

EFFECT OF CIGARETTE SMOKE EXPOSURE ON THE EXPRESSION OF MUTANT P53 IN TRANSITIONAL CELLS OF THE BLADDER

An Experimental Study on *Rattus norvegicus*

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ABSTRACT

*We studied the effect of exposure to cigarette smoke on the expression of mutant p53 transitional epithelial cells of the bladder to prove the relation of smoking and bladder cancer. In this experimental study in rat's (*Rattus norvegicus*) we used post test control group design. Forty-four rats were allocated randomly into 4 groups; the first 2 groups were exposed to cigarette smoke for 30 days and 60 days respectively, while the other 2 were control groups. All animals were euthanatized and cystectomy was done at the end of the experiment. Bladder slices were examined using HE stain to confirm the histopathologic structure of the bladder, then immunohistochemistry (IHC) stain was applied using p53 monoclonal antibody (Pab 1801). A positive result to IHC stain indicates that the expression of mutant p53 supposed to play a role in the pathophysiology of bladder cancer. Normal transitional cells were found in all animals in each with HE stain, while the IHC stain showed negative results in all control groups and in the 30 days group. However, a positive result on mutant p53 was found in the 60 days group. This study shows that 60 days of cigarette smoke exposure can alter p53 expression in transitional epithelial cells of the bladder.*

Keywords: mutant p53, cigarette smoke, transitional epithelium

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INTRODUCTION

Bladder cancer in one of most common malignancies in urology. From all genitourinary tract malignancies in male, it holds the second highest prevalence rate after prostate cancer. In the United States, between 1985 and 2000 the prevalence rate increased 33% annually, either in men or women. The incidence rate was 0.02% of total population. Men are 2.5 times more often affected than women. In 2002, 53,200 new cases of bladder cancer were found in the United States, with mortality rate of 12,200 in men and 8,100 in women (year 2000). Bladder cancer is the seventh highest cause of death in that country. The high mortality rate is due to the fact that it frequently is diagnosed in advanced stage, resulting in more complicated management and poor prognosis (Edward 2002). In 2002, 49,531 new cases of bladder cancer are reported. In Dr Soetomo Hospital, Surabaya, between 2002, 2003, and 2004, it was reported that bladder cancer is the most frequent urological malignancy, comprising 70% of the total malignant urogenital cases. Its prevalence tended to increase from one year to another, i.e. 48, 54, and 78 cases.

The emergence of bladder cancer is often related to cigarette smoking (25-60%) (Andrew & Siroky 2004), exposure to chemical substances in working place (25%) or congenital abnormalities, such as glutathione s-transferase and N-acetyltransferase [NAT-2] polymorphism. Cancer occurs due to the presence of multigene defects, one of which is tumor suppressor gene, such as p53 (Edward 2002). There is positive correlation between the number of duration of cigarette smoking and the emergence of bladder cancer, even though it requires a latent period of 16-22 years, which one of its causes is slow urothelial turnover, although it may quickly proliferate as a response to injury. This indicates that, as compared to unexposed patients, tumor can be induced in earlier age among the patients exposed to carcinogen (Griffiths 1999).

Generally, smoking habit cannot be cured due to the risk of addiction. Advertisement displaying images that cigarette smokers are macho, handsome and healthy, reinforces such habit. The exposure to cigarette smoke likely triggers mutation in p53 gene, which in turn affect the loss of control in cell division cycle or carcinogenesis in various organs, such as the lung, trachea, and bladder (Donatella et al, 1997; Izzoti et al

1999). Cigarette smoke is heterogeneous aerosol from the burning of tobacco and its wrap. Chemical substances present in the cigarette are consisting of nicotine, tar, carbon monoxide, and free radicals (NO₂ and NO) (Halliwell 1999).

Amino aromatic existing in the tar can be easily absorbed through the skin and mucosal membrane, metabolized in the liver and excreted with the urine. Individuals' capability are different in detoxifying those mutagens, which is due to the nature of enzymatic polymorphism as slow acetylators and rapid acetylators in hepatic cells. Detoxification in hepatic cells acts through the enzymes N-acetyl transferase, glutathione transferase and cytochrome P450. This chemical reaction produces hydroxylamine that will be secreted along with the urine. Hydroxylamine in the bladder is highly reactive, causing defect in the DNA of bladder mucosal epithelial cells (Ichabod 2000). In the research by Alberto Izzotti et al. in 1999 it was found that the change of DNA in the bladder of experimental animals (rats) exposed to cigarette smoking started to occur since the second week and remained high after fifth week. Cigarette smoke contains urothelial carcinogens, approximately 82 types of amino aromatics (Raine et al. 2001). However, in human and rats only β -Naphthylamine and 4-aminobiphenyl that have highly effect on the emergence of bladder cancer. The compounds of β -Naphthylamine and 4-aminobiphenyl can be immediately found in the blood and bladder translational epithelium right after being exposed to cigarette smoke (Wallace 2001).

