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Homozygous Deletion of P16 Gene in Primary Bladder and Breast Carcinomas in Egyptian Cancer Patients

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ABSTRACT

Bladder and breast cancers are the most common types of cancer affecting males and females in Egypt. Bladder cancer in Egypt is different from Western countries due to its association with the presence of bilharziasis. Genetic changes in bladder and breast cancers in Egypt are still not clearly understood. In the present study, tissues from 170 primary bladder cancer patients (bilharzial and non bilharzial) and 42 primary invasive ductal carcinoma of breast with different grades and stages were examined for homozygous deletion at P16 tumor suppressor gene, the gene that controls cell cycle growth, using comparative multiplex PCR technique. Our results showed high frequency of deletion in bladder and breast carcinomas (41% and 43%, respectively). Homozygous deletion at P16 observed in different histopathological types of bladder cancer was slightly higher in squamous cell carcinoma (40.4%) than in the transitional type (33.9%). Both bilharzial and non bilharzial bladder carcinomas showed almost the same percentage of deletion at P16 gene (40.5% and 40.7%). Also, homozygous deletion at P16 gene was detected in both positive and negative lymph node metastasis of bladder cancer (47.6% and 39.6%). In breast carcinoma, P16 deletion detected in grade 3 was slightly higher (53.9%) than in grade 2 (37.9%) and was much higher in positive lymph node metastasis (48.6%) than in negative lymph nodes (14.8%). This study showed that (1) P16 gene may be involved in the initiation and progression of bladder carcinoma, (2) Bilharziasis has no role in the inactivation of P16 gene, and finally (3) P16 gene may be involved in the progression and metastasis of breast carcinoma.

Key Words: *P16 gene - Bilharziasis - Bladder cancer - Breast cancer - Homozygous deletion.*

INTRODUCTION

The $p16^{INK4}$ is known as inhibitor of cyclin dependent kinase 4, and is also known as Multi Tumor Suppressor gene 1 (MTS-1). It is a tumor suppressor gene located at chromosomal region 9p21 **[12,16]** and encodes P16 protein which is a cell cycle regulatory protein that binds to cyclin-dependent kinase 4 (CDK4) and inhibits the catalytic activity of the CDK4/ cyclin D complex. The CDK/cyclin D complex controls passage through the G1 phase into the S phase of the cell cycle by phosphorylation of cellular factors like retinoblastoma protein **[21]**. High frequency of homozygous deletion of the P16 gene has been reported in many cell lines derived from a variety of human tumors including melanomas **[18]**, lung cancer **[17]**, leukemia **[5]**, and transitional cell carcinoma of bladder **[22,3]**.

Bladder carcinoma is one of the foremost oncologic problems in many tropical and subtropical countries. In Egypt, it is one of the most common tumors among men and is strongly associated with bilharzial infestation **[30]**. Bilharziasis-associated bladder carcinoma is histopatholgically different from that reported in Western countries and commonly presents as squamous cell carcinoma **[6]**. It differs from transitional cell carcinoma not only in its histology, geographic distribution, and risk factors, but also in its clinical behavior and progress. At the time of diagnosis, squamous cell carcinoma is more advanced than transitional cell carcinoma with deep muscle invasion. Squamous cell carcinoma is also more aggressive than transitional cell carcinoma with a lower overall 5-year survival rate **[7]**. The molecular mechanism of this type of tumor is poorly understood.

Breast cancer is one of the most common cancers affecting females of the Western countries **[28]**. Almost 200,000 new cases are diagnosed each year in the United States alone, resulting in more than 40,000 deaths/year. In Egypt, breast cancer is considered to be number one among females (27.3%) **[14]**. Breast carci-

noma, like other neoplastic diseases, develops and progresses in consequence of the accumulation of genetic alterations. Several previous studies on primary breast carcinoma showed loss of heterozygosity at different chromosomal regions, which suggests that several tumor suppressor genes may participate in the development and/or progression of breast cancer [4,13].

In this study, we intend to investigate whether or not P16 gene plays a role in the tumorigenesis of bilharziasis-associated bladder and breast carcinomas. Homozygous deletion of p16 gene will be examined in the DNA samples of bladder and breast carcinomas using comparative multiplex PCR technique and correlation will be made between P16 deletion and various clinico-pathological parameters.

