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Enhancive effect of *N*,*N*'-dinitrosopiperazine on inducing precancerous lesion on nasal and/or nasopharyngeal epithelia of TgN(p53mt-LMP1)/HT mice*

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Abstract: Objective: To investigate the enhancive effect of *N*,*N*'-dinitrosopiperazine (DNP) on induced carcinogenesis in nasal and/or nasopharyngeal epithelia among TgN(p53mt-LMP1)/HT transgenic mice to examine the underlying mechanism for the development of nasopharyngeal carcinoma (NPC). Methods: TgN(p53mt-LMP1)/HT transgenic mice and the same strain of C_{57}BL/6J wild-type mice both at the age of 5 months were randomly divided into 2 groups in parallel, respectively, i.e., TgN(p53mt-LMP1)/HT cancerous lesion-inducing group (TI), TgN(p53mt-LMP1)/HT control group (TC), C_{57}BL/6J cancerous lesion-inducing group (CI), and C_{57}BL/6J control group (CC). TI and CI mice were treated only with DNP for 16 weeks, twice each week, while TC and CC mice were given the same volume of saline as controls. At the end of treatment, animals were sacrificed to collect epithelial tissue samples from nasal cavity and nasopharynx for pathohistological evaluation by haematoxylin and eosin (HE) staining and for determination on the expression of TRAF2, c-Jun, and p16 by immunohistochemistry. Results: Atypical hyperplasia was more significant in the samples of TI than in those of TC, CI, and CC, with the rates of lesions being 90%, 10%, 0, and 0 (P<0.01) respectively, though DNP was used alone in a much shortened inducing period at less dosage and without the use of carcinogenic promoter 12-*O*-tetradecanoylphorbol-13-acetate as usual. The expressions of tumor necrosis factor (TNF) receptor-associated factor 2 (TRAF2) and c-Jun in these samples were significantly up-regulated in TI (P<0.01), while the expression of p16 was significantly lower in TI than in the other groups (P<0.01). Conclusion: TgN(p53mt-LMP1)/HT mice hold inherited constitutional defect in immune surveillance function, which can be aggravated by environmental carcinogens, such as DNP used even though in a much less strength. The enhanced carcinogenesis-inducing effect of DNP on TgN(p53mt-LMP1)/HT mice should be closely associated with abnormal signaling of activator protein-1 (AP-1) pathway, especially up-regulated expressions of TRAF2 and c-Jun, and down-regulated expression of p16.

Key words: Nasal epithelia, Nasopharyngeal epithelia, Precancerous lesions, *N*,*N*'-dinitrosopiperazine (DNP), Activator protein-1 (AP-1) pathway, Signal transduction

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INTRODUCTION

The development of nasopharyngeal carcinoma (NPC) is closely related with the infection of Epstein-Barr (EB) virus and contact of chemical carcinogenesis, such as the substances containing too much nitrosamine. However, the exact etiology and pathogenesis for NPC developing have not been clearly made sure yet, and there are many blind-spots and controversies present between the development of NPC and the status of nasopharyngeal precancerous lesions. In the previous work, we constructed a strain of mouse model (TgN(p53mt-LMP1)/HT mouse) with precancerous lesions naturally developed in nasal and/or nasopharyngeal epithelia by transferring exogenous human mutant p53 gene (p53mt) and EB

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virus latent membrane protein 1 (LMP1) into its genome, and this kind of model can be used to examine the possible association of pathogenesis for NPC with inherent genome defect and exogenous EB virus infection (He *et al*., 2006; 2005). It has been found that the precancerous lesions will occur in nasal and nasopharyngeal epithelia with significantly elevated incidence as compared with that of controls. However, it would take a longer time period to develop, with the rate of precancerous lesions being 50% at the age of 11 months and 75% at the age of 18 months. In the present study, we investigated the speeding-up interactive effects of the chemical carcinogen *N*,*N*'-dinitrosopiperazine (DNP) on the constitutional conditions caused by transferring genes into the TgN(p53mt-LMP1)/HT transgenic mice. Here, DNP, as a mimic agent of environmental carcinogens, is responsible for the speeded up carcinogenesis that takes place in nasopharyngeal epithelia in the mice of generation 4 (G_4). Particularly, the treatment procedure was carried out only with DNP inducing, without the use of carcinogenic promoting agent tetradecanoylphorbol acetate (TPA) as in routine program. Then, we looked into the speeding-up effects of DNP on the course of carcinogenesis specially held by this strain of transgenic mouse and its signal transduction pathway focused on AP-1.

