factors have been involved in the pathology of bladder cancer, which include cigarette smoking (American Cancer Society 2006); synthetic nitrogen fertilizers (Mensing et al. 2003); organophosphate-based pesticides (Webster et al. 2002); aromatic amines (Xifeng et al. 2007); pelvic irradiation, A cyclophosphamide, chronic cystitis, and schistosomiasis (American Cancer Society 2006); human papilloma virus (LaRue et al. 1995); and some occupational disease and genetic factors (American Cancer Society 2006).

The importance of these risk factors in the occurrence of bladder cancer differs according to the populations. Risk factors like family history, pesticide exposure, consanguinity between parents, and chronic cystitis play an important role than bilharziasis and smoking in the development of bladder cancer in our country (Zarzour et al. 2008). During the twentieth century, fertilizers and insecticides have been very popular to be used due to the increase of the population to minimize the loss of plants and to increase the yields of the crop. Pesticides is the most widely used method of controlling the majority of agricultural pests (Hunter 1989). Since the 1960s, the amount of pesticides in Egypt and Africa has increased about fivefold. During the last 40 years, about 1 million tons have been injected into the Egyptian environment (Amr 1999).

Persistence of organochlorine compounds in the environment, and their accumulation in the living organisms, is a major factor in causing the bad effects on specific organs. The risks to all populations from the use and misuse of pesticides are one of the most important health and environmental problems mostly in the third world countries (Amr 1999).

RAS gene family is performed from four functional genes [Harvey ras (H-ras), Kristen ras (K-ras) A and B, and Neuroblastoma ras (N-ras)] that encode closely related proteins which are localized in the internal part of the cell membrane and have intrinsic GTPase activity, which regulates their cellular activity. The main action of these family proteins is to activate the downstream kinases belonging to mitogen-activated protein kinase pathway, which lead to continuous mutation signals (Nanda et al. 2010).

Some studies have detected mutation of *RAS* mutations of different types in human bladder tumors (Cattan et al. 2000; Jebar et al. 2005; and Zhu et al. 2004). These studies show different types and range of mutation frequencies. It is not clear whether these differences are related to the exposure to pesticides or not.

P_{53} protein is almost undetectable because it is rapidly degraded. Once activated, the protein escapes the degradation and accumulates in the nucleus. Simultaneously, it is turned from a latent one to an active by some changes which activate its capacity to transactivate target genes. Many genotoxic and non-genotoxic agents may lead to this activation of P_{53}. These agents include those which lead to single- or double-strand breaks in DNA. Once the activation occurs, it can produce several cellular changes (Zeimet et al. 2000 and Tokino and Nakamura 2000).

According to that, the aim of the present study is to investigate the frequency of specific point mutations of the *RAS* gene family in a group of Egyptian patients suffering from bladder cancer who have been directly exposed to organophosphates. A secondary aim is to check if indirect exposure would cause the same mutation in the k-ras gene or not. The third aim is to find the relation between direct and indirect exposures to pesticides and level of P_{53} and the bladder cancer.

Research methods and techniques

Type of the study

This is a prospective case-control analytic study.

Outcome measures (primary/secondary)

-Detection of organophosphates in patients with bladder cancer as a risk factor (by questionnaire to evaluate the exposure and analysis of cholinesterase enzyme in blood to confirm the exposure to organophosphates)

-Evaluating the level of P_{53} as an indicator for cancer in the blood of the patient

-Detection of K-ras gene and incidence of its mutation in tissue of all patients with bladder cancer

-Comparing the incidence of K-ras mutation between those exposed to organophosphate and those who were not exposed to it

Sample size analysis

Sample size will be calculated by using the Epi Info program.

Patient suffering from bladder cancer and asking for treatment in South Egypt Cancer Institute in a period of 1 year was asked to share in the research after taking informed consent from them and to fill a questionnaire. Questions focused on occupation and lifestyle (including any method for

organophosphate exposure) and duration of employment or duration of exposure.

Method

This is a case-control hospital-based study measuring the correlation between the exposure to organophosphorus compounds (by history, and confirmed with the level of cholinesterase enzyme) with different pathological types of bladder cancer occurring in patients from different upper Egypt governorates, and level of P_{53} and K-ras gene. The matching control group was selected to match for residence, age and sex. They were selected from those with nonmalignant lesion in bladder as (bladder polyp).

