附件一

行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告 □期中進度報告

活化的Notch受體結合蛋白及其下游基因調控之研究

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中　　華　　民　　國 93 年 10 月 1 日

中文摘要

Notch 接受子對於生物的發育過程扮演很重要的調控角色。Notch1 接受子的活化是經由 Notch1 接受子細胞外區域與鄰近細胞的 ligand 結合而引發，造成 Notch1 接受子經過蛋白切斷而釋放出 Notch1 接受子細胞內區域 (Notch1 intracellular domain, N1IC)。N1IC 移動至細胞核中與轉錄因子 CBF1 結合，活化 Notch 訊息傳遞的下游基因。本實驗室經由 yeast two-hybrid 方法尋找與 N1IC 結合的細胞內蛋白，發現 β_{II}-tubulin 與 N1IC 在細胞內結合。本研究工作先在 K562 以及 HeLa 細胞株的細胞核偵測到 β_{II}-tubulin 蛋白，並且進一步證實在 HeLa 細胞株的細胞核中，細胞核內的 β_{II}-tubulin 會與 N1IC 結合。以 taxol 處理 K562 以及 HeLa 細胞株後，經由 CBF1 媒介的 Notch1 訊息傳遞會被活化；以 colchicine 處理細胞則不影響經由 CBF1 媒介的 Notch1 訊息傳遞。此外，以 taxol 處理 K562 以及 HeLa 細胞株，會造成細胞核內 β_{II}-tubulin 及 α-tubulin 蛋白量的上升，但是以 colchicine 處理細胞則不影響細胞核中的 β_{II}-tubulin 及 α-tubulin 蛋白含量。而且經由 taxol 處理而進入細胞核的 β_{II}-tubulin 及 α-tubulin，48 小時後仍穩定存在細胞核中。以上結果顯示細胞核內 β_{II}-tubulin 與 Notch-1 接受子在癌細胞細胞核內有結合關係，並且可以調控 Notch1 訊息傳遞。

英文摘要

The Notch signal pathway plays important roles in proliferation, apoptosis, and differentiation. Abnormalities in Notch signaling are linked to many human diseases. After ligand binding, Notch signaling is activated through the cleavage of Notch receptors to release and translocate the Notch intracellular domain into the nucleus. The Notch1 receptor intracellular domain (N1IC), the activated form of the Notch1 receptor, can modulate downstream target genes *via* both CBF1-dependent and -independent pathways.

To further dissect the Notch1 signaling pathway, we screened the N1IC-associated proteins using a yeast two-hybrid system and identified nuclear β_{II}-tubulin as a candidate for the N1IC-associated proteins. It was suggested that the presence of β_{II}-tubulin in nuclei might be correlated with the cancerous state of cells. However, the function of β_{II}-tubulin locating in the nucleus is still unknown. Herein, we show that the complex of α- and β_{II}-tubulin is associated with N1IC in cancer cells by a co-immunoprecipitation analysis. The ankyrin (ANK) domain of the Notch1 receptor alone was sufficient to associate with β_{II}-tubulin. Furthermore, α- and β_{II}-tubulin were localized in the nucleus and formed a complex with N1IC. Treatment with taxol increased the amounts of nuclear α- and β_{II}-tubulin in K562 and HeLa cells and promoted the CBF1-dependent transactivation activity of N1IC. We also demonstrate that nuclear β_{II}-tubulin was bound on the CBF1-response elements *via* the association with N1IC. These results suggest that nuclear β_{II}-tubulin can modulate Notch signaling through interaction with N1IC in cancer cells.

關鍵詞(keywords): N1IC; Notch1; β_{II}-tubulin; CBF1; taxol

報告內容

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Nuclear β_{II}-Tubulin Associates with the Activated Notch Receptor to Modulate Notch Signaling

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ABSTRACT

The Notch signal pathway plays important roles in proliferation, apoptosis, and differentiation. Abnormalities in Notch signaling are linked to many human diseases. After ligand binding, Notch signaling is activated through the cleavage of Notch receptors to release and translocate the Notch intracellular domain into the nucleus. The Notch1 receptor intracellular domain (N1IC), the activated form of the Notch1 receptor, can modulate downstream target genes via CBF1-dependent and -independent pathways. To further dissect the Notch1 signaling pathway, we screened the N1IC-associated proteins using a yeast two-hybrid system and identified nuclear β_{II}-tubulin as a candidate for the N1IC-associated proteins. It was suggested that the presence of β_{II}-tubulin in nuclei might be correlated with the cancerous state of cells. However, the function of β_{II}-tubulin locating in the nucleus still is unknown. Herein, we show that the complex of α- and β_{II}-tubulin is associated with N1IC in cancer cells by a coimmunoprecipitation analysis. The ankyrin domain of the Notch1 receptor alone was sufficient to associate with β_{II}-tubulin. Furthermore, α- and β_{II}-tubulin were localized in the nucleus and formed a complex with N1IC. Treatment with Taxol increased the amounts of nuclear α- and β_{II}-tubulin in K562 and HeLa cells and promoted the CBF1-dependent transactivation activity of N1IC. We also show that nuclear β_{II}-tubulin was bound on the CBF1 response elements via the association with N1IC. These results suggest that nuclear β_{II}-tubulin can modulate Notch signaling through interaction with N1IC in cancer cells.

INTRODUCTION

Genes of Notch receptors encode evolutionarily conserved transmembrane receptors to regulate cell fate decisions during development (1). Many reports have documented that the Notch signal pathway modulates proliferation, apoptosis, and differentiation (1–3). Notch signaling also has been implicated in human cancers and in cancers induced by retroviral insertions in mice (4, 5). The Notch intracellular domain, the activated form of the Notch receptor, may function as an oncogene or tumor suppressor to promote or suppress tumorigenesis (for a review, see ref. 6). The effects of Notch signaling are cell type dependent, and these pathways that mediate growth and transformation may proceed through cross-talk with other signal pathways, such as transforming growth factor β (7) and epidermal growth factor receptor–mitogen-activated protein kinase signaling pathways (8).

After ligand binding, Notch signaling is activated through cleavage of Notch receptors, which allows the release and translocation of the Notch intracellular domain into the nucleus. The Notch intracellular domain modulates downstream target genes via CBF1-dependent and -independent pathways (9).

A few Notch1 intracellular domain (N1IC)–associated cellular factors have been found, and we recently also reported the association of transcription factor Yin Yang 1 (YY1) with N1IC (10). Only few downstream target genes of N1IC have been identified, such as the HES family (11), Nrarp (12), HERP2 (13), cyclin D1 (14), activator protein (15), the pre–T-cell receptor α ($pT\alpha$) gene (16), and acid α-glucosidase (17). However, the mechanisms controlling Notch1 signaling remain poorly understood.

