Activation of Akt/GSK3β and Akt/Bcl-2 signaling pathways in nickel-transformed BEAS-2B cells

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Abstract. The Akt signaling pathway has been implicated in a wide range of cellular functions involving cell survival and proliferation, angiogenesis, metabolism and cell migration. Accumulating evidence suggests that Akt perturbations play an important role in human malignancy. Here, we investigated Akt perturbation in nickel-transformed cells. Chronic treatment of human bronchial epithelial BEAS-2B cells with low doses of nickel chloride resulted in cell transformation demonstrated by anchorage-independent (AI) growth, increased cell growth and alterations of cell growth pattern. Western blot assays show that phosphorylation of Akt at Ser473, but not that of p38, JNK and ERK, was increased in nickel-transformed cells compared with controls. Inhibition of Akt or PI3K by pharmacological or biochemical interference suppressed nickel AI growth and cell growth of transformed cells. Activation of Akt led to inhibition of GSK3β by phosphorylation at Ser9 in nickel-transformed cells. In addition, two major anti-apoptotic proteins of the Bcl family, Bcl-2 and Bcl-XL, were increased in nickel-transformed cells. By employing the small interfering RNA technique (siRNA), our results showed that siRNA Akt attenuated the expression of Bcl-2 and Bcl-XL in nickel-transformed cells, indicating that induction of Bcl-2 and Bcl-XL was likely mediated through Akt. ROS generation was decreased in nickel-transformed cells compared with controls. Moreover, down-regulation of retinoblastoma protein (Rb) was observed in nickel-transformed cells. Taken together, these findings demonstrate that activation of Akt, followed by GSK3β inhibition and Bcl-2, Bcl-XL up-regulation and decrease of ROS generation, along with a synergistic effect of Rb down-regulation may cause apoptosis resistance, contributing to the overall mechanism of nickel carcinogenesis.

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Introduction

The serine/threonine kinase Akt is known as protein kinase B (PKB). Since its discovery as an oncogene of the mouse leukemia virus AKT8 and a homolog of protein kinase C, much progress has been made to explore its diverse cellular roles (1,2). It is involved in regulating, such as cell size/growth, cell proliferation and survival, glucose metabolism, genome stability and neo-visualization (3). Enhancing the survival and growth of cells is one of critical roles for Akt. Therefore, Akt activation has been observed not only in many human cancers including breast, endometrium, prostate, lung, pancreatic, liver, ovarian and colorectal cancers (4,5), but also in some malignant transformation (6-8).

Nickel is a ubiquitous environmental transition metal and is widely used in industrial and medical processes (9). Epidemiological and laboratory studies have implicated nickel compounds as carcinogens (10). As results, nickel compounds have been classified as established carcinogen to humans (Group 1) by the International Agency for Research on Cancer (IARC) in 1990 (10). Due to its weak mutagenesis, epigenetic changes have been implicated in nickel carcinogenesis (11-13). Disruption of cellular iron homeostasis by interfering with iron-dependent enzymes and generation of reactive oxygen species (ROS) also reportedly contribute to nickel carcinogenesis (14). In addition, induction of the hypoxia signaling pathway reportedly represents a key mechanism in nickel-initiated carcinogenesis (13,15). Despite the progress, the molecular mechanisms underlying nickel-induced cell transformation, which is an essential step in tumor development, remains poorly understood.

Given the established role of Akt implicated in cancer and cell transformation, we investigated whether the Akt pathway is activated in nickel-transformed cells. In this study, we demonstrate that chronic exposure of low dose soluble nickel chloride ($NiCl_2$) induced transformation of human bronchial epithelial BEAS-2B cells. Akt, but not MAPK, was activated in nickel-transformed cells, followed by glycogen synthase kinase 3β (GSK3β) inhibition and up-regulation of the Bcl-2, Bcl-XL protein expression, and decrease of ROS generation. These processes may lead to apoptosis resistance, contributing to nickel-induced carcinogenesis.

Materials and methods

Cell culture and other reagents. Immortalized, non-tumorigenic human bronchial epithelial cells, BEAS 2B were used in this study (15). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Calbiochem) supplemented with 10% fetal bovine serum (FBS), 5% penicillin/streptomycin and 2 mM L-glutamine (Invitrogen) at 37˚C in a humidified atmosphere with 5% CO_2. Nickel cholroride ($NiCl_2$), LY294002 and 5-(2-benzothiazolyl)-3-ethyl-2-[2-(methylphenylamino) ethenyl]-1-phenyl-1H-benzimidazolium iodide (B2311) were purchased from Sigma. Antibodies against Akt, GSK3β, phospho-JNK, JNK, phospho-ERK, ERK, phospho-p53, p53 and β-actin were purchased from Santa Cruz Biotechnology, PTEN, Bcl-XL, phospho-Akt at Ser473, phospho-GSK3β at Ser9, phospho-mTOR at Ser2448, mTOR, phospho-Bad at Ser136, Bad, phospho-p38, p38, Rb from Cell Signaling, Bcl-2 from Dako, dephospho-β-catenin, β-catenin from Calbiochem and phospho-GSK3β at Tyr216 from Applied Biological Materials. Dihydroethidium (DHE) and 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA) were from Invitrogen.

Establishment of nickel transformed cell line and soft agar colony formation assay. BEAS-2B cells were chronically and continuously cultured without or with $NiCl_2$ at concentration of 25, 50, 75 and 100 μM, respectively. Cell transformation assay was checked biweekly by performing soft agar colony formation assay after 2-month treatment with nickel. For soft agar colony formation assay, specifically, $2x10^4$ cells were plated in 2 ml of top agar medium (DMEM with 10% FBS and 0.3% agar) and layered over 2 ml of bottom agar medium (DMEM with 10% FBS and 0.5% agar) in 6-well plates in triple and were incubated at 37˚C for 3-4 weeks. Formed colonies ≥0.5 mm were counted. Chronically nickel-treated BEAS-2B cells had the ability of anchorage-independent (AI) growth after 6-month treatment.

