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4-Aminobiphenyl and Nitric Oxide Synergistically Modified Human DNA: It's Implication in Bladder Cancer

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Abstract

Bladder cancer is a disease of continuing public health significance. Some known risk factors for bladder cancer development include smoking, occupational exposure, genetic susceptibility, infectious diseases and radiation therapy. Smoking and occupational exposure have strongly implicated aromatic amines as being carcinogenic for the bladder, with 4-aminobiphenyl (4-ABP) being one of the most potent. 4-ABP is a major etiological agent of human bladder cancer, and its metabolites are able to form DNA adducts that may induce mutation and initiate bladder carcinogenesis. Binding characteristics and specificity of bladder cancer anti-DNA antibodies were analyzed by direct binding and inhibition enzyme-linked immunosorbent assay. The data show preferential binding of bladder cancer antibodies to 4-ABP-NO-modified DNA in comparison with native native DNA. A band shift assay further substantiated the enhanced recognition of 4-ABP-NO-modified DNA by anti-DNA antibodies. Cancer antibodies exhibited enhanced binding with the modified human DNA as compared to the native form. Lymphocyte DNA from cancer patients showed appreciable recognition of anti-4-ABP–NO-DNA IgG as compared to the DNA from healthy subjects. The 4-ABP-NO- modified DNA presents unique epitopes which may be one of the factors for the autoantibody induction in bladder cancer patients.

The results suggest that 4-ABP-NO-modification of self-antigen(s) can generate neoepitopes capable of inducing bladder cancer characteristic autoantibodies. The preferential binding of 4-ABP-NO-modified DNA bladder cancer anti-DNA antibodies points out the likely role of oxidatively modified in the initiation/progression of bladder cancer. Moreover, oxidatively modified genomic DNA antigen, appear to be more suitable as a trigger for bladder cancer. The aim of this study was to evaluate whether biomarkers of environmental tobacco smoke exposure [i.e., 4-aminobiphenylDNA (4-ABP-DNA) adducts] were common pathway for the predictive of the risk of cancers.

Keywords: 4-aminobiphenyl; Nitric oxide; Bladder cancer; Human DNA

Introduction

4-Aminobiphenyl (4-ABP) is a well-studied aromatic amine and a known bladder carcinogen in both experimental animals and humans and a possible breast carcinogen. Cigarette smoking is a major cause of cancer of the urinary bladder. In countries where a long history of cigarette consumption exists, the proportion of bladder cancers attributed to this risk factor is estimated to be about 50% for men and 30% for women [1-3]. The relative risk for bladder cancer among cigarette smokers ranges in most reports from 1.5- to 3.0-fold higher than among nonsmokers, with higher values reported in certain populations [4]. In line with the gender disparity in bladder cancer incidence, the mortality rate of this disease is also more than three-fold higher in men than in women [5]. It has been hypothesized that the carcinogenicity of tobacco smoke for the bladder may be due to the presence of various aromatic amines, including the known human bladder carcinogens 4-aminobiphenyl and 2-naphthylamine [6,7]. Current evidence indicates that aromatic amines exert their carcinogenic effects on the bladder only after a number of metabolic and distribution steps. The amounts of 4-aminobiphenyl have been reported in unfiltered mainstream, filtered mainstream and side-stream cigarette smoke, respectively: 2.4 to 4.6 ng/cigarette; 0.2 to 23 ng/cigarette; and up to 140 ng/cigarette [8,9].

4-ABP is metabolically activated to several electrophilic intermediates was initially detected by evaluating DNA adducts and higher levels were observed in cigarette smokers than non-smokers [10,11]. The predominant one is N-(2' deoxyguanosine-8-yl) 4-ABP accounting for 70% of the total adduct formed [12]. As DNA adducts may lead to somatic point mutations, it is reasonable to assume that activated aromatic amines may lead to bladder-tumor development by inducing mutations in key genes such as the *TP53* tumor suppressor gene [13,14] and the *H-RAS* gene [15] both involved in bladder carcinogenesis. The *NAT2* slow-acetylates or genotype accounts for a greater risk for cancer of the urinary bladder in individuals exposed to 2-naphthylamine or 4-aminobiphenyl [16]. The major 4-ABP-DNA adducts identified in human bladder and lung is *N*-(deoxyguanosin-8-yl)-4-ABP [17]: other adducts include *N*-(deoxyadenosin-8-yl)-4-ABP and *N*-(deoxyguanosin-*N2*-yl)-4-ABP [18]. *N*-(deoxyguanosin-8-yl)-4-ABP has also been detected in female breast tissue of both smokers and non-smokers [17,19] indicating that 4-ABP-reactive intermediates are distributed systemically and/or that multiple organs are capable of activating 4-ABP or its metabolites. Increased levels of 4-ABP-haemoglobin adducts are associated with cigarette smoking [11], and occupational exposure to 4-ABP is associated with an increased risk for cancer of the urinary bladder [20]. 4-ABP also increased the mutation frequency in the bladder, liver, and bone marrow of mice. In human bladder cells treated with *N*-hydroxy-4-ABP, preferential sites of adduct formation in *TP53* were at codons 175, 248, 280, and 285, which are mutational hotspots for cancer of the urinary bladder [14]. There is sufficient evidence in humans for the carcinogenicity of 4-aminobiphenyl. There is strong mechanistic evidence indicating that the carcinogenicity of 4-aminobiphenyl in humans operates by

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a genotoxic mechanism of action that involves metabolic activation, formation of DNA adducts, and induction of mutagenic and clastogenic effects. Metabolic activation to DNA- reactive intermediates occurs by multiple pathways including N-oxidation in the liver, O-acetylation in the bladder, and peroxidative activation in the mammary gland and other organs. These adducts have proven useful in monitoring integrated exposures over several months [10]. In this study, attempts have been made to ascertain the extent of involvement of ABP-nitric oxide modified human DNA in cancer development. Comparative immunobinding of antibodies, present in sera of bladder cancer patients with history of smoking, with native and 4-ABP-NO- modified-human DNA has been studied.

