Chinese Medical Journal 2001; 114(9): 958-961

Growth inhibition of gastric cancer cells by all-trans retinoic acid through arresting cell cycle progression

WU Qiao 吴 乔, CHEN Zhengming 陈正明 and SU Wenjin 苏文金

Keywords: all-trans retinoic acid · gastric cancer cells · cell cycle

Objective To investigate the mechanism of all-trans retinoic acid (ATRA) on the regulation of the cell cycle in gastric cancer cells.

Methods The protein level was detected by Western blot. Immunoprecipitation was used in protein kinase activity determination. Cell growth and cell cycle phase were examined by MTT assay and flow-cytometric analysis, respectively.

Results ATRA could effectively induce G_0/G_1 arrest and inhibit cell growth in certain human gastric cancer cell lines. ATRA might induce $p21^{WAF1/CIP1}$ expression in ATRA-sensitive cell lines through p53-dependent and p53-independent pathways. Induction of $p21^{WAF1/CIP1}$ caused decrease in CDK_4 and CDK_2 activities independent of CDK_4 and CDK_2 protein expression levels. In addition, the dephosphorylated form of Rb protein increased because of the down-regulation of CDK_4 and CDK_2 activities by ATRA.

Conclusions Growth inhibition on gastric cancer cells by ATRA occurs through the regulation of relevant proteins leading to the arrest of cell cycle progression.

Chin Med J 2001; 114(9): 958-961

The cell cycle includes a variety of highly ordered processes that ultimately result in cell duplication. Abnormalities in cell cycle regulation lead to uncontrolled cell proliferation and the transformation into cancer cells.[1-2] Regulation of the G_1 - S transition is the most important event in cell cycle progression. Various kinds of proteins are involved in this transition. Among them, cyclin-dependent kinases (CDKs) and cyclins are most important.

$p16^{INK4}$ and $p21^{WAF1/CIP1}$, also termed CKIs (cyclin-dependent kinase inhibitors), are two critical negative regulators of CDK phosphorylation. $p16^{INK4}$ is a specific inhibitor of CDK_4 and CDK_6. Mutation and/or deletion of the $p16^{INK4}$ gene occurs in many types of tumors.[3] $p21^{WAF1/CIP1}$ inhibits most types of CDKs, including CDK_4 and CDK_2, and its transcription can be induced through p53-dependent and p53-independent pathways.[4] The inhibition of CDK activity by $p16^{INK4}$ and $p21^{WAF1/CIP1}$ results in the dephosphorylation of Rb protein, thereby arresting cell cycle progression.[5] The cyclin-CDK-CKI-Rb pathway, therefore, plays an important role in the regulation of cell cycle.

All-trans retinoic acid (ATRA) may regulate the proliferation and differentiation in many cell types.[6] The pathways on which ATRA acts in cancer cells are various and different.[7,8] One of them is the regulation of cell cycle. However, there has been relatively little comprehensive and systematic study addressing the function of the cyclin-CDK-CKI-Rb pathway in gastric cancer cells. Here, we report that the effect of ATRA on the regulation of cell cycle progression in gastric cancer is due to the up-regulation of $p21^{WAF1/CIP1}$, which results in the decrease in CDK_4 and CDK_2 kinase activities, the expression of cyclin E, and then, the dephosphorylation of Rb protein. These results reveal that the mechanism of growth inhibition by ATRA in gastric cancer cells is through the regulation on some relevant proteins to hinder the cell cycle from further progressing.

Key Laboratory of Ministry of Education for Cell Biology and Tumor Cell Engineering, The School of Life Sciences, Xiamen University, Xiamen 361005 (Wu Q, Chen ZM and Su WJ)

Correspondence to: Dr. Wu Qiao, Key Laboratory of Ministry of Education for Cell Biology and Tumor Cell Engineering, The School of Life Sciences, Xiamen University, Xiamen 361005, China (Tel: 0592-2182542. Fax: 0592-2086630. Email: xgwu@xmu.edu.cn)

This work was supported by the National Outstanding Youth Science Foundation of China (B type, No. 39825502) and the National Natural Science Foundation of China (No. 39880015).