Colony was picked up from 75 μM $NiCl_2$ treated BEAS-2B cells with sterilized pipette and trypsinized in a 6-well plate for ~2 min and then trypsinization process was stopped by adding 2 ml DMEM. Cells were cultured in 6-well plates and later transferred to flask when cell density reached 90% confluent. Cells derived from the colony were cultured and defined as nickel-transformed cells. These nickel-transformed cells were rechecked for colony formation to confirm their transformation properties and used for experiment later on. Paralleled cultured BEAS-2B cells without $NiCl_2$ treatment were used as control cells.

Cell counting assay. Cell counting was carried out to assess cell proliferation between nickel-transformed BEAS-2B cells and control cells. Both control and nickel-transformed cells were seeded in each well of 6-well plates and grown for various time-points as indicated. Then cells were washed by PBS, trypsinized and then cell counting was performed using Beckman Coulter.

Western blotting. NuPAGE Bis-Tris electrophoresis system from Invitrogen company was used to perform Western blot analysis. The total cellular samples were washed once with ice cold PBS and lysed in 1X RIPA buffer supplemented with 50 mmol/l DTT (Fisher Biotech), then loaded with NuPAGE LDS sample buffer. The protein concentration was determined by Coomassie Protein Assay Reagent (Pierce). The total cellular protein extracts were separated by SDS-PAGE and transferred to nitrocellulose membrane. Membranes were blocked with 5% fat-free dry milk in 1X TBS containing 0.05% Tween-20 and incubated with antibodies (all primary antibodies diluted at 1:1000, except 1:2000 for GSK3β and actin, 1:200 for phospho-p38). Protein bands were detected by incubation with horseradish peroxidase-conjugated antibodies (1:4000, Kirkegaard and Perry Laboratories) and visualized with enhanced chemiluminescence reagent (Perkin-Elmer Life Sciences). Band density was performed using AlphaImager HP Imaging System from Alpha Innotech.

Cell transfection. To block Akt activation, the specific small interference RNA (siRNA) targeted Akt (which inhibits expression of Akt1 and 2, not Akt3) and control were purchased from Cell Signaling Co. Cell transfection was performed using Lipofectamine™ RNAiMAX from Invitrogen Co. Transfection procedure was followed by the protocol provided by the transfection reagent manufacturer. Briefly, control siRNA and siRNA Akt were incubated with Lipofectamine™ RNAiMAX in OPTI-MEM I for 20 min at room temperature and then added to cells in maintenance media without antibiotics (final concentration of both control siRNA and Akt siRNA were 100 nM each). Media were replaced with maintenance media with antibiotics 24 h later after transfection. Experiments were performed ~48 h following transfection.

ROS measurement. ROS production was detected by using the fluorescent probe CM-H_2DCFDA (Molecular Probes) or DHE (Molecular Probes). CM-H_2DCFDA is oxidized to green fluorescent DCF (dichlorofluorescein) by H_2O_2 and DHE is oxidized to red fluorescent ethidium by $O_2^{\bullet-}$ (16). Cells were loaded with 5 μM CM-H_2DCFDA or 10 μM DHE for 30 min, respectively, at 37˚C, 5% CO_2 in PBS. After washing twice, mean fluorescence intensity was determined as ROS generation by flow cytometry FACS Calibur (BD Bioscience, San Jose, CA).

Statistical analysis. For statistical analysis, values were presented as mean ± SE. Statistical differences between controls and treated groups were determined by One Way ANOVA. Differences were considered statistically significant for $P<0.05$.

Results

Chronic exposure of low dose nickel induces transformation of BEAS-2B cells. We first tested if low dose of nickel chloride could induce transformation of BEAS-2B cells *in vitro*. BEAS-2B cells were continuously exposed to 0, 25, 50, 75 and 100 μM nickel chloride for 6 months. During the period, cell transformation was checked biweekly by performing soft agar colony formation assay. Our study showed that BEAS-2B cells treated with different concentration of nickel gained the ability of anchorage-independent (AI) growth after 6-month treatment, while control cells did not acquire the ability to grow in soft agar or died shortly after plating (Fig. 1A). Fig. 1B demonstrates that different concentrations of nickel all conferred AI growth capacity of BEAS-2B cells.