Materials and Methods

Human placental DNA, 4-aminobiphenyl (4-ABP), nuclease S1, alkaline phosphatase conjugate, p-nitrophenyl phosphate, Tween-20, protein-A agarose and ethidium bromide were purchased from Sigma Chemical Company, USA. Sodium nitroprusside, chloroform, sodium hydroxide, isoamyl alcohol, sodium chloride and EDTA were from Qualigens, India. Flat bottom maxisorp ELISA modules were purchased from NUNC, Denmark. All other reagents/chemicals were of highest analytical grade available.

Purification of DNA

Commercially obtained, highly polymerized human placental DNA and calf thymus DNA were purified free of proteins, RNA and single stranded regions as described by Ali et al., 1985 [21]. The DNA (2 mg/ml) was dissolved in 0.1 x SSC (15 mM sodium citrate and 150 mM sodium chloride), pH 7.3 and mixed with an equal volume of chloroform-isoamyl alcohol mixture (24:1). The sample was extracted in a stoppered measuring cylinder for 1 hour with regular slow shaking. The aqueous layer containing DNA was separated from the organic layer and re-extracted by mixing with fresh mixture of chloroform-isoamyl alcohol. The DNA in the aqueous layer was precipitated with two volumes of cold absolute ethanol and collected on a glass rod. After air drying, the DNA was dissolved in acetate buffer, (30 mM each of sodium acetate and zinc chloride), pH 5.0, and treated with nuclease S1 (150 units/mg DNA) at 37°C for 30 minutes to remove single stranded regions. The reaction was stopped by adding one tenth volumes of 200 mM EDTA, pH 8.0. Nuclease S1 treated DNA was extracted twice with the mixture of chloroform-isoamyl alcohol and finally precipitated with two volumes of cold ethanol. The precipitated DNA was dissolved in phosphate buffered saline (PBS) (10 mM sodium phosphate containing 150 mM sodium chloride), pH 7.4.

Modification of human placental DNA by 4-ABP and nitric oxide

The modification was carried out by incubating 15.15 μM of human placental DNA in 50 mM phosphate buffer, pH 7.4 with 1.3 mM of 4-ABP in the presence of 8 mM sodium nitroprusside (SNP), a nitric oxide donor, at 37°C for three hours. At the end of incubation, the sample was extensively dialyzed against phosphate buffer, pH 7.4.

Collection of sera

Eighty blood samples were collected from bladder cancer patients [22] each who had a history of smoking as well as non-smoker patients from the J.N. Medical College Hospital, A.M.U, Aligarh. Sera from age and sex-matched normal healthy individuals (n=25) served as control. Specifically, there were no cases of lupus or other connective tissue disorders. Normal human sera (with matched age and sex) were obtained from healthy subjects (both smokers and non-smokers). Samples were collected in a glass test tube and left to clot for 30 min at 37°C. Serum was separated by centrifugation at 3000 rpm for 10 min. Serum samples were heated at 56°C for 30 min to inactivate complement proteins and stored in aliquots at –20°C with 0.1% sodium azide as preservative.

Isolation of IgG by Protein-A- agarose column

Serum IgG was isolated by affinity chromatography on Protein A-agarose column [23]. Serum (0.3 ml) diluted with equal volume of PBS, pH 7.4 was applied to column (12 mm x 45 mm) equilibrated with the same buffer. The wash through was recycled 2-3 times. Unbound IgG was removed by extensive washing with PBS, pH 7.4. The bound IgG was eluted with 0.58% acetic acid in 0.85% sodium chloride (Goding, 1976) and neutralized with 1 ml of 1M Tris-HCl, pH 8.5. Three ml fractions were collected and read at 251 and 278 nm. The IgG concentration was determined considering 1.40 OD280= 1.0 mg IgG/ml. The isolated IgG was then dialyzed against PBS, pH 7.4 and stored at -20 °C with 0.1% sodium azide.

Enzyme linked immunosorbent assay (ELISA)

Antibodies were detected by ELISA using polystyrene microtitre plates as solid support [24]. One hundred microlitre of 2.5 μg/ml antigen in TBS, pH 7.4 was coated in test wells of microtitre plates, incubated for 2 hr at 37°C and overnight at 4°C. The antigen coated wells were washed three times with TBS-T to remove unbound antigen. Unoccupied sites were blocked with 150 μl of 1.5% BSA in TBS for 4-5 hrs at room temperature. The plates were washed once with TBST-T and antibody (100 μl/well) to be tested were diluted in TBS and added to each well. After 2 hr incubation at 37°C and overnight at 4°C, the plates were washed four times with TBS-T and an appropriate anti-immunoglobulin alkaline phosphatase conjugate was added to each well. After incubation at 37°C for 2 hr, the plates were washed four times with TBS-T and three times with distilled water and developed using p-nitrophenyl phosphate substrate respectively. The absorbance was recorded at 410 nm on an automatic microplate reader. Each sample was run in duplicate. The control wells were treated similarly but were devoid of antigen. Results were expressed as a mean of A test-A control