METHODS

MTT assay

The human gastric cancer cell lines, BGC-823, SGC-7901 and MKN-45, were purchased from the Institute of Cell Biology, Shanghai, China. The MGC80-3 cell line was established by the Cancer Research Center, Xiamen University, China. All cells were maintained in RPMI-1640 medium, supplemented with 10% FCS, 1 mmol/L glutamine, and 100 U/ml penicillin. Cells were seeded at 1000 cells/well in 96-well plates, treated with ATRA (Sigma, USA) at various concentrations for one week, and stained with MTT (Sigma, USA) for the assay.[9]

Cell cycle analysis

Cells were trypsinized and collected, followed by 70% ice-cold ethanol fixation for one hour. Fixed cells were stained for 30 min (protecting from light) with 50 μg/ml propidium iodide (Sigma, USA) containing 1.0 mg/L DNase-free RNase A, and subsequently analyzed by FACScater-plus Flow Cytometer.

Cell lysis and Western blot

Cells were lysed in RIPA buffer with protease inhibitors (PMSF, aprotinin and leupeptin). Total proteins 100 μg were separated by 10% SDS-PAGE, and transferred to nitrocellulose membranes. Proteins were incubated at room temperature with the primary antibodies and the corresponding secondary antibodies (Santa Cruz, USA), 2 - 4 h for each incubation, and then visualized by the ECL detection system (Amersham, USA).

Immunoprecipitation and kinase activity

Cell lysate was prepared as mentioned above. Protein 500 μg was incubated with anti-CDK_4 or anti-CDK_2 antibody (Santa Cruz, USA) at 4 ℃ for 2 h, and then precipitated by protein A-Sepharose beads for an additional 1 h. The kinase reaction was carried out by resuspending the beads in 20 μl kinase buffer, then reacted with 3 μg Histone H_1 as its substrate and 5 μCi ^{32}P-γ-ATP at 30 ℃. Thirty minutes later, 2X SDS sample buffer was added, electrophoresed on 12% SDS-PAGE gels, and examined by autoradiography.

RESULTS

Regulation of cell cycle is the pathway in growth inhibition of ATRA on gastric cancer cells

The effect of ATRA on four gastric cancer cell lines was determined by MTT assay first. As shown in Fig. 1, ATRA effectively inhibited the growth of MGC80-3, BGC-823 and SGC-7901 cells in an ATRA-dose dependent manner. In contrast, ATRA did not show any clear effect on the growth of MKN-45 cells, even at the concentration of 10^{-6} mol/L. Flow-cytometric analysis was then performed to analyze the status of cell cycle progression in response to ATRA. When treated with ATRA only for 12 h, MGC80-3, BGC-823 and SGC-7901 cells were significantly arrested at the G_0/G_1 phase; the percentage of cells in the S-phase decreased significantly. In contrast, apparent change in cell cycle phase distribution of MKN-45 cells did not occur even when treated with ATRA for as long as 3 days (Table).

Table. Cell cycle phase distribution of gastric cancer cell lines (%)

ATRA	MGC80-3				BGC-823				SGC-7901				MKN-45			
(h)	0	12	24	72	0	12	24	72	0	12	24	72	0	12	24	72
G_0/G_1	35.7	52.9 *	61.7 *	67.5 * *	29.9	50.8 *	56.9 * *	78.2 * *	40.7	51.4 *	52.1 *	63.3 *	40.8	42.9	49.4	48.6
S	54.0	17.5	10.7 *	21.6 *	61.4	23.5	16.0 * *	15.0 * *	48.9	23.9	20.3	30.3 *	38.1	34.4	32.8	33.5
G_2/M	10.3	29.5	27.6 *	10.9 *	8.7	25.7	27.1 *	6.8 *	10.4	24.7	27.6 *	6.4 *	21.1	22.6	17.8	18.0

The values represent the results of 2 independent duplicate experiments. * $P < 0.05$, * * $P < 0.01$, vs control.

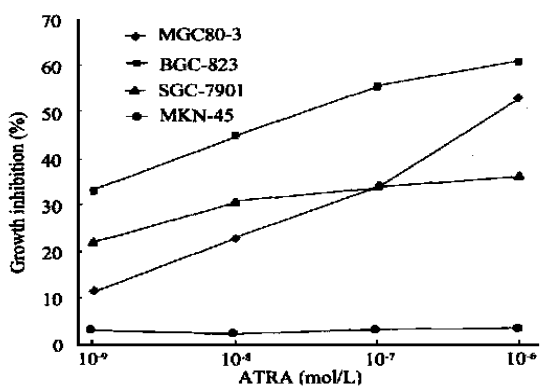

Fig. 1. Growth inhibitory effect of ATRA on gastric cancer cell lines. Cells were treated with different concentration of ATRA indicated (10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} mol/L, respectively) for 7 days, then measured with MTT method. The data shown are representative of three independent experiments.