MATERIALS AND METHODS

Animals

In this study, 5-month-old adult TgN(p53mt-LMP1)/HT mice of G_4 were used. They came from our previously prepared transgenic mice (License No. SLAC-001; SLAC is the abbreviation of Shanghai Laboratory Animal Center) (He *et al*., 2006; 2005). The peer C_{57}BL/6J wild-type mice were purchased from the Institute of Laboratory Animal Research, Chinese Academy of Medical Sciences (License No. SCXK Beijing 2004-0001). There were half males and half females in both types of mice. All the mice were kept in individual ventilated caging system (IVC)-independent natural air cages with two in each standard raising box at (23±1) °C and with a humidity of (50±10)%. Solid standard food and free-style drinking water from plastic bottles were supplied. Beddings, cages, water and other items for the animals were treated by high pressure steam sterilization at 126 °C for 20 min, and food was dried at 115 °C for 10 min.

Main reagents

Mouse monoclonal antibody against p16 was purchased from Beijing ZSGB-BIO Co., China; rabbit anti-human polyclonal antibody against c-Jun and immunohistochemical staining strept-avidin-biotin complex (SABC) kit were purchased from Wuhan Boster Technology Co., Ltd., China; rabbit polyclonal antibody against TRAF2 was from Santa Cruz Co., USA.

Animal grouping and cancer-inducing by chemicals

TgN(p53mt-LMP1)/HT mice and C_{57}BL/6J wild-type mice were both randomly divided into two groups in parallel, i.e., TgN(p53mt-LMP1)/HT transgenic mice cancerous lesion-inducing group (TI), TgN(p53mt-LMP1)/HT transgenic mice control group (TC), C_{57}BL/6J wild-type mice cancerous lesion-inducing group (CI), and C_{57}BL/6J wild-type mice control group (CC). There are 20 animals in each group other than TI in which only 10 mice were used. The animals in TI and CI were all treated only with DNP (40 mg/kg each time) subcutaneously injected in the forelimb axillary for 16 weeks, twice each week, without the use of carcinogenic promoting agent TPA and the additional inducing period as usual once the treating course of DNP finished, while those in TC and CC were injected with normal saline in the same volume and the same length of treating period. The carcinogenesis-inducing period was lasted for 16 weeks for all the animals in each group.

Sample preparation

Immediately by the end of treatment, all the mice in each group were sacrificed, and the tissue samples of nasal and nasopharyngeal mucosae were collected, respectively, and fixed in a solution of 40 g/L polyformaldehyde for more than 24 h. Then, these samples were treated with routine procedures to prepare tissue embedding blocks and series sections at a thickness of 4 μm for pathohistological evaluation and immunohistochemical assays.

Evaluation on the pathohistological changes in the epithelia of nasal and nasopharyngeal mucosae

Tissue sections were treated with haematoxylin and eosin (HE) staining procedures, and pathohistological changes in the epithelia of nasal and nasopharyngeal mucosae were observed under an optic microscope. Histological changes in the epithelia were classified into four grades, i.e., normal status, simple hyperplasia to mild atypical hyperplasia, moderate to severe atypical hyperplasia (precancerous lesions), and cancerous lesion (Cai *et al*., 1985; Min *et al*., 2003; Li and Cai, 1987).

Detection on the expressive activities of tumor necrosis factor (TNF) receptor-associated factor 2 (TRAF2), c-Jun, and p16

To target the responsible genes in AP-1 signal transduction pathway, the expressive activities of TRAF2, c-Jun and p16 were determined in the specimens of nasal and nasopharyngeal tissues by immunohistochemical SABC staining procedures. Sections treated with phosphate buffered saline (PBS) and those known to be positive to these indicators, such as esophagus cancer tissue sample for TRAF2, bladder cancer tissue sample for c-Jun, and cervical cancer tissue sample for p16, were served as negative and positive controls, respectively. The experimental procedures were carried out in accordance with the manufacturer's instructions.