Competition ELISA

The antigenic specificity of the antibodies was determined by competition ELISA [25]. Varying amounts of inhibitors (0-20 μg/ml) were mixed with a constant amount of antiserum or IgG. The mixture was incubated at room temperature for 2 hr and overnight at 4°C. The immune complex thus formed was coated in wells instead of the serum. The remaining steps were the same as in direct binding ELISA. Percent inhibition was calculate using the formula

$$\text{Percent inhibition} = (1\text{- A-inhibited/A-uninhibited}) \times 100$$

Isolation of DNA from human lymphocytes

Blood samples were collected in EDTA vials from different bladder cancer patients and normal healthy individuals. Lymphocyte DNA was isolated as per the manufacturer's instructions [26]. To 500 μl of Qiagen protease 5 ml of blood was added and then buffer AL (6 ml) was mixed followed by vigorous shaking. Mixture was incubated at 70°C for 10 min. Then 5 ml of ethanol (98%) was added to the sample and mixed by inverting the tube, followed by vigorous shaking. The solution was then carefully transferred onto QIAamp Maxi column placed in a 50 ml centrifuge tube and was centrifuged at 1850 x g (3000 rpm) for 3

min. Filtrate was discarded and 5 ml of Buffer AW2 was added and again centrifuged at 4500 x g (5000 rpm) for 1 min. Again 5 ml of AW2 buffer was added and centrifuged at 4500 x g (5000 rpm) for 15 min discarding the filtrate. Then 600 µl of buffer AE was poured directly onto the membrane of the column which was later incubated for 5 min, and centrifuged at 4500 x g (5000 rpm) for 2 min. Eluent containing DNA was reloaded onto the membrane of the column, and was again incubated for 5 min, and centrifuged at 4500 x g (5000 rpm) for 5 min. Eluent containing DNA was air dried and dissolved in PBS, pH 7.4.

Gel retardation assay

For the visual detection of antigen antibody binding and immune complex formation, gel retardation assay was performed [27]. A constant amount of antigen (native and modified DNA) was incubated with varying amounts of IgG in PBS, pH 7.4 for 2 hr at 37°C and overnight at 4°C. One-tenth volume of 'stop mix' dye (0.125% bromophenol blue, 30% Ficoll-400, 500 mM EDTA in TAE buffer) was added to the mixture and electrophoresed on 1% agarose for 2 hr at 30 mA in TAE buffer (40 mM Tris-acetate and 2 mM EDTA), pH 7.9. The gels were stained with ethidium bromide (0.5 µg/ml), visualized under light and photographed.

Statistical analysis

Data are presented as mean ± SD. Statistical significance of control versus test was computed using student's t–test (Statgraphics, Origin 6.1). A p value of ≤0.05 was considered statistically significant.

Results and Discussion

Characterization of human placental DNA

The arylamine, 4-aminobiphenyl (4-ABP), a tobacco smoke constituent and an environmental contaminant is an established carcinogen. Higher 4-ABP-DNA adducts have been reported in smokers than in non-smokers [28]. A freshly generated smoke contains 600 µg of nitric oxide (NO) per cigarette in the gas phase. Nitric oxide is known to cause deamination of nitrogenous DNA bases leading to single strand breaks. Our previous study [29] has shown structural alterations in human placental DNA (15.15 µM) subjected to modification by 1.3 mM of 4-ABP in presence of 8 mM SNP (nitric oxide donor). The changes were studied by UV and fluorescence spectroscopy, Circular dicroism, HPLC and agarose gel electrophoresis. Analysis of the data revealed that carbonyl and nitrotyrosine contents were significantly increased in 4-ABP-NO-modified human DNA.

Binding of autoantibodies in bladder cancer patients to native and 4-ABP-NO modified human DNA

A total of eighty serum samples were collected from bladder cancer patients attending J.N. Medical College and hospital, A.M.U., Aligarh after the informed consent. Forty samples were from the patients with a history of smoking while the other forty samples belonged to non-smoker group. Sera from age and sex-matched normal healthy individuals (n=25) served as control. All sera were diluted to 1:100 in TBS (pH 7.4) and subjected to direct binding ELISA to ascertain antibody binding with human DNA and 4-ABP-NO modified human DNA. In smoker group of bladder cancer patients, higher binding with 4-ABP-NO modified DNA was observed in all the samples. However, for further studies we selected only those samples in which the binding with 4-ABP-NO-DNA was more than double the binding with native DNA. Out of the total of 40 samples from smoker group, 24 samples (60%) were selected for further studies (Figure 1). Similarly, we analyzed the serum binding of 40 samples from bladder cancer patients in non-smoker group. Only 13 samples (32.5%) showed more than double binding with 4-ABP-NO-DNA, and hence chosen for further studies (Figure 1).