To further dissect the Notch1 signaling pathway, we used a yeast two-hybrid system to search the N1IC-associated proteins and found nuclear β_{II}-tubulin as one of the candidates. β-Tubulin is one of the structural subunits of microtubules, which consists of the heterodimer of α- and β-tubulin. In most normal cells, tubulin resides only in the cytosol and not in the nucleus. Although the existence of nuclear β_{II}-tubulin could not be detected in biopsy samples of normal human tissues (18), the β_{II} isotype of tubulin recently was found in the nuclei of several tumor cells (19–21). These observations suggest that the presence of nuclear β_{II}-tubulin may be correlated with the cancerous state of cells (20).

Taxol, an antitumor drug, exhibits higher specificity for β_{II}-tubulin than for other isotypes. In a concentration-caused cellular apoptosis, Taxol could irreversibly deplete the nuclear β_{II}-tubulin content in rat C6 glioma cells (20). Nuclear β_{II}-tubulin was found to exist as $\alpha\beta_{II}$ dimers instead of assembled microtubules (19). However, the biological function of β_{II}-tubulin locating in nuclei still is unknown. In this study, we show that the association of nuclear β_{II}-tubulin with N1IC can modulate CBF1-dependent gene expression.

MATERIALS AND METHODS

Plasmids and Plasmid Construction. The expression construct of pcDNA-HA-N1IC contains cDNA encoding the amino acid residues 1764 to 2444 of the intracellular domain of the human Notch1 receptor with an NH_2-terminal hemagglutinin tag (10). The fusion protein plasmids pGST-ANKΔEP and pGST-ANK express glutathione *S*-transferase (GST) fusion proteins with amino acid residues 1821 to 2095 and 1821 to 2205 of the human Notch1 receptor, respectively. Reporter plasmids 4×wtCBF1Luc and 4×mtCBF1Luc were described previously (10, 22).

Yeast Two-Hybrid Screening. Plasmid pBTM116-N1-ANKΔEP, which encodes the ankyrin (ANK) domain of the Notch1 receptor (amino acid residues 1821 to 2095), was used as a bait in two-hybrid screening of human testis cDNA library (Clontech, Palo Alto, CA) according to the Matchmaker two-hybrid system protocol (Clontech). Owing to the ability of this plasmid construct to autonomously activate the *LacZ* reporter gene, positive yeast clones were only selected by histidine prototrophy from 5.6×10^6 transformed colonies in the presence of 20 mmol/L 3-amino-1,2,4-triazole.

Cell Culture and Transfection. All of the cell lines, including human erythroleukemia K562 cells, acute T-cell lymphoblastic leukemia SUP-T1 cells, and cervical carcinoma HeLa cells, were cultured in Roswell Park Memorial Institute 1640 and Dulbecco's modified Eagle's medium with 10% fetal bovine serum. The stable K562 cell lines expressing HA-N1IC (K562/HA-N1IC) and their control cells (K562/pcDNA3) were established previously (10). Taxol (Sigma-Aldrich, St. Louis, MO) at indicated concentrations in dimethyl sulfoxide or an equal volume of dimethyl sulfoxide was added for 24 hours, followed by washing with PBS three times and further incubated in the

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absence of Taxol for 24 hours or 48 hours. Cycloheximide (Sigma-Aldrich) was used at 25 μg/mL. For transient transfection of the luciferase reporter assay, K562 cells or HeLa cells (1×10^6) were seeded onto six-well plates and transfected using the SuperFect transfection reagent (Qiagen, Valencia, CA), and luciferase activities were measured as described previously (10).

For chromatin immunoprecipitation (ChIP) experiments, the K562/HA-N1IC cells (5×10; ref. 6) were transfected with 5 μg of reporter plasmids 4×wtCBF1Luc; cells were harvested 24 hours after transfection.

Coimmunoprecipitation. To prepare whole-cell lysates, cells were lysed in NETN buffer [50 mmol/L Tris-HCl (pH 7.9), 150 mmol/L NaCl, 0.5 mmol/L EDTA, and 0.5% NP40] containing protease and phosphatase inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 100 mmol/L sodium fluoride). Two alternative buffers, buffer A [20 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 2 mmol/L $MgSO_4$, and 0.5% NP40] and buffer B (PBS containing 0.5% NP40), containing protease and phosphatase inhibitors also were used as indicated. Cell lysates briefly were centrifuged to remove cell debris and then immunoprecipitated with protein A–Sepharose-bound anti–β_{II}-tubulin antibody (Sigma-Aldrich) as described previously (10). Western blot analysis was performed with anti-Notch1 COOH-terminal (Santa Cruz Biotechnology, Santa Cruz, CA), anti–β_{II}-tubulin, and anti–α-tubulin antibodies (Santa Cruz Biotechnology).

Glutathione *S*-Transferase Pull-Down Assay. N1IC proteins expressed as GST-ANKΔEP and GST-ANK fusion proteins from the expression constructs of pGST-ANKΔEP and pGST-ANK were induced and purified as described previously (10). Whole-cell extracts of K562/HA-N1IC cells were prepared in NETN buffer as described previously. A 50% (v/v) slurry of glutathione-agarose resin prebound with 0.5 μg of GST or GST fusion proteins was incubated with 500 μg of whole-cell extracts for the pull-down assay at 4°C for 2 hours as described elsewhere (10).

Subcellular Fractionation and Sucrose Gradient Analysis. To prepare the nuclear extracts, cell pellets were suspended and lysed in a hypotonic buffer; after centrifugation, the nuclear pellets were resuspended in a high-salt buffer as described previously (10). Nuclear extracts of K562/HA-N1IC or K562/pcDNA3 cells were loaded on the top of a 10% to 60% (w/v) sucrose gradient for centrifugation (10). The gradients were fractionated into 0.5-mL fractions each from the top, and aliquots of each fraction were subjected to Western blot analysis to detect β_{II}-tubulin and N1IC proteins. We also prepared the protein standards (catalase, 11.3 S, M_r 232,000; thyroglobulin, 19.4 S, M_r 669,000) to be run on a sucrose gradient.

Oligoprecipitation and Chromatin Immunoprecipitation. The 5′-biotinylated oligonucleotides and the protocol for oligoprecipitation were as described by Yeh *et al.* (10). The procedure for ChIP of K562/HA-N1IC cells transfected with luciferase reporter plasmids using protein A–Sepharose-bound anti–β_{II}-tubulin antibody and the specific primers for PCR amplification also was described previously (10).