Data analysis

All the data were processed with SPSS 14.0 statistical software. The measurement data were shown as mean±*SD* and analyzed by one-way analysis of variance (ANOVA), and the counting data were analyzed using Fisher's exact test, at the significant level of hypothesis testing of $P<0.05$.

RESULTS

Comparison on the pathohistological changes in nasal and nasopharyngeal epithelia among different groups

Among the animals in TI, 4 cases were detected pathohistologically with neutrophils infiltration, arrangement disorder of epithelial cells, and cell proliferation with moderate atypical hyperplasia in the nasal mucosa, 1 with severe atypical hyperplasia in the nasal epithelia, 4 with neutrophils infiltrating and moderate atypical hyperplasia companied with focal atypical squamous cell metaplasia in the nasopharyngeal mucosa, and 1 with neutrophils infiltrating and focal epithelial hyperplasia in the nasopharyngeal mucosa, with an incidence of 90% with precancerous lesion in this group. However, only 2 mice (10%) were detected with mild to moderate atypical hyperplasia in nasal mucosal epithelia among the mice of TC. In contrast, only a few deeply-stained epithelial cells were found in 2 mice, and no obvious lesions occurred to other mice except for mild chronic inflammatory changes among the animals in CI, while no any significant lesions were identified in the nasopharyngeal and nasal mucosae in CC (Fig.1). The incidence of precancerous lesion in the nasal and nasopharyngeal epithelia was 90% and 10% for groups TI and TC, respectively, while no typical precancerous lesions occurred in either CI or CC ($P<0.01$).

Expressive activities of TRAF2, c-Jun, and p16 in the nasal and nasopharyngeal epithelia

1. Expressive level of TRAF2

The results of immunohistochemical assays showed that the positive reaction of TRAF2 was mainly located in the plasma in brown particles or plaque, and was weakly expressed in the normal nasal and nasopharyngeal epithelia and highly expressed in tumor cells. High expressive level of TRAF2 could be detected in the epithelial cytoplasm of nasopharyngeal and nasal mucosae with precancerous lesion in group TI (Fig.2). Its expressive levels in nasopharyngeal and nasal epithelia were determined in a lowering order from TI to TC, CI, and CC. When compared with the other groups, the expression of TRAF2 in TI was significantly increased (with its average gray value decreased) ($P<0.01$). In comparison with CI and CC, the expressive activity of TRAF2 was also increased significantly in TC ($P<0.01$) (Table 1).

2. Expressive level of c-Jun

The positive reaction of c-Jun was found to be mainly located in the nuclei, shown as brown particles or plaque. c-Jun was lowly expressed in normal nasal and nasopharyngeal epithelia, but highly expressed in tumor cells. Higher expressive level of c-Jun was

Table 1 Comparison on the expressive levels of TRAF2 in nasal and nasopharyngeal epithelia among different groups (mean±*SD*)

Group	Nasal epithelia		Nasopharyngeal epithelia	
	n	*AGV*	*n*	*AGV*
TI	10	122.86±11.29	8	123.43±12.49
TC	20	149.21±9.71**	18	150.75±9.95**
CI	20	157.48±6.49$^{**\Delta\Delta}$	20	158.48±6.90$^{**\Delta\Delta}$
CC	20	162.07±7.42$^{**\Delta\Delta\#}$	20	163.39±7.06$^{**\Delta\Delta\#}$
F value		51.758		42.547
P value		0.000		0.000

AGV: average gray value; $^{*}P<0.05$, $^{**}P<0.01$ when compared with TI; $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$ when compared with TC; $^{\#}P>0.05$ when compared with CI

determined in the epithelial nuclei of nasopharyngeal and nasal mucosae with precancerous lesion in TI, shown as black staining in positive cells. There were significantly more positive cells in TI than in TC, CI, and CC (Fig.3). Image analysis showed that the expressive levels of c-Jun were arranged in a decreasing order from TI to TC, CI, and CC. When compared with the other groups, the expressive level of c-Jun in TI was significantly increased (with the average gray value decreased) ($P<0.01$). Moreover, when compared with CI and CC, the expressive level of c-Jun in TC was increased significantly as well ($P<0.01$) (Table 2).