Competitive binding assay of serum autoantibodies from bladder cancer patients (smoker group)

Specific binding of circulating autoantibodies in the smoker group of bladder cancer patients for native and 4-ABP-NO modified human DNA was analyzed by competitive-inhibition ELISA. Out of the 24 sera chosen from the smoker group, the observed maximum inhibition with 4-ABP-NO modified human DNA was in the range of 44.1% to 59.4% while with the native DNA it ranged from 21.0% to 29.2%. Mean inhibition for all the samples tested with native DNA as inhibitor

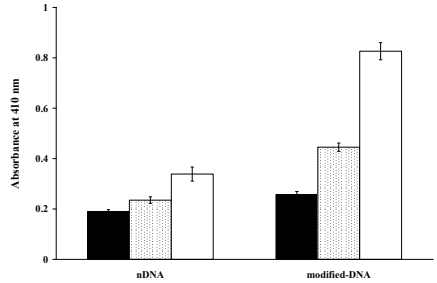

Figure 1: Direct binding ELISA of serum autoantibodies from bladder cancer patients of smoker group with native and 4-ABP-NO-modified-human DNA .The histograms represent mean ± SD of 40 bladder cancer patients of smoker samples. Bars represent mean ± SD of 25 control normal human sera and 25 normal human smoker sera serve as a negative control binding with corresponding antigens (2.5 µg/ml).

	Maximum percent inhibition at 20 µg/ml	
Cancer Sera	**Native human DNA**	**4-ABP-NO modified human DNA**
01	26.8	52.8
02	28.8	44.8
03	29.2	45.6
04	21.0	44.1
05	26.1	46.7
06	27.5	48.7
07	28.1	46.2
08	24.2	45.6
09	26.5	46.1
10	26.2	46.1
11	28.6	49.2
12	25.7	45.2
13	27.6	46.1
14	24.7	50.2
15	25.5	44.4
16	25.9	45.1
17	28.1	46.2
18	25.5	47.1
19	22.1	59.4
20	28.4	49.1
21	26.7	53.1
22	24.3	51.2
23	29.0	57.2
24	25.1	54.6
Mean ± SD	26.34 ± 2.1%	48.63 ± 4.25%
The microtitre plates were coated with 4-ABP-NO modified human DNA (2.5 µg/ml).		

Table 1: Competitive inhibition data of serum autoantibodies in smoker group of bladder cancer patients.

was 26.34 ± 2.1% while for 4-ABP-NO modified human DNA, it was 48.63 ± 4.25% Table 1 summarizes the inhibition data of anti-DNA antibodies binding to native and 4-ABP-NO modified human DNA. The results indicate appreciable recognition of 4-ABP-NO modified human DNA by the autoantibodies in the group.

Binding of IgG from bladder cancer patients (smoker group) to native and 4-ABP-NO modified human DNA

Purified IgG from bladder cancer patients in the smoker group, were subjected to direct binding ELISA on a microtitre plate coated with 4-ABP-NO modified human DNA to evaluate the amount of IgG required for antigen saturation. The average saturation value for modified human DNA was obtained at 50 µg/ml of IgG. Therefore, for smoker group, IgG concentration was kept constant (50 µg/ml) in all further experiments unless indicated. Direct binding profile of one IgG sample has been presented as a representative profile in Figure 2. The binding specificity of the isolated IgG, towards native and 4-ABP-NO modified human DNA, was evaluated by inhibition ELISA. The IgG was mixed with varying amounts of native or 4-ABP-NO modified human DNA (0-20 µg/ml) and incubated for 2 hr at 37°C and overnight at 4°C and used in the assay as described in the methods section. The observed antibody (IgG) inhibition ranged from 53.6-67.7% when modified human DNA was employed as inhibitor, while with the native human DNA it varied from 25.4-33.2%; maximum inhibitor concentration being 20 µg/ml in both the cases. The mean of inhibitions with 4-ABP-NO modified human DNA as inhibitor was calculated to be 52.8 ± 4.2%, while with native human DNA, as inhibitor, it was 33.8 ± 2.1%. Table 2 summarizes the inhibition data of isolated IgGs from bladder cancer patients in the smoker group. Appreciably high binding of affinity purified IgG towards 4-ABP-NO modified human DNA indicates specific recognition of 4-ABP-NO modified epitopes on the DNA molecules by autoantibodies in bladder cancer patients.

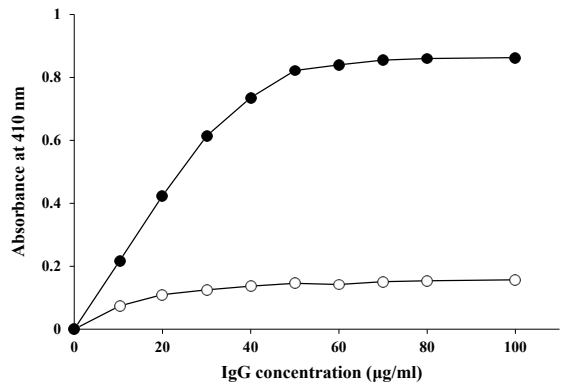

Figure 2: Direct binding ELISA of affinity purified bladder cancer IgG (●) from smoker group and normal human IgG (○). Microtitre wells were coated with 4-ABP-NO modified human DNA (2.5 µg/ml).

	Maximum percent inhibition at 20 µg/ml	
Cancer IgG	**Native human DNA**	**4-ABP-NO modified human DNA**
01	25.4	63.5
02	26.7	54.2
03	30.1	55.1
04	28.4	57.2
05	32.3	59.2
06	31.1	56.1
07	29.0	63.1
08	33.2	64.8
09	31.8	53.6
10	29.4	61.1
11	32.1	52.3
12	27.6	67.7
13	33.6	52.9
14	28.1	64.4
15	31.3	62.2
16	26.1	66.1
17	29.4	65.2
Mean ± SD	33.8 ± 2.1%	52.8 ± 4.2%
The microtitre plates were coated with 4-ABP-NO modified human DNA (2.5 µg/ml).		