Effect of ATRA on cyclin D_1/CDK_4 and cyclin E/CDK_2 complexes

As determined by Western blotting, cyclin D_1, CDK_4 and CDK_2 proteins were expressed in these four gastric cancer cell lines, and ATRA did not show any effect on their expressions (data not shown). On the other hand, cyclin E was down-regulated by ATRA in ATRA-sensitive cells but not in ATRA-resistant MKN-45 cells (Fig. 2). The kinase activity of CDK_2 and CDK_4 was further analyzed after immuno-precipitation. The results shown in Fig. 3 reveal that in MGC80-3 and BGC-823 cells, CDK_4 activity increased when cells were treated for 6 h, but finally lowered dramatically at 24 h. The same change happened with SGC-7901 cells, just moderate. However, in ATRA-resistant MKN45 cells, no apparent alteration of CDK_4 activity could

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be observed. The overall inhibitory effect of ATRA on CDK_2 kinase activity was found to be in a time-dependent manner in ATRA-sensitive cell lines, but not in ATRA-resistant MKN45 cells.

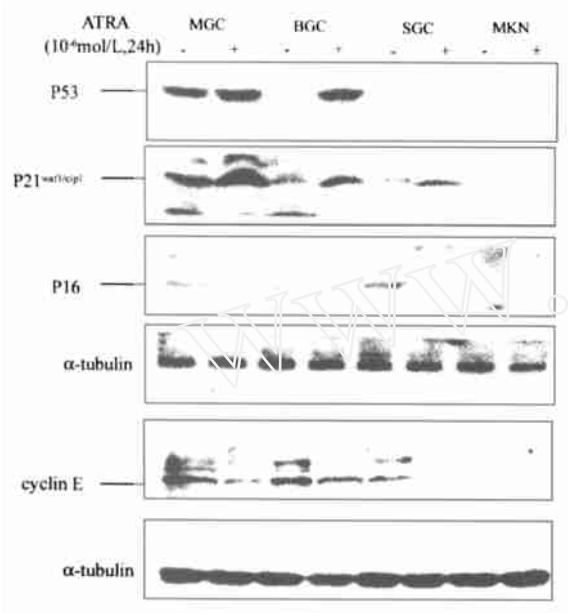

Fig. 2. Expressions of cyclin E, p53, $p21^{WAF1/CIP1}$ and $p16^{INK4}$ in gastric cancer cell lines. Cells were analyzed by Western blotting. α-tubulin was used as a control to normalize the amount of protein tested. C: positive control.

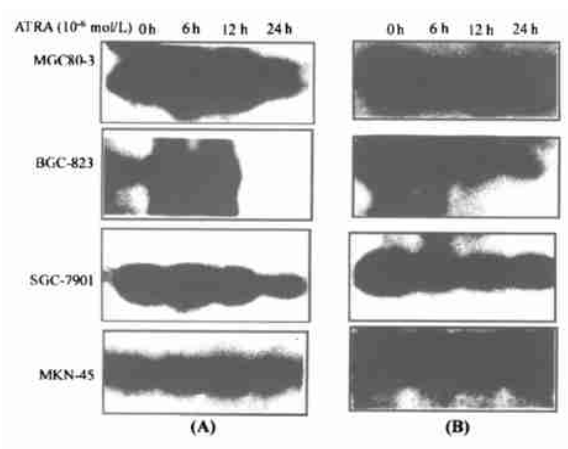

Fig. 3. Inhibition of CDK_4 (**A**) and CDK_2 (**B**) activity by ATRA. Activities of CDK_4 and CDK_2 were analyzed by immunoprecipitation assay.

Effect of ATRA on the expressions of $p16^{INK4}$, $p21^{WAF1/CIP1}$ and p53

As ATRA-sensitive cells were exposed to ATRA, the $p16^{INK4}$ protein level, contrary to our expectation, was down-regulated. Nevertheless, the $p21^{WAF1/CIP1}$ protein level was up-regulated. Neither of these two proteins could be detected in MKN45 cells (Fig. 2). To determine whether induction of $p21^{WAF1/CIP1}$ by ATRA was mediated by p53, we analyzed p53 expression in response to ATRA. Fig. 2 shows that ATRA could enhance or induce p53 expression in MGC80-3 or BGC-823 cells respectively, but not in that of SGC-7901 cells.