Table 2 Comparison on the expressive levels of c-Jun in nasal and nasopharyngeal epithelia among different groups (mean±*SD*)

Group	Nasal epithelia		Nasopharyngeal epithelia	
	n	*AGV*	*n*	*AGV*
TI	10	106.69±10.32	8	108.61±10.11
TC	20	148.53±11.48**	18	151.06±10.79**
CI	20	156.07±7.72$^{**\Delta}$	20	157.02±7.29$^{**\Delta}$
CC	20	161.14±10.03$^{**\Delta\Delta\#}$	20	162.09±7.55$^{**\Delta\Delta\#}$
F value		73.760		75.754
P value		0.000		0.000

AGV: average gray value; $^{*}P<0.05$, $^{**}P<0.01$ when compared with TI; $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$ when compared with TC; $^{\#}P>0.05$ when compared with CI

3. Expressive level of p16

The positive reaction of p16 was mainly located in the nuclei and plasmas, shown as brown particles or plaque. Its expressive level was found higher in normal nasal and nasopharyngeal epithelia and lower in tumor cells. Among the mice of TI, the nuclei and plasmas were stained mildly and the number of positive cells was significantly less than those in TC, CI, and CC groups, respectively in each visual field (Fig.4). The expressive levels of p16 in nasopharyngeal and nasal epithelia were arranged in a decreasing order from TI to TC, CI, and CC. When compared with the other groups, the expression of p16 in TI was significantly decreased (with the average gray value increased) ($P<0.01$). When compared with CI and CC, the expressive level of p16 in TC was also decreased significantly ($P<0.05$) (Table 3).

Table 3 Comparison on the expressive levels of p16 in nasal and nasopharyngeal epithelia among different groups (mean±*SD*)

Group	Nasal epithelia		Nasopharyngeal epithelia	
	n	*AGV*	*n*	*AGV*
TI	10	156.64±11.84	8	158.92±10.08
TC	20	136.40±6.69**	18	135.47±8.02**
CI	20	133.22±11.18**	20	132.21±9.83**
CC	20	127.62±11.80$^{**\Delta\Delta\#}$	20	127.12±12.39$^{**\Delta\#}$
F value		17.773		18.834
P value		0.000		0.000

AGV: average gray value; $^{*}P<0.05$, $^{**}P<0.01$ when compared with TI; $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$ when compared with TC; $^{\#}P>0.05$ when compared with CI

DISCUSSION

NPC has a strong genetic susceptibility, and its development is closely related with the interaction of EB virus infection and chemical carcinogen inducement with inherited risk factors within the body of host. Tang *et al*.(1999) indicated that the constitutional status of individuals was associated with their genetic susceptibility to NPC and should be dependent upon one's own genetic factors inherited from their kinship leading to a pathological constitution status. These individuals with such a pathological constitution status will be at high risk of precancerous lesion on the basis of unusual inheritance. Therefore, the pattern of one's own constitution will play a very important role in the developing process of carcinogenesis induced in this way. They may have defected immune surveillance function and should be easily infected with EB virus activated further by chemical carcinogen intake to initiate the process of cellular malignant transformation. This should be the interactive result of