Table 2: Competitive inhibition data of IgG isolated from bladder cancer patients (smoker group).

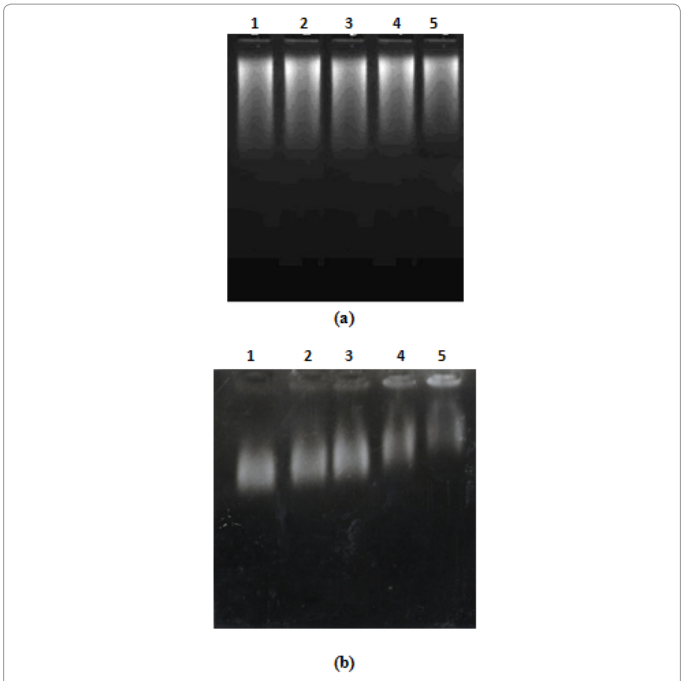

Figure 3: Band shift assay of IgG from bladder cancer patient (smoker group) using (a) native human DNA and (b) 4-ABP-N0 modified human DNA. Electrophoresis was carried out on 0.8% agarose gel for 2 hrs at 30 mA. (a) Native human DNA (0.5 µg, lane 1) was incubated with 20, 40, 60 and 80 µg of IgG from bladder cancer patient (lanes 2-5) for 2 hrs at 37°C and overnight at 4°C. (b) 4-ABP-NO modified human DNA (0.5 µg, lane 1) was incubated with 20, 40, 60 and 80 µg of IgG from bladder cancer patient (lanes 2-5) under identical conditions.

Band shift assay

Band shift assay was performed to visually detect the interaction of native or 4-ABP-NO modified human DNA with the purified IgG from smoker group of bladder cancer patients. Constant amount of antigens were incubated with increasing concentration of IgG for 2 hr at 37°C and overnight at 4°C. The resultant immune complex of 4-ABP-NO-DNA with the purified IgG caused a proportional band retardation and increase in the band intensity near the wells in agarose gel electrophoresis. However, native DNA did not show any visible retardation in the mobility which indicates that immune complex formation, under identical conditions, did not occur with native DNA (Figure 3).

Probing 4-ABP-NO mediated DNA damage in bladder cancer patients belonging to smoker group using anti-4-ABP-NO modified DNA antibodies

DNA was isolated from lymphocytes of various bladder cancer

patients (n=12) in the smoker group. The purity and concentration of DNA preparation was ascertained by A260 and A280 measurements. DNA isolated from lymphocytes of normal healthy smokers (n=8) was used as a control for this study. Anti-4-ABP-NO-DNA IgG was used to probe the 4-ABP-NO and related arylamines mediated lesions in the DNA from bladder cancer patients in smoker group through solid phase competitive immunoassay. Immune complexes, formed by incubating isolated DNA (0-20 µg/ml) with anti-4-ABP-NO modified DNA IgG (40 µg) were used in competitive-inhibition ELISA. The coating antigen on microtitre plate was 4-ABP-NO modified DNA (2.5 µg/ml).

For bladder cancer patients the inhibition in the activity of anti-4-ABP-DNA antibodies ranged from 54.7% to 70.1% with the mean inhibition being 61.32 ± 3.2%. While for healthy smokers, the inhibition

Sera group	Number of samples tested	Maximum percent inhibition at 20 µg/ml	Mean ± SD
Bladder cancer	12	54.7, 70.1, 67.4, 63.6, 61.4, 57.7, 56.4, 59.2, 64.4, 62.2, 65.8, 61.8	61.32 ± 3.2
Normal human	8	27.9, 24.4, 21.5, 31.2, 26.6, 23.3, 22.4, 25.2	25.31 ± 3.8
The microtitre plates were coated with 4-ABP-NO modified human DNA (2.5 µg/ml).			

Table 3: Detection of 4-ABP-NO mediated lesion in DNA of bladder cancer patients. Binding of anti-4-ABP-NO modified DNA IgG to DNA isolated from lymphocytes of bladder cancer patients and normal healthy individuals in the smoker group.

