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# Chemical Carcinogenesis and Nasopharyngeal Carcinoma

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## 1. Introduction

### 1.1 Epidemiology of nasopharyngeal carcinoma

The non-viral exposure that is most consistently and strongly associated with risk of nasopharyngeal carcinoma (NPC) is the consumption of salt-preserved fish, a traditional staple food in several NPC-endemic areas [1]. In studies of the Chinese population, the relative risk of NPC associated with weekly consumption of salt-preserved fish generally ranges from 1.4 to 3.2, whereas the risk for daily consumption ranges from 1.8 to 7.5 [2-7]. This indicates that consumption frequency of salt-preserved fish is associated with the risk of NPC. However, elevated NPC risk is also associated with other preserved food items, including meats, eggs, fruits, and vegetables [4-15].

In southern China, intake of salted and other preserved foods is particularly high among boat-dwelling fishermen and their families, this is also the population subgroup at highest risk of developing NPC [16]. Salt-preserved foods are a dietary staple in all NPC-endemic populations [14, 17]. Furthermore, salted fish is a traditional weaning food and is fed early and frequently to infants, especially in the Cantonese population [4, 14] and in families of lower socioeconomic status [3, 18]. Childhood exposure, especially at weaning, appears to be more strongly related to NPC risk than exposure during adulthood [3, 4, 14, 15, 17, 19-21]. This dietary association may partly explain the international distribution of NPC incidence.

The carcinogenic potential of salt-preserved fish is supported by experiments in rats, which develop malignant nasal and nasopharyngeal carcinoma [22-24]. The process of salt preservation is inefficient, allowing fish and other foods to become partially putrefied [25, 26]. As a result, these foods accumulate significant levels of nitrosamines, which are known carcinogens [25, 27-29]. Consumption of salted fish is a significant source of nitrosamines. Total volatile N-nitrosamines, consisting of N-dimethylnitrosamine, N-diethylnitrosamine, N-nitrosopyrrolidine, and N-nitrosopiperidine, are present in salted fish at concentrations of

0.028 to 4.54 mg/kg [25] and are converted into carcinogenic N-nitrosocompounds including N,N'-dinitrosopiperazine after food intake [26]. N-nitrosodimethylamine is the predominant volatile nitrosamine in salted fish. In addition, some bacteria can also induce conversion of nitrate to nitrite, which forms important carcinogenic N-nitroso compounds [26]. Experiments in rats have demonstrated the carcinogenicity of nitrosamines and N-nitroso compounds such as diethylnitrosamine (DEN), dimethylbenzanthracene anthracene (DMBA), and Dinitrosopiperazine (DNP) [6, 15, 30].

## 2. Carcinogens related to the etiology of human NPC

NPC occurs most frequently in Southeast Asia and Africa. The highest incidence rate is reported to be in the southern provinces of China, and NPC contributes to a high mortality rate among Chinese people [31]. There are many articles and publications focusing on the viral and hereditary factors associated with NPC but few on chemical factors such as environmental carcinogens. The importance of each factor may vary between different tumors and in different areas of the world [32]. Moreover, it appears that a multiple-factor concept of cancer etiology may be relevant to human NPC and chemical carcinogens should be taken into consideration within this context. Although numerous chemical agents are suspected to be related to human tumors, the discussion here will be limited to certain polycyclic hydrocarbons, nitrosamines, and some related compounds that might play a more intimate role in the etiology of human NPC [33].

### 2.1 Polycyclic aromatic hydrocarbons

Since Pott's [34] observation on scrotal cancer and the first demonstration of the induction of cancer in animals by painting coal tar on the skin, the importance of hydrocarbons in human carcinogenesis has been extended to include a possible role as a risk factor in human NPC.

Some clinicians have paid attention to hydrocarbon as one of the etiological factors of human NPC due to the continued exposure of their patients to coal dust. Furthermore, Schoental et al [35] reported that the incidence rate of NPC was higher among the inhabitants of the mountainous district than in those living at low altitude in the flatlands. It was subsequently found that the inhabitants of districts with a higher incidence of NPC warmed themselves by burning firewood. In low and poorly ventilated living rooms, the accumulation of sooty fumes results in environmental pollution. In the high incidence region, studies found that the benzo(a)pyrene concentration reached a level of 85-29  $\mu\text{g}/1,000 \text{ m}^3$ , and that of benzanthrene reached 79-515  $\mu\text{g}/1,000 \text{ m}^3$ , so it was believed that NPC in this district is related to exposure to polycyclic aromatic hydrocarbons [36].

Fong YY et al [37] tried to instill the oil extraction of soot obtained from the houses of NPC patients into the nasal cavities of the mice three times a week, but this did not induce NPC. The control group was treated with methylcholanthrene and developed nasal cavity tumors but not NPC. He concluded that nasopharyngeal mucosa is not sensitive to chemical carcinogens. Similarly, Lo et al [38] injected carcinogenic agents methylcholanthrene and benzo(a)pyrene into the nasopharyngeal region of rabbits, rats, mice, and dogs, and did not observe any positive results. In contrast, Pan et al [72, 73] inserted DMBA crystals into ectopically implanted nasopharyngeal tissues of homologous mice, and two cases of squamous carcinoma were induced in 20 mice. This result implied that nasopharyngeal

epithelium could be induced to develop carcinoma, and was not insusceptible to these carcinogens.

Toth B et al [39] injected benzo(a)pyrene and dimethylbenzanthrene into AKR mice through the posterior nasal orifice. The induced tumors were mainly located at the hard palate and nasal cavity. Pan et al [72] developed a method for inducing NPC in rats. Long thin polyethylene tubes loaded with benzo(a)pyrene, DMBA, or 3-methylcholanthrene (MC) were inserted into the nasopharyngeal cavities of rats under anesthesia, resulting in squamous carcinoma of the nasopharynx. One animal developed a cancerous ulcer on the mucosa of the nasopharynx. This nasopharynx cancer was a grade III squamous cell carcinoma, which malignant cells grew upwards protruding into the cavity or downwards into the stroma. The incidence rates of the respective groups were as high as those in the group of rats treated with DMBA or DEN.

## 2.2 Nitrosamines

Since Magee [40] first described the toxicity and carcinogenicity of dimethylnitrosamine in rats, the carcinogenicity of nitroso-compounds in different animals has aroused increasing interest and received intensive investigation. A series of reports confirmed that nitroso-compounds could induce a variety of malignant tumors in a great number of different animal species [41-44]. Moreover, nitroso-compounds may result from the interaction of nitrites and secondary amines. These precursors are produced from nitrosamines by bacterial action in the acidic environment of stomach or alkaline intestinal contents [45, 46]. The putative role of nitroso-compounds in the induction of NPC has fascinated many researchers. Ho [47] raised the tentative assumption that the high incidence of NPC in Hong Kong was due to the ingestion of salted fish as main protein source. An appreciable amount of dimethylnitrosamine was reportedly detected in salted fish in the markets of Hong Kong [48]. However, a control survey carried out in Guangzhou demonstrated no such relationship between NPC and the intake of salted fish [4].