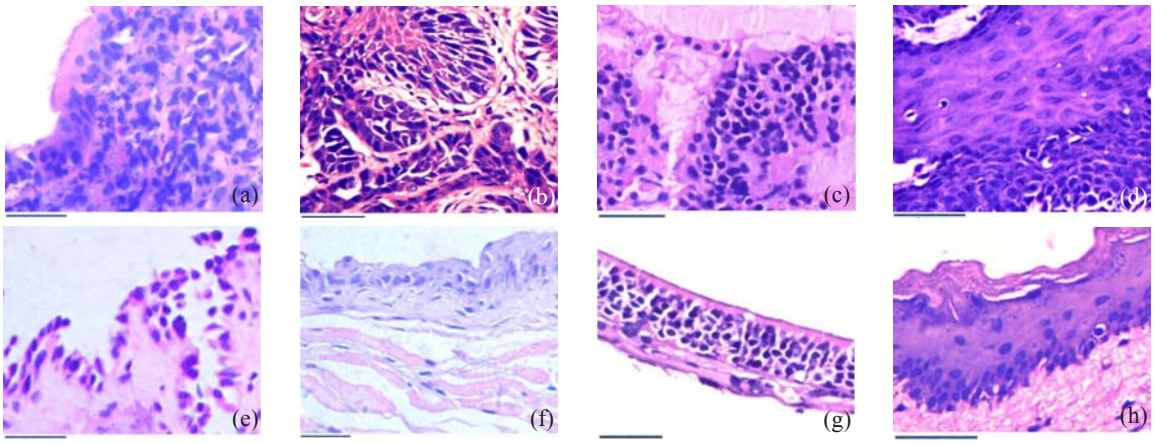

Fig.1 Pathohistological characteristics in the nasal and nasopharyngeal mucosae of the mice among different groups (HE staining, scale bar=50 μm). (a) Obvious atypical hyperplasia of nasal mucosa in TI; (b) Obvious atypical hyperplasia of nasopharyngeal mucosa in TI; (c) Simple hyperplasia of nasal mucosa in TC; (d) Simple hyperplasia of nasopharyngeal mucosa in TC; (e) A few deeply-stained nuclei of nasal mucosa in CI; (f) Mild chronic inflammation of nasopharyngeal mucosa in CI; (g) No obvious abnormality of nasal mucosa in CC; (h) No obvious abnormality of nasopharyngeal mucosa in CC

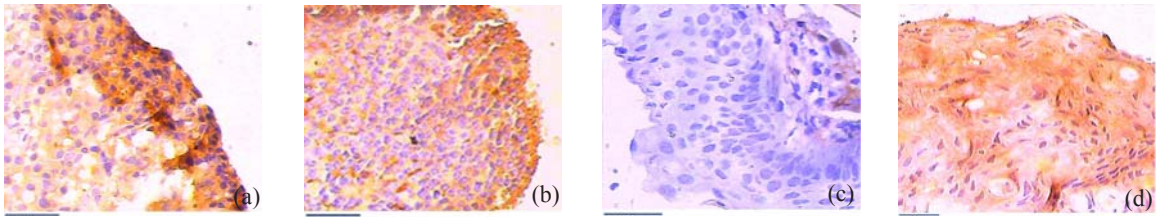

Fig.2 The expression of TRAF2 in nasal and nasopharyngeal epithelia among different groups (HE staining, scale bar=50 μm). (a) Nasopharyngeal epithelia of TI; (b) Nasal epithelia of TI; (c) Negative control (with the first antibody substituted by PBS); (d) Esophagus cancer epithelia as positive control

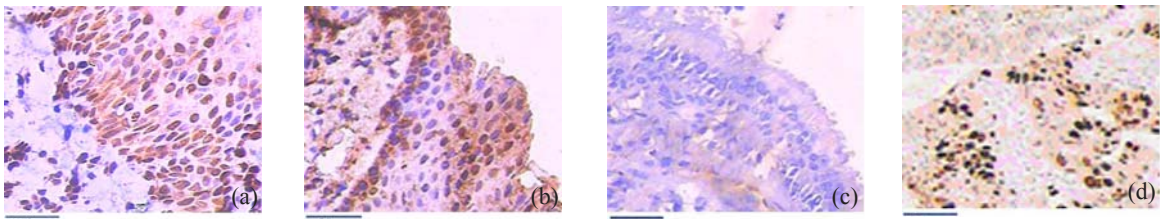

Fig.3 The expression of c-Jun in nasal and nasopharyngeal epithelia in different groups (HE staining, scale bar=50 μm). (a) Nasopharyngeal epithelia of TI; (b) Nasal epithelia of TI; (c) Negative control (with the first antibody substituted by PBS); (d) Bladder cancer as positive control