Effect of ATRA on alteration of Rb protein phosphorylation state

CDK may phosphorylate the Rb protein. In ATRA-sensitive cells, total Rb protein expression tended to be decreased by ATRA in a time-dependent manner, accompanied by increase in the ratio of dephosphorylated-state pRb to phosphorylated-state ppRb (Fig. 4). Again, Rb protein was not detectable in ATRA-resistant MKN45 cells.

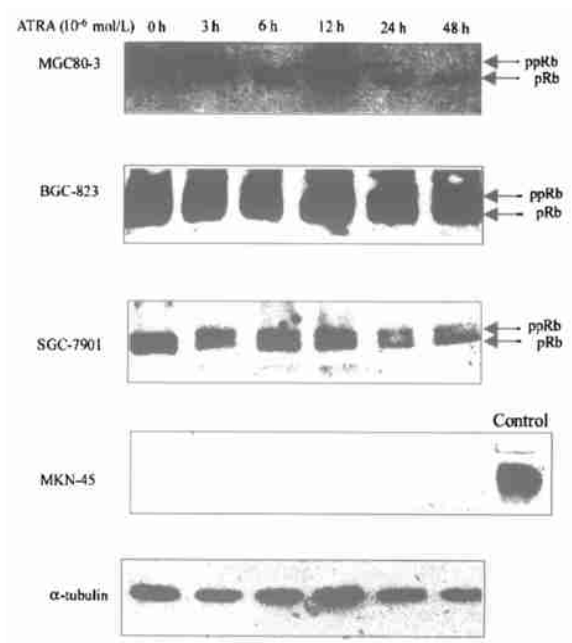

Fig. 4. Expression of Rb protein in gastric cancer cell lines. Cells were analyzed by Western blotting. α-tubulin was used as a control to normalize the amount of protein tested.

DISCUSSION

In this study, we demonstrated that ATRA could effectively inhibit growth of certain gastric cancer cells through its blockage of the cell cycle progression from the G_0/G_1 to the S phase. Since the G_1 to S phase transition is mainly regulated by cyclin D_1/CDK_4 and cyclin E/CDK_2 complexes,[2-3] it was suggested that the expression level and/or kinase activity of these proteins might be regulated by ATRA treatment. We found that, in ATRA-sensitive gastric cancer cells, only the expression of cyclin E protein was apparently suppressed by ATRA (Fig. 2), and the kinase activity of both CDK_2 and CDK_4 was inhibited by ATRA (Fig. 3), suggesting that cyclin E expression and CDK activity might be the targets of ATRA action. These

results differ from previous reports stating that ATRA can inhibit the expression of cyclin D_1 in breast cancer cells,[11] and the 9-cis RA can inhibit CDK kinase avtivity in some gastric cancer cell lines,[12] indicating that different mechanisms may be involved in regard to the effect of retinoic acid on various cancer cells. We expected that the dephosphorylated form of Rb protein (pRb), which is the target of CDK, should increase because of the down-regulated CDKs activity, and it was indeed the case. Thus, the cyclin-CDK-Rb pathway may be critical for ATRA in its regulation on gastric cancer cell cycle progression.

$p21^{WAF1/CIP1}$ and $p16^{INK4}$ are the major negative regulators of CDK_4 and CDK_2. In our study, the $p21^{WAF1/CIP1}$ protein was up-regulated by ATRA in a time-dependent manner in ATRA-sensitive cells. Further analysis indicated that induction of $p21^{WAF1/CIP1}$ was mediated through both p53-dependent and p53-independent pathways in ATRA-sensitive gastric cancer cell lines, which may correspond to the fact that MGC80-3 and BGC-823 cells express functional wild-type p53, while SGC-7901 cells express a p53 mutant gene.[13] Recently, it was reported that the $p21^{WAF1/CIP1}$ promoter contains a RA-response element located at the -1200 bp of $p21^{WAF1/CIP1}$,[14] which could mediate the induction of $p21^{WAF1/CIP1}$ by ATRA. It implies that ATRA may directly act on such a RA-response element in mediating the up-regulation of $p21^{WAF1/CIP1}$ expression. In addition, $p21^{WAF1/CIP1}$ is related to at least two of its targets in the G1 phase, cyclin D_1 and CDK_4. The binding of $p21^{WAF1/CIP1}$ with its targets should inhibit the activity of cyclin D_1/CDK_4 complexes.[15,16] Consistent with this point, we also demonstrated the decrease in CDK_4 and CDK_2 kinase activities by ATRA. Taken together, these results suggested that the decrease in CDK_4 and CDK_2 activities by ATRA treatment might not be due to its direct inhibition on cyclin D1, CDK_4 and CDK_2 expressions, but might function through its effect of increasing the level of $p21^{WAF1/CIP1}$ expression. The resulting protein product then interacts with cyclin D_1/CDK_4 and cyclin E/CDK_2 complexes, suppresses their activity and finally leading to G_0/G_1 arrest.