RESULTS

β_{II}-Tubulin Is a Candidate of Notch1 Receptor Intracellular Domain–Associated Proteins. To date, molecular mechanisms regulating the Notch signal pathway remain obscure. To understand the Notch1 signaling pathway, we used the yeast two-hybrid system to search the protein(s) that interact with the activated Notch1 receptor (N1IC). The truncated fragment of N1IC, ANKΔEP (23), was expressed to screen N1IC-associated cellular factors from a human testis cDNA library in the presence of 3-amino-1,2,4-triazole. The cytoskeletal protein β_{II}-tubulin was one of the candidates for the N1IC-associated proteins. The COOH-terminal region of β_{II}-tubulin (accession no. BC019829) encompassing amino acid residues from 197 to 445 was identified as the region associated with the ANK domain of the Notch1 receptor (ANKΔEP) in the yeast two-hybrid system (data not shown).

The Notch1 Receptor Intracellular Domain Associates with β_{II}-Tubulin in Cancer Cells. To confirm the association of N1IC with β_{II}-tubulin in cancer cells, whole-cell extracts of K562 cells (K562/pcDNA3), HA-N1IC protein-expressing K562 cells (K562/HA-N1IC), and SUP-T1 cells were used for coimmunoprecipitation by antimouse IgG or anti–β_{II}-tubulin antibodies (Fig. 1*A* and *B*). The HA-N1IC fusion protein was coimmunoprecipitated with β_{II}-tubulin in K562/HA-N1IC and SUP-T1 cells by anti–β_{II}-tubulin antibody. This interaction between β_{II}-tubulin and N1IC also was observed in Jurkat cells using anti-Notch1 COOH-terminal antibody for immunoprecipitation (data not shown). α-Tubulin also was coimmunoprecipitated with β_{II}-tubulin; this might be because of the nuclear β_{II}-tubulin existing as $\alpha\beta_{II}$ dimers (19).

To avoid the binding artifacts generated by the denaturation of tubulins, either HEPES buffer (buffer A) or PBS (buffer B) also was used for the coimmunoprecipitation instead of NETN buffer. The interaction between β_{II}-tubulin and N1IC also was found in K562/HA-N1IC cells (Fig. 1*C*). Therefore, these results suggest that the Notch1 receptor intracellular domain associated with the heterodimer form of α- and β_{II}-tubulin in cells.

The ANK Domain of the Notch1 Receptor Is Sufficient to Associate with the β_{II}-Tubulin. The *in vitro* GST fusion protein pull-down assay was used to dissect the region of N1IC required for the association with β_{II}-tubulin. Partially purified GST and GST fusion proteins of N1IC were analyzed by SDS-PAGE and

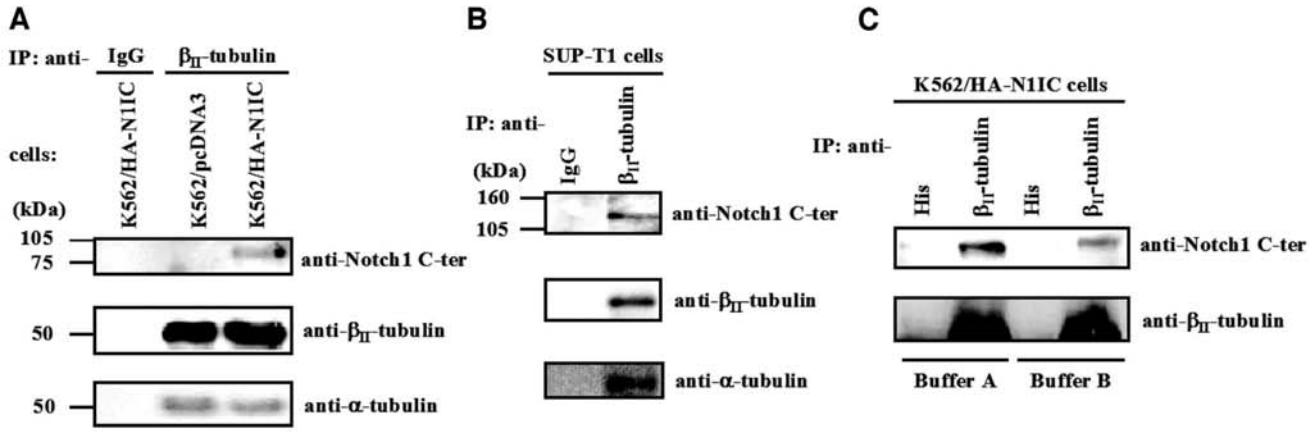

Fig. 1. Human N1IC associates with β_{II}-tubulin in cancer cells. *A* and *B*. Whole-cell extracts of K562 cells (K562/pcDNA3), HA-N1IC protein-expressing K562 cells (K562/HA-N1IC) (*A*), and SUP-T1 cells (*B*) were immunoprecipitated (*IP*) with antimouse IgG or anti–β_{II}-tubulin antibodies. The precipitated proteins were resolved by SDS-PAGE and analyzed by Western blot analysis using the anti-Notch1 COOH-terminal (*C-ter*) antibody (*top*), anti–β_{II}-tubulin antibody (*middle*), or anti–α-tubulin antibody (*bottom*). *C*. Whole-cell extracts of HA-N1IC protein-expressing K562 cells (K562/HA-N1IC) prepared by buffer A or buffer B were immunoprecipitated with anti-His or anti–β_{II}-tubulin antibodies. The precipitated proteins were analyzed by Western blot analysis using the anti-Notch1 C-ter antibody (*top*) and anti–β_{II}-tubulin antibody (*bottom*).

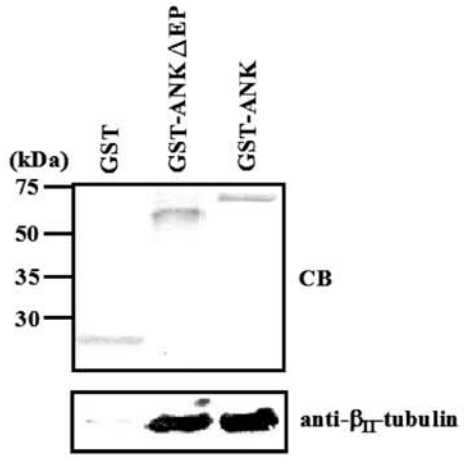

Fig. 2. ANK domain of the Notch1 receptor is sufficient to associate with β_{II}-tubulin. Whole-cell extracts of K562/pcDNA3 cells were used for the pull-down assay with purified GST, GST-ANKΔEP, and GST-ANK fusion proteins. The pull-down pellets were resolved by SDS-PAGE and analyzed by Western blot analysis using anti–β_{II}-tubulin antibody (*bottom*). The same amounts of inputs of various GST fusion proteins were resolved by SDS-PAGE and analyzed by Coomassie Blue (*CB*) staining (*top*).