This study included 100 cases newly diagnosed with histopathologically proven bladder cancer and 200 controls who attended the outpatient clinic. An informed consent was obtained from all participants after the explanation of the aim of the study. A well-structured questionnaire asking about the exposure to organophosphates was offered to the patients. Diagnosis of the cases was based on histopathological examination of the tumor. Patients diagnosed with bladder cancer who have received radio- and/or chemotherapy prior to the study were excluded. Personal interview was done to collect the following data: socio-demographic data (name, age, sex, occupation, residence) and clinical data of cases (Karimianpour et al. 2008).

Surgical specimens were collected and stored at − 80 °C; the patients were selected from those admitted in Assiut University Hospital.

Sample collection and testing

Surgically resected specimens were collected, and part of it were sent to the pathology laboratory in order to know the type of the mass; the samples were used to determine the mutation in k-ras gene.

Five milliliters of blood was collected from patients and controls on EDTA in vacutainer tubes to measure the level of cholinesterase enzyme and P_{53}.

Cholinesterase enzyme level was measured from blood samples which were collected using plain vacutainers tubes and proceeded quickly in the lab to prevent damage associated with storage according to the method of Knedel and Kin (1967).

P_{53} were measured using the human p_{53} ELISA kit from the blood of the patient according to the kit method (www.glorybioscience.com).

DNA extraction

The DNA was extracted from the primary tumor tissue and adjacent noncancerous tissues using the DNA extraction kit (proteinase K and phenol extraction) and then stored at 4 °C, for examining the mutations in the K-ras gene according to Nanda et al. (2010). In polymerase chain reaction (PCR), primer was designed from previous studies for amplifying sequences around codons 12 and 13 of KRAS; the primer sequences used were as follows: 5′-ACTGAATATAAACT TGTGGTAGTTGGACCT-3′ and 5′-TTCTCCATCAATTA CTACTTGCTTCCTGTA-3′ (Przybojewska et al. 2000).

Statistical analysis

The statistics were done using SPSS program version 15, to explore the risk factors for those patients especially the exposure to organophosphates and explore the relation between organophosphate, level of p53, the k-ras gene mutation, and the occurrence of bladder cancer.

Ethical aspects

An informed consent was taken from all the participants involved in the study before participation. The consent was taken in written form after giving the patient's full information about the research and after clarifying that there will be no hazards for him if he refused to share in the research as regards the services offered for him in the hospital. All ethical statements of the institute were followed to maintain the confidentiality of the results.

Results

This study was done to prove or reject the hypothesis of that exposure to organophosphates in pesticides which can lead to mutation in certain genes that lead to bladder cancer in patients who had been proved to have cancer by pathology and also to prove or reject the hypothesis that the P_{53} level correlates with the occurrence of cancer. The study was done on 100 patients of bladder cancer in Assiut University Hospital.

As shown in Table 1 summary of the data related to the patients and the control group, it seems that in our era, the occurrence of bladder cancer is more in males than in females. The median age group of the patient group was 57 years old (from 49 to 66 years old). There are many risk factors that related to the appearance of bladder cancer among the patients as shown in Tables 1 and 2: the presence of bladder stones ($p < 0.001$), positive family history of bladder cancer ($p < 0.001$), exposure to organophosphates (0.001), smoking (0.005), occurrence of recurrent cystitis (0.001), and bilharziasis (0.013). Table 2 shows the logistic regression of the significant risk factors that contribute in the development of the bladder cancer.

Table 3 shows the relation between level of acetylcholinesterase enzyme, the pesticide exposure, types of tumors, and occurrence of mutation in the K-ras gene in patients and in the control group. The table shows that there was a highly significant relation between these multiple factors. The most

Table 1 Basic characteristic of bladder cancer patients and controls at presentation (*T* test)

		Patients		Controls		*p* value
Type of pathology		100		200		
		UC	90	Polyp	37	
		SCC	4	Stone	103	
		Adenocarcinoma	6	Ulcer	7	
				TVP	34	
				TURP	19	
Median (IQR)		57 (49, 66)		56 (49, 66)		0.811
Sex	Male	89%		86%		0.466
	Female	11%		14%		
	Total	100%		100%		
% Rural residence		83		82		0.830
% Illiterates		65		60.5		0.075
% Farmers		43		42		0.652
% Exposure to pesticides		60		29		<0.001**
% Smokers		71		54		0.005*
% Bilharziasis		69		54		0.013*
% History of bladder stones		38		18.5		<0.001**
% Recurrent cystitis		33		16.5		<0.001**
% Positive family history of bladder cancer		32		7		<0.001**
% Parents' consanguinity		19		8.5		0.008

UC urothelial carcinoma, *SCC* squamous cell carcinoma, *TVP* transvesical prostatectomy, *TURP* transurethral prostatectomy

*Statistically significant

**Highly significant

common type of cancer bladder which is the UC type is highly correlated with the pesticide exposure and occurrence of the K-ras mutation.

