

## NEOPLASTIC CELL APOPTOSIS IN NUDE MICE TRANSPLANTS WITH NASOPHARYNGEAL CARCINOMA CELL LINES

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### ABSTRACT

**Objective:** To observe the morphological features of neoplastic cell apoptosis developed in nude mice transplants with nasopharyngeal carcinoma (NPC) cell lines, CNE-1 and CNE-2, and to investigate the roles of p53, bcl-2 and bax playing in the process of apoptosis. **Methods:** CNE-1 and CNE-2 cell lines were inoculated and passed in nude mice for 3 generations. The cell apoptosis was detected on H & E and TUNEL staining slides. The expression of p53, bcl-2 and bax were detected by using immunohistochemistry. p53 gene alteration was assayed in cell lines and transplants by PCR-SSCP. **Results:** A considerable number of neoplastic cells underwent apoptosis in CNE-1 and CNE-2 transplant tissues. The "shrinkage necrosis" and apoptotic bodies were the main appearances of apoptosis. The p53 alteration was detected in exon 8 by PCR-SSCP and p53 protein accumulation observed in the cell smears and nude mice transplant tissue sections. All the transplant tissue sections of 3 passages showed bcl-2 negativity and bax overexpression. **Conclusion:** The neoplastic cells of CNE-1 and CNE-2 transplants underwent death mainly taking the way of apoptosis. The "shrinkage necrosis" and apoptotic bodies were the main morphological features of apoptosis seen in those transplants. The apoptosis in CNE-1 or CNE-2 nude mice transplant is highly probable through a p53-independent and bax-mediated pathway.

**Key words:** Nasopharyngeal carcinoma, Nude mice transplant, Apoptosis

We know that tumor growth speed is critically influenced by the ratio of neoplastic cell proliferation and

cell death, and there are two patterns of cell death, namely, necrosis and apoptosis. What is the proportion of necrosis and apoptosis seen in nude mice transplants of nasopharyngeal carcinoma (NPC) cell lines, CNE-1 and CNE-2? Does the apoptosis play an important role in neoplastic cell death? If so, what is the pathway of apoptosis developed in those transplants?

### MATERIALS AND METHODS

#### Materials

Nasopharyngeal carcinoma cell lines, CNE-1 and CNE-2 were cultured in RPMI1640 plus 10% FCS medium under the condition of 37°C and 5% CO<sub>2</sub>. Either CNE-1 or CNE-2 cells, 1×10<sup>6</sup> in number was injected to the subcutaneous tissue of nude mice at the back of the neck. When the tumor mass grew up to an appropriate volume (about 1×1×1 cm<sup>3</sup>), those nude mice bearing tumors were killed. A small portion of the neoplastic tissue cut from the tumor mass was re-inoculated to another nude mice, and the remaining tumor growths were taken for DNA extraction and tissue sectioning. The CNE-1 and CNE-2 cell lines were thus inoculated in nude mice for 3 passages. DNA was extracted from fresh tissue and tissue sections were made from 10% formalin-fixed paraffin-embedded blocks.

#### *In situ* Cell Death Detection by TUNEL Method

TUNEL [TdT-mediated fluorescein-dUTP Nick End Labeling] method was performed for detection and quantification of apoptotic cells by use of Boehringer Mannheim "In Situ Cell Death Detection Kit, AP (Cat. No. 1684809)". The working procedure was carried out following the instructions enclosed in the kit. The positive signals showed dark purple in color. The average number of apoptotic cells per high power field (5×40) was designated as TUNEL index (TI).

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### Immunohistochemical Staining

LSAB immunohistochemistry was adopted for detecting the bcl-2 (Clone 124, DAKO M0887), bax (Bax 1-19, Santa Cruz, SC-930) and p53 (Clone Do-7, DAKO, M7001). The working dilutions were 1:60, 1:100 and 1:100 for bcl-2, bax and p53, respectively. The sections were pretreated in citrate buffer (pH 6.0) with microwave to retrieve the antigens. Positive and negative controls were performed while doing the procedure. p53 positive signals were localized within the nuclei, and more than 10% positive neoplastic cells found on a tissue section were termed accumulation of p53 protein. Bcl-2 and bax positive signals appeared on membrane and in cytoplasm.

Once a bcl-2 or bax positive neoplastic cell was found on a tissue slide, overexpression of bcl-2 or bax was termed.

### PCR-SSCP

#### Primers Used for Amplification of p53 Exons

The sequences of primers used for amplification of exon 5, 6, 7, and 8 of p53 gene were shown as follows in Table 1.