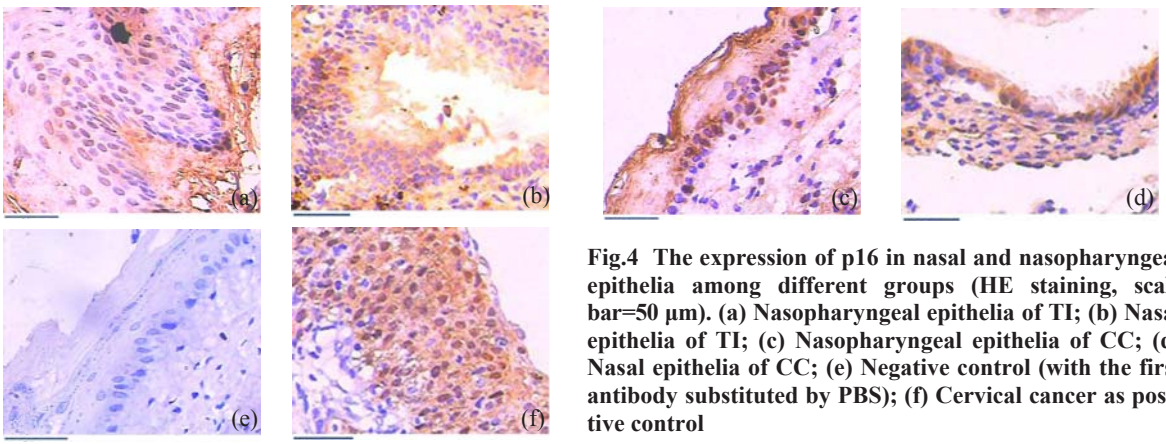

Fig.4 The expression of p16 in nasal and nasopharyngeal epithelia among different groups (HE staining, scale bar=50 μm). (a) Nasopharyngeal epithelia of TI; (b) Nasal epithelia of TI; (c) Nasopharyngeal epithelia of CC; (d) Nasal epithelia of CC; (e) Negative control (with the first antibody substituted by PBS); (f) Cervical cancer as positive control

environmental carcinogens with genetic susceptibility of individuals in a pathological constitution, i.e., the interaction with one's own internally defected immune surveillance function, leading to activation of carcinogenesis signaling pathway. So, TgN(p53mt-LMP1)/HT transgenic mice have been prepared based on such a hypothesis. In this strain of transgenic mice, the mutant p53 will result in breakdown of restricting points on the spindles so as to induce abnormality of chromosomes, decreasing DNA damage repair capacity, defecting immune surveillance function, and inhibiting apoptosis inducing activity. Therefore, such a pathological process taking place in this strain of transgenic mice may be mimicking the pathogenesis induced by internal risk factors, such as the genetic susceptibility of individuals with pathological constitution at high risk to carcinogenesis. On the other hand, LMP1, one of the expressive products by EB virus, has been confirmed as a tumor protein and holds significant pathological effect on cellular biological behaviors, such as cellular growth, proliferation, differentiation, transformation, and apoptosis activities in nasopharyngeal epithelia, invasive and metastatic potentialities of NPC cells (He and Tian, 2005). Here, LMP1 will bring about significant pathological effect on cellular biological behaviors of host, especially during the process of carcinogenesis. Once such a pathological risk factor is added into, or combined with, the prevalent constitution status within the body of host with genetic susceptibility to NPC, the process of carcinogenesis on the basis of epithelial transformation in the nasopharynx may be accelerated and initiated internally on the basis of host's own defected constitutional status, particularly the defected immune surveillance function.

In the present study, it has been confirmed that the naturally developing process of precancerous lesion in nasal and/or nasopharyngeal epithelia was speeded up markedly in this strain of transgenic mice model of TgN(p53mt-LMP1)/HT. When these transgenic mice were treated further with chemical carcinogen DNP, the incidence of precancerous lesion was largely promoted to a higher level (90%) in TI, even though DNP was used in a way without combination with carcinogenic promoting agent TPA and only in a very short period of inducing course. In contrast, this kind of precancerous lesion did not occur in the nasal and/or nasopharyngeal mucosae of the same type of wild mice (0%) in CI, even though they were treated with DNP in the same way. This indicates that the initiation and development of cell transformation and carcinogenesis within the body need a potentially pathological constitution basis for further evolvement, i.e., genetic susceptibility as the basic internal risk factor for carcinogenesis, since two transgenic mice (10%) in TC were detected with such a kind of lesion (even though being mild to moderate atypical hyperplasia) in these targeting tissues. As reported in previous work, naturally developed precancerous lesion in nasal and/or nasopharyngeal epithelia could be determined in many of the transgenic mice several months later since birth (He *et al*., 2008a; 2008b), which suggested that one kind of pathological constitution status susceptible to carcinogenesis has been laid down within their bodies with a trend to evolve further during the process of malignant developing, while a longer period was needed to develop into such a condition naturally. There should be a very strong interactive effect on these hosts who integrated the internally presented pathological constitution factor with externally added cancer-inducing factor to initiate the developmental process of cellular transformation and carcinogenesis, and this process can be largely speeded up by additional simply use of carcinogenic agent DNP in these transgenic mice. The transferred exogenous genes of p53mt and LMP1 should have brought about the effect of carcinogenic promoting agent on TgN(p53mt-LMP1)/HT mice in this kind of pathological process.