Generally, the saliva of NPC patients has a higher nitrite content and lower nitrate content than that of normal individuals. In addition, the urinary nitrite content of NPC patients was higher than those of normal controls. These differences were statistically significant in Sihui County [49]. It was suggested that nitrate content of saliva from NPC patients might be reduced by microorganisms in oral cavity; however, the precise mechanism needs to be further investigated. Yi Z et al [50] analyzed nitrate levels in saliva and urine samples collected from 75 NPC patients. The nitrate content in the urine sample of these patients was considerably lower than that of normal subjects, whereas the nitrite content was significantly higher in the urine samples of NPC patients. It was proposed that NPC patients might possess certain reduction mechanisms that could reduce nitrates into nitrites, thus resulting in increased urinary excretion of nitrites and enhanced endogenous synthesis of nitroso-compounds.

There were no reports on experimental nasopharyngeal carcinoma induced by nitroso-compounds as carcinogens until 1972. Huang et al [24] reported that out of 22 white rats fed with salted fish, four developed nasal tumors but none developed nasopharyngeal cancer. However, in 1972, Pan et al [72, 73] successfully induced NPC in rats using nitroso-compounds, thus providing new clues in the investigation of the cause of NPC.

### 3. Establishment of an animal model of NPC with chemical carcinogens

#### 3.1 General principles of establishing a NPC animal model

An important approach in studying the etiology and pathogenesis of malignant tumors is to establish various animal models. As it is impossible to carry out experiments directly on patients, a simple method to induce tumor in animals with high incidence provides a valuable research tool to simulate the human cancer. The following is a discussion on the general principles of establishing a NPC animal model.

##### 3.1.1 Animal selection

There is a great variation in the susceptibility of different animal species to carcinogens. It is better to choose animals with a low incidence of spontaneous tumors and a predicted high incidence of induced tumor. Some scholars originally believed that animal nasopharyngeal epithelium was not susceptible to aromatic hydrocarbons. Nonetheless, Wang successfully induced experimental “NPC in situ” in mice [51]. However, because rat nasopharynx is analogous to that of humans and rats are readily available, the rat is the preferred model animal. The rats used in these experiments were of mixed breeds, but they very rarely suffered from spontaneous tumors.

As a preferred model animal, the anatomical and histological characteristics of rat nasopharyngeal organs were studied in detail. The anatomical location of rat nasopharynx has been clearly defined [52], but some confusing terms are used in these reports to describe the same structure. For example, rat’s nasopharynx is tubular, and some authors called it a nasopharyngeal duct. Actually, rat’s nasopharynx is histologically similar to that of humans; both are lined with two kinds of epithelia, stratified squamous epithelium and pseudostratified ciliated columnar epithelium, and have orifices of Eustachian tubes near oropharyngeal end. The term “nasopharyngeal duct” may be confused with another tubular structure lying above the hard palate between the nasopharynx and posterior naris, which is lined completely with ciliated columnar epithelium. This structure is often called the nasopharyngeal tube, and is in fact in the posterior part of nasal cavity. Therefore, if the induced tumor was located above the hard palate, it should be classified as a nasal tumor rather than a nasopharyngeal tumor. Certain terms used in these reports, such as “cancer of nasal cavities,” “cancer of nasal turbinates,” “ethmoid cancer,” and “cancer of nasal sinuses,” actually describe malignant tumors developed from nasal turbinates, which are often different from NPC in histological appearance.

Leaton-Jones P et al [52] described histology of normal rat nasopharyngeal epithelium in 1971. Subsequently, Albin N et al [53] have systematically studied serial sections of rat nasopharynxes at different ages. They demonstrated that nasopharyngeal epithelium consists of three different kinds of epithelium: pseudostratified ciliated columnar epithelium, stratified squamous epithelium, and transitional epithelium. In adult rats approximately two-thirds of nasopharynx towards cephalic end is lined with ciliated columnar epithelium. The third near oropharyngeal end is a mixed-type epithelium, with increasing abundance of stratified squamous epithelium towards oropharyngeal end. The cover epithelium on roof and lateral sides of nasopharynx is mainly ciliated columnar epithelial cells, with predominantly continuous stratified squamous epithelium at bottom. The nasopharynx of newborn rats is mainly lined with ciliated columnar epithelium, which

is poorly differentiated. Stratified squamous epithelium appears 10 days after birth. The nasopharyngeal epithelium of 60-day-old rats is similar to that of adult rats.

### **3.1.2 Selection of carcinogens and induction methods**

Generally, potent carcinogen with short induction time is chosen as tumor-inducing agent, for example, benzo(a)pyrene for lung cancer induction [54] and aflatoxin for liver cancer [55]. Human NPC is too complicated to determine one or two factors because there are many factors involved in NPC development, such as viral and genetic factors, chemical carcinogens in the environment [56]. Moreover, the etiology of human cancers can be complicated, and might not be attributed to a single factor or initiation event [2]. The combined action of chemical carcinogens, viruses, and genetic factor, and also co-carcinogens should be discussed.

## **3.2 Establishment of experimental NPC and further studies**

### **3.2.1 Establishment of the NPC animal model**

After establishing the optimal carcinogens and method of administration, we succeeded in the induction of experimental NPC in rats using DNP [57]. This suggests that chemical carcinogens might be among etiologic factors of NPC. Furthermore, subcutaneous injection of DNP could induce NPC without complications of liver cancer. Therefore, DNP showed organ specificity for nasopharyngeal epithelium. Moreover, DNP-induced NPC exhibited a consistently high incidence rate, thereby paving the way for a subsequent study of DNP in which the carcinogenesis of experimental NPC in rats was further investigated, including atypical cytokinetics in carcinogenesis of the nasopharyngeal epithelium, DNA damage and repair of the nasopharyngeal epithelium by DNP and its relation to tissue specificity, and changes in enzyme activities during carcinogenesis. The results of these studies are discussed below.

### **3.2.2 Susceptibility of rat nasopharyngeal epithelium to the carcinogens DEN and DMBA**

The induction of NPC in rats by treatment with DEN and DMBA is summarized in Table 1 [72, 73]. These studies showed that the highest incidence of NPC was achieved in the DEN instillation group, and that NPC with less differentiated cells at early stages developed in the rats receiving DEN instillation and DMBA insertion. Occasional lymphatic emboli and multiple metastases to lung were observed. The data suggest some synergic effect between these chemical compounds.