Coomassie Blue staining (Fig. 2). Whole-cell extracts of K562/pcDNA3 cells were prepared for the pull-down assay by GST and GST fusion proteins. Both fusion proteins of GST-ANKΔEP and GST-ANK were shown to associate with endogenous β_{II}-tubulin of K562/pcDNA3 cells. Therefore, the ANK domain of the Notch1 receptor alone was sufficient to associate with β_{II}-tubulin. This is consistent with the result of two-hybrid screening, in which the COOH-terminal half of β_{II}-tubulin was identified as the prey of ANKΔEP protein.

Nuclear β_{II}-Tubulin Exists in Cancer Cells. The β_{II} isotype of tubulin was found in the nuclei of several tumor cells, including HeLa cells, and the presence of nuclear β_{II}-tubulin might be correlated with the cancerous state of cells (20). To determine the existence of nuclear β_{II}-tubulin in the hematopoietic tumor cells, the nuclear and cytosolic extracts of K562 and SUP-T1 cells were used for Western blot analysis (Fig. 3). β_{II}-Tubulin was present in the nuclei and the cytoplasm of K562 and SUP-T1 cells and HeLa cells. In the same blotting membranes, nucleolar B23 only was detected in the nuclear fractions, whereas glyceraldehyde-3-phosphate dehydrogenase only was observed in cytosolic fractions. Therefore, the existence of nuclear β_{II}-tubulin from the possibility of contamination of cytosolic proteins in the nuclear extracts of these cells could be excluded.

The Intrinsic Activated Notch1 Receptor Associates with Endogenous β_{II}-Tubulin in Nuclear Complexes. The Notch1 receptor intracellular domain previously had been shown to form a high molecular weight complex with cellular factors in the nuclei (10, 24). To gain further insights into the physiology of the interaction between N1IC and β_{II}-tubulin, their distributions in nuclei were investigated by sucrose gradient analysis. The nuclear extracts of K562/pcDNA3- and HA-N1IC–expressing K562/HA-N1IC cells were prepared for sucrose gradient ultracentrifugation. The collected fractions were analyzed by Western blot analysis using anti-Notch1 COOH-terminal and anti–β_{II}-tubulin antibodies sequentially. The sucrose gradient analysis profile showed that N1IC existed in both lower molecular weight fractions (fractions 3 to 7) and higher molecular weight fractions (fractions 14 to 16; Fig. 4*A*), whereas β_{II}-tubulin was detected in fractions 2 to 15. In the absence of the activated Notch1 receptor (*i.e.,* K562/pcDNA3 cells), β_{II}-tubulin showed the same sucrose gradient profile as that of K562/HA-N1IC cells.

To confirm the association between N1IC and nuclear β_{II}-tubulin, coimmunoprecipitation was applied to the nuclear extracts of K562/pcDNA3 and stable N1IC-expressing K562/HA-N1IC cells. β_{II}-tubulin was immunoprecipitated in the nuclear fractions of these two cells using anti–β_{II}-tubulin antibody (Fig. 4*B*). After stripping and reprobing this immunoprecipitated blot, N1IC, but not transcription factor YY1, also was detected in the nuclear fraction of K562/HA-N1IC cells. Furthermore, α-tubulin also was coimmunoprecipitated in this analysis, presumably through association with β_{II}-tubulin. These results showed that ectopically expressed N1IC was associated with endogenous β_{II}-tubulin in nuclei by the analysis of coimmunoprecipitation.

To check whether intrinsic N1IC interacts with endogenous β_{II}-tubulin in the nuclei of cancer cells, nuclear extracts of SUP-T1 cells were prepared for coimmunoprecipitation using the anti–β_{II}-tubulin antibody. Fig. 4*B* shows that N1IC could be coimmunoprecipitated with β_{II}-tubulin in the nuclei of SUP-T1 cells. This N1IC–β_{II}-tubulin association also was found in nuclei of HeLa cells (data not shown).

Contents of Nuclear α- and β_{II}-Tubulin Were Elevated by Nonapoptotic Concentrations of Taxol in Cancer Cells. To evaluate the effect of Taxol on β_{II}-tubulin, nuclear and cytosolic extracts of K562 and HeLa cells were prepared for Western blot analysis after treatment with Taxol for 24 hours. The K562 cell, a human chronic myelogenous leukemia cell line, expresses Bcr-Abl, which mediated high resistance to Taxol-induced apoptosis and showed higher concentrations of Taxol (μmol/L range) were used (25). Nevertheless, Taxol at low concentrations (10 nmol/L for 20 hours) already induced mitotic block in HeLa cells by suppressing the dynamics of spindle microtubules (26). To confirm whether Taxol inhibited the cell proliferation in these conditions, HeLa cells were treated with various concentrations of Taxol for 24 hours. As described previously (27), cell numbers of HeLa cells were not affected in the presence of Taxol at lower concentrations (5 and 10 nmol/L; Fig. 5*A*). However, the numbers of survival cells were decreased after treatment with higher concentrations of Taxol (25 and 50 nmol/L) in HeLa cells.

The Taxol-treated K562 cells showed dramatic quantity increment of α- and β_{II}-tubulin in the nuclei without affecting both contents in the cytosol (Fig. 5*B*). Furthermore, the amounts of nuclear α- and β_{II}-tubulin also were elevated without affecting cytosolic counterparts apparently in Taxol-treated HeLa cells (Fig. 5*C*). Previous treatment of HeLa cells with cycloheximide did not affect the increment of nuclear tubulins by Taxol. Therefore, this phenomenon was not because of *de novo* protein synthesis. This increase of nuclear β_{II}-tubulin induced by Taxol was sustained within 2 days after the removal of Taxol (Fig. 5*D*). However, the effect on the augmentation of nuclear tubulins was not observed in the presence of colchicine, an antimicrotubule agent (data not shown). Analyzing using confocal microscopy, the nuclear β_{II}-tubulin was increased in K562/HA-N1IC

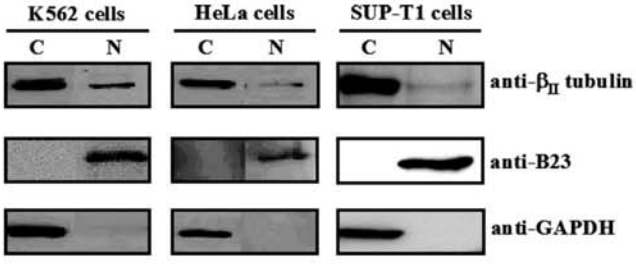

Fig. 3. Nuclear β_{II}-tubulin is present in cancer cells. Cytosolic extracts (*C*) and nuclear extracts (*N*) of K562 cells (*left*), HeLa cells (*middle*), and SUP-T1 cells (*right*) were prepared for Western blot analysis using the anti–β_{II}-tubulin antibody. The same membranes were stripped and reprobed with anti-B23 (a nuclear marker) or anti–glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) antibodies as indicated.