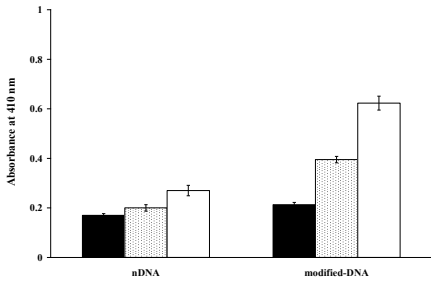

Figure 4: Direct binding ELISA of serum autoantibodies from bladder cancer patients in non-smoker group with native (▨) and 4-ABP-NO-modified-human DNA (▯).The histograms represent mean ± SD of 40 bladder cancer patients of non-smoker samples. Bars represent mean ± SD of 25 control normal human sera (■) serve as a negative control binding with corresponding antigens (2.5 µg/ml).

	Maximum percent inhibition at 20 µg/ml	
Cancer Sera	**Native human DNA**	**4-ABP-NO modified human DNA**
01	27.2	42.6
02	29.4	49.6
03	30.7	34.3
04	26.2	35.7
05	31.4	42.1
06	29.1	41.4
07	29.6	46.6
08	27.4	40.3
09	23.2	44.1
10	25.6	39.2
11	29.5	47.6
12	25.2	43.2
13	27.4	45.7
Mean ± SD	27.83 ± 2.2%	42.49 ± 4.8%
The microtitre plates were coated with 4-ABP-NO modified human DNA (2.5 µg/ml).		

Table 4: Competitive inhibition data of serum autoantibodies in non-smoker group of bladder cancer patients.

in the activity of anti-4-ABP-NO-DNA-IgG by the isolated lymphocyte DNA ranged from 21.5% to 27.9%; the mean inhibition being 25.31 ± 3.8%. The results have been summarized in Table 3. Significantly high recognition of the lymphocyte DNA from bladder cancer patients by the experimentally induced antibodies against the modified DNA is a clear indicator of epitope sharing between the human DNA modified *in vitro* by 4-ABP-NO and the genomic DNA of bladder cancer patients. This leads to the conclusion that 4-ABP-NO generates neo-epitopes on the DNA molecule that are recognized as 'alien' or non-self by the immune system resulting in autoantibody generation in bladder cancer patients. The strong binding of autoantibodies as well as significantly high level of recognition of the lymphocyte DNA from bladder cancer patients having history of smoking to 4-ABP-NO modified human DNA is an evidence towards the involvement of modified bases and single strand regions in disease pathogenesis.

Detection of antibodies against native and 4-ABP-NO modified human DNA in bladder cancer patients (non-smoker group)

Forty serum samples from bladder cancer patients in the non-smoker group were analyzed for binding to native and 4-ABP-NO modified human DNA by direct binding ELISA. As shown in Figure 4, only 32.5 percent (13 samples) exhibited enhanced binding with 4-ABP-NO modified human DNA as compared to the native DNA. However, binding in non-smoker group of bladder cancer was found to be appreciably low in comparison to the observed results for the smoker group of cancer patients. The specific binding of serum antibodies to native and 4-ABP-NO modified human DNA was studied by competition inhibition solid phase assay. An inhibition in the range of 23.2% to 30.7% and 34.3% to 49.6% was recorded with native and 4-ABP-NO modified human DNA respectively. Mean inhibition for all the samples tested with native human DNA was 27.8 ± 2.2%, while for 4-ABP-NO modified human DNA, it was 42.49 ± 4.8%. The inhibition studies results have been summarized in Table 4.

Binding of IgG from non-smoker group of bladder cancer patients to native and 4-ABP-NO modified human DNA

Purified IgG from the non-smoker group of bladder cancer patients, were subjected to direct binding ELISA on microtitre plate coated with native human DNA and 4-ABP-NO modified human DNA respectively to evaluate the amount required for antigen saturation in each case. The saturation for modified human DNA was obtained at 80 µg/ml of IgG. Therefore, for non-smoker bladder cancer, IgG concentration was kept constant (80 µg/ml) in all further experiments. Direct binding profile of one IgG sample, from non-smoker group, is shown in Figure 5.

The specific binding of the IgG isolated from non-smoker bladder cancer patients was ascertained in competitive inhibition ELISA wherein an inhibition in the range of 24.8% to 33.7% and 44.7% to 51.4% was recorded with native and 4-ABP-NO modified human DNA respectively (Figure 5). Mean inhibition for all the IgG samples inhibited by native human DNA was computed to be 28.55 ± 2.7%, while for 4-ABP-NO modified human DNA, it was 47.2 ± 3.3%. Table 5 summarizes the inhibition data of IgG from non-smoker bladder cancer group.

Probing 4-ABP-NO-mediated DNA damage in bladder cancer patients belonging to non-smoker group using anti-4-ABP-NO-modified DNA antibodies

DNA was isolated from the lymphocytes of various bladder cancer

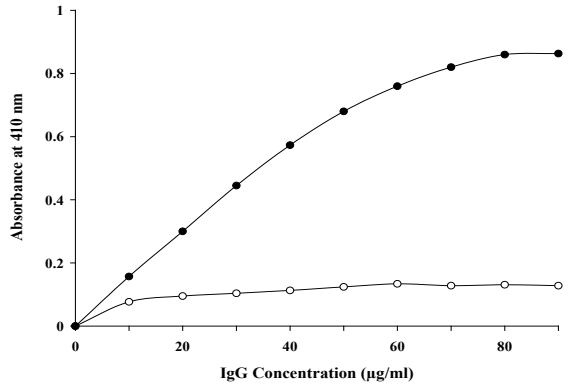

Figure 5: Direct binding ELISA of affinity purified bladder cancer IgG (●) from non-smoker group and normal human IgG (○). Microtitre wells were coated with 4-ABP-NO modified human DNA (2.5 µg/ml).