Incidence of NPC in the DEN instillation group was higher than that of the group treated with *s.c.* injection of DEN and empty plastic tube insertion. This suggested that DNE was most effective on nasopharyngeal epithelium. The group with empty tube insertion was examined to exclude any possible carcinogenic action of the polyvinyl chloride tube. The empty tube itself occasionally induced NPC (two cases of NPC in 23 rats). Incidence of NPC in this group was quite lower than other groups [72, 73]. Therefore, it was suggested that NPC development in these groups was mainly caused by the action of carcinogens.

| Group    | Administration route                            | Examined animals | Number of tumors | Incidence of NPC (%) |
|----------|---|------------------|------------------|----------------------|
| Saline   | Subcutaneous                                    | 10               | 0                | 0                    |
| DEN      | Instillation                                    | 27               | 17               | 63                   |
| DMBA     | Loaded-tube insertion                           | 45               | 18               | 40                   |
| DEN+DMBA | DEN:subcutaneous<br>DMBA: loaded-tube insertion | 27               | 16               | 59                   |

Table 1. NPC induced in rats by DEN and DMBA

The induced tumors resembled human nasopharyngeal cancers but were of a well-differentiated type, including squamous cell carcinoma (Grade I and II), occasional multiform cell type, and papillary or basal cell carcinomas [57, 72, 73]. The serial sections of nasopharynx enabled us to trace number, sites, and distribution, as well as histogenesis of experimental NPC. This model could be helpful in further study on nasopharyngeal carcinogenesis. The finding that chemical carcinogens such as nitrosamine and aromatic hydrocarbon compounds are capable of inducing nasopharyngeal carcinoma in rats suggests that chemical carcinogens might be one of the etiological factors of human nasopharyngeal carcinoma.

3.2.3 Induction of nasopharyngeal carcinoma by nitroso-compounds

DEN, DNP, and cyclic nitrosocompounds including nitrosomorpholin could induce NPC. The next step was to facilitate studies on pathomorphology, histogenesis, and carcinogenesis of experimental NPC. Two methods were used to minimize the occurrence of hepatoma in DEN-treated rats: the rats were injected intramuscularly with vitamin B<sub>12</sub> twice a week and given glucose in their drinking water at the time of DEN administration. DEN was sometimes given through rectal instillation because it causes little damage to this organ. Rats were treated with cyclic nitroso-compounds by two treatment methods, *s.c.* injection and nasopharyngeal instillation. The results of these studies are shown in Table 2 [72, 73].

| Groups                              | n  | NPC     |                |          | animals tumor | incidence of NPC (%) |
|-------------------------------------|----|---------|----------------|----------|---------------|----------------------|
|                                     |    | In situ | Early invasive | Invasive |               |                      |
| Saline                              | 30 | 0       | 0              | 0        | 0             | 0                    |
| DEN Inst.                           | 98 | 11      | 37             | 21       | 69            | 70                   |
| DEN Inj.                            | 16 | 1       | 3              | 0        | 4             | 25                   |
| DEN Rectal Adm.                     | 23 | 1       | 5              | 0        | 6             | 26                   |
| DEN Nasal Inst.<br>+Vit.B12-glucose | 42 | 4       | 24             | 11       | 39            | 93                   |

Inst, instillation; Inj, injection; Adm, administration; Vit, vitamin

Table 2. Induction of NPC by DEN administered by various routes

The data showed that DEN instillation resulted in the highest incidence of NPC. The highest incidence of tumor was in the group treated with DEN and vitamin B<sub>12</sub>, followed by the group given nitrosomorpholin carcinogen. Two tumors with a nodular and ulcerated appearance were visible to the naked eye, one in DEN group and one in nitrosomorpholin group. Both cases were squamous cell carcinomas, partly involving or arising from the soft palate. These tumors invaded surrounding stroma, nerve bundles, and salivary glands without lymph node metastasis.

A few other points were of interest: (a) many squamous carcinomas occurred in neighborhood of auditory pharyngeal tubes or extended along tube walls, indicating that pathological lesions occurred in nasopharyngeal cavity. (b) In nasal cavities of some rats with extensive tumor, the tumor was found only in nasopharyngeal region without invasion of the nasal cavities or the base of the skull. Under naked-eye examination, no tumor was visible in the esophagus or other organs except the liver. (c) Subcutaneous injection of cyclic nitrosamines induced a considerably high incidence of nasopharyngeal tumors, providing some clues to organ specificity of carcinogens. (d) It is worth noting that administration of vitamin B<sub>12</sub> markedly increased the incidence of nasopharyngeal cancer ( $p<0.01$ ), but significantly decreased the incidence of hepatoma. Poirier et al [58] observed that vitamin B<sub>12</sub> enhanced hepatic carcinogenesis and shortened the animals' lives. In the present experiments, vitamin B<sub>12</sub> increased the incidence of NPC in the DEN group but had the opposite effect in the DNP group. The precise mechanism of this complicated relationship between the carcinogen and nutritional factors needs further study. (e) Vitamin B<sub>12</sub>, piperazine, and morpholin are usually used in clinics or in pharmaceutical chemistry. Some of them are precursors of the carcinogenic substance and some have an enhancing effect on the development of cancer.

Further investigation into experimental induction of NPC showed that N-nitrosomorpholin and DNP could induce NPC in rats, and subcutaneous injection of DNP could induce NPC without complications of liver cancer. These results are summarized in Table 3 [72, 73].

| Group | n  | Sex | NPC | Nasal cavity | Esophageal | Other                                |
|-------|----|-----|-----|--------------|------------|--------------------------------------|
| 1     | 12 | M   | 8   | 6            | 3          | Soft palate 1                        |
| 2     | 13 | F   | 10  | 8            | 5          | Tongue root 1, soft palate2          |
| 3     | 18 | M   | 18  | 11           | 10         | Tongue root1,soft palate 4, maxilla1 |
| 4     | 20 | M   | 18  | 14           | 14         | Tongue root 1, soft palate 5         |

M, male; F, female

Table 3. Types and site distribution of tumors induced by DNP

A total of 54 rats developed NPC in this experiment. In addition, 39 cancers of the nasal cavities, 32 esophageal tumors, and a few other tumors were observed. Upon postmortem examination, six rats were found to have gross tumor masses in nasopharynx, and two of these had a cauliflower-like tumor mass on the soft palate, representing co-existing squamous cell carcinoma of the soft palate. The induced nasopharyngeal tumors were all squamous cell carcinomas, most of which were well-differentiated (Fig. 1). The cancer

pattern observed in this experiment was somewhat different from that of NPC induced by diethylnitrosamine. The cancer cells invaded the stroma in trabecular or branched cords, but more frequently grew intraepithelially to form patches of masses before invading the stroma. A lymphatic cancer embolus consisting of poorly differentiated cancer cells was found in a case of invasive NPC. No metastatic foci could be found in the lymph nodes of this animal [72, 73].