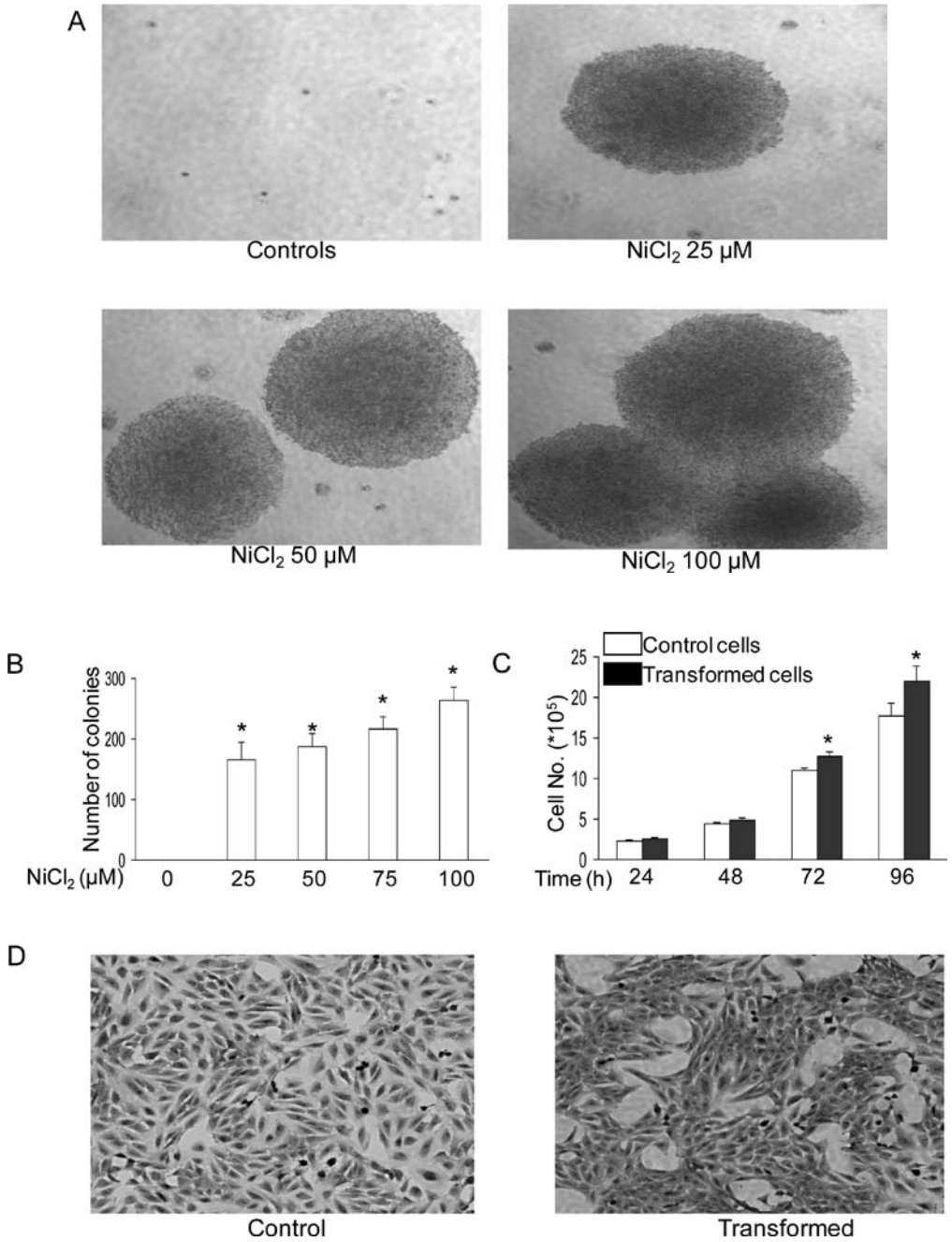

Figure 1. Low dose of $NiCl_2$ induces transformation of BEAS-2B cells. (A) Different low dose of $NiCl_2$ all induced colony formation in soft agar. The data shown here are typical examples of colonies formed in soft agar from three independent experiments. Photomicrographs of colonies were taken 3 weeks after plating (x10). Cell treatment is described in Materials and methods in detail. (B) Quantitative analysis of colony formation in soft agar. Bars represent the averages (±SD) of three independent experiments. The number of colonies in soft-agar was counted 3 weeks after plating. *P<0.05, significance between control cells and nickel-transformed cells. (C) Comparison of proliferation growth rate between control cells and nickel-transformed cells. Cells ($1.5x10^5$) were plated in 6-well plates and cultured for indicated time-point, then cells were washed by PBS, typsinized and counted using Beckman Coulter. *P<0.05 significance between control cells and nickel-transformed cells. (D) Alterations in cell growth pattern between control cells and nickel-transformed cells. Cells ($2x10^5$) were plated in 60-mm dishes and cultured for 48 h, and then pictures were taken using phase contrast microscope (x4) and representative images of three independent experiments are shown.

As described in Materials and methods in detail, cells isolated from colony grown in soft agarose were defined as nickel-transformed cells and utilized for later experiments. These nickel-transformed cells were re-examined for AI growth to confirm their ability of growing in soft agar. As expected, these nickel-transformed cells formed colonies in soft agar (data not shown). One of the key characteristics of transformed cells is their increased ability to survive when comparing to their normal counterparts. Fig. 1C shows that nickel-transformed cells also proliferated faster than control cells. Morphological changes have been reported in some nickel-treated cells (17,18). Cells undergo from epithelial-like to less epithelial or fibroblast-like, but in nickel-transformed BEAS-2B cells, morphological alterations were not obvious compared with control cells (Fig. 1D). However, the pattern of cell growth changed for those nickel-transformed

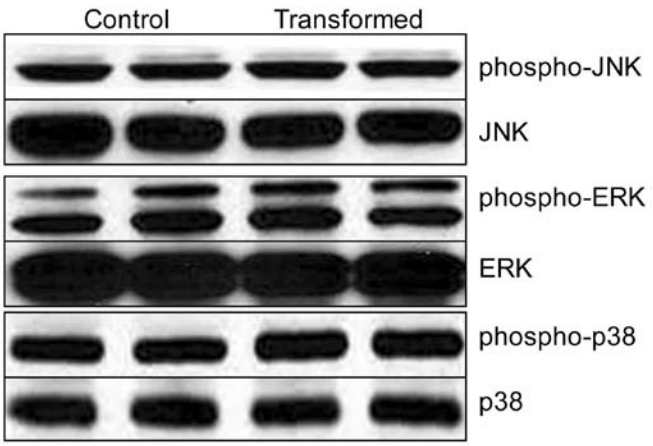

Figure 2. Analysis of the MAPK activation in nickel-transformed cells. Total cell lysates from both control and nickel-transformed cells were subjected to immunoblot analysis with antibody specific for the phosphorylated form of p38, JNK and ERK, respectively. The same blots were also stripped first and reprobed with antibody to p38, JNK and ERK to confirm that equal amounts of these proteins were loaded. All immunoblot analyses performed in the current study are representatives of at least three independent experiments.

BEAS-2B cells. They grew in clustered and aggregated forms (Fig. 1D).

Akt pathway, not the MAPK pathway, is activated in nickel-transformed BEAS-2B cells. Among the complicated signaling network, Akt and MAPK pathways are two well-defined signaling pathways involved in cell transformation. We first examined potential involvement of the MAPK pathway. Phosphorylation of endogenous p38, ERK and JNK, which represents the activated states of the kinases, was analyzed by immunoblotting. No significant difference was found between nickel-transformed and control cells (Fig. 2), suggesting that the MARK pathway was not activated in nickel-transformed BEAS-2B cells.