An epidemiological study in a public hospital in Helsinki, Finland, revealed 40% positive p53 mutation in cigarette-smokers bladder cancer patients. It indicated that the alteration of p53 gene functioned as a predictor of bladder cancer (Kirsty & Annamaria 1996). The alteration of bladder tumor in lower stage, low recurrence rate, and good prognosis, which comprised 53% of all bladder cancer cases, is related to the mutation of fibroblast growth factor receptor 3 (FGFR 3). Whereas, twenty percent of bladder cancer that has been invaded the muscle with poor prognosis is related to the mutation of p53 gene (Bas et al. 2004). However, the effect of cigarette smoke exposure to the change of p53 gene remains unclear. No studies have been undertaken to prove the effect of cigarette smoke exposure on the change of p53 gene expression in bladder translational epithelium. This study attempted to disclose such effect by using mutant p53 protein as the parameter since the protein was regarded identical with mutant p53 gene expression. As it was impossible to conduct such research in human beings, the author used white rats (*Rattus norvegicus*) as experimental animal (Izzotti et al. 1999).

MATERIALS AND METHODS

This was an experimental study using male adult Wistar strain rats (*Rattus norvegicus*) as experimental animals. The animals aged 2.5 - 3 months with bodyweight of 100-200 grams. This study used post-test control group design. Forty-four rats were divided randomly into four groups. The first two groups were sacrificed on day 31 and the remaining two groups on day 61. Eleven rats in the first group were exposed to cigarette smoke, and the other 11 rats, serving as control, were not. Eleven rats in the second group were also exposed, while the other 11 were not.

Cigarette smoke exposure was given 6 hours a day, divided into the first 3 hour and the second 3 hours with one hour interval within a glass box of 25 x 12 x 15 cm for 30 days and 60 days. The dose of cigarette smoke exposure (the mainstream smoke) was 1 cigarette burned in 10 minutes. Total blows was 10 times, with interval between blows was 1 minute. Each blow contained 50 cc (Terumo Syringe : 50 cc). Subsequently, rats bladder specimen was washed with physiological NaCl and was kept within plastic pot containing 10% formalin, fixated in paraffin blocs, and HE preparations were made to ascertain the presence of bladder transitional epithelium. If it was confirmed, then IHC preparations were made, which were subsequently examined using light microscope in 400 x magnification to observe the presence of mutant p53 protein within bladder mucosal cells.

The positive mutant p53 expression was counted in each IHC preparation in the rats' bladder transitional epithelium, as shown by the presence of dark brown cells, which indicated positive reaction against p53 monoclonal antibody Ab-2 Ab-2 (Clone Pab 1801) (Helene 2000). Data were tabulated and subjected to normality test using one sample Kolmogorov-Smirnov test, followed with independent sample t2 test for comparison between treatment groups and observation times.

RESULTS

The result of homogeneity test on rats' bodyweight in four experimental groups using One-way Anova analysis is presented in Table 1.

Table 1 shows that rats' body weight in this study was not significantly different ($p = 0.933$). Therefore, any change in mutant p53 gene expression in this study should be related to the exposure to cigarette smoke. To determine the type of hypothetical test using in this study, data distribution normality test to four sample

groups should be undertaken at the first place using one-sample Kolmogorov Smirnov test (one sample K-S), whose result can be seen in Table 2.