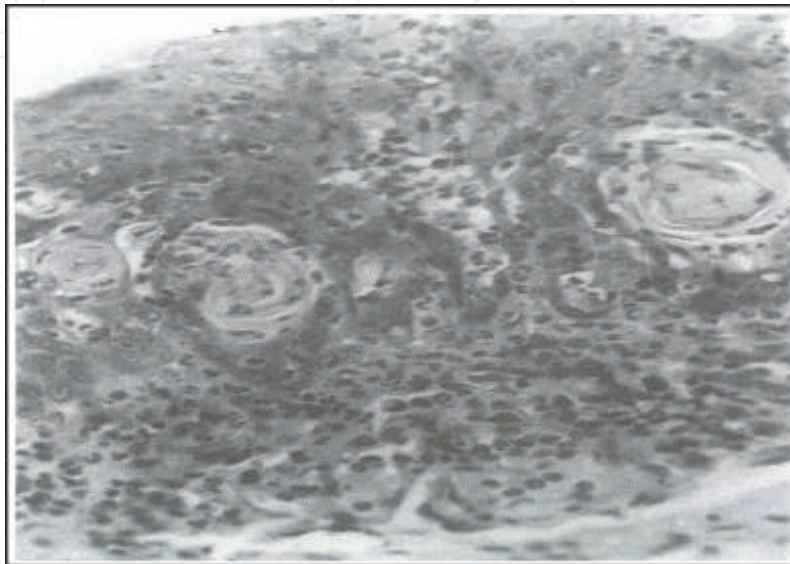


Fig. 1. DNP- induced early nasopharyngeal cancer (Original magnification,  $\times 200$ ). Wistar rats were injected subcutaneously with DNP in a stumped needle at a dosage of 40 mg/kg, twice a week and 38 times in total to accumulation dose of 99.5–122 mg per rat. The rats were sacrificed to collect nasopharyngeal samples at 308 days after DNP injections. Nasopharyngeal samples were histopathologically examined under microscope 200.

Most of nasal cavity tumors developed from nasal turbinates. Occasionally, tumors destroyed nasal bone and bulged out as a local prominence. The soft tumor mass with a pink-grey tint frequently destroyed cribriform plate and invaded or replaced olfactory bulb, but it was well demarcated from brain tissue. The tumors of nasal cavity were of different histological types, including squamous cell carcinoma, adenocarcinoma, and the so-called “olfactory neuroepithelial tumor.” The olfactory neuroepithelial tumors had a pleomorphic histology that suggested that these tumors originated from epithelia. On gross examination, most of esophageal tumors were multiple papillomas, that developed mainly from the upper and middle parts of esophagus; these papillomas were histologically confirmed as squamous cell carcinoma [73].

Some remaining points need to be clarified: (a) the induced rat NPC was of a squamous cell type. Most of tumors were well-differentiated and developed from base and lateral side of nasopharynx, and some co-existed with squamous carcinoma of soft palate mucosa. There was rarely distant metastasis; in this regard NPC induced by nitroso-compounds was somewhat different from human NPC. (b) DNP-induced NPC and cancer of nasal cavities and esophagus in rats resulted from a subcutaneous effect. (c) Nitroso-compounds often exist as cis- and trans-isomers, and rapid axial-equatorial conversion of dinitrospiperazin and the respective carcinogenicity of these isomers should be explored in the future.

#### 4. Morphologic and histogenetic studies of experimental NPC

In the normal rat, cephalic and middle portion of nasopharyngeal cavity is covered by ciliated columnar epithelium, while caudal portion is covered by squamous and columnar epithelium.

##### 4.1 Pathology of the experimental NPC

In the soft palates of tumor-bearing rats, nodules ranging from 1-2 mm to 1 cm in diameter were observed by palpation. Some of the invasive tumors were ulcerative cauliflowerlike or nodular in appearance when the soft palate was cut open, cancerous ulcer with elevated irregular edge was observed when cancerous ulcer with elevated irregular edge was cut open [24]. Under the microscope, cancer cell foci in different stages of differentiation could be found in many serial sections, indicating the multiple growth pattern of malignancy (Fig.1. DNP- induced early nasopharyngeal cancer. Wistar rats were injected subcutaneously with DNP in a stumped needle at a dosage of 40 mg/kg, twice a week and 38 times in total to accumulation dose of 99.5–122 mg per rat. The rats were sacrificed to collect nasopharyngeal samples at 308 days after DNP injections. Nasopharyngeal samples were histopathologically examined under microscope 200). A localized solitary malignancy was observed in rats that received carcinogen for a short period. Tumors often developed at the base of nasopharynx or at the junction of the base and lateral side of nasopharyngeal wall. Carcinomas in situ, and early invasive or infiltrative carcinomas were observed, and were of a well-differentiated squamous cell type [73].

The diagnostic criterion of experimental NPC is generally that of human nasopharyngeal cancer, but the structural characteristics of rat nasopharynx must also be taken into consideration. For example, the epithelium at nasopharynx base, especially at nasopharynx roof next to skull base, consists of two or three layers of cells surrounded by bony plates[59]. The observed malignancy may be composed of basal cells with thin solid strands of cancer cells.

Carcinoma in situ, early invasive carcinoma, and infiltrative carcinoma were observed in tumor-bearing rats. Carcinoma in situ might arise from epithelium of normal thickness, or from highly hyperplastic or atrophic epithelium (Fig.2. DEN- induced nasopharyngeal cancer. Wistar rats were injected subcutaneously with DEN in a stumped needle at a dosage of 40 mg/kg, twice a week and 38 times in total to accumulation dose of 89.8–119.3 mg per rat. The rats were sacrificed to collect nasopharyngeal samples at 365 days after DEN injections. Nasopharyngeal samples were histopathologically examined under microscope). It often originates from columnar epithelium and evolves through squamous cell metaplasia, atypical hyperplasia, and Grade I and II anaplasia. The early invasive lesions were of two types: arising from basal layer with normal looking superficial layers, or extending into stroma (primarily seen in the DEN group). Downward growth of the lesion, which formed trabecular, branching, rounded, or small square nests, infiltrated into the stroma. The neoplasms were usually moderately or even poorly differentiated squamous carcinomas. This downward growth of neoplasms, which often occurs in human NPC, may cause difficulty in early detection or in producing a good smear of exfoliative cells for cytological diagnosis [57,72,73].

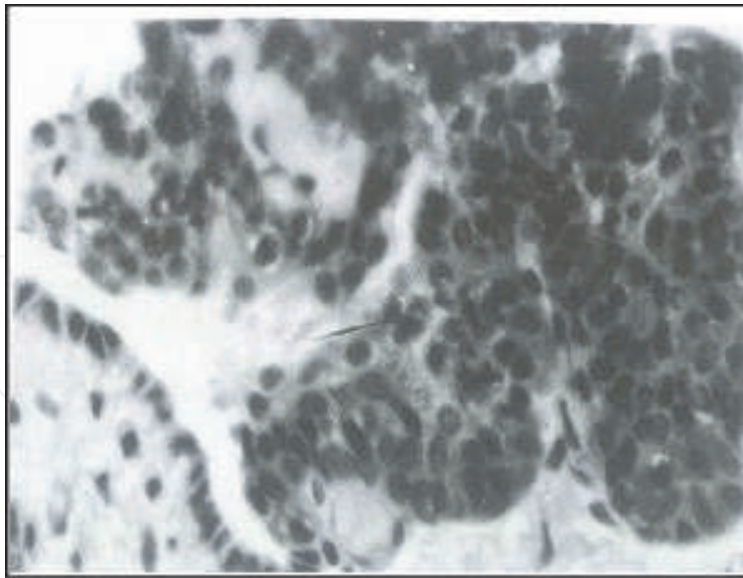


Fig. 2. DEN- induced nasopharyngeal cancer (Original magnification,  $\times 400$ ). Wistar rats were injected subcutaneously with DEN in a stumped needle at a dosage of 40 mg/kg, twice a week and 38 times in total to accumulation dose of 89.8–119.3 mg per rat. The rats were sacrificed to collect nasopharyngeal samples at 365 days after DEN injections. Nasopharyngeal samples were histopathologically examined under microscope 400.