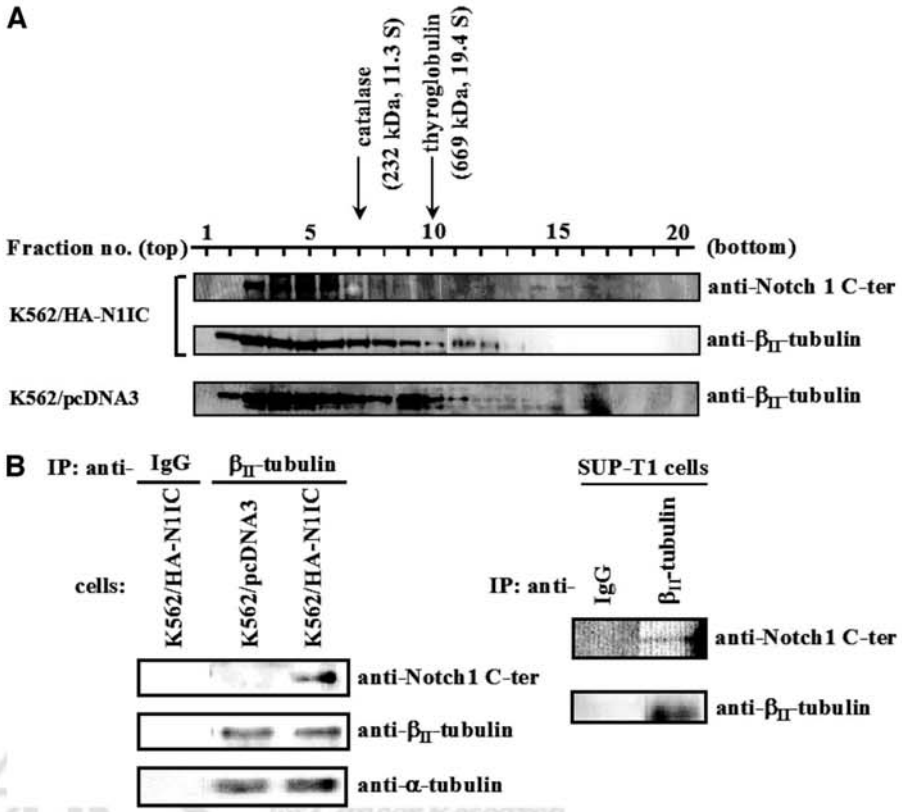

Fig. 4. N1IC associates with endogenous β_{II}-tubulin in nuclei of cancer cells. *A*. Nuclear extracts from K562/pcDNA3 and K562/HA-N1IC cells were subjected to sucrose gradient centrifugation. N1IC and β_{II}-tubulin were visualized by Western blot analysis with anti-Notch1 COOH-terminal (*C-ter*) and anti–β_{II}-tubulin antibodies, respectively. Arrows indicate the native molecular masses of known standards. *B*. Nuclear extracts of K562/pcDNA3 cells, K562/HA-N1IC cells (*left*), and SUP-T1 cells (*right*) were prepared for immunoprecipitation by antibodies against mouse IgG or β_{II}-tubulin. The immunoprecipitates were resolved by SDS-PAGE and analyzed by Western blot analysis using anti-Notch1 C-ter, anti–β_{II}-tubulin, or anti–α-tubulin antibodies.

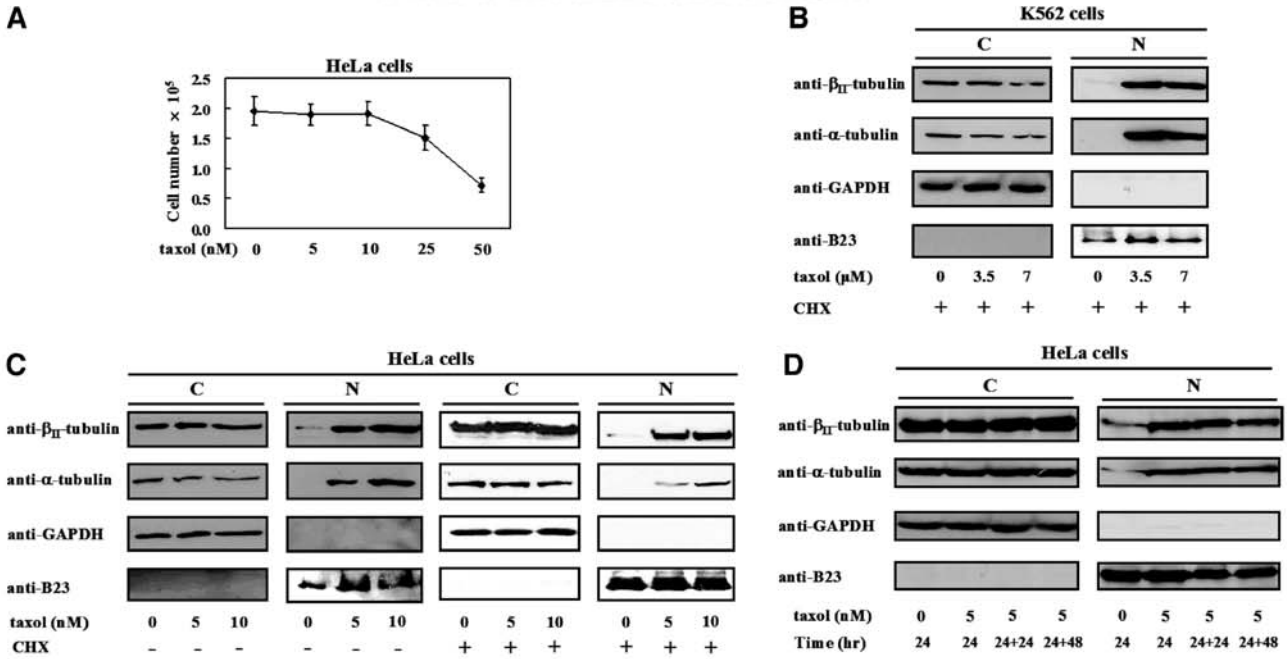

Fig. 5. Contents of nuclear α- and β_{II}-tubulin were elevated by Taxol in cancer cells. *A*. HeLa cells (1×10^5) were seeded in the presence of Taxol for 24 hours, and the cell number then was counted by trypan blue exclusion. *B* and *C*. After treatment with various concentrations of Taxol, the cytosolic and nuclear fractions of K562 cells (*B*) and HeLa cells (*C*) were prepared for Western blot analysis using anti–β_{II}-tubulin antibody. The same membranes were stripped and reprobed with anti–α-tubulin, anti–glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), or anti-B23 antibodies as indicated. Cycloheximide (*CHX*; 25 μg/mL) was added 30 minutes before Taxol treatment to block *de novo* protein synthesis. *D*. After treatment with 5 nmol/L Taxol for 24 hours, HeLa cells were washed by PBS three times (*24*) and further incubated in the absence of Taxol for 24 hours (*24 + 24*) or 48 hours (*24 + 48*). The cytosolic and nuclear fractions were prepared for Western blot analysis.