$p16^{INK4}$ is known as a tumor suppressor gene. Its deletion, mutation or other abnormal expressions have frequently been found in many types of cancer cells.[5] In this study, we found unexpectedly that the $p16^{INK4}$ protein level apparently decreased during ATRA treatment in ATRA-sensitive cells. The possible explanation may be that some abnormalities exist in the $p16^{INK4}$ gene of these cells. Since $p16^{INK4}$ specifically binds to CDK_4, and $p21^{WAF1/CIP1}$ can bind to both CDK_4 and CDK_2, thereby inhibiting their kinase activities, it may be reasonable that CDK_4 activity, when affected by both $p16^{INK4}$ and $p21^{WAF1/CIP1}$, first increases and subsequently decreases. The decrease in CDK_2 activity may be caused both by the decrease in the positive factor cyclin E and by the increase in the negative factor $p21^{WAF1/CIP1}$.

REFERENCES

1. Hartwell LH, Kastan MB. Cell cycle control and cancer. Science 1994;266:1821-1828.
2. Morgan DO. Principles of CDK regulation. Nature 1995;374:131-134.
3. Nobori T, Miura K, Wu DJ, et al. Deletions of CDK_4 inhibitor gene in multiple human cancers. Nature 1994;368:753-756.
4. Datto MB, Li Y, Panus JF, et al. Transforming growth factor beta induces the cyclin-dependent kinase inhibitior p21 through a p53-independent mechanism. Proc Natl Acad Sci U S A 1995;92:5545-5549.
5. Wolfel T, Hauer M, Schneider J, et al. A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. Science 1995;269:1281-1284.
6. Lotan R. Effect of vitamin A and its analogs (retinoids) on normal and neoplastic cells. Biochem Biophys Acta 1981;605:33-91.
7. Wu Q, Chen ZM, Su WJ. Mechanism of inhibition on avtivator protein-1 activity by all-trans retinoic acid in gastric cancer cells. Chin Med J 2000;113:972-976.
8. Chen YQ, Wu Q, Chen ZM, et al. Effect of all-trans retinoic acid on growth of xenograft tumor and its metestasis in nude mice. Chin Med J 2000;113:345-349.
9. Wu Q, Dawson MI, Zheng Y, et al. Inhibition of trans-retinoic acid-resistant human breast cancer cell growth by retinoid X receptor-selective retinoids. Mol Cell Biol 1997;17:6598-6608.
10. Wilcken NC, Sarcevic B, Musgrove EA, et al. Differential effects of retinoids and antiestrogens on cell cycle progression and cell cycle regulatory genes in human breast cancer cells. Cell Growth Differ 1996;7:65-74.
11. Zhou Q, Stetlerstevenson M, Steeg PS. Inhibition of cyclin D expression in human breast carcinoma cells by retinoids in vitro. Oncogene 1997;15:107-115.
12. Naka K, Yokozaki H, DOMEN T, et al. Growth inhibition of cultured human gastric cancer cells by 9-cis retinoic acid with induction of CDK inhibitor WAF1/CIP1/SDI1/p21 protein. Differentiation 1997;61:313-320.
13. Zhang QY, Lu YY, Li Z, et al. p53 gene mutation in chinese gastric carcinoma cell lines. Chin Bioche J 1995;11:311-315.
14. Liu M, Iavarone A, Freedman LP, et al. Transcriptional activation of the human $p21^{WAF1/CIP1}$ gene by retinoic acid receptor. J Biol Chem 1996;271:31723-31728.
15. Harper JW, Adami GR, Wei N, et al. The p21 CDK-interacting protein cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 1993;75:805-816.
16. Xiong YG, Hannon J, Zhang H, et al. p21 is a universal inhibitor of cyclin kinases. Nature 1993;366:701-704.