Arising from a dysplastic squamous epithelium, cancer protruded into nasopharyngeal cavity at the one hand and invaded the stroma at the other. This growth pattern occurred more frequently in the DNP group, and sometimes formed papillary carcinomas [57,72,73]. The infiltrative carcinomas were usually of a squamous cell type, although basal cell carcinoma was observed occasionally. This kind of tumor growth could be seen in many serial sections. The cancer cells forming trabecular, solid masses, or patches were not well differentiated at the periphery but had cornified cells in the central portion. Cancer cells invaded salivary glands, muscle, and nerve bundles with occasional lymphatic emboli but without widespread metastases to the lymph nodes. The primary site or origin of the infiltrative tumor was usually difficult to recognize, but could occasionally be traced by serial sectioning.

#### 4.2 The histogenesis of experimental NPC

In early lesions, dysplasia occurred in the ciliated columnar, transitional, or squamous epithelium, and was possibly preceded or accompanied by squamous metaplasia.  $^3\text{H}$ -thymidine autoradiography showed that a single dose of dinitrospiperazine could cause DNA damage of the squamous or transitional cells followed by unscheduled DNA synthesis [74]. This may give some clues to NPC carcinogenesis. Hyperplastic, dysplastic, and neoplastic foci often co-existed without sharp demarcation. The cancer cell population was more localized and clearly demarcated from the surrounding normal epithelium or stromal tissues. Inflammatory infiltration into the surrounding normal stroma was rarely found except in ulcerative carcinomas.

NPC was present in multiple serial sections due to the extension of a large tumor mass or multiple diffusely scattered lesions. Some of the irregular cords or trabeculae of tumor cells,

arising from different portions of the epithelium, might finally fuse together to form a large solid mass of tumor [57, 72, 73]. It was evident that most of the lesions were multicentric rather than unicentric in origin. Sometimes the same cancer cell focus contained not only undifferentiated fusiform cells and small cornified epithelial pearls, but also small cysts containing mucinous material. These kinds of lesions may be seen in human NPC and are considered to represent the biphasic differentiation of nasopharyngeal epithelium.

### 4.3 Statistical analysis of NPC carcinogenesis

The well-known two-stage concept suggested by Berenblum et al [60] was developed from experimental study of carcinogenesis. They proposed that carcinogenesis might be divided into two different but related stages, a stage of specific initiation and a stage of relatively non-specific promotion. However, on the basis of epidemiological data and statistical studies of human cancer, carcinogenesis is generally considered a multi-hit/multi-step process. The multistage theory of carcinogenesis proposed by Amitage and Dell is representative of this concept [61]. Therefore, it is interesting to analyze the carcinogenesis of experimental NPC by means of mathematical statistics.

Yao et al [73] reported the results of statistical analysis of experimental NPC induced by DNP. The corrected cumulative percentage of dead rats with NPC was calculated and fitted with Weibull and lognormal distributions. The data fitted both distributions well as verified by  $\chi^2$ -test, but the value of  $\chi^2$  was smaller in the Weibull distribution than in the lognormal distribution. The mathematical expression of NPC carcinogenesis in rats according to Weibull distribution was as follows:

$$G=1-e^{-2.55 \times 10^{-4} (t-192)^{1.69}}$$

G, corrected cumulative percentage of the dead rats bearing NPC; t, time in days

Peto [62] and Emmelot et al [63] suggested that Weibull's distribution represents the number of "hits" or "stages" in cancer development. Therefore, the above mathematical expression seems to indicate that rat NPC development experienced two hits. Using retrospective survey data of cancer mortalities in Hunan Province, Yao [73] analyzed the age distribution of NPC mortality and proposed that the development of NPC needs three hits. Hence, the relationship and differences between carcinogenesis in experimental NPC and human NPC need to be explored further.

### 4.4 Carcinogenesis mechanism of experimental NPC

#### 4.4.1 Atypical cytokinetics of nasopharyngeal epithelium in rats treated with DNP

Cytokinetic studies may be helpful in elucidating the mechanism of carcinogenesis and providing important data for improving chemotherapeutic regimens for malignancies. In 1981, Chen et al [74] studied the cytokinetics of nasopharyngeal epithelium of rats treated with DNP using stathmokinetic and autoradiographic techniques. The mitotic blocking agent selected was vincristine, which had an optimal dose of 0.83 mg/kg body weight and a blocking effect that lasted 10 to 12 h. The metaphase chromosomes of mitotic cells appeared as deeply stained rosette-like structures under the microscope. The cytokinetic parameters of normal and DNP-treated rats were measured *in vivo* using  $^3\text{H}$ -TdR labeling in

combination with stathmokinetics. After DNP treatment there were many hyperplastic foci in nasopharyngeal squamous epithelium. Within the hyperplastic foci and apparently normal nasopharyngeal epithelium there was a significant increase in the number of labeled basal cells, but no change in labeling index (LI) of the transitional epithelium lining the lateral side of nasopharynx.

In normal rats, LI was highest in the squamous epithelium lining nasopharynx lateral side, followed by the base and then the transitional epithelium. The differences between these were highly significant. After DNP treatment, LI of both the lateral and base side increased, and there was no difference between them. This suggested that LI of the bottom side increased more significantly than that of the lateral side. In the normal rats,  $^3\text{H}$ -TdR labeled cells in nasopharyngeal squamous epithelium were confined to the basal layer (Fig.3. Autoradiaograph of nasopharyngeal squamous epithelium of normal rat, showing labeled cells in basal cell layer. Rats were treated with vincristine at 0.83 mg/kg body weight for 12 h, and then  $^3\text{H}$ -TdR was injected for 1 h. The rats were sacrificed to collect nasopharyngeal samples. The nasopharyngeal samples were histopathologically examined. Cells with  $^3\text{H}$ -TdR labeling were observed under microscope.). After DNP treatment, there was a significant increase in the number of labeled basal cells. Moreover, a few cells in the prickle cell layer were found to be labeled, indicating that the proliferation compartment had expanded. This phenomenon suggested that stem cells in the G0 stage might enter the proliferation stage and the two daughter cells of mitosis had proliferative ability. S phase of nasopharyngeal epithelium located at the lateral bottom side was prolonged from 6.7 to 9 h after DNP treatment. The time of cell cycle can be calculated according to the formula if the proliferation of nasopharyngeal epithelium in normal and DNP-treated rats remains in steady state.