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cells and colocalized with N1IC in nucleus after treatment with 3.5 μmol/L Taxol for 24 hours (data not shown). These results suggest that Taxol may increase the nuclear import of α- and β_{II}-tubulin in K562 and HeLa cells.

Enhancement of Luciferase Reporter Activity Transactivated by the Notch1 Receptor Intracellular Domain after Treatment with Taxol. To elucidate the biological function of the association between N1IC and nuclear β_{II}-tubulin in the Notch signaling pathway, a luciferase reporter assay was performed. K562 cells were cotransfected with a luciferase reporter plasmid containing four copies of wild-type CBF1 response elements (4×wtCBF1Luc) and an N1IC-expressing construct, pcDNA3-HA-N1IC, or their control vectors in the presence of various concentrations of Taxol. The transfected cells were harvested and assayed for luciferase activity 24 hours after transfection. Although Taxol had been shown to alter gene expression (28–30), it did not affect the activity of the luciferase reporter gene containing CBF1 response elements (Fig. 6*A*). In the absence of Taxol, N1IC enhanced the expression of the reporter gene containing CBF1 response elements by ~12-fold in K562 cells. This promotion of luciferase activity was further elevated ~1.5- and 4-fold in the presence of 3.5 and 7 μmol/L Taxol, respectively. Enhancement of luciferase activity of approximately twofold to threefold by Taxol also was observed in HeLa cells (Fig. 6*B*). These effects of Taxol were not detected in the luciferase reporter plasmid containing four copies of the mutant CBF1 response elements (4×mtCBF1Luc) in either cell line (data not shown). Moreover, the activation of CBF1-dependent luciferase reporter activity in K562 cells and HeLa cells was not observed in the presence of colchicine (data not shown). These results imply that Taxol augments the CBF1-dependent transactivation activity of N1IC.

The β_{II}-Tubulin–N1IC-Associated Complex Binds on the Wild-Type CBF1 Response Element, but not on the Mutant One. The aforementioned data show that Taxol treatment increases the quantity of nuclear β_{II}-tubulin and also turns on CBF1-dependent Notch signaling in K562 and HeLa cells. We surmised that nuclear β_{II}-tubulin might participate in modulation of the Notch signal pathway. Therefore, oligoprecipitation was performed to study the interaction between the β_{II}-tubulin–N1IC-associated complex and the CBF1 recognition DNA sequence. In K562/HA-N1IC cells, N1IC and β_{II}-tubulin could be precipitated, together with the 5′-biotinylated wild-type CBF1 response element, by streptavidin-agarose beads but not with the mutant one (Fig. 6*C*). Furthermore, the ChIP assay also was used to examine the specific association of β_{II}-tubulin and N1IC with targeted DNA sequence in the context of living cells. K562/HA-N1IC cells were transiently transfected with reporter plasmid 4×wtCBF1Luc. Twenty-four hours after transfection, the cells were treated with formaldehyde to cross-link DNA and protein and then harvested for the ChIP assay using anti-His and anti–β_{II}-tubulin antibodies. The amplified PCR product of 470 bp was only present in the sample immunoprecipitated by anti–β_{II}-tubulin antibody (Fig. 6*D*). These data imply that nuclear β_{II}-tubulin indirectly binds on the wild-type CBF1 response element via associating with N1IC, which directly interacts with CBF1, and this association may enhance the transactivation activity of N1IC.

DISCUSSION

The role and regulation of Notch signal pathway are complicated and not yet fully understood. Notch signaling integrates with several other signal pathways during development. Many of these integrations are cell-type and -context dependent, suggesting that these are either directly or indirectly modulated by cell-specific cofactors. The activated Notch1 receptor recently was shown to form a high molecular weight complex containing Mastermind-like 1, CBF1, and transcription factor YY1 in the nucleus (10, 24). These associations modulate the transactivation activity of N1IC in regulating the Notch signal pathway.

In this study, we identified β_{II}-tubulin as an N1IC-associated protein, and this association modulates Notch signaling. The biological function of β_{II}-tubulin locating in nucleus still is unclear, although it was suggested that nuclear β_{II}-tubulin might be correlated with the cancerous state of cells (20). The relationship between the intracellular domain of the human Notch1 receptor and β_{II}-tubulin was investigated. We show herein that N1IC associated with β_{II}-tubulin in the

