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Identification of Differentially Expressed Genes in Nickel(Ii)-Treated Normal Rat Kidney Cells

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ABSTRACT : **Nickel(II) compounds are carcinogenic metals which induce genotoxicity and oxidative stress through the generation of reactive oxygen species. In search of new molecular pathways toward understanding the molecular mechanism of nickel(II)-induced carcinogensis, we performed mRNA differential display analysis using total RNA extracted from nickel(II) acetate-treated normal rat kidney cells (NRK-52E). Cells were exposed for 3 days to 160 and 240 uM nickel(II) concentrations. cDNAs corresponding to mRNAs for which expression levels were altered by nickel(II) were isolated, sequenced, and followed by a GenBank Blast homology search. Specificity of differential expression of cDNAs was determined by RT-PCR and Western blot analysis. Two of them (SH3BGRL3 and FHIT) were down-regulated and one (metallothionein) was up-regulated by nickel(II) treatment. The expression of these mRNAs were nickel(II) concentration-dependent. The levels of FHIT and metallothionein proteins were also consistent with the results for mRNAs. Overall, although the fundamental questions related to function of these genes in nickel(II)-mediated carcinogenicity are not answered, our study suggests that they can be interesting candidates for studies of molecular mechanisms of nickel(II) carcinogenesis.**

Key words : **Nickel(II), Metallothionein, FHIT, SH3BGRL3, Carcinogenicity, NRK-52E cells, Oxidative stress**

Introduction

The transition metal nickel(II) which is known as an important industrial and environmental pollutant is a nonessential heavy metal. Nickel(II) is known to produce a variety of health hazards in human and experimental animals due to it ability to induce toxic effects in various organs and tissues, following either acute and chronic exposure. It is carcinogenic to human, and is potent inducer of tumor in animals. It induces local sarcoma, lung and renal carcinoma in experimental animals (Sunderman, 1984; Kasprzak *et al.*, 1990). Human epidemiological data suggest that it causes lung and sinonasal cancer (Kasprzak *et al.*, 1990). Whereas nickel (II) is not generally mutagenic (Biggart and Costa, 1986), it is genotoxic and can neoplastically transform the cultured mammalian cells. Nickel(II) binding to DNA and nuclear proteins may cause promutagenic oxidative damages (Kasprzak, 1995) that include DNA base modification, DNA single-strand breaks, cross-linking of DNA-protein, rearrangements and depurinations in a wide variety of in vitro cell culture, and whole animal experimental systems.

In addition to the genotoxic potential, epigenetic effects of nickel(II) may also contribute to carcinogenicity of this metal (Kasprzak, 1995; Oller *et al.*, 1997). Nickel (II) is a redox active metal and indirect damage due to generation of reactive oxygen species (ROS) is probably important in nickel(II) carcinogenicity (Kasprzak, 1995). Furthermore, nickel(II) impairs cellular defense through inhibition of catalase and glutathione peroxidase (Rodriguez and Kasprzak, 1992). The formation of ROS in cells may lead to oxidative stress and related activation of the oxidative damage-inducible gene, which may account for the ability(II) to act as a carcinogen. Nickel has been extensively studied with respect of gene induction. These include inactivation of senescence genes (Klein and Costa, 1997), and antiangionetic thrombospondin gene (Salnikow *et al.*, 1997), silencing of a telomere marker gene (Broday *et al.*, 1999), induction of the hypoxia-regulated gene cap43 (Zhou *et al.*, 1998), NF-kB (Huang *et al.*, 2002) and HIF-1 transcription factor gene (Salnikow *et al.*, 2003). Nevertheless, changes in gene expression in response to the oxidative stress mediated by nickel(II) are still unclear. So, the identification of mRNAs differentially transcribed, or modified before

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and after nickel(II) treatment is of fundamental importance for the understanding of molecular basis of nickel(II) carcinogenicity.

In this study, we followed our initial observation (Lee *et al.*, 1999) of nickel(II)-induced gene expression in Chinese hamster ovary (CHO) cells with additional study in NRK-52E cells derived from normal rat kidney, which is the main target of nickel(II) toxicity and carcinogenicity.

Materials and Methods

Cell culture and treatments

NRK-52E cell line used in this study was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured at 37°C in humidified atmosphere containing 5% CO_2 in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum, 1 mM glutamine, 100 units of penicillin/ml and 100 ug of streptomycin/ml. At 24 h before the addition of nickel(II) acetate (J.T. Baker, Phillipsburg, NJ, USA), freshly dissolved in the above medium, the cells were seeded in 162 cm^2 culture flasks. Cells cultured in the presence or absence of nickel(II) acetate (160 and 240 uM) for 3 days were used for the differential display analysis.