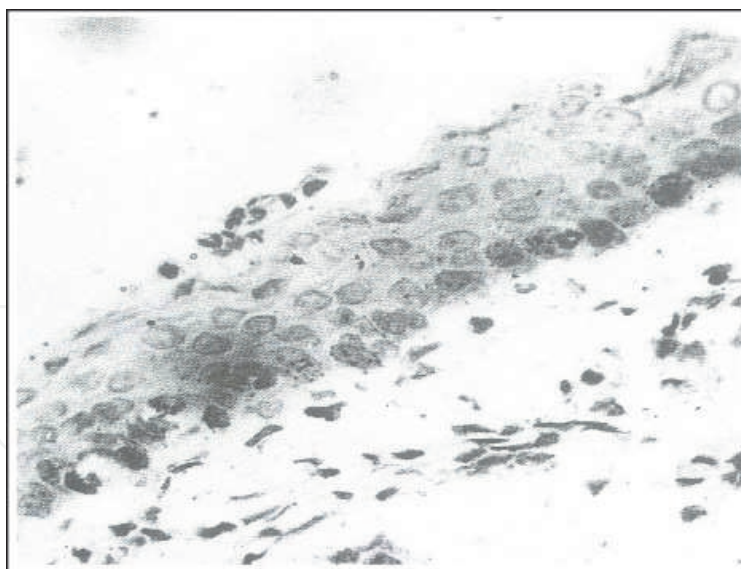


Fig. 3. Autoradiaograph of nasopharyngeal squamous epithelium of normal rat, showing labeled cells in basal cell layer (Original magnification,  $\times 400$ ). For mitotic blocking of nasopharyngeal epithelium, rats were treated with vincristine at 0.83 mg/kg body weight for 12 h.  $^3\text{H}$ -TdR (0.5 mCi/kg bodyweight) was intraperitoneally injected into the rats for 1 h. The rats were sacrificed to collect nasopharyngeal samples. The nasopharyngeal samples were histopathologically examined. Cells with  $^3\text{H}$ -TdR labeling were observed under microscope 400

Carcinogenesis is a multistep process. Cytokinetic parameters may be changed with morphological progression from normal epithelium, hyperplasia, atypical hyperplasia, to carcinoma. Atypical cytogenetics, increased LI, expansion of the proliferation compartment, and prolongation of S phase were observed in nasopharyngeal epithelium after DNP treatment.

#### **4.4.2 Effect of DNP on $^3\text{H}$ -thymidine incorporation into DNA of rat nasopharyngeal epithelium**

The adult rats were sacrificed at 4 hrs after a single injection of DNP intramuscularly, and then the tissue fragments of nasopharynx, esophagus, kidney and liver were cultured in  $^3\text{H}$ -thymidine - 199 medium for 10 hrs. Autoradiography was performed to examine  $^3\text{H}$ -thymidine labeled epithelial cells. The experimental data indicated that LI of nasopharyngeal epithelium markedly decreased to 2.40% in the experimental group with DNP treatment compared with the saline control, which suggested that a single dose of DNP inhibits DNA synthesis of nasopharyngeal epithelium. This inhibition may be result of DNA damage, providing a key link in carcinogenesis process of experimental nasopharyngeal cancer [73].

#### **4.4.3 Organotropic action of nitroso-compound carcinogenesis**

Le et al [75] injected 0.25% DNP solution (15 mg/kg bodyweight) into the dorsum of rats. Animals were sacrificed by cervical dislocation at 4 h, 79 h, and 124 h after injection.  $^3\text{H}$ -TdR (0.5 mCi/kg bodyweight) was injected intraperitoneally 1 h before the animals were sacrificed. The nasopharynx, esophagus, and forestomach were sectioned and processed histologically and autoradiographically. A count of 1,000 consecutive basal cells was performed in the epithelia of nasopharynx, esophagus, and fore stomach of each animal. The labeled cells (>5 silver grains/nucleus) were scored and the LIs were expressed as percentages of the mean value for the controls of the same experiment. LI of squamous epithelia in the base of nasopharynx and esophagus significantly decreased 4 h after DNP injection and recovered gradually by the 3rd and 5th days; the LI of squamous epithelium in nasopharynx base was actually significantly higher than that of the control at 5 days after DNP injection. LI in the squamous epithelium of forestomach did not significantly change at 4 h or on the 3rd day, but declined significantly 5 days after DNP treatment.

#### **4.4.4 Unscheduled DNA Synthesis (UDS)**

UDS was detected autoradiographically. 30, 50 or 80 mg/kg in 0.5% DNP was injected subcutaneously into the dorsum of rats [64] and an equivalent amount of saline was injected into rats of the control group. The rats were sacrificed 2 h after injection and small epithelial tissue fragments of the nasal concha, soft palate, esophagus, and forestomach, as well as the basal and lateral side of nasopharynx, were removed immediately. The tissues were processed histologically and autoradiographically. It is easy to identify the nuclei of S phase cells by their extremely heavy labeling. UDS was considered present if there were at least nuclei, each covered by 5-20 silver grains, in a cluster and the number of grains covering the nuclei was no more than that of the background (Fig.4. UDS autoradiograph of nasopharyngeal squamous epithelium. DNP was injected subcutaneously into the dorsum

of rats at 80 mg/kg. The rats were sacrificed 2 h after injection, and the epithelial tissue fragments of nasal concha were immediately removed. The tissues samples were processed histologically and autoradiographically). The results showed that at 4 h after subcutaneous injection of DNP (30 mg/kg), DNA synthesis was inhibited in the squamous epithelia of nasopharynx base and esophagus, both of which are tumor prone [75]. This finding is in agreement with reports of Mirvish [65] et al that some carcinogens inhibited DNA synthesis in their respective target organs. While UDS induction was not detected in the forestomach, there are no references indicating that carcinoma of the stomach was induced by DNP. It was also noted that UDS was autoradiographically present only in the epithelia of organs prone to develop cancer after DNP treatment whereas no fibroblasts in the stroma were positive for UDS. These findings suggest that DNP fails to induce any local and distant sarcoma in the rats, and may be related to selective activation of carcinogens in the target organs epithelia with subsequent DNA damage and repair.

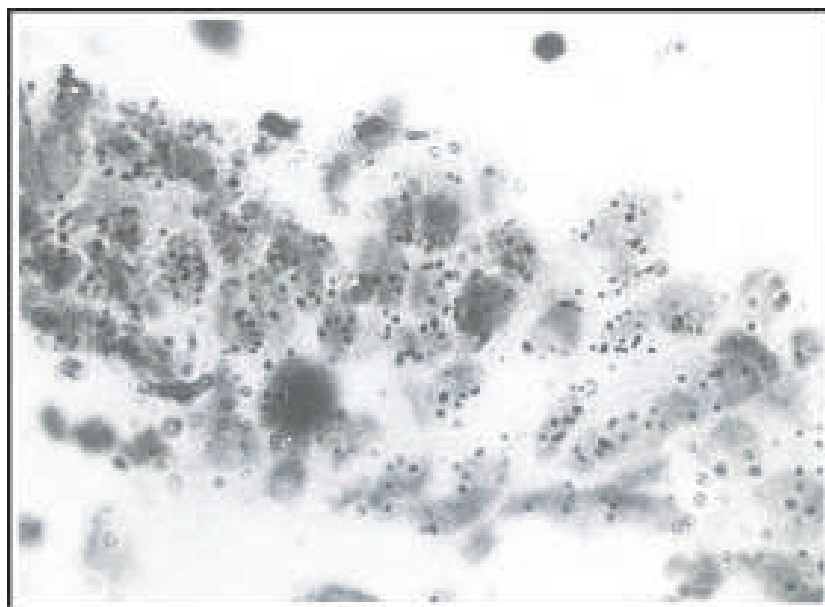


Fig. 4. UDS autoradiograph of nasopharyngeal squamous epithelium. DNP was injected subcutaneously into the dorsum of rats at 80 mg/kg. The rats were sacrificed 2 h after injection, and the epithelial tissue fragments of nasal concha were immediately removed. The tissues samples were processed histologically and autoradiographically. UDS was considered present when nuclei with 5-20 silver grains UDS positive (Original magnification,  $\times 1000$ )