PATIENTS AND METHODS

Tissue samples from one hundred and seventy bladder cancers and 42 invasive ductal carcinomas of the breast obtained from patients diagnosed at NCI, Cairo University, were included in this study. Bladder tumors were histopathologically graded according to WHO classification [25] and staged according to TNM staging system [15]. Breast tumors were classified according to TNM classification [1]. In this study, bladder cases included: Squamous cell carcinoma (n=104), transitional cell carcinoma (n=56), adenocarcinoma (n=8), and undifferentiated carcinoma (n=2). Bilharziasis-associated bladder cancer specimens were 111 and 59 were free of bilharsiasis. Bilharsiasis was diagnosed by the presence of calcified bilharzial ova in the tumor tissues during examination for histopathology. Staging of bladder tumors included: T_1 (n=2), T_2 (n=3), T_3a (n=70), T_3b (n=82), T_4a (n=10), and T_4b (n=3). Histologic grading of bladder tumors included: Grade 1 (n=15), grade 2 (n=110), and grade 3 (n=45). Twenty one cases showed lymph node positive and 149 were negative for lymph nodes.

For breast carcinoma, all the tumors were invasive ductal carcinoma, 29 were grade 2 and 13 were grade 3. Seven cases showed negative lymph nodes and 35 and positive lymph nodes. At least 70% of the cells present in each specimen included in this study were tumor cells as confirmed by histopathological examination.

DNA extraction and PCR:

High molecular weight DNA was isolated from tumors and corresponding normal tissues following the technique described by Sambrook et al [20]. Comparative multiplex PCR was used according to Walker et al [26] to assess homozygous deletion in the region of p16 (MTSl) gene. The primer pair for exon 2 of the P16 gene and one control primer pair for a gene located on chromosome 9q (D9S196) used as internal control, were amplified in the same tube of the PCR reaction. Primers were obtained from Gibco BRL, UK and PCR was performed 10 µl containing 100 ng genomic DNA, 5 pmol of each of the 4 primers, 50 mM KCI, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 1 unit of Taq DNA polymerase (Gibo, BRL, UK), 100 uM of each deoxyribonucleotide triphosphate. PCR was carried out in a DNA thermal cycler (Perkin Elmer Cetus, 480), 30 amplification cycles were performed. Each cycle consists of denaturation at 94˚C for 1 min., annealing at 55˚C for 1 min., and extension at 72˚C for 2 min. A final extension step of 5 min. at 72˚C was added. The PCR products were electrophoresed on 2% agarose gel after ethidium bromide staining. The gel was scanned using. Dual-Wavelength Flying Spot Densitometer PN 206 (Shimadzu Co., Japan). The intensities of the MTSl band and the D9S196 control were determined and expressed as a ratio (MTSl/D9S196). The homozygous deletion was scored if the intensity of P16 was lost or highly reduced (the ratio is less than 0.3).

Statistical Analysis:

The statistical significance of the correlation between homozygous deletion of P16 in both bladder breast and each of the clinicopathological parameters was determined by Fisher's exact test using Basica Epistate V2.02 (Richardson, Texas).

RESULTS

1- Homozygous deletion of P16 in bladder cancer:

Tumor DNA from 170 primary bladder cancers was examined for homozygous deletion of P16 (MTSl) using multiplex PCR. Deletion was detected in 69 of the 170 cases examined (40.6%). It was observed in different stages of bladder cancer with the following distribution: One case T_1, one case T_2, 26 cases T_3a, 34 cases T_3b, 5 cases T_4a, and 2 cases T_4b (Table 1). Homozygous deletion was detected in all histo-

pathological types with the following frequency: 42 out of 104 (40.4%) squamous carcinomas, 19 out of 56 (33.9%) transitional carcinomas, 6 out of 8 (75%) adenocarcinomas, and finally 2 out of 2 (100%) in undifferentiated carcinomas (Table 1).

Homozygous deletion was also detected in all grades of bladder cancer with the following frequency: 8 out of 15 (53%) grade I, 43 out of 110 (39%) grade II, and 18 out of 45 (40%) grade III. For bilharziasis-associated bladder cancer: 45 out of 111 (40.7%) of bilharzial bladder cancers showed deletion, while 24 out of 59 (40.6%) non bilharzial bladder cancers revealed deletion. Regarding lymph node metastasis, homozygous deletion was observed in 10 out of 21 cases (47.6%) with positive lymph nodes, and in 59 out of 149 cases (39.6%) with negative lymph nodes.

In this study, the correlation between the presence of bilharziasis and the type of tumor at one side and the presence of deletion at P16 on the other side, showed that 24 out of 59 squamous cell carcinomas with bilharziasis (40.7%) exhibited deletion, and 20 out of 45 squamous type without bilharziasis (44.4%) exhibited deletion. Deletion was detected in 15 out of 40 of the transitional type with bilharziasis (37.5%), while 4 out of 16 transitional type without bilharziasis (25%) reveald the deletion. Five out of the 6 adenocarcinomas with bilharziasis (83%) showed deletion, one out of two adenocarcinomas without ilharziasis (50%) showed deletion. Finally, the two undifferentiated carcinomas with bilharziasis (100%) showed deletion. These results are shown in table (1).