It has been shown that the development of precancerous lesion should be a result of abnormal expression of a series of associated genes. As one of the important nuclear transcription factors, AP-l is involved in malignant cellular transformation and tumorogenesis. It has also been confirmed that AP-l is one of the focused targets with the LMPl-inducing effects on the epithelial cells of a host. As shown in NPC cells, CTAR2 domain of LMP1 can activate stress-activated protein kinase (SAPK)/extracellular signal-regulated protein kinase (ERK) kinase (SEK) through the routes of tumor necrosis factor (TNF) receptor-associated death domain (TRADD) and TRAF to cause a series of cascades activated further, such as abnormal activation of AP-1 and the formation of c-Jun/JunB heterologous dimmer, which can weaken the activity of p16 promoter and reduce the

expression of p16 in turn (Cao *et al*., 2002; Berry *et al*., 2001; Song *et al*., 2004; Chang *et al*., 2003). Moreover, DNA damage, induced by all kinds of chemical carcinogen present in the environment, may induce telomerase activation and p16/p15 genes inactivation, which has been confirmed by transforming nasopharyngeal epithelia into precancerous lesion at the very early stage of carcinogenesis development. Usually, TRAF2, as an adapter protein, can directly react with a number of cell surface receptors. Then, it will mediate cascade signaling activity in its downstream to regulate the balance of cellular survival and death. It holds apoptosis-inhibiting effect once its expressive activity is up-regulated. In addition, c-Jun, as an expressive product of oncogene and one of the components of AP-1 complex, is involved at the early stage in the network of signal transduction of biosynthesis and cell proliferation, which plays an important role in cell proliferation, differentiation and transforming processes. The ultimate effects or elements in this network of signal transduction are to regulate cell cycle. Here, associated cells are much easier to be involved in the inducing process of carcinogenesis, once cell cycle regulation is in a status of abnormality or imbalance (Johnson *et al*., 2000). Moreover, p16 is a cyclin-dependent kinase (CDK) inhibitor, and can integrate itself with CDK4 or CDK6 by competing with cyclin D to inhibit the activities of these two kinases. Then, inhibition of tumor cell growth is induced by blocking progression in cell cycle (Xue *et al*., 2002). Once p16 is inactivated, its negatively regulatory effect on CDK may be lost. As the result of such an inactivation, too many cells will be promoted into a situation of over-crossing the checking point in phases G1/S so as to accelerate the proliferating activities of these cells, which may finally lead to tumorogenesis development.

As shown in this study, TgN(p53mt-LMP1)/HT mice held via inheritance abnormal expression of key targeting genes in AP-1 signaling pathway evolved from transferred p53mt-LMP1 genes, and this kind of pathogenesis can be aggravated largely by the further inducing effect of DNP, even though in a much less dosage and a very short treating period. This suggested that the expressive activities of TRAF2 and c-Jun have been up-regulated and that of p16 down-regulated in TI when compared with CI and CC in the same strain without p53mt-LMP1 genes transferred. This might have resulted in abnormal progress of cell cycle, acceleration of cell proliferation, and production of apoptosis-inhibiting capacity in turn. When excessive cells crossed over the checking point of G1/S phases, carcinogenesis or malignant transformation should become much easier to take place in the epithelial cells on nasal and/or nasopharyngeal mucosae, as represented by moderate to severe atypical hyperplasia and/or metaplasia. Therefore, the present study has provided evidence to support not only the theory of carcinogenesis of NPC induced by the integrated effects of genetic susceptibility and environmental risk factor EB virus infection on the constitutional basis laid down by gene transferring, but also a primary annotation about the theoretic hypothesis on the pathogenesis of precancerous lesion development in nasal and/or nasopharyngeal epithelia at molecular level with the use of transgenic technology, i.e., the genetic susceptibility dependent upon hereditarily-decided constitutional status to NPC developing aggravated excessively by environmental risk factors.

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