	Maximum percent inhibition at 20 µg/ml	
Cancer IgG	**Native human DNA**	**4-ABP-NO modified human DNA**
01	25.4	47.4
02	30.6	51.4
03	27.1	46.2
04	33.7	44.7
05	28.2	49.6
06	31.3	46.9
07	26.4	50.2
08	24.8	47.2
09	29.5	48.8
Mean ± SD	28.55 ± 2.7%	47.2 ± 3.3%
The microtitre plates were coated with 4-ABP-NO modified human DNA (2.5 µg/ml).		

Table 5: Competitive inhibition data of IgG isolated from non-smoker group of bladder cancer patients.

Sera group	Number of samples tested	Maximum percent inhibition at 20 µg/ml	Mean ± SD
Bladder cancer	08	47.1, 49.2, 45.7, 44.4, 42.1, 51.6, 48.2, 42.3	46.325 ± 3.1
Normal human	07	21.8, 22.4, 26.7, 20.2, 19.1, 24.2, 23.1	22.5 ± 2.9
The microtitre plates were coated with 4-ABP-NO modified human DNA (2.5 µg/ml).			

Table 6: Detection of 4-ABP-NO mediated lesion in the DNA of bladder cancer patients. Binding of anti-4-ABP-NO modified DNA IgG to DNA isolated from lymphocytes of bladder cancer patients and normal healthy individuals in non-smoker group.

patients in non-smoker group. The purity and concentration of DNA preparation was ascertained by A260 and A280 measurements. DNA isolated from lymphocytes of normal healthy individuals was used as control for this study. Anti-4-ABP-NO modified DNA IgG was used as a probe to detect the damage/lesions caused by 4-ABP-NO or may be by certain related 4-ABP metabolites or even other related aryl amines mediated lesion in the DNA isolated from bladder cancer patients in non-smoker group through solid phase immunoassay. Immune complexes, formed by incubating isolated DNA (0-20 µg/ml) and anti-4-ABP-NO modified DNA IgG were used in competitive-inhibition ELISA. The coating antigen on the microtitre plates was 4-ABP-NO modified DNA (2.5 µg/ml).

DNA was isolated from 8 bladder cancer patients and 7 healthy individuals in non-smoker group. For bladder cancer patients, the observed inhibition in the activity of experimentally induced anti-4-ABP-NO modified DNA antibodies ranged from 42.1% to 51.6% with the mean inhibition being 46.32 ± 3.1%. While, for normal healthy individuals, the inhibition in the activity of anti-4-ABP-NO-DNA-IgG ranged from 19.1% to 26.7% with the mean inhibition being 22.5 ± 2.9%. The results have been summarized in Table 6.

Bladder cancer auto-antibodies in smoker group showed preferential binding to 4-ABP-NO modified human DNA as compared to the native form. Higher recognition of 4-ABP-NO modified human DNA by autoantibodies of bladder cancer patients, in the smoker group, is a clear indication of 4-ABP-NO induced DNA damage in these patients. It could, therefore, be one of the factors for the autoimmune response leading to the induction of circulating anti-DNA autoantibodies in bladder cancer patients with a habit of smoking. Antibodies from non-smoker group of bladder cancer patients exhibited low to moderate binding with 4-ABP-NO modified human DNA. Cancer patients are known to have strand breaks and other lesions, that could be the result of exposure to aryl amines and nitric oxide, thus presenting epitopes that are recognized to some extent, by experimentally induced antibodies against 4-ABP-NO damaged DNA. This shows that 4-ABP-NO modified DNA could be an antigenic stimulus for these auto-antibodies.

Ethics statement

This study was duly permitted by the Bio-ethical Committee (8290/CAH/21-02-2007), and the Institutional Ethical Committee (1548/FM/18-01-2007), duly registered under Committee for the Purpose of Control and Supervision of Experiments on Humans (CPCSEH), India only for the research work. Registration no. 405/RO/E/2003/CPCSEH) at the Jawaharlal Nehru Medical College, Faculty of Medicine, Aligarh Muslim University. The mode of consent was duly approved by the ethical committee. All patients' record has been maintained through proper channel.

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References

1. Hartge P, Silverman D, Hoover R, Schairer C, Altman R, et al. (1987) Changing cigarette habits and bladder cancer risk: a case-control study. J Natl Cancer Inst 78: 1119-1125.
2. International Agency for Research on Cancer (1985) Tobacco Smoking, IARC Monographs on the evaluation of the carcinogenic risk of chemicals to humans (IARC, Lyon, France) Vol. 83.
3. Schifflers E, Jamart J, Renard V (1987) Tobacco and occupation as risk factors in bladder cancer: a case-control study in southern Belgium. Int J Cancer 39: 287-292.
4. Vineis P, Estève J, Terracini B (1984) Bladder cancer and smoking in males: types of cigarettes, age at start, effect of stopping and interaction with occupation. Int J Cancer 34: 165-170.
5. Cook MB, McGlynn KA, Devesa SS, Freedman ND, Anderson WF (2011) Sex disparities in cancer mortality and survival. Cancer Epidemiol Biomarkers Prev 20: 1629-1637.
6. Lower GM, Nilsson T, Nelson CE, Wolf H, Gamsky TE, et al. (1979) N-acetyltransferase phenotype and risk in urinary bladder cancer: approaches in molecular epidemiology. Preliminary results in Sweden and Denmark. Environmental Health Perspectives 29: 71-79.
7. Bhattacharya A, Klaene JJ, Li Y, Paonessa JD (2015) The inverse relationship between bladder and liver in 4-aminobiphenyl-induced DNA damage. Oncotarget 6: 836-845.
8. Patrianakos C, Hoffmann D (1979) Chemical studies on tobacco smoke. LXIV. On the analysis of aromatic amines in cigarette smoke. J Anal Toxicol 3: 150–154.