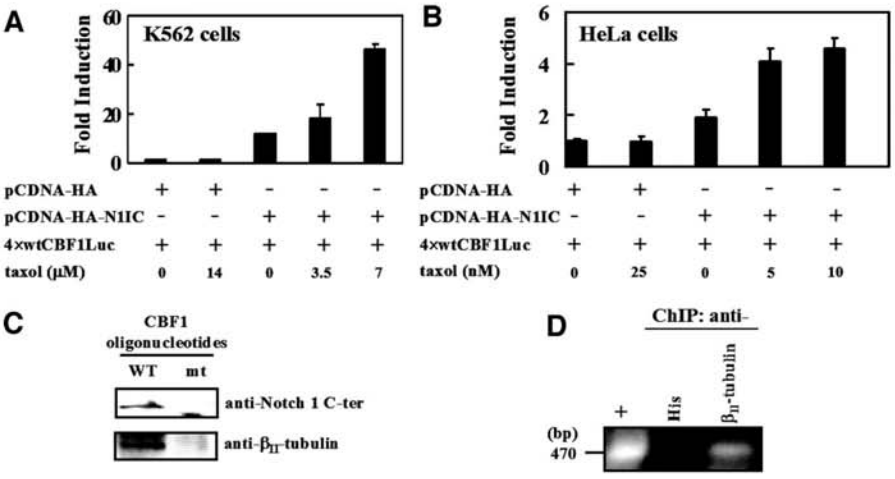

Fig. 6. CBF1-mediated transactivation activity of N1IC is modulated by Taxol. *A* and *B*. Reporter plasmid containing wild-type CBF1 response elements was cotransfected with HA-N1IC protein-expressing plasmid or its control vector into K562 cells (*A*) or HeLa cells (*B*) in the presence of various concentrations of Taxol. After 24 hours, luciferase activity was determined from whole-cell extracts, and the basal promoter activity of the reporter construct was set to unity. Mean values and SDs from at least four independent experiments are shown. *C*. The β_{II}-tubulin–N1IC-associated complex binds on the wild-type CBF1 response element but not on the mutant one. The nuclear extract of K562/HA-N1IC cells was incubated with the 5′-biotinylated double-stranded oligonucleotides of wild-type (*WT*) or mutant (*mt*) CBF1 response elements and then precipitated with streptavidin-agarose beads. The precipitated proteins were resolved by SDS-PAGE and analyzed by Western blot analysis using anti-Notch1 COOH-terminal (*C-ter*) antibody (*top*) or anti–β_{II}-tubulin antibody (*bottom*). *D*. K562/HA-N1IC cells were transiently transfected with reporter plasmid containing wild-type CBF1 response elements. Twenty-four hours after transfection, transfected cells were harvested for ChIP assay using anti-His and anti–β_{II}-tubulin antibodies. The immunoprecipitated DNA was used to amplify a 470-bp PCR product by specific primers for the region of the CBF1 response element in the reporter plasmid. (+) PCR-positive control uses 4 ng of 4×wtCBF1Luc plasmid as the DNA template.

nuclei of cancer cells and that Taxol treatment induced the increment of nuclear tubulins and increased the CBF1-dependent transactivation activity of N1IC. This is the first report regarding the involvement of nuclear β_{II}-tubulin in CBF1-dependent Notch signaling. These data suggest that nuclear β_{II}-tubulin may be involved in the control of tumorigenesis through the interaction with activated Notch1 receptor, which has been implicated in cancers (5, 6).

It was reported that the distribution of nuclear β_{II} was highly dependent on the type of cancer (21). These findings and our results suggest that nuclear β_{II}-tubulin could be a useful diagnostic agent in cancers. In addition to clarifying the biological function of nuclear tubulin in cancer cells, this study also raises the possibility that regulation of tumor formation could be regulated by nuclear β_{II}-tubulin through Notch signaling. We will further dissect the roles of nuclear β_{II}-tubulin in tumorigenesis of various cancers.

An intriguing similar situation is that the cytoskeletal protein actin also has been detected in the cell nucleus, and it has been tentatively implicated in gene expression (31–35). Percipalle *et al.* (36) recently also showed that the actin-ribonucleoprotein interaction is involved in transcription by RNA polymerase II.

Taxol not only binds to tubulin to promote microtubule assembly and to stabilize microtubules by bundle formation (37–39) but also to modulate gene expression (28–30). Furthermore, Taxol markedly enhances the nuclear factor κB and activator protein transcription factors binding to their response elements in the interleukin-8 promoter, which in turn up-regulates the *IL-8* gene in Taxol-responsive ovarian cancer cells (40). To clarify whether elevation of nuclear β_{II}-tubulin by Taxol of nonapoptotic concentration was caused by activation of gene expression or nuclear translocation, cycloheximide was used to block the *de novo* protein synthesis. Elevation of nuclear β_{II}-tubulin by Taxol also was observed in the presence of this inhibitor (Fig. 5*B* and *C*). The amount of nuclear β_{II}-tubulin in the cytosolic fraction also showed no apparent variation with the various treatments. These results may exclude the possibility that Taxol activates β_{II}-tubulin gene expression and suggest that Taxol enhances the nuclear import of β_{II}-tubulin. This observation in K562 and HeLa cells is different from that of rat C6 glioma cells; Taxol in an apoptotic concentration has been shown to deplete nuclear β_{II}-tubulin in C6 glioma cells (20).

How does β_{II}-tubulin enter nuclei of cancer cells? The COOH-terminal region of tubulin is less conserved among various isotypes (41); therefore, this isotype-specific region may be involved in the nuclear entrance of β_{II}-tubulin. The nuclear β_{II}-tubulin, most likely in the form of $\alpha\beta_{II}$ dimer, was suggested to bind with the β_{II}-interacting protein to remain itself in the nucleus (21). In the experiment of yeast two-hybrid system, we found that the COOH-terminal region of β_{II}-tubulin (amino acid residues 197 to 445) is sufficient to associate with N1IC. It is possible that N1IC with the nuclear localization signal may be involved in the nuclear localization of β_{II}-tubulin through their association.

Alternatively, it also has been speculated that β_{II}-tubulin remains attached to chromatin after cell division and then is trapped in the nucleus during interphase (42). However, cell cycle arrest induced by Taxol cannot enhance nuclear localization of β_{II}-tubulin through attachment to chromatin from the mole-per-liter phase into interphase. This implies that there must be other mechanisms through which β_{II}-tubulin enters the nucleus.