The primers of exon 5, 6 and 7 were kindly provided by Professor Nancy Roab-Traub, Lineberg Cancer Research Center, North Carolina University. Jinhua Tech. Co., Guangzhou manufactured the primer of exon 8.

Table 1. The sequences of exon 5, 6, 7, and 8 of p53 gene<sup>[1]</sup>

Exon	Sequence	Length
5	TTCCTCTTCCTGCAGTACT AGCTGCTCACCATCGCTAT	209 bp
6	GCCCTCTGATTCCCTCACTGA GCCACTGACAACCACCCTTA	170 bp
7	TGTTGTCTCCTAGGTTGGCT CAAGTGGCTCCTGACCTGGA	139 bp
8	CCTATCCTGAGTAGTGGTAA TCCTGCTTGCTTACCTCGCT	164 bp

### PCR

50 µl PCR reaction mixture contained 0.5-1.0 µg sample DNA, 0.2 mol/L dNTPs, 30 pmol primers, and 0.3-unit super Taq enzyme. 5 µl 10× PCR buffer was also added into the mixture. The cycles used were as follows: 95°C 1 minute, 55°C 1 minute, 72°C 1 minute (10 cycles). Another 30 cycles were as follows: 95°C 30 seconds, 55°C 1 minute, 72°C 1 minute. The entire procedure required 40 cycles. This was followed by an extension at 72°C for 10 minutes using PTC-15 tempcycler. SW480 cell line DNA was used as positive control and DNA from the p53-deletion cell line Saos2 was used as negative control.

### SSCP

30 µl product of PCR were denatured in the alkaline solution of 1.5 mol/L NaCl and 0.5 mol/L NaOH for 20 minutes at 54°C, then added 3 µl 10 × loading buffer. These samples were added to the polyacrylamide gel for electrophoresis with 300 volts for 3-5 hours at 4°C.

### Staining

Taken out of the gel and put it into a solution containing 0.5 µg/ml EtBr, shaken and stained for 20-30

minutes, and observed the position of DNA single strand swimming in the gel.

### RESULTS

CNE1 and CNE2 nasopharyngeal carcinoma cell lines were inoculated into the subcutaneous tissue of nude mice and tumor growths could be observed with the naked eye on the 6th and 9th day. The CNE-1 nude mice transplant tissues of 3 generations were taken on the 22nd day and the CNE-2 on the 16th day. Figure 1 shows a living nude mouse bearing a tumor growth.

Several foci of neoplastic cell death could be observed either in CNE-1 or CNE-2 nude mice transplant tissue sections. A considerable number of neoplastic cells underwent "shrinkage necrosis", and a few cells underwent coagulation necrosis. Neoplastic cells that underwent so-called "shrinkage cells" were those that appeared smaller in cell size with condensed nucleus and eosinophilic cytoplasm. Some typical apoptotic bodies could also be found within the so-called "shrinkage necrosis" foci (Figure 2). Karyolysis and karyorrhexis but not karyopyknosis were the main appearance of coagulation necrosis seen on the coagulated necrosis foci. The cells undergoing coagulation necrosis seemingly kept their outline. A variable number of apoptotic cells could be found in the tissue areas where no aggregation of died

cells was found. The apoptotic cells might show margination, fragmentation or condensation of nuclear chromatin with intensively eosinophilic cytoplasm. Those cells were early apoptotic cells. Apoptosis bodies appeared as small round or oval eosinophilic masses with or without basophilic nuclear materials contained. In sections, apoptosis bodies were often to be found within intercellular space or halo (Figure 3). Apoptotic bodies phagocytosed by neighboring cells (might be neoplastic cells or macrophages) could often be seen. The so-called shrinkage cells and apoptotic bodies demonstrating TUNEL signals could easily be detected by TUNEL method (Figure 4). In addition, some mitotic figures of neoplastic cells could at the same time be seen within the "non-death areas".

The range of TUNEL index examined microscopically on all the transplant tissues taken from 3 nude mice passages of CNE-1 and CNE-2 was 142.5-182.9/HPF; the mean value being  $170.00 \pm 17.55$ /HPF.

P53 protein accumulation could be detected not only in CNE-1 and CNE-2 cell smears but in all of the nude mice transplant tissues (Figure 5). The vast majority of neoplastic cells underwent bax overexpression, but on the contrary, bcl-2 positive cells had never been found in any transplant tissues of CNE-1 and CNE-2 nude mice passages.