#### 4.4.5 Induction of lactic dehydrogenase, its isozymes, and acid esterase in NPC by DNP

To investigate enzymatic and isozymatic changes in NPC development, concentrations of urea and pyruvate for the demonstration of H-type and M-type isozymes were determined [76]. The reaction intensity was scored as grade 0-4, depending on the size and color of the formazan granules. The data were statistically analyzed by the ranked data method. There was no significant difference in total activity between squamous and ciliated columnar epithelium, but the reaction in transitional epithelium was more intense. All normal epithelia were mainly of the M-type. In the late embryonic stage, nasopharynx was well developed but covered with

less differentiated epithelium. In the medial stage, undifferentiated flat cuboid cells located below brain vesicle between the optic cups on both sides of bony plate were designated as primordial respiratory epithelium. The activity of lactic dehydrogenase and its isozymes in nasopharynx during the embryonic and neonatal stages was as follows: In the medial stage, enzymatic activity of the undifferentiated epithelium was clearly higher than that of the late embryonic stage and neonatal rats, but no significant difference was found between the latter two groups. In the nasopharyngeal epithelium of medial, late embryonic, or neonatal stages, the isozymes were mainly of M-type.

After DNP treatment for 7 months, different kinds of lesions were present in the nasopharyngeal epithelium, such as hyperplasia, dysplasia, carcinoma in situ, and early invasive growth. There were 14 cases of NPC in 24 rats, and some of these had more than one lesion, a total of 22 cancer foci composed of three carcinomas in situ, 14 early infiltrative lesions, and five papillary carcinomas. The histochemical results demonstrated that there was no difference in enzymatic activity of squamous epithelium in normal and experimental groups, while hyperplastic and dysplastic squamous epithelium showed higher enzymatic activity than the normal cells. During these stages, the increased isozymes were mainly of the M-type. In the 22 cancer foci, total activity of lactic dehydrogenase and isozymes (including both M- and H-types) increased to a much higher level than that of the normal tissue, and total activity and isozyme types resembled the undifferentiated cells of the 14- to 17-day-old embryos. At this stage there was also a high level of H-type isozyme activity. Nasopharyngeal epithelium in hyperplastic, dysplastic, or neoplastic stages showed an appreciable increase in the enzymes activity. During the first and second stages, the isozyme was initially mainly M-type, and then H-type isozymes. Since the lactic dehydrogenase in normal nasopharyngeal epithelium was mainly of M-type, the increase in H-type isozyme activity might represent abnormal gene expression during the course of carcinogenesis.

There were two different types of enzymatic change in carcinogenesis course induced by DNP. Lactic dehydrogenase activity increased throughout the stages from hyperplasia and dysplasia up to neoplasia, while acid nonspecific esterase activity significantly increased in the hyperplastic stage, and then decreased or even disappeared in the neoplastic lesions. This suggests that nonspecific esterase is related to the development, diagnosis, and prognosis of carcinoma.

Esterase activity was histochemically detected in the different sites of nasopharynx, with stronger activity in the base wall than in other sites. The activity was different in various types of nasopharyngeal epithelia; stronger activity was observed in the stratified squamous and transitional epithelium than in the ciliated columnar epithelium, but the intensity was similar in the squamous and transitional epithelium. The deposits of esterase in squamous epithelium were diffusely distributed in the cytoplasm of basal and spinous cells, which were mainly localized at the margin of cilia and cytoplasm of cells. The former were distributed diffusely, while the latter took the shape of dot-like granules. Very weak activity was observed in the goblet cells and the reaction in transitional epithelium was similar to that in the squamous epithelium.

There were hyperplastic lesions in the squamous epithelium of all animals treated with DNP for 7 months. The enzymatic activity was markedly decreased compared with the surrounding epithelia, squamous epithelium of the control animals, or hyperplastic lesions. No difference was found between dysplasia and neoplasia. In short, enzymatic activity

increased in hyperplasia but was decreased in dysplasia and neoplasia, and sometimes even totally disappeared. Similarly, the esterase activity of human NPC was markedly decreased compared with the surrounding epithelia of the cancer foci and the squamous or ciliated columnar epithelia of the nasopharynx in the control cases, and decreased to varying extents in different cancer cells of various cases or in different foci of the same case. The reactivity remained in well-differentiated cancer cells, but entirely disappeared in poorly differentiated cancer cells.

## **5. Molecular and signal transduction activated by chemical carcinogens**

### **5.1 Biomolecular and signal pathways activated by DEN**

Activation of  $\beta$ -catenin is the central effector of canonical Wnt pathway. DEN-induced tumorigenesis was examined in hepatic  $\beta$ -catenin conditional knockout ( $\beta$ -cat KO) mice.  $\beta$ -cat KO mice show a paradoxical increase in susceptibility to DEN-induced tumorigenesis. This accelerated tumorigenesis is due to increased injury and inflammation, unrestricted oxidative stress, fibrosis, and a compensatory increase in hepatocyte proliferation secondary to PDGFR $\alpha$ /phosphoinositide 3-kinase (PIK3CA)/Akt activation and c-Myc overexpression. Loss of  $\beta$ -catenin impairs the ability of liver to counteract DEN-induced oxidative stress and enhances tumorigenesis through PDGFR $\alpha$ /PIK3CA/Akt signaling. [66].

C/EBP $\alpha$  is a transcription factor that regulates liver quiescence. Phosphorylation of C/EBP $\alpha$  at serine 193 (S193-ph) is upregulated in older mice and is thought to contribute to age-associated liver dysfunction. DEN treatment of knock-in mice expressing a phospho-mimetic aspartic acid residue in place of serine at position 193 (S193D) of C/EBP $\alpha$  induces the formation of liver cancer, and actually results in earlier development of liver tumors. DEN/phenobarbital treatment is associated with specific degradation of both the S193-ph and S193D isoforms of C/EBP $\alpha$  through activation of the ubiquitin-proteasome system (UPS) [66].

The role of PBP/MED1 [peroxisome proliferator-activated receptor-binding protein (PBP)/mediator subunit 1 (MED1)] in DEN-induced hepatocarcinogenesis was also examined. The carcinogenic process of PBP/MED1D mice was initiated by injection of DEN and initiated cells were promoted with phenobarbital. These mice revealed a striking proliferative response in the few residual PBP/MED1-positive hepatocytes that escaped Cre-mediated deletion of the PBP/MED1 gene. No proliferative expansion of PBP/MED1 null hepatocytes was noted in the PBP/MED1DLiv mouse livers. Multiple hepatocellular carcinomas developed in the DEN-initiated PBP/MED1fl/fl and PBP/MED1DLiv mice [67].