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REFERENCES

1. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. Science 1999;284:770–6.
2. Miele L, Osborne B. Arbiter of differentiation and death: Notch signaling meets apoptosis. J Cell Physiol 1999;181:393–409.
3. Kopan R. Notch: a membrane-bound transcription factor. J Cell Sci 2002;115:1095–7.
4. Ellisen LW, Bird J, West DC, et al. TAN-1, the human homolog of the Drosophila Notch gene, is broken by chromosomal translocation in T lymphoblastic neoplasms. Cell 1991;66:649–61.
5. Pear WS, Aster JC, Scott ML, et al. Exclusive development of T cell neoplasms in mice transplanted with bone marrow expressing activated Notch alleles. J Exp Med 1996;183:2283–91.
6. Radtke F, Raj K. The role of Notch in tumorigenesis: oncogene or tumour suppressor? Nat Rev Cancer 2003;3:756–67.
7. Rao P, Kadesch T. The intracellular form of notch blocks transforming growth factor ã-mediated growth arrest in Mv1Lu epithelial cells. Mol Cell Biol 2003;23:6694–701.
8. Yoo AS, Bais C, Greenwald I. Crosstalk between the EGFR and LIN-12/Notch pathways in *C. elegans* vulval development. Science 2004;303:663–6.
9. Baron M. An overview of the Notch signalling pathway. Semin Cell Dev Biol 2003;14:113–9.
10. Yeh TS, Lin YM, Hsieh RH, Tseng MJ. Association of transcription factor YY1 with the high molecular weight Notch complex suppresses the transactivation activity of Notch. J Biol Chem 2003;278:41963–9.
11. de la Pompa JL, Wakeham A, Correia KM, et al. Conservation of the Notch signalling pathway in mammalian neurogenesis. Development 1997;124:1139–48.
12. Krebs LT, Deftos ML, Bevan MJ, Gridley T. The. Nrarp gene encodes an ankyrin-repeat protein that is transcriptionally regulated by the Notch signaling pathway. Dev Biol 2001;238:110–9.
13. Iso T, Sartorelli V, Chung G, Shichinohe T, Kedes L, Hamamori Y. HERP, a new primary target of Notch regulated by ligand binding. Mol Cell Biol 2001;21:6071–9.
14. Ronchini C, Capobianco AJ. Induction of cyclin D1 transcription and CDK2 activity by Notch^{ic}: implication for cell cycle disruption in transformation by Notch^{ic}. Mol Cell Biol 2001;21:5925–34.
15. Chu J, Jeffries S, Norton JE, Capobianco AJ, Bresnick EH. Repression of activator protein-1-mediated transcriptional activation by the Notch-1 intracellular domain. J Biol Chem 2002;277:7587–97.
16. Reizis B, Leder P. Direct induction of T lymphocyte-specific gene expression by the mammalian Notch signaling pathway. Genes Dev 2002;16:295–300.
17. Yan B, Raben N, Plotz P. The human acid α-glucosidase gene is a novel target of the Notch-1/Hes-1 signaling pathway. J Biol Chem 2002;277:29760–4.
18. Roach MC, Boucher VL, Walss C, Ravdin PM, Ludueña RF. Preparation of a monoclonal antibody specific for the class I isotype of β-tubulin: the β isotypes of tubulin differ in their cellular distributions within human tissues. Cell Motil Cytoskeleton 1998;39:273–85.
19. Walss C, Kreisberg JI, Ludueña RF. Presence of the β_{II} isotype of tubulin in the nuclei of cultured mesangial cells from rat kidney. Cell Motil Cytoskeleton 1999;42:274–84.
20. Xu K, Ludueña RF. Characterization of nuclear β_{II}-tubulin in tumor cells: a possible novel target for taxol. Cell Motil Cytoskeleton 2002;53:39–52.
21. Yeh IT, Ludueña RF. The β_{II} isotype of tubulin is present in the cell nuclei of a variety of cancers. Cell Motil Cytoskeleton 2004;57:96–106.
22. Hsieh JJ, Henkel T, Salmon P, Robey E, Peterson MG, Hayward SD. Truncated mammalian Notch1 activates CBF1/RBPJk-repressed genes by a mechanism resembling that of Epstein-Barr virus EBNA2. Mol Cell Biol 1996;16:952–9.
23. Oswald F, Täuber B, Dobner T, et al. p300 acts as a transcriptional coactivator for mammalian Notch-1. Mol Cell Biol 2001;21:7761–74.
24. Jeffries S, Robbins DJ, Capobianco AJ. Characterization of a high-molecular-weight Notch complex in the nucleus of Notch^{ic}-transformed RKE cells and in a human T-cell leukemia cell line. Mol Cell Biol 2002;22:3927–41.
25. Lu Y, Jamieson L, Brasier AR, Fields AP. NF-κB/RelA transactivation is required for atypical protein kinase Cι-mediated cell survival. Oncogene 2001;20:4777–92.
26. Jordan MA, Toso RJ, Thrower D, Wilson L. Mechanism of mitotic block and inhibition of cell proliferation by taxol at low concentrations. Proc Natl Acad Sci USA 1993;90:9552–6.
27. Jordan MA, Wendell K, Gardiner S, Derry WB, Copp H, Wilson L. Mitotic block induced in HeLa cells by low concentrations of paclitaxel (Taxol) results in abnormal mitotic exit and apoptotic cell death. Cancer Res 1996;56:816–25.
28. Ding AH, Porteu F, Sanchez E, Nathan CF. Shared actions of endotoxin and taxol on TNF receptors and TNF release. Science 1990;248:370–2.
29. Bogdan C, Ding A. Taxol, a microtubule-stabilizing antineoplastic agent, induces expression of tumor necrosis factor α and interleukin-1 in macrophages. J Leukoc Biol 1992;52:119–21.
30. Makino K, Yu D, Hung MC. Transcriptional upregulation and activation of p55Cdc via p34^{cdc2} in Taxol-induced apoptosis. Oncogene 2001;20:2537–43.
31. Scheer U, Hinssen H, Franke WW, Jockusch BM. Microinjection of actin-binding proteins and actin antibodies demonstrates involvement of nuclear actin in transcription of lampbrush chromosomes. Cell 1984;39:111–22.
32. Zhao K, Wang W, Rando OJ, et al. Rapid and phosphoinositol-dependent binding of the SWI/SNF-like BAF complex to chromatin after T lymphocyte receptor signaling. Cell 1998;95:625–36.

33. Sotiropoulos A, Gineitis D, Copeland J, Treisman R. Signal-regulated activation of serum response factor is mediated by changes in actin dynamics. Cell 1999;98:159–69.
34. Rando OJ, Zhao K, Crabtree GR. Searching for a function for nuclear actin. Trends Cell Biol 2000;10:92–7.
35. Zhang S, Köhler C, Hemmerich P, Grosse F. Nuclear DNA helicase II (RNA helicase A) binds to an F-actin containing shell that surrounds the nucleolus. Exp Cell Res 2004;293:248–58.
36. Percipalle P, Fomproix N, Kylberg K, et al. An actin-ribonucleoprotein interaction is involved in transcription by RNA polymerase II. Proc Natl Acad Sci USA 2003;100: 6475–80.
37. Schiff PB, Fant J, Horwitz SB. Promotion of microtubule assembly in vitro by taxol. Nature 1979;277:665–7.
38. Schiff PB, Horwitz SB. Taxol stabilizes microtubules in mouse fibroblast cells. Proc Natl Acad Sci USA 1980;77:1561–5.
39. Kumar N. Taxol-induced polymerization of purified tubulin. Mechanism of action. J Biol Chem 1981;256:10435–41.
40. Lee LF, Haskill JS, Mukaida N, Matsushima K, Ting JP. Identification of tumor-specific paclitaxel (Taxol)-responsive regulatory elements in the interleukin-8 promoter. Mol Cell Biol 1997;17:5097–105.
41. Ludueña RF. Multiple forms of tubulin: different gene products and covalent modifications. Int Rev Cytol 1998;178:207–75.
42. Walss-Bass C, Kreisberg JI, Ludueña RF. Mechanism of localization of β_{II}-tubulin in the nuclei of cultured rat kidney mesangial cells. Cell Motil Cytoskeleton 2001;49: 208–17.

計畫成果自評

本研究工作之研究內容與原計畫相符，而且達成預期目標，同時研究成果已經在學術期刊發表（Cancer Research）。