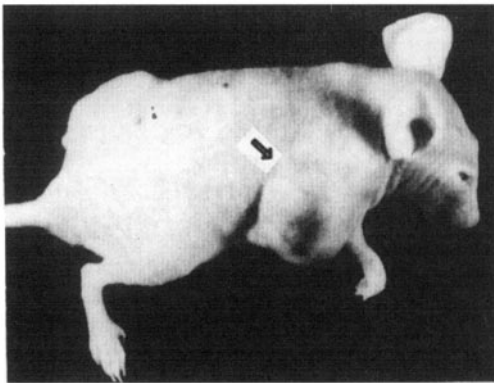


Fig. 1. A living nude mouse bearing a tumor growth.

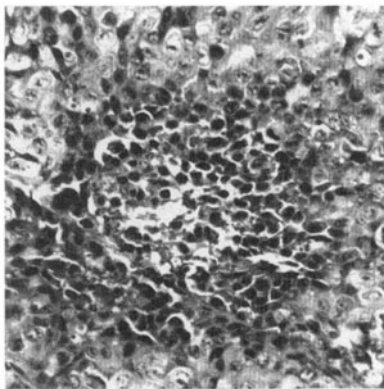


Fig. 2. A focus of "shrinkage necrosis", H & E 5×10.

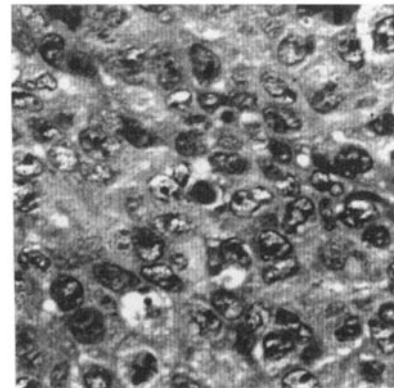


Fig. 3. Apoptosis body within intercellular space in "non-death area", H & E 5×20.

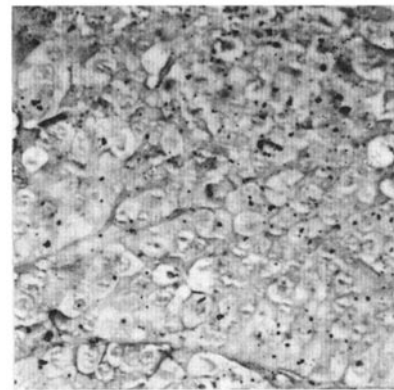


Fig. 4. Numerous apoptotic bodies, TUNEL 5×10.

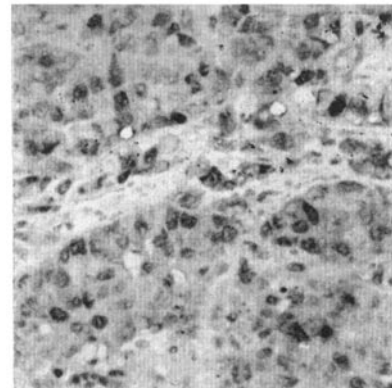


Fig. 5. p53 protein accumulation in the nude mice transplant. P53 (DO7), IHC 5×10.

The p53 gene alteration was detected in exon 8 by PCR-SSCP in the CNE-1, CNE-2 cell lines as well as their nude mice transplant tissues (Figure 8, 9). There was no aberrant gel shift found in exon 5, 6, and 7.

## DISCUSSION

After inoculation of CNE-1 and CNE-2 cells into the subcutaneous tissue of nude mice, the authors found that

the tumor mass grew gradually and the neoplastic cell proliferation and apoptosis could be found in the same tissue section and even on the same microscopic field. This means that the growth of tumor was resulted from the imbalance of proliferation and apoptosis in the neoplastic cell population. The tumor mass enlarges itself depending on a higher proliferative activity and a relatively lower cell death rate. That is to say, the ratio of neoplastic proliferation and cell death can figure out the growth speed of the transplant. As we know there are two patterns of cell death, namely, apoptosis and necrosis.<sup>[2]</sup> What pattern took place in nude mice tumor transplant? Was it necrosis or apoptosis? It is noted herein that most of neoplastic cells in the CNE-1 and CNE-2 transplants underwent death mainly taking the pattern of apoptosis. Nuclear chromatin changes including margination, fragmentation and condensation, apoptotic bodies and apoptotic bodies phagocytosed by neighboring cells were the common features seen. However, the authors would like to point out that the so-called "shrinkage necrosis" was the prominent appearance seen in the transplants. That is to say, most of the apoptotic cells became smaller in size with a condensed nucleus and intensely eosinophilic cytoplasm. It was worthy to note that those apoptotic cells were abundant and often crowded each other to form so-called foci of "shrinkage necrosis". This is markedly different from what the authors saw in human nasopharyngeal carcinoma tissues where the apoptotic cells were often scanty in number and scattered randomly. Therefore, the mechanism involved in apoptosis developed in nude mice transplants is worthy further investigation.