Table 4 shows the relation between the acetylcholinesterase level (*AChE*) and the P_{53} level in the serum of bladder cancer patients and control group. It shows that there was a significant inhibition of *AChE* in patient group in comparison with that of the control group ($p < 0.001$). Inhibition of *AChE* level is related to high level of organophosphates in blood. In the same table, there is highly significant elevation of P_{53} level in those with bladder cancer in comparison with the control group ($p < 0.001$).

Table 5 shows the relation between inhibition of the *AChE* level and the k-ras mutation. Most of the patients with *AChE* inhibition had a k-ras mutation in 65 patients (65%) with *p* value < 0.001.

Table 2 Logistic regression for the significant risk factor in patients with bladder cancer

Variables	Odds ratio	Standard errors (SE)	*Z*	*P* value	95% confidence interval	
Pesticide exposure	2.715411	0.8721926	3.11	0.001**	1.446867	5.096151
Bladder stones	2.527015	0.7669903	3.05	0.001**	1.393972	4.581011
Family history	3.264406	1.321565	2.92	0.002**	1.476396	7.217808
Recurrent cystitis	1.881407	0.5931815	2.00	0.003**	1.01417	3.490236
Smoking	2.393731	0.7687472	2.72	0.005**	1.275592	4.491991
Bilharziasis	1.488446	0.4502449	1.31	0.013*	0.8227166	2.692873
Consanguinity	1.375484	0.7374396	0.59	0.552	0.4809499	3.933789
Sex (male)	0.4281686	0.2102419	− 0.23	0.466	0.1635509	1.120926
Residence (rural)	0.8852298	0.3369343	− 0.32	0.830	0.41983	1.866546

*Statistically significant

**Highly significant

Table 3 Relation between exposure to pesticide, the acetylcholinesterase enzyme level, type of bladder cancer, and occurrence of the mutation in K-ras (multivariate analysis)

		Type of tumor	Patient							Control						
			K-ras mutation	Exposed		Not exposed		Total		*P* value	K-ras mutation	Exposed		Not exposed		Total		*P* value
				No.	%	No.	%	No.	%			No.	%	No.	%	No.	%	
AChE (U/L)	Abnormal level "decreased"	UC	Mutation	33	33	0	0	60	60	0.001**	Mutation	1	0.5	0	0	15	7.5	0.001**
			No mutation	24	24	3	3											
		SCC	Mutation	0	0	0	0	2	2									
			No mutation	1	1	1	1				No mutation	12	6	2	1			
		Adenocarcinoma	Mutation	1	1	0	0	3	3									
			No mutation	1	1	1	1											
	Normal level	UC	Mutation	1	1	0	0	30	30		Mutation	0	0	0	0	185	92.5	
			No mutation	0	0	29	29											
		SCC	Mutation	0	0	0	0	2	2									
			No mutation	1	1	1	1				No mutation	45	22.5	140	70			
		Adenocarcinoma	Mutation	0	0	0	0	3	3									
			No mutation	0	0	3	3											
	Total			62	62	38	38	100	100			58	29	142	71	200	100	

Normal level AChE in children, men, and women is equal to 3500–8500 U/L. Abnormal level AChE is equal to or less than 3500 U/L. Assay range of the P53 kit is 20–6000 pg/ml

UC urothelial carcinoma, *SCC* squamous cell carcinoma

** Highly significant

Table 4 Relation between presence of bladder cancer and abnormal *AChE* and serum P_{53} levels (*T* test)

		Patients		Mean level	Control		Mean level	P value
		No.	%		No.	%		
AChE (U/L)	Abnormal level "decreased"	65	65	1500 ± 750	15	7.5	2500 ± 540	0.001**
	Normal level	35	35	5000 ± 1200	185	92.5	5500 ± 954	0.001**
		100	100		200	100		
Serum P_{53} before surgery (pg/ml)	High level	71	71	5100 ± 250	9	4.5	1050 ± 500	0.001**
	Low level	29	29	1550 ± 200	191	95.5	720 ± 150	0.001**
		100	100		200	100		

Normal level AChE in children, men, and women is equal to 3500–8500 U/L. Abnormal level AChE is equal to or less than 3500 U/L. Assay range of the P53 kit is 20–6000 pg/ml

** Highly significant

Discussion

Identification of the risk factors that lead to bladder cancer may decrease the incidence of the cases and give our era an alarm to decrease the unsafely excessive use of the organophosphates. Early screening by using an ideal marker (P_{53}) may improve the diagnosis and prognosis. In this study, we found that inhibition of *AChE* level was associated with the occurrence of mutation in the K-ras gene. Also, high level of P_{53} was associated with the occurrence of bladder cancer.