Statistical analysis showed no significant association between deletion of P16 gene and any of the clinicopathological parameters examined (type of tumor, grade, stage, presence of bilharziasis, and lymph nodes metastasis).

Figure (1). Shows the frequency of homozygous P16 gene deletion in different cases of bladder cancer as compared to the control using comparative multiplex polymerase chain reaction (PCR). The upper bands represent internal control (D9S196), the lower bands represent P16 gene. Tumor samples 2,4,6,8 and 10 showed homozygous deletion of P16 gene whereas tumor samples 1,3,5,7,9 and 11 showed retained P16 gene.

2- Homozygous deletion of P16 in breast carcinoma:

Tumor DNA from 42 primary invasive ductal carcinoma of breast was examined for homozygous deletion at P16 gene. The overall percentage of deletion detected was 42.9% (18 out of 42 cases). Eleven of 29 (37.9%) of grade 2 showed homozygous deletion and 7 of 13 (53.9%) grade 3 showed deletion. Also, 17 of 35 (48.6%) that were lymph node positive exhibited homozygous deletion and only one case of 7 (14.3%) lymph node negative cases exhibited homozygous deletion (Table 2). Statistically, no significant correlation was observed between homozygous detetion at P16, and histopathological tumor type, grade, and lymph node metastasis. Fig. (2) shows homozgous deletion of P16 gene in some representative samples from breast cancer.

Figure (2). Comparative multiplex polymerase chain reaction (PCR) showing homozygous deletion of P16 gene in 9 breast carcinomas. The upper bands represent internal control (D9S196), the lower bands represent P16 gene. Tumor samples 2, and 4 showed homozygous deletion of P16 gene whereas tumor samples 1,3,5 and 9 showed retained P16 gene.

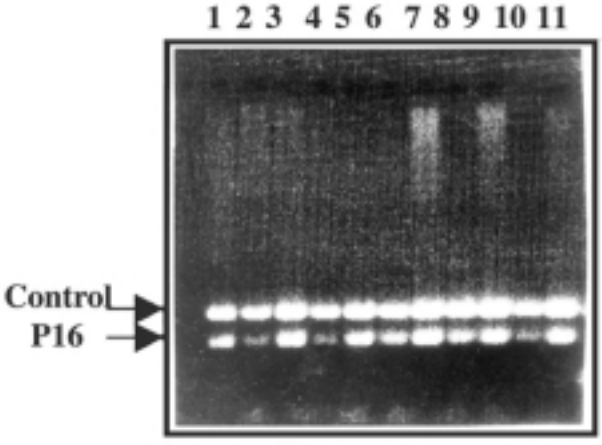

Fig. (1): Comparative multiplex polymerase chain reaction (PCR) showing homozygous deletion of P16 gene in 11 bladder carcinomas. The upper bands represent internal control (D9S196), the lower bands represent P16 gene. Tumor samples 2,4,6,8 and 10 showed homozygous deletion of P16 gene where tumor samples 1,3,5,7,9 and 11 showed retained P16 gene.

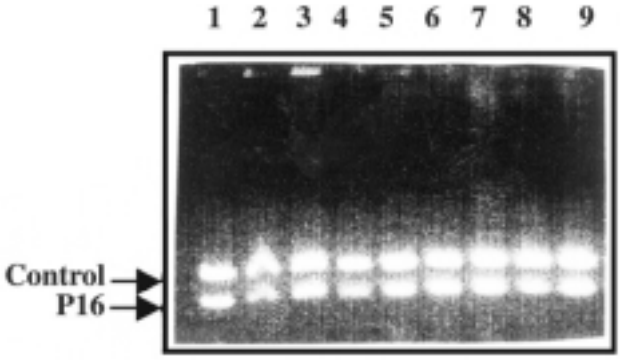

Fig. (2): Comparative multiplex polymerase chain reaction (PCR) showing homozygous deletion of P16 gene in 9 breast carcinomas. The upper bands represent internal control (D9S196), the lower bands represent P16 gene. Tumor samples 2 and 4 showed homozygous deletion of P16 gene where tumor samples 1,3,5,6,7,8,9 showed retained P16 gene.

Table (1): Correlation between homozygous deletion of P16 gene and main clinicopathological parameters in bladder carcinoma.