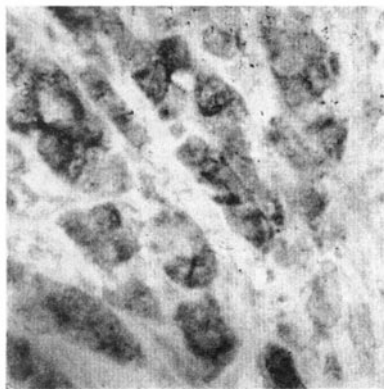


Fig. 6. Bax overexpression of the neoplastic cells. Bax, IHC 5x40.

The authors assumed that cell apoptosis developed in nude mice transplants would not be mediated by immune response because the nude mice is a kind of severe immunodeficient animal. The authors think that the neoplastic cell apoptosis taking place in nude mice transplants might result from deregulation of apoptosis-associated genes presented in CNE-1 or CNE-2 cell lines.

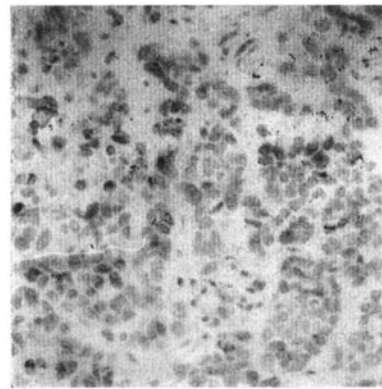


Fig. 7. Bcl-2 negativity in the nude mice transplants. Bcl-2, IHC 5x10.

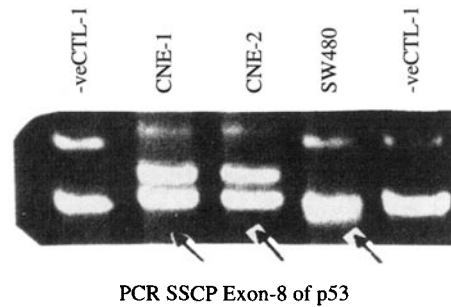


Fig. 8. The p53 gene alteration was detected in exon 8 by PCR-SSCP in CNE-1, CNE-2 and SW480 cell lines.

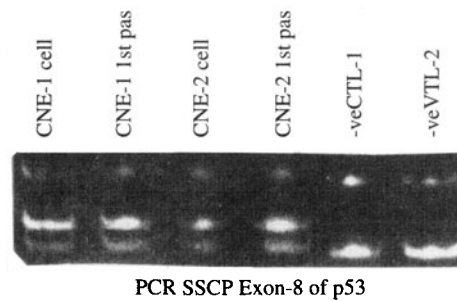


Fig. 9. The p53 gene alter in exon 8 in CNE-1, CNE-2 cell lines and the first generation transplant tissues. PCR-SSCP

What is the pathway of cell apoptosis mediated in these transplants? It is popularly known that the wild-type p53 protein is able to induce apoptosis of cells with DNA damage by the way of up-regulating expression of bax and forming more bax/bax homodimers.<sup>[3]</sup> This is called p53-dependent pathway of apoptosis. Indeed, human neoplastic cells in a variety of malignancies took this pathway to induce apoptosis.<sup>[3]</sup> The results of this study showed that not only the CNE-1 and CNE-2 cells but also all of the transplant tissues had p53 protein accumulation. In addition, alteration of exon 8 of p53 gene was also demonstrated either in cells or in transplants as Spruck et

al. (1992) reported previously.<sup>[4]</sup> They found a point mutation at code 280 in exon 8. These two cell lines could encode wild and mutant type p53 protein simultaneously. However, only mutant-type p53 protein could be accumulated in the tissue and play its role in regulating cell proliferation and apoptosis.<sup>[5]</sup> It is well known that the mutant p53 protein had lost its function to activate the promoter of bax gene,<sup>[6]</sup> so we think that the neoplastic cell apoptosis reported herein was not mediated by p53 protein. It was p53-independent. In other words, the neoplastic cell apoptosis developed in CNE-1 and CNE-2 nude mice transplants was not taking the p53 dependent pathway, it took the p53-independent pathway. O'Neill et al.<sup>[7]</sup> also reported that carcinoma cell apoptosis occurred independently of p53 overexpression in non-small cell lung carcinoma in 1996.