Using the same strategy, we analyzed the activation state of endogenous Akt protein in both nickel-transformed and control cells by immunoblotting. There was obvious difference in the phosphorylation of Akt at Ser473, which represents activated Akt (19), between transformed and control cells (Fig. 3A). B2311, an Akt inhibitor that inhibits Akt phosphorylation/activation, has also been used (20). Our results showed that treatment of nickel-transformed cells with B2311 has reduced cell growth and decreased colony formation in soft agar (Fig. 3D and E). Thus, Akt was constitutively activated in nickel-transformed BEAS-2B cells.

Akt activation regulated by PI3K, not PTEN. Akt is a major effector of phosphoinositide 3-kinase [PI(3)K] (21). We tested whether Akt phosphorylation in nickel-transformed cells is mediated through PI3K. As shown in Fig. 3B, LY294002, a well-established PI3K inhibitor, partially suppressed the nickel-induced Akt phosphorylation, implying that nickel-induced constitutive activation of Akt is, at least in part, mediated via PI3K. We also examined the effect of PI3K inhibitor, LY294002, on cell proliferation and colony formation of nickel-transformed cells. Our results demonstrated that inhibition of PI3K dramatically attenuated the cell proliferation and colony formation of nickel-transformed cells (Fig. 3D and E).

PTEN (phosphatase with tensin homology) is a lipid phosphatase and a tumor suppressor that negatively regulates the Akt signaling pathway (22). Since Akt activation is partially mediated by PI3K, we tested if PTEN is involved in the Akt pathway in nickel-transformed cells. Our results showed similar expression of PTEN protein among nickel transformed and control cells (Fig. 3C).

Downstream targets of Akt are involved in nickel-transformed cells. Akt plays diverse roles through its substrates (21). The protein kinase mTOR (mammalian target of rapamycin) is phosphorylated at Ser2448 via the PI3 kinase/Akt signaling pathway (23). Dysregulation of Akt/mTOR pathway has been documented in a variety of transformed cells and human tumors (24). We then investigated the activation level of mTOR by performing Western blot analysis. However, both phosphorylation of mTOR at Ser2448 and mTOR were not significantly different between nickel-transformed and control cells (Fig. 4B).

However, phosphorylation of GSK3β at Ser9, another substrate and effector of Akt, was elevated in nickel-transformed BEAS-2B cells (Fig. 4A). GSK3β is a multifunctional serine/threonine kinase found in all eukaryotes. It is now known that GSK3β functions not only in diverse cellular processes including proliferation, differentiation, motility and survival, but also in neoplastic transformation and tumor development. Full activity of GSK3β generally requires phosphorylation at tyrosine (Tyr216), and conversely, phosphorylation at serine (Ser9) inhibits GSK3β activity (25). However, in nickel-transformed cells, phosphorylation of GSK3β at tyrosine (Tyr216) remained unchanged compared with parental cells (Fig. 4A). Therefore, our study demonstrated the inhibition of GSK3β activity in nickel-transformed cells.

Amounting evidence suggests that the Wnt/β-catenin signaling pathway is often involved in oncogenesis and cancer development. GSK-3β is an essential component and regulator of the pathway, and activation of this kinase results in inhibition of the Wnt signaling pathway. Because GSK3β was inhibited in nickel-transformed cells, we examined if β-catenin is also activated in nickel-transformed cells. Dephosphorylated β-catenin and total β-catenin were expressed in both nickel-transformed and the control cells at similar levels (Fig. 4C).

Up-regulation of Bcl-2 and Bcl-XL in nickel- transformed cells mediated by Akt pathway. The Bcl-2 family of proteins plays an instrumental role in the regulation of apoptosis. This family includes both proapoptotic proteins such as Bad and Bax as well as anti-apoptotic proteins such as Bcl-2 and Bcl-XL. Bad is one of well known substrates of Akt. Phosphorylation of Bad by Akt at Ser136 can suppress apoptosis and promote survival (26). However the state of both phosphorylated Bad at Ser136 and total Bad showed no difference between nickel-transformed and control cells (data not shown). Since Bcl-2 and Bcl-XL are overexpressed in a variety of cancers (27), we investigated whether Bcl-2 and Bcl-XL were involved in nickel-induced cell transformation. Our study showed that protein expression of both Bcl-2 and Bcl-XL was up-regulated in nickel-transformed