(Received February 28, 2001)
本文编辑: 刘 晖

Chin Med J 2001; 114(9): 954-957

军事医学科学院基础医学研究所 北京 100850　宋　伦　沈倍奋　黎　燕

目的　研究蛋白激酶 ERK 对人骨髓瘤细胞系 Sko-007 中转录因子 STAT3 在 IL-6 刺激下诱导活化的调控作用。
方法　首先分别采用凝胶阻滞电泳(EMSA)和免疫沉淀(IP)方法观察 IL-6 刺激前后 Sko-007 细胞中 STAT3 和 ERK 的诱导活化情况。然后将 ERK 反义寡核苷酸(ERK-AS)转染入 Sko-007 细胞用来特异性抑制 ERK 的表达及功能，并同时观察 STAT3 诱导激活情况的改变。最后采用免疫共沉淀方法检测 STAT3 与 ERK 之间是否存在直接相互结合作用。
结果　STAT3 和 ERK 在 Sko-007 细胞中都能够被 IL-6 诱导激活；ERK-AS 转染后 STAT3 的诱导活化信号明显减弱。同时，STAT3 和 ERK 可在 IL-6 刺激后发生直接相互结合作用。
结论　Sko-007 细胞中 ERK 可直接结合并参与 IL-6 刺激作用下 STAT3 的完全诱导活化。

关键词　STAT3　ERK　IL-6　结合　调控

全反式视黄酸通过抑制细胞周期进程抑制胃癌细胞生长

Growth inhibition of gastric cancer cells by all-trans retinoic acid through arresting cell cycle progression

Chin Med J 2001; 114(9): 958-961

福建厦门大学生命科学院细胞生物学与肿瘤细胞工程教育部重点实验室 厦门 361005　吴　乔　陈正明　苏文金

目的　探讨 ATRA 调节胃癌细胞生长的作用机理。
方法　Western blot 测定蛋白表达水平，免疫沉淀测定蛋白激酶活性，MTT 方法检测细胞生长和流式细胞术分析细胞周期。
结果　ATRA 有效地诱导细胞滞留 G_0/G_1 期并抑制胃癌细胞生长。ATRA 通过依赖 P53 和非依赖 P53 途径诱导 ATRA 敏感细胞的 $P21^{WAF1/CIP1}$ 表达，由此导致 CDK_4 和 CDK_2 活性下降，但对 CDK_4 和 CDK_2 蛋白表达没有影响。另外，由于 ATRA 抑制 CDK_4 和 CDK_2 活性，导致 Rb 蛋白去磷酸化水平上升。
结论　ATRA 通过调节细胞周期进程的相关蛋白而抑制胃癌细胞生长。

关键词　全反式视黄酸(ATRA)　胃癌细胞　细胞周期

具有鼻咽癌相关抗原内影像的抗独特型单克隆抗体研究

Monoclonal anti-idiotype antibody bearing the internal image of nasopharyngeal carcinoma associated antigen

Chin Med J 2001; 114(9): 962-966

中南大学湘雅医学院肿瘤研究所 长沙 410078　李官成　胡锦跃　周国华　朱建高　孙去病

目的　制备和鉴定具有鼻咽癌相关抗原内影像的抗独特型单克隆抗体(Ab2)。
方法　用鼻咽癌单抗(Ab1)免疫小鼠，免疫鼠脾细胞与骨髓瘤细胞 SP2/0 融合获得杂交瘤细胞系。用 Sandwich ELISA 和结合抑制试验筛选阳性克隆。为进一步证实 Ab2 是否具有鼻咽癌相关抗原的内影像，用 Ab2 免疫小鼠获得抗血清，以 ELISA 和竞争抑制试验检测 Ab3。用迟发性变态反应和 T 细胞增殖试验检测 Ab2 诱导的细胞免疫反应。
结果　选用两株抗鼻咽癌单抗 FC2、HNL5(Ab1)为免疫原，制备两株抗相应 Ab1 独特型的抗独特型抗体 2H4 和 5D3(Ab2)。2H4、5D3 能分别与 FC2、HNL5 特异性结合，并能抑制相应 Ab1 与靶细胞 HNE2 的反应，诱导产生能与相应 Ab1 竞争结合靶抗原的抗抗独特型抗体(Ab3)。并能诱发特异性迟发型超敏反应(DTH)和细胞增殖反应。
结论　抗独特型抗体 2H4、5D3 具有鼻咽癌相关抗原的内影像(Ab2β)，能模拟鼻咽癌相关抗原诱导体液和细胞免