It is a crucial finding of this study that bax was overexpressed in almost all of the CNE-1 and CNE-2 cells in nude mice transplants. The overexpressed bax protein within tumor cells would form bax/bax homodimers, which will change the mitochondrial permeability and then induce cell apoptosis.<sup>[8]</sup> This pathway of apoptosis is bax-mediated. As is known, overexpression of bax protein might result from up-regulation of wild-type p53 or not, and therefore, there are two kinds of bax-mediated apoptosis, namely p53-dependent and p53-independent. Because the p53 detected in CNE-1 and CNE-2 nude mice transplants had lost its function of up-regulating bax, the bax mediated apoptosis developed in those transplants should be categorized as a pathway termed p53-independent bax-mediated. This kind of apoptotic pathway has been reported previously in experimental researches. Strobel et al. (1996)<sup>[9]</sup> and Janson et al. (1997)<sup>[10]</sup> reported that paclitaxel or butyrate could induce cell apoptosis through p53-independent and bax-enhanced pathways. However, bax overexpression might not definitely induce apoptosis since other factors might be involved. Han et al. (1995)<sup>[11]</sup> and Ishida et al. (1997)<sup>[12]</sup> reported that bax overexpression might not induce cell apoptosis in human intestinal-type gastric carcinoma. Accordingly, it might be necessary to further investigate what factor plays its role in increasing the bax protein expression in nude mice transplants of CNE-1 and CNE-2 cell lines.

In this research the authors studied the mechanism of neoplastic cell apoptosis developed in nude mice transplants of NPC cell lines. However, the human NPC tissue where tumor-host interaction exists is greatly different from the cell line transplant of nude mice with severe immunodeficiency. The authors' unpublished data shows that about 90% of neoplastic cells expressed bcl-2 (75/83) and almost all of the neoplastic cells expressed

bax in 83 NPCs. As reported,<sup>[4]</sup> the p53 gene mutation developed in NPCs is infrequent. Therefore it is necessary to clarify the mechanism of neoplastic cell apoptosis in human NPCs.

## REFERENCES

- [1] Effert P, Hegamyer G, Cheng YJ, et al. An infrequent point mutation of the p53 gene in human nasopharyngeal carcinoma. *Proc Natl Acad Sci USA* 1992; 89:6516.
- [2] Kerr JF, Wyllie AH, Currie AR. Apoptosis: A basic biological phenomenon with wide-ranging implication in tissue kinetics. *Br J Cancer* 1972; 26:239.
- [3] Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* 1993; 74:609.
- [4] Spruck CH, Tsai YC, Huang DP, et al. Absence of p53 gene mutations in primary nasopharyngeal carcinomas. *Cancer Res* 1992; 52:4787.
- [5] Sun Y, Dong Z, Nakamura K, et al. Dosage-dependent dominance over wild-type p53 of a mutant p53 isolated from nasopharyngeal carcinoma. *FASEB J* 1993; 7: 944.
- [6] Miyashita T, Reed JC. Tumour Suppressor p53 is a direct transcriptional activator of human bax gene. *Cell* 1995; 80:293.
- [7] O'Neill AJ, Staunton MJ, Gaffney EF. Apoptosis occurs independently of bcl-2 and p53 over-expression in non-small cell lung carcinoma. *Histopathology* 1996; 29: 45.
- [8] Pastorino JG, Chen ST, Tiffany M, et al. The overexpression of Bax produces cell death upon induction of the mitochondrial permeability transition. *J Biol Chem* 1998; 273: 7770.
- [9] Strobel T, Swanson L, Korsmeyer S, et al. Bax enhances paclitaxel-induced apoptosis through a p53-independent pathway. *Proc Natl Acad Sci USA* 1996; 93:14094.
- [10] Janson W, Brandner G, Siegel J. Butyrate modulates DNA- damage-induced p53 response by induction of p53-independent differentiation and apoptosis. *Oncogene* 1997; 15:1395.
- [11] Han Z, Chatterjee D, He DN, et al. Evidence for a G2 checkpoint in p53-independent apoptosis induction by X-irradiation. *Mol Cell Biol* 1995; 15:5849.
- [12] Ishida M, Gomyo Y, Ohfuji S, et al. Evidence that expression of a mutated p53 gene attenuates apoptotic cell death in human gastric intestinal-type carcinomas *in vivo*. *Jpn J Cancer Res* 1997; 88:468.