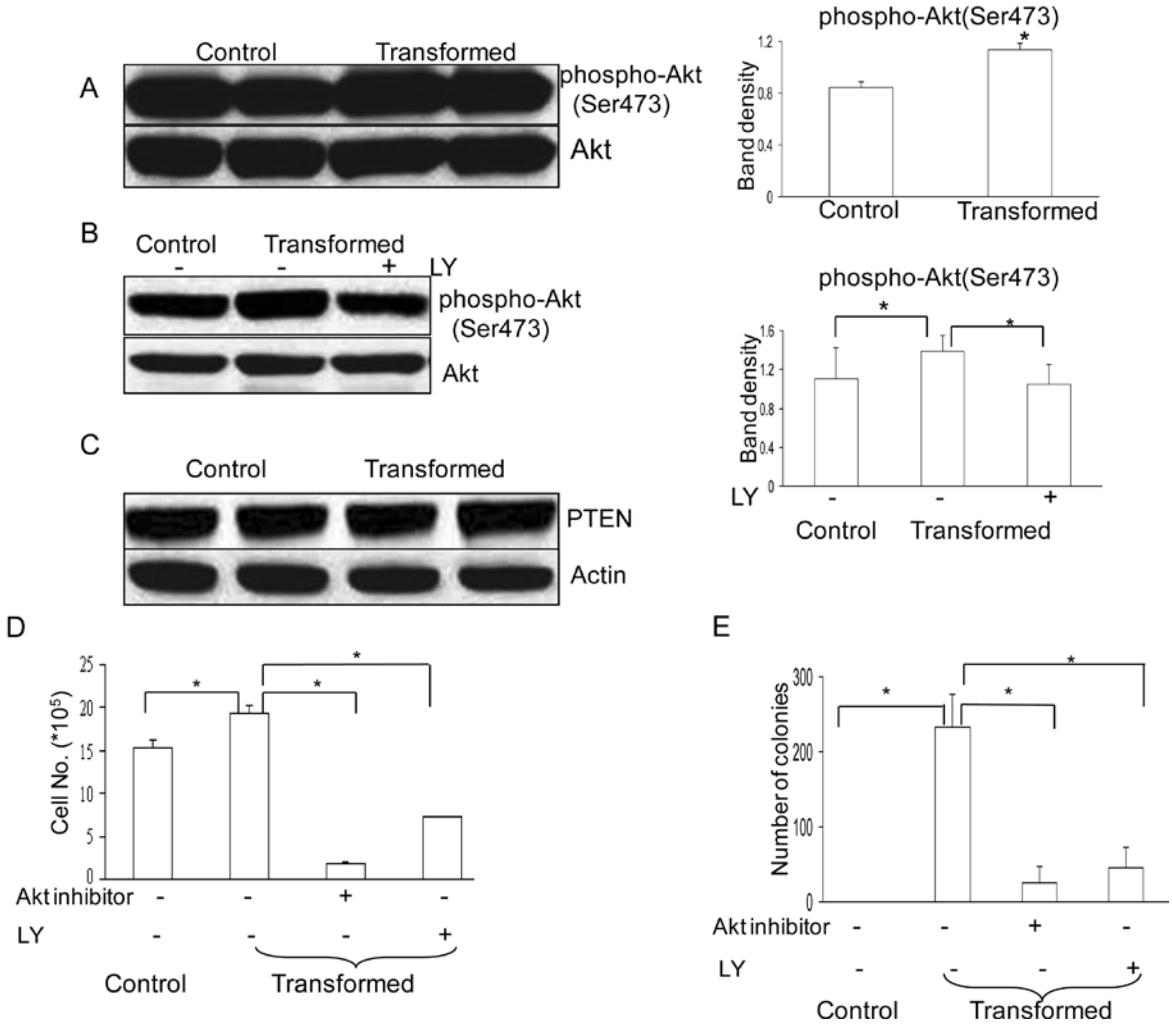

Figure 3. Activation of the PI3K/Akt in nickel-transformed cells. (A) Activation of Akt in nickel-transformed cells. Total cell lysates from both control and nickel-transformed cells were subjected to immunoblot analysis with antibody specific for the phosphorylated form of Akt. The same blot was also stripped first and reprobed with antibody to Akt to confirm that equal amounts of proteins were loaded. Quantitative analysis of band density is shown in the right panel normalized to Akt. Each bar represents the mean ± SE of three independent experiments. *P<0.05, significance between control cells and nickel-transformed cells. (B) Effects of PI3K inhibitor on Akt activation. Nickel-transformed cells were treated with or without LY294002 at 10 μM for 48 h. Then, total cell lysates from these cells as well as from control cells were subjected to immunoblot analysis with antibody specific for the phosphorylated form of Akt. The same blot was also stripped first and then reprobed with antibody to Akt to confirm that equal amounts of proteins were loaded. Quantitative analysis of band density is shown in the right panel. *P<0.05, significance between control cells and nickel-transformed cells or between the indicated two groups. (C) Protein expression of PTEN in nickel-transformed cells. Total cell lysates from both control and nickel-transformed cells were subjected to immunoblot analysis with antibody specific for PTEN. The same blot was also stripped first and reprobed with antibody to actin to confirm that equal amounts of proteins were loaded. (D) Effects of PI3K inhibitor and Akt inhibitor on proliferation of nickel-transformed cells. Cells ($1.5x10^5$) were plated in 6-well plates. Nickel-transformed cells were cultured for 96 h with or without existence of PI3K inhibitor, LY294002 at 10 μM and Akt inhibitor, B2311 at 1 μm. Then cells were washed by PBS and typsinized, followed by cell counting using Beckman Coulter. *P<0.05, significance between control cells and nickel-transformed cells or between the indicated two groups. (E) Suppression of nickel-induced soft agar colony formation by PI3K inhibitor and Akt inhibitor. Nickel-transformed cells were treated with PI3K inhibitor, LY294002 at 10 μM and Akt inhibitor, B2311 at 1 μM for 72 h, and then soft agar colony formation assay was performed according to methods described in Materials and methods. *P<0.05, significance between control cells and nickel-transformed cells or between the indicated two groups.

cells compared with the controls (Fig. 5). Furthermore, by using small interference RNA (siRNA) specific for Akt, we investigated whether up-regulation of Bcl-2 and Bcl-XL was mediated by Akt signaling pathway. Our results demonstrated that expression of both Bcl-2 and Bcl-XL proteins was apparently attenuated by siRNA Akt, implying that nickel-induced Bcl-2 and Bcl-XL overexpression was regulated via the Akt pathway (Fig. 6).

Decrease of ROS generation in nickel-transformed cells. ROS, key molecules in signaling cascades, have been investigated in great detail on their involvement in cancer and cell transformation over the last 10-15 years. A significant ROS generation has been found in many types of cancer cells (28). A review from the Cotter's group at the University College Cork in Ireland has emphasized the effect of ROS on PI3K/Akt signaling pathway in tumors (29). Consequently, we investigated ROS generation in nickel-transformed cells. However, we found that ROS generation, instead of increase, decreased in nickel-transformed cells compared with their counterparts (Fig. 7).