	Factor	Category	Homozygous deletion n (%)		*p*-value
1-	Histology	SCC*	42/104	(40.4)	>0.05
		TCC♦	19/56	(33.9)	
		Adenocarcinoma	6/8	(75)	
		Undifferentiated	2/2	(100)	
2-	Grade	I	8/15	(53)	>0.05
		II	43/110	(39)	
		III	18/45	(40)	
3-	Stage	T^1	1/2	(50)	>0.05
		T^2	1/3	(33)	
		T^3a	26/70	(37)	
		T^3b	34/82	(42)	
		T^4a	5/10	(50)	
		T^4b	2/3	(66.6)	
4-	Bilharziasis				
		present	45/111	(40.5)	>0.05
		absent	24/59	(40.7)	
5-	Lymph node metastases				
		present	10/21	(47.6)	>0.05
		absent	59/149	(39.6)	

* SCC: squamous cell carcinoma ♦TCC: transitional cell carcinoma
p-value <0.05 is considered significant n - number of cases

Table (2): Correlation between homozygous deletion at P16 gene and various clinicopathological parameters in breast carcinomas.

Factor	Homozygous deletion n (%)		*p*-value
1- *Grade*			
II	11/29	(37.9)	>0.05
III	7/13	(53.9)	
2- *Lymph node metastases*			
a. present	17/35	(48.6)	>0.05
b. absent	1/7	(14.3)	

p-value <0.05 is considered significant
n - number of cases

DISCUSSION

Alteration of P16 gene is a common event in certain human primary tumors **[16]** and has contributed to the development and progression of cancers. Homozygous deletion of P16 was detected in different types of primary tumors including astroctyoma **[26]**, lung carcinoma **[17]**, and bladder carcinoma **[3,22]**. In bladder cancer, the frequency of deletion of P16 differs from one study to another. In Tamimi et al study **[23]**, deletion was only detected in 14% of cases that had been studied, while Cairns et al **[3]** and Williamsons et al **[27]** reported a percentage of 20% and 35% of cases, respectively. In the present study, 170 bladder tumor specimens (bilharzial

and non bilharzial) and 42 invasive ductal carcinoma of breast have been examined for homozgyous deletion of P16 using multiplex PCR, where about 41% and 43% deletions in bladder and breast carcinomas were detected respectively.

It was found that histopatholgical types of bladder tumors play an important role in this issue, where squamous cell carcinoma is more aggressive than transitional cell carcinoma with lower overall 5-year survival rate [7]. At the genetic level, most of the studies focused on transitional cell carcinoma and little was known about squamous cell carcinoma. In the present study, squamous cell carcinoma showed a slightly higher frequency of P16 deletion (40.4%), as compared to transitional cell carcinoma (33.9%), but this difference is still statistically not significant (p value >0.05). Our results agree with other previous studies [8,24], which reported that homozygous deletion at P16 was higher in squamous cell carcinoma than in transitional cell carcinoma.

Numerous mechanisms have been proposed to clarify the role of bilharziasis in the induction of bladder cancer [29]. One of the explanations showed that bilharzial eggs can cause mechanical irritation to the urothelial lining [2], along with other factors eg. chronic urinary tract infections can potentiate the action of environmental mutagens such as N-nitroso-compounds [10] which can ultimately lead to methylation then G to A mutations in the DNA [19]. In this study, the homozygous deletion at P16 was observed in both bilharzial and non bilharzial bladder cancer (40.5% and 40.7%, respectively) which explains that P16 inactivation is not associated specifically with the presence of bilharziasis. These results are in agreement with others [8,23,24] who reported that bilharziasis-associated bladder cancer did not exhibit specific P16 deletion than non-bilharzial type.

Also, homozygous deletion at P16 was observed in different stages (T_1, T_2, T_3, and T_4) and different grades [1, 2 & 3] of bladder cancer which suggests that P16 may be involved in the initiation and progression of bladder cancer. Some studies reported that deletion at 9p21 region may involve not only inactivation of P16 gene but also several genes reside at the same region. P15 gene which lies beside P16 gene may be one of the genes that are inactivated by deletion especially that it has similar function as P16 [11]. They concluded that homozygous deletion may be an efficient mechanism for the simultaneous inactivation of both genes. coincident deletion of P16 and P15 genes has been reported in T-cell lineage acute lymphoblastic leukemias [9].

Homozygous deletion at P16 in breast cancer also showed high frequency (42.9%) and all the cases were invasive ductal carcinoma. The frequency of deletion was higher in grade 3 (53.9%) than in grade 2 (37.9%). None of the examined samples were grade 1. These results may indicate that deletion of P16 gene is associated with high tumor grades. Also, presence of positive lymph node metastasis showed high frequency (48.6%) than cases with no lymph node metastasis (14.3%) which may explain that inactivation of P16 may be involved in the metastasis of the tumor not in its development.

In conclusion, homozygous deletion at P16 gene was observed at high frequency in both bladder and breast cancer. In bladder cancer, P16 may be involved in initiation and progression of the tumor. However, no role of bilharziasis was observed for the inactivation of P16 gene. In breast cancer, homozygous deletion at P16 was observed in metastatic and high grade tumors.

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