DEN may activate PDGFR $\alpha$ /PIK3CA/Akt signaling through  $\beta$ -Catenin, and mediate C/EBP $\alpha$  phosphorylation through the ubiquitin-proteasome system (UPS), and regulate PBP/MED1, and involve nasopharyngeal carcinogenesis.

### **5.2 Biomolecular and signal pathways activated by DMAB**

When K5-protein kinase C- $\alpha$  (PKCA) mice (transgenic mice that overexpress PKCA in the epidermis) were initiated with DMBA and promoted with a low dose of 12-O-tetradecanoylphorbol-13-acetate (TPA), 58% of the mice developed skin papillomas that progressed to carcinoma. CXCR2 is expressed by keratinocytes and transformation by

oncogenic ras (a hallmark of DMBA initiation) or TPA exposure induced all CXCR2 ligands. Ras induction of CXCR2 ligands was mediated by autocrine activation of epidermal growth factor receptor and nuclear factor-KB, and potentiated by PKCA. Oncogenic ras also induced CXCR2 ligands in keratinocytes that were genetically ablated for CXCR2. *In vitro*, CXCR2 was found to be essential for CXCR2 ligand-stimulated migration of ras-transformed keratinocytes and for ligand activation of the extracellular signal-regulated kinase (ERK) and Akt pathways. Both cell migration and activation of ERK and Akt were restored by CXCR2 reconstitution of CXCR2 null keratinocytes [68].

Constitutive activation of signal transducer and activator of transcription 3 (Stat3) has been described in a variety of human malignancies and has been suggested to play an important role in carcinogenesis. The epidermis of inducible Stat3-deficient mice treated with 4-hydroxytamoxifen (TM) showed a significant increase in apoptosis induced by DMBA and reduced proliferation following exposure to TPA. In two-stage skin carcinogenesis assays, inducible Stat3-deficient mice treated with TM during the promotion stage showed a significant delay in tumor development and a significantly reduced number of tumors compared with control groups. Inducible Stat3-deficient mice treated with TM before initiation with DMBA also showed a significant delay in tumor development and a significantly reduced number of tumors compared with control groups [69].

### 5.3 Biomolecular and signal transduction targeted by DNP

DNP displays some degree of organ specificity for nasopharyngeal epithelium in inducing rat NPC. To clarify the mechanism underlying this DNP organotropic action, a rat NPC model was constructed using DNP, and atypical hyperplastic nasopharyngeal and NPC tissue was obtained from rats at different stages of tumorigenesis. Differential protein expression was screened using proteome analysis and further confirmed by immunoblotting. Expression of heat shock protein 70 (HSP70) and Mucin was increased in the atypical hyperplasia and NPC cells, and we therefore postulated that DNP might up-regulate these genes. In further studies to determine whether DNP does regulate HSP70 and Mucin, we treated HENE cells (cultured from biopsies of normal nasopharyngeal tissue) with 2  $\mu$ M and 4  $\mu$ M DNP and showed that expression of HSP70 and Mucin increased in dose-dependent manner. To confirm the specificity of DNP, we used arsenite as a control because its carcinogenicity has previously been proven [70]. Expression of HSP70 and Mucin was not induced by arsenite. We therefore think that HSP70 and Mucin might be specific and important targets of DNP [57].

DNP induced expression of phosphorylated ezrin at threonine 567 (phos-ezrin Thr567) in a dose- and time-dependent manner in 6-10B nasopharyngeal carcinoma cells (Fig.5 Effects of DNP on ezrin phosphorylation at Thr 567. 6-10B cells were treated with 2 or 4  $\mu$ M DNP for 24 h (A), and treated with 4  $\mu$ M DNP for 12 or 24 h (B), and ezrin and phos-ezrin expression were assayed with immunoblotting). Furthermore, DNP-induced expression of phos-ezrin Thr567 was dependent on increased Rho kinase and PKC activity. The activation of Rho kinase and PKC occurred through binding to Rho kinase pleckstrin-homology (PH) and promotion of PKC translocation to the plasma membrane. Ezrin is associated with induction of filopodia growth in 6-10B cells, and further studies showed that DNP induces filopodia formation in 6-10B NPC cells and also increases invasion and motility of these cells. This indicated that DNP is involved in NPC metastasis, and DNP-mediated NPC metastasis was

indeed confirmed in nude mice. However, DNP did not effectively induce motility and invasion of DNP-treated NPC cells containing ezrin mutated at Thr 567. Similarly, motility and invasion were not induced in DNP-treated NPC cells transfected with si-RNAs against Rho or PKC. These findings indicate that DNP induces ezrin phosphorylation at Thr567, increases motility and invasion of cells, and promotes tumor metastasis. DNP may therefore be involved in NPC metastasis through regulation of ezrin phosphorylation at Thr567 [71].

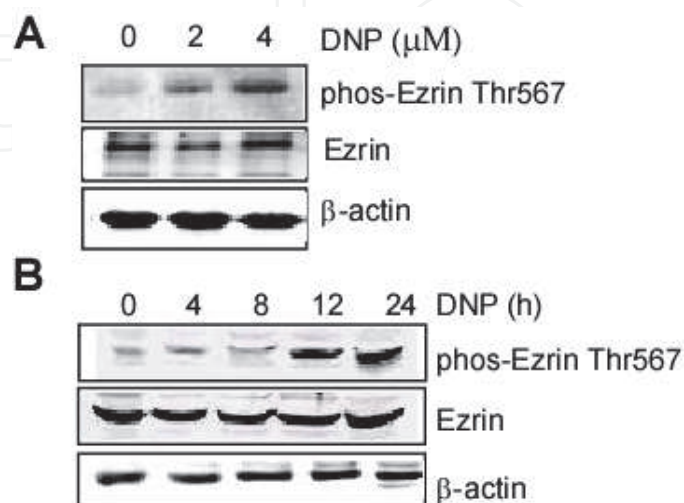


Fig. 5. Effects of DNP on ezrin phosphorylation at Thr 567. 6-10B cells were treated with 2 or 4  $\mu$ M DNP for 24 h (A), and treated with 4  $\mu$ M DNP for 12 or 24 h (B), and ezrin and phos-ezrin expression were assayed with immunoblotting.

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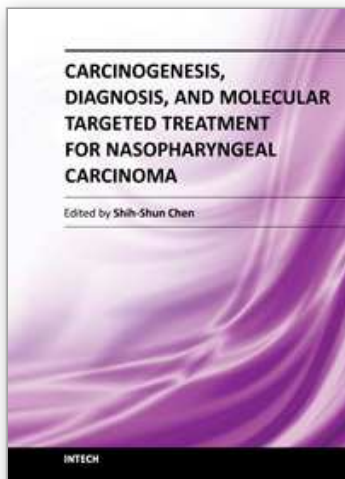
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