Down-regulated Rb protein, not p53 in nickel-transformed cells. Similar to carcinogenesis, cell transformation is also a multistep process. Activation of oncogenes and inactivation

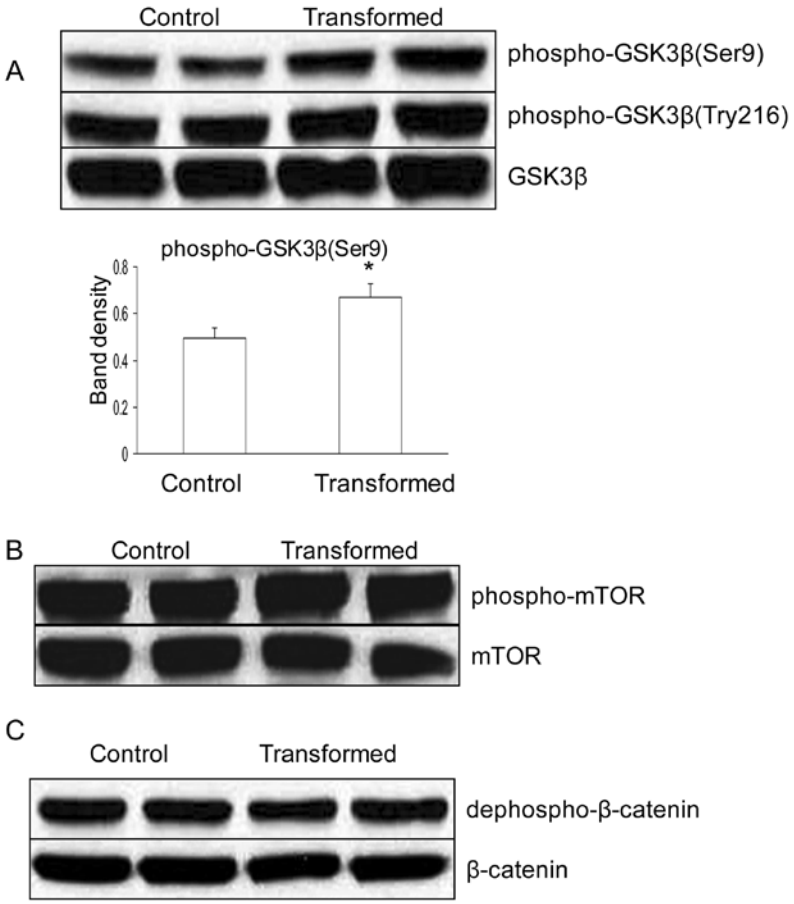

Figure 4. Downstream targets of Akt in nickel-transformed cells. (A, B) GSK3β, not mTOR, is involved in the Akt pathway in nickel-induced transformation. Total cell lysates from both control and nickel-transformed cells were subjected to immunoblot analysis with antibody specific for the phosphorylated form of mTOR and GSK3β at Ser9 and Tyr216, respectively. The same blots were also stripped first and reprobed with antibody to mTOR and GSK3β to confirm that equal amounts of these proteins were loaded. (C) β-catenin is not involved in nickel-induced cell transformation. Total cell lysates from both control and nickel-transformed cells were subjected to immunoblot analysis with antibody specific for the dephosphorylated form of β-catenin. The same blots were later stripped first and reprobed with antibody to β-catenin to confirm that equal amounts of these proteins were loaded.

of tumor suppressor genes are involved in the process of cell transformation (30). Rb and the p53, two tumor suppressors, are the most prominent among the regulators disrupted in cancer cells. Western blot analysis showed down-regulation of Rb protein in nickel-transformed BEAS-2B cells compared with their counterparts (Fig. 8), suggesting a synergistic role for Rb, along with activation of Akt pathway in nickel-transformed cells. Whereas, alterations of both phosphorylated p53 and p53 expression were not detected in nickel-transformed cells compared with their parental cells (Fig. 8).

Discussion

Both soluble and insoluble nickel compounds have been reported to be able to induce cell transformation in mouse and human cells including mouse embryo fibroblasts, HOS cells, and human bronchial epithelial (16HBE) cells, with relatively high concentrations of $NiCl_2$ (1 or 2 mM) (31,32). Here we show that various concentrations of low dose $NiCl_2$ ranging from 25 to 100 μM, were able to induce transformation of BEAS-2B cells as confirmed by AI growth, one of characteristics for transformed cells, which is a traditionally accepted criterion to confirm cell transformation. Moreover, nickel-transformed cells showed

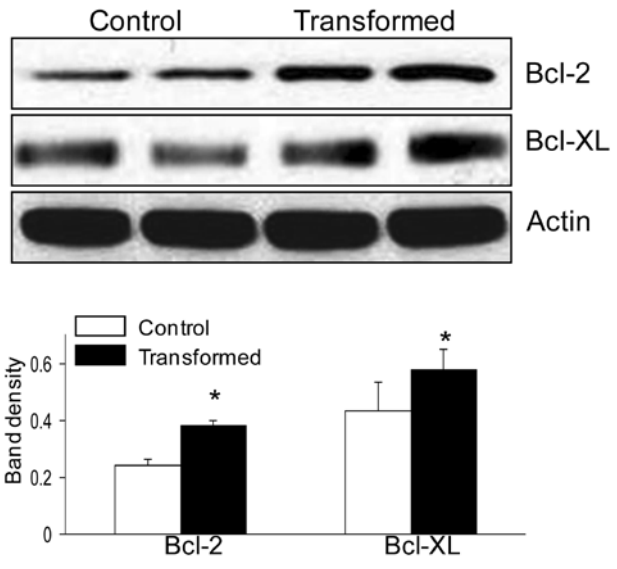

Figure 5. Up-regulation of Bcl-2 and Bcl-XL in nickel-transformed cells. Total cell lysates from both control and nickel-transformed cells were subjected to immunoblot analysis with antibody specific for Bcl-2 and Bcl-XL. The same blots were also stripped first and reprobed with antibody to actin to confirm that equal amounts of these proteins were loaded.