Table 1. Homogeneity test on body weight (grams) between treatment groups

Group	Body weight (gram) (X ± SD)	ANOVA	
		F value	P value
Control 30 days	173.18 ± 11.24	0.143	0.933
Control 60 days	174.55 ± 11.92		
Exposed 30 days	175.45 ± 12.93		
Exposed 60 days	176.36 ± 11.42		

Table 2. Normality test of mutant p53 protein expression in each group

Groups	Positive cell count (X ± SD)	F value	P value
Control 30 days	Undetected	0	0
Control 60 days	Undetected	0	0
Exposed 30 days	Undetected	0	0
Exposed 60 days	0.64 ± 0.80	1.093	0.183

Table 2 shows that rats bodyweight in the 60-day exposure group had normal distribution ($p > 0.05$). Therefore, the test for conducting subsequent analysis was parametric statistical test. Independent sample t2 test was conducted to find the difference between mutant p53 expression in control and exposed groups, either in 30- or 60-day observation time. The result can be seen in Table 3.

Table 3. The difference of mutant p53 expression between exposed and control groups in 30- and 60-day observation

Observation	Treatment group		T test	
	Control	Exposed	F value	P value
30 days	Undetected	Undetected	0	0
60 days	Undetected	0.64 ± 0.804	42.609	0.017

Table 3 shows that there is significant difference between 60-day exposed group and 60-day control group ($p = 0.017$). Comparative test could not be conducted between 30-day exposed and 30-day control groups since there were no value changes in those groups, indicating no difference in mutant p53 expression in both groups. Independent sample t 2 test was used to find difference in p53 expression in 30- and 60-day exposed groups. The result is displayed in Table 4.

Table 4. The difference of mutant p53 expression between 30-day and 60-day exposed groups

Group	Mutant p53 expression		T test	
	30-day Observation	60-day Observation	F value	P value
Exposed	Undetected	0.64 ± 0.809	42.609	0.017

Table 4 shows that there was significant difference in mutant p53 expression in exposed groups between 30-day and 60-day observation ($p = 0.017$).

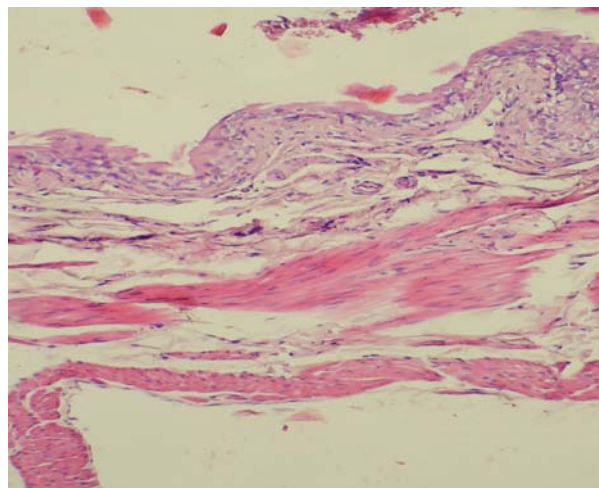


Figure 1. Histopathological staining with HE. *Rattus norvegicus* bladder transitional epithelium is apparent. M 100 x

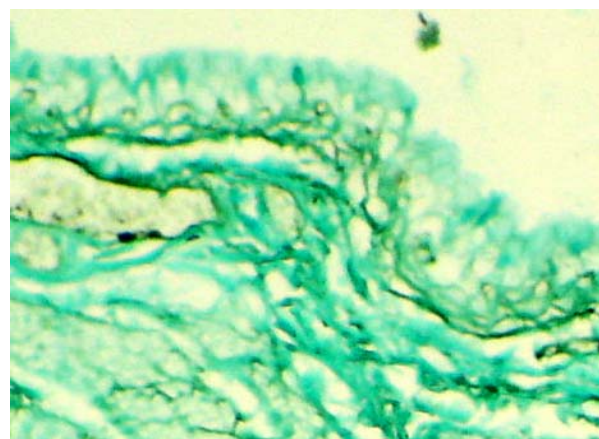


Figure 2. Immunohistochemical staining. It is apparent that bladder transitional epithelium of *Rattus norvegicus* not exposed to cigarette smoke does not demonstrate mutant p53 protein expression (M 400 x).