Many studies were done to evaluate the risk factors that lead to bladder cancer among people, but few try to know the mechanism by which this factors lead to bladder cancer. The present study tries to find a correlation between the K-ras mutation and exposure to organophosphates. Lee et al. (2004) and Koutros et al. (2015) in their study found that there was a highly significant correlation between the pesticide exposure and occurrence of bladder cancer especially in those who never smoke to exclude the smoking factor. Many other studies were done to find the relation between cancers and some types of pesticides like atrazine; these studies were done in different era with different types of users and did not find a good relation between atrazine exposure and bladder cancer (Rusiecki et al. 2004; Freeman et al. 2011). This may give an alarm about the method and amount of organophosphorous used by Egyptian farmers.

Few studies were done to know how pesticides can cause different types of cancers; in the present study, the authors searched for K-ras gene mutation and found that, on the one hand, there was a significant correlation between pesticide exposure and mutation in K-ras gene and, on the other hand, there is a correlation between the k-ras mutation and the occurrence of the bladder cancer. This was indicated by the increase in the level of P53 in the blood of the bladder cancer patients exposed to the organophosphorus compounds and the significant decrease in the cholinesterase enzyme level. Barbacid (1990) agreed with the present study results that the ras gene have a role in different types of neoplasia. Noaishi et al. (2011) agreed with the present results and found mutation in the K-ras gene in the peripheral blood of Egyptian workers occupationally exposed to pesticides. Also, Ahrendt et al. (2001) found a correlation between cigarette smoking and mutation of the k-ras gene in patients with lung cancer; Riely et al. (2009), Roberts et al. (2010), Karachaliou et al. (2013), Rau et al. (2016), and Li et al. (2016) found an association between Kars mutation and non-small cell lung cancer. Alguacil et al. (2002) found that occupational exposure to organic solvents can also lead to k-ras mutation in pancreatic cancer, and Xiong et al. (2016) found that k-ras can be mutated in endometrial carcinoma. So, K-ras is an important indicator gene which can be affected easily by toxin exposure; few studies were done to find the relation between exposure to pesticides and k-ras mutation. Many studies were done to

Table 5 Relation between inhibition of *AChE* and *K-ras* mutation (*T* test)

		Patients		K-ras mutation ($n = 35$)		*P* value	Control		K-ras mutation ($n = 1$)		*P* value
		No.	%	No.	%		No.	%	No.	%	
AChE level	Normal level	35	35	1	2.85	0.723	185	92.5	0	0	0.712
	Abnormal level "decreased"	65	65	34	52.30	0.001**	15	7.5	1	0.5	0.921
	Total	100	100	35			200	100			

**Highly significant

explain the mechanisms by which pesticide exposure can develop cancer. These include immunotoxicity (Galloway and Handy 2003), gene mutations (Menozzi et al. 2004), oxidative stress (Abdollahi et al. 2004; and John et al. 2001), and proliferation in cells (Cabello et al. 2001). In this study, authors found a relation between the k-ras gene and exposure to pesticides as a correlated factor to produce cancer in the bladder.

P_{53} level and its relation with the appearance of cancer were studied in previous studies, but few of them reported its relation with the bladder cancer; in this study, the authors found a significant correlation between high level of P_{53} and appearance of bladder cancer. P_{53} as a marker is a noninvasive method if it is found in high level, giving an alarm to physicians that there is something wrong with his patient. Shim et al. (1998) in their study suggested that mutation in P_{53} protein can be used as a biomarker in the management of patients with colorectal cancer. Also, Calaf et al. (2009) suggested that pesticide exposure can alter P_{53} and c-Ha ras and induce malignancy of breast cells through genetic factors. Nigro et al. (1989), Levine et al. (1991), and Callahan (1992) all agree with the results of the present study that P_{53} play a role in the development of many types of cancers. Also, Du et al. (2016) found that in their study, there is a correlation between P_{53} over estimation with progression of T1 non-muscle invasive bladder cancer (NMIBC) patients due to the heterogeneity and other limitations.

Conclusion

In conclusion, the present study supports the hypothesis of direct relationships between organophosphorus exposure and the activation of the K-ras gene and P_{53} level. However, future studies are needed on larger number of patients with follow-up, P_{53} level, and acetylcholinesterase level to confirm that excision of the cancer and correction of the exposure to organophosphates can improve the level of P_{53} and help in prognosis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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