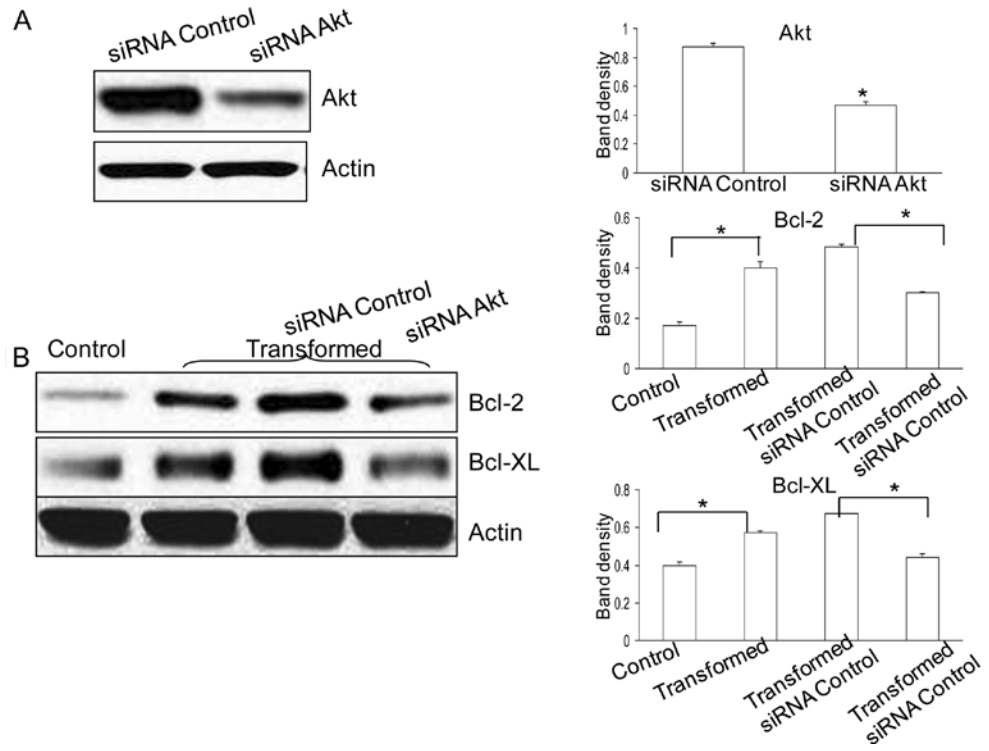

Figure 6. Up-regulation of Bcl-2 and Bcl-XL in nickel-transformed cells is mediated through Akt pathway. (A) siRNA Akt decreases Akt expression. Nickel-transformed cells were transfected with Akt siRNA (100 nM) and control siRNA (100 nM) for 48 h. The protein expression was measured by immunoblot analysis. The results demonstrated are representative of three separate experiments. (B) siRNA Akt attenuated protein expression of Bcl-2 and Bcl-XL in nickel-transformed cells. Nickel-transformed cells were transfected with 100 nM Akt siRNA and control siRNA for 48 h. Total cell lysates from these cells were subjected to immunoblot analysis with antibody specific for Bcl-2 and Bcl-XL. The same blots were also stripped first and reprobed with antibody to actin to confirm that equal amounts of these proteins were loaded. Data are representative of three separate experiments.

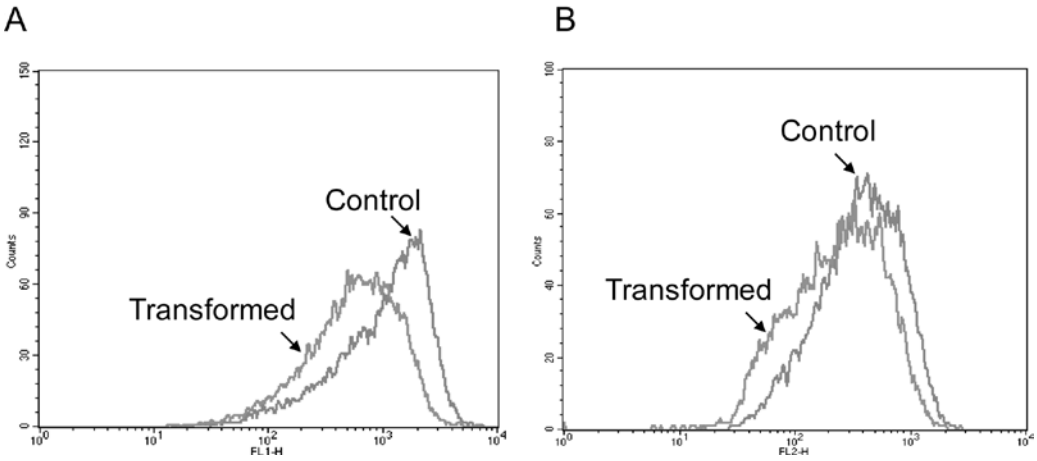

Figure 7. Generation of ROS in both nickel-transformed cells and controls. Both nickel-transformed and control cells ($4x10^5$) were seeded in a 60-mm dish. When cell density was about 90% confluent, the cells were labeled with CM-H2DCFDA (A) and DHE (B), respectively, followed by flow cytometry as described in Materials and methods. The rightward shift of the overlay reflects ROS generation. Illustrated overlays are representatives of at least three separate experiments.

accelerated growth and altered pattern of cell growth. Similar to our observations, Lin and Costa have observed this phenomenon in immortalized human osteoblastic (HOS) cells transformed by NiS (17). Nickel-transformed BEAS-2B cells grew in clustered pattern which might be a growth advantage by providing more space for nickel-transformed cells to grow to high densities.

The mechanism by which nickel induces cell transformation remains unclear. Previous studies by others have revealed the importance of gene expression and epigenetic alterations in nickel-induced cell transformation (14,31-35). However, little weight has been given to the signaling pathway alterations in nickel-induced cell transformation.