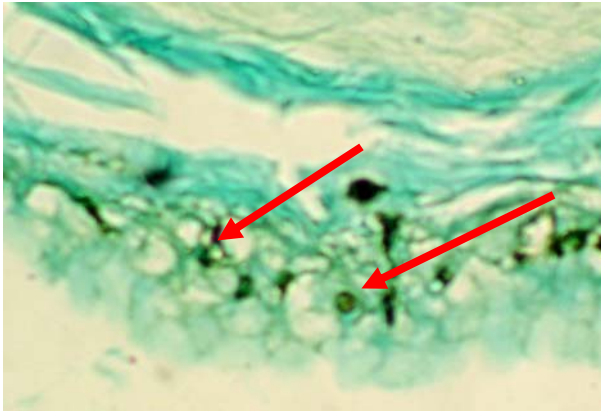


Figure 3. Immunohistochemical staining. The bladder transitional epithelium of *Rattus norvegicus* exposed to cigarette smoke showed dark brown cells, indicating positive expression of mutant p53 protein (arrow). (LAB staining, magnification 400 x).

DISCUSSION

As can be seen in Table 2, observation of the difference in mutant p53 expression in exposed and control groups in 30- and 60-day observation revealed significant difference in 60-day treatment ($p = 0.017$) between exposed and control group. However, 30-day treatment group was not subjected to statistical test since there were no altered values between exposed and control groups. This proved that in control groups (both 30-day and 60-day) the change of mutant p53 expression did not occur, as it could be observed after being stained immunohistochemically. In normal condition, or in which carcinogenesis-resulting confounding factors have been eliminated (in this study, the control groups), it can be ascertained that p53 mutation will never occur. Normal p53/wild type protein cannot be presented due to its short half-life with its fixation time (about 10 minutes) (Neal et al. 1999). Cigarette smoke exposure for 30 days had also not revealed changes in mutant p53 expression, since, according to Izzotti et al. (1999), the change of DNA/nucleotide 108 in rats' bladder started to show significant increase after 4-week exposure.

Group with cigarette smoke exposure for 60 days showed significant difference ($p = 0.017$) as compared to the control. This indicated that cigarette smoke exposure was able to act as carcinogenic or reactive substance in altering the expression of mutant p53. Whereas, the control group, which was not exposed to anything, showed no change in mutant p53 expression. Table 4 shows significant difference between 60-day and 30-day exposed groups ($p = 0.017$). This also confirmed the study by Izzotti et al. (1999) who showed

that the change of DNA/nucleotide 108 started to increase rapidly in week 4 exposure, and its count would remain high if the exposure was continued, proliferating the p53 mutation.

In bladder cancer, the duration of cigarette smoke exposure plays an important factor in p53 mutagenesis, which is the final event in such process (Helena et al. 2000). However, positive mutant p53 expression in the staining was only found in one or two cells in IHC slides of 60-day exposed group. This was due to the importance of exposure duration in carcinogenesis process. The longer the exposure, the higher the possibility of alteration in cancer cells. Lilinfield suggests a consistent correlation between the number and duration of smoking with the emergence of bladder cancer, requiring a latent period of 16-22 years. This is because of extremely slow turnover of bladder mucosal cells, even though they are able to proliferate rapidly as a response against injury.

In an epidemiological study on 51 bladder cancer patients in a hospital in Quebec, Canada, Helena et al. (2000) found strong correlation between the duration of cigarette smoking and p53 mutation in bladder cancer, with a median of 49.5 years. The factor of duration was also suggested by Mauderly et al. (2004). In a laboratory study they had proved the change in malignancy at pulmonary bronchoalveolar tissue as much as 11% in 753 experimental rats that were exposed to cigarette smoke for more than 30 months. The change of mutant p53 expression found in this study confirmed that mutant p53 expression can be one of the predictors of bladder cancer, which may lead to the improvement of therapeutic curability and to predict the type of invasiveness of the bladder cancer that can be used in considering the response on adjuvant therapy that will be given to the patient.

CONCLUSION

In normal *Rattus norvegicus* transitional epithelium, the mutant p53 expression is absent. Bladder translational epithelium in *Rattus norvegicus* exposed to cigarette smoke inhalation for 30 days has not revealed changes of mutant p53 expression, while inhalation exposure of cigarette smoke for 60 days has proved to be able in inducing the change in mutant p53 gene expression, marked by the presence of positive expression. This study has proved that the change of mutant p53 expression depends on the duration of cigarette smoke exposure. The longer the exposure the more the changes of mutant p53 protein. Therefore, it can be used as genetic-based molecular marker that has diagnostic value for the occurrence of cancer in bladder mucosa.

An experimental study should be undertaken using PCR method in the same duration of smoke exposure in experimental rats as a comparative study to IHC method. The research should be in longer duration and continuous to obtain bladder cancer histopathological feature.

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