9. Hoffmann D, Djordjevic MV, Hoffmann I (1997) The changing cigarette. Prev Med 26: 427-434.

10. Bryant MS, Skipper PL, Tannenbaum SR, Maclure M (1987) Hemoglobin adducts of 4-aminobiphenyl in smokers and nonsmokers. Cancer Res 47: 602-608.

11. Bryant MS, Vineis P, Skipper PL, Tannenbaum SR (1988) Hemoglobin adducts of aromatic amines: associations with smoking status and type of tobacco. Proc Natl Acad Sci USA 85: 9788-9791.

12. Talaska G, Al-Juburi AZ, Kadlubar FF (1991) Smoking related carcinogen-DNA adducts in biopsy samples of human urinary bladder: Identification of N-(deoxyguanosin-8-yl)-4-aminobiphenyl as a major adduct. Proc Natl Acad Sci USA 88: 5350-5354.

13. Sørlie T, Martel-Planche G, Hainaut P, Lewalter J, Holm R, et al. (1998) Analysis of p, p16MTS, p21WAF1 and H-ras in archived bladder tumours from workers exposed to aromatic amines. Br J Cancer 77: 1573-1579.

14. Feng Z, Hu W, Rom WN, Beland FA, Tang MS (2002) 4-aminobiphenyl is a major etiological agent of human bladder cancer: evidence from its DNA binding spectrum in human p53 gene. Carcinogenesis 23: 1721-1727.

15. Boulalas I, Zaravinos A, Karyotis I, Delakas D, Spandidos DA (2009) Activation of RAS family genes in urothelial carcinoma. J Urol 181: 2312-2319.

16. Yu MC, Skipper PL, Tannenbaum SR, Chan KK, Ross RK (2002) Arylamine exposures and bladder cancer risk. Mutat Res 506-507: 21-28.

17. Lin D, Lay JO Jr., Bryant MS, Malaveille C, Friesen M (1994) Analysis of 4-aminobiphenyl-DNA adducts in human urinary bladder and lung by alkaline hydrolysis and negative ion gas chromatography-mass spectrometry. Environmental Health Perspectives 102: 611–616.

18. Beland FA, Beranek DT, Dooley KL, Heflich RH, Kadlubar FF (1983) Arylamine-DNA adducts in vitro and in vivo: their role in bacterial mutagenesis and urinary bladder carcinogenesis. Environmental Health Perspectives 49: 125–134.

19. Faraglia B, Chen SY, Gammon MD, Zhang Y, Teitelbaum SL, et al. (2003) Evaluation of 4-aminobiphenyl-DNA adducts in human breast cancer: the influence of tobacco smoke. Carcinogenesis 24: 719-725.

20. Beyerbach A, Rothman N, Bhatnagar VK, Kashyap R, Sabbioni G (2006) Hemoglobin adducts in workers exposed to benzidine and azo dyes. Carcinogenesis 27: 1600-1606.

21. Ali R, Dersimonian H, Stollar BD (1985) Binding of monoclonal anti-native DNA autoantibodies to DNA of varying size and conformation. Mol Immunol 22: 1415-1422.

22. Arnett FC, Edworthy SM, Bloch DA (1988) Revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 315–324.

23. Goding JW (1978) Use of staphylococcal protein A as an immunological reagent. J Immunol Methods 20: 241-253.

24. Dixit K, Khan MA, Sharma YD, Moinuddin, Alam K (2011) Peroxynitrite-induced modification of H2A histone mimics epitopes which are strongly bound by human anti-DNA autoantibodies: Role of peroxynitrite-modified-H2A in SLE induction and progression. Human Immunol 72: 219–225.

25. Khan MA, Dixit K, Moinuddin, Arif Z, Alam K (2014) Studies on peroxynitrite-modified H1 histone: implications in systemic lupus erythematosus. Biochimie 97: 104-113.

26. Habib S, Ahmad S, Dixit K, Moinuddin, Ali A (2013) Peroxynitrite modified DNA may be an antigenic trigger for antibodies in various cancers of gynecologic origin. Hum Immunol 74: 1239-1243.

27. Khan MA, Dixit K, Uddin M, Malik A, Alam K (2012) Role of peroxynitrite-modified H2A histone in the induction and progression of rheumatoid arthritis. Scand J Rheumatol 41: 426-433.

28. Flamini G, Romano G, Curigliano G, Chiominto A, Capelli G, et al. (1998) 4-Aminobiphenyl-DNA adducts in laryngeal tissue and smoking habits: an immunohistochemical study. Carcinogenesis 19: 353-357.

29. Moinuddin, Dixit K, Ahmad S, Shahab U, Habib S, et al. (2014) Human DNA damage by the synergistic action of 4-aminobiphenyl and nitric oxide: an immunochemical study. Environ Toxicol 29: 568-576.