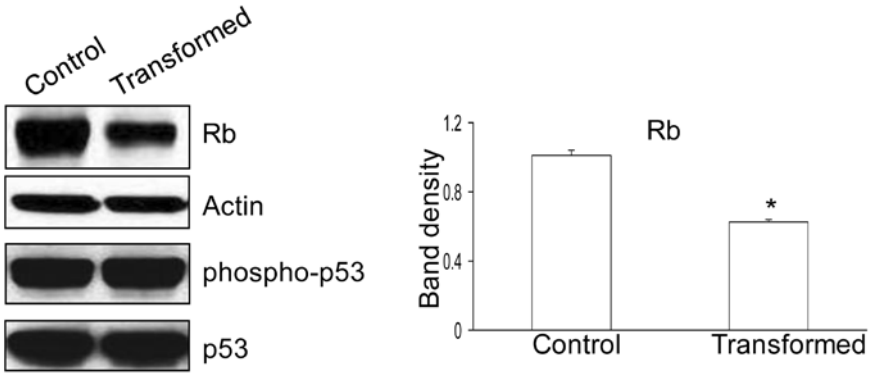

Figure 8. Down-regulation of Rb, not p53, is involved in nickel-induced cell transformation. Total cell lysates from both control and nickel-transformed cells were subjected to immunoblot analysis with antibody specific for Rb and phospho-p53, respectively. The same blots were also stripped first and reprobed with antibody to actin and p53, repectively to confirm that equal amounts of these proteins were loaded. Bars shown in the right panel are quantitative analysis of band density of Rb protein normalized to actin.

Studies reveal that activation of Akt pathway is required for cells undergoing transformation elicited by obl oncogene, FLJ10540 (6,36). We found that the PI3K/Akt pathway was constitutively activated in nickel-transformed BEAS-2B cells. By utilizing pharmacological inhibition method, we confirmed that LY294002 and B2311, inhibitor of PI3K and Akt, respectively, significantly suppressed proliferation of nickel-transformed cells, demonstrating the involvement of PI3K/Akt pathway in nickel-induced transformation. The lipid phosphatase activity of PTEN is associated with down-regulation of multiple downstream components of the PI3K pathway, including Akt and its downstream targets. Level of PTEN is often correlated to activation of Akt. However, our results show that PTEN protein expression, which reflects PTEN activation, remains similar between nickel-transformed and control cells, probably suggesting that PTEN is not involved in activation of PI3K/Akt pathway.

Many substrates of Akt have been identified, mTOR is one of them. Activation of mTOR regulated by Akt is highly implicated in cancer. It has reported that Akt/mTOR pathway is also involved in cell transformation induced by EGF in JB6 cells (37). But in nickel-transformed BEAS-2B cells, elevated activation of mTOR was not observed. Akt enhances the survival of cells by blocking the function of pro-apoptotic proteins and processes and thereby contributes to apoptosis resistance. GSK3β was the first substrate identified to be phosphorylated and inhibited by Akt in 1995 (38). Akt can phosphorylate constitutively active GSK3β within the N-terminal on serine 9 and block its apoptotic signaling. Phosphorylation on tyrosine 216 residue (Tyr216) is necessary for the functional activity of GSK3β (25). Research has revealed the implication of Akt/GSK3 pathway in growth regulation of myeloma cells, pancreatic cancer cells, vascular smooth muscle cells and IGF-1-induced skeletal myotube hypertrophy (37-40). Our current study shows that phosphorylation of GSK3β at Ser9 but not Tyr216 was apparently increased in nickel-transformed cells.

GSK3β can regulate diverse substrates and signaling pathways. One of the most important impacts of GSK3β on neoplastic transformation of tumor development is likely mediated by its influence on Wnt/β-catenin signaling. Phosphorylation of β-catenin by active GSK3β targets β-catenin for ubiquitin-mediated proteasomal degradation (39). Activation of Wnt signaling inhibits GSK3β and stabilizes cytoplasmic β-catenin, qualifying β-catenin as a proto-oncogene (40). Since GSK3β was inhibited in nickel-transformed cells, we speculate a possible crosstalk between the Akt and Wnt pathways in nickel-induced cell transformation converging on GSK3β. However, we did not observe obvious difference in the amounts of both dephosphorylated β-catenin and β-catenin expression between transformed and control cells.

Many members of the human Bcl-2 family proteins have been identified and alterations in their expression and function contribute to the pathogenesis and progression of human cancers (27). For instance, the Bcl-2 gene is elevated in perhaps half of all human cancers (27). Our study demonstrates that expression of Bcl-2 and Bcl-XL, two anti-apoptotic proteins of the Bcl family was elevated in nickel-transformed cells. Furthermore, to examine the role of Akt on expression of both Bcl-2 and Bcl-XL, siRNA specific for Akt was carried out. Results show that expression of both Bcl-2 and Bcl-XL was attenuated by siRNA Akt, indicating that expression of Bcl-2 and Bcl-XL was mediated through the Akt pathway. It has been reported that Akt-dependent Bcl-XL overexpression was involved in acidic preconditioning protection of endothelial cells from apoptosis (41), while Akt could also up-regulate Bcl-2 expression and mediated cell survival signal in PC cells (42). Others have observed a synergistic effect between PI3K/Akt and Bcl-XL in controlling cell death (43).

Transformation is a complicated process and many factors are involved including oncogenes and suppression genes. Several lines of evidence support the hypothesis that tumor suppressor genes or oncogenes play synergistically a role in cell transformation induced by various inducers. For example, Myc has reported synergized with Ras and PI3-kinase to induce cell transformation (44). Here we found protein expression of Rb was down-regulated in nickel-transformed cells. Rb was the first identified tumor suppressor gene. It is mutated or expressed at very low levels in several tumor types, including retinoblastoma and osteosarcoma, as well as small cell lung, colon, prostate, bladder and breast carcinoma (45). Deregulation of Rb has also contributed to cell transformation (46). Similar to our observation, another study also found the

hypophosphorylated Rb protein in NiS-transformed HOS cells (47). Even though abnormality of p53 is frequently observed in most human cancer, both phosphorylated p53 and total p53 remained unchanged between nickel-transformed cells and their counterparts.

In summary, our study shows that nickel is able to induce transformation of BEAS-2B cells. In nickel-transformed cells, Akt and not MAPK was activated, followed by GSK3β inhibition and up-regulation of Bcl-2, Bcl-XL, and decrease of ROS generation. These processes will lead to the apoptosis resistance, contributing to the overall mechanism of nickel-induced carcinogenesis.

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