mRNA differential display

Total RNA was isolated by the acid guanidinium thiocyanate/phanol/chloroform method from NRK-52E cells cultured for 3 days in medium containing 160 uM nickel(II) acetate and those cultured in medium from which nickel(II) acetate had not been added. Possible DNA contamination was removed by treating the RNA with RNase-free DNase I (Boehringer Mannheim, Indianapolis, IN) for 30 min at 37°C. After phenol/chloroform/isoamyl alcohol (25:24:1) extraction and ethanol precipitation, RNA was resuspended in DEPC-treated H_2O. Differential display analysis was carried out as described previously (Lee *et al.*, 1999). Briefly, 400 ng of DNA-free total RNA was reverse-transcribed using $T_{11}VV$ primer where V represents a degenerative mixture of dA, dG and dC. Amplification reactions were performed in 20 ul volume containing 10 mM Tris-HCl, pH 8.3, 10 mM KCl, 5 mM $MgCl_2$, 200 uM of each deoxyribonucleoside triphosphate (dNTP), 0.5 uM of primer and 1 unit of Stoffel fragment (Perkin-Elmer, Norwalk, CT). Each PCR amplification was carried out on a thermal cycler (Perkin-Elmer DNA Thermal Cycler 480) for a total of 40 cycles at 94°C for 30 sec, at 40C for 60 sec, and at 72°C for 60 sec, followed by 7 min post-extention at 72 °C. PCR amplification products were analyzed by electro-phoresis in nondenaturing 10% polyacrylamide mini-gels. After silver staining, PCR bands of interest ranging 150 to 600 bp were recovered from gel and reamplified in a 50 ul reaction mixture with identical pair of primers used in mRNA differential display reaction.

Subcloning and DNA sequencing

Amplified cDNA fragments were resolved by gel electrophoresis using 6% polyacrylamide gel. The gel-purified cDNAs were directly inserted into the TA cloning vector of pGEM-T system according to manufacturer's instruction (Promega, Madison, WI, USA). DNA inserts from positive colonies were amplified with T7 and SP6 universal primers and sequenced using an ABI Prism BigDye Terminator Cycle Sequencing kit and an ABI 310 sequencer (Perkin Elmer). DNA sequences were compared to the GenBank databases utilizing the Blast program available to the web site, http://www.ncbi.nlm.nih.gov.

Reverse transcription-polymerase chain reaction

Selected 3 genes of interest showing expression differences in DD-PCR were further examined by RT-PCR. Synthesis of the first-stranded cDNA was performed by use of GeneAmp RNA PCR kit (Perkin Elmer). A 20 ul-reaction mixture, containing 2 ug total RNA, 2.5 U MuLV reverse transcriptase, 2.5 uM oligo $d(T)_{16}$, 1 U RNase inhibitor, 1 mM dNTPs, 5 mM $MgCl_2$, and 10X PCR buffer, was incubated at 37°C for 1 h and then heated to 94°C for 10 min to denature the reverse transcriptase. PCR was performed in 100 ul volume using 20 ul of cDNA mixture plus 2.5 U of AmpliTaq polymerase, 2 mM $MgCl_2$, 10X buffer, 0.2 mM dNTPs, and 0.15 uM of each primer under following conditions: 94C for 1 min; followed by 30 cycles of 94°C for 30 sec, 55°C for 40 sec, and 72°C for 40 sec; followed by 7 min at 72°C. The following 3' and 5' primers of FHIT, metallothionein, and SH3BGRL3 were used for PCR. Primer sequences were: FHIT, sense 5-CCATGTCATTTAGATTCGGC-3 and antisense 5-CATGCCTGAAAGTAGGCCCT-3; metallothionein, sense 5'-GAATGGACCCCAACTGCT C-3' and antisense 5'-GCAGCAGCACTGTTCGT CA-3'; SH3BGRL3, sense

5'-CATCTTCTG GGTCCATAGCG-3 and antisense 5'-GCTTTGCTCCTTCTTCTCTTG-3'. The GAPDH mRNA, amplified in parallel, served as an internal and amplification control. PCR products were electrophoresed through a 1.5% agarose gel in Tris-borate EDTA buffer and stained with ethidium bromide.

Western blotting

Cells were lysed in 5 volumes of RIPA buffer (1X PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 g/ml phenylmethylsulfonyl fluoride) for 30 min on ice. Cell lysates containing 60 ug of protein were denatured by boiling for 5 min in sample buffer and fractionated by electrophoresis on 16% SDS-polyacrylamide gel. The fractionated proteins were electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA). The membrane was incubated with the 1:1,000 dilution of rabbit anti-FHIT antibody or 1:2,000 dilution of goat anti-metallothionein antibody (Santa Cruz Inc., Santa Cruz, CA, USA) for 1 h in Tris-buffered saline (Bio-Rad, Hercules, CA, USA) containing 0.1% bovine serum albumin, followed by the incubation with 1:5,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit secondary antibody or mouse anti-goat secondary antibody (Santa Cruz Inc.). The signal was visualized using an Enhanced Chemiluminescence detection kit (Amersham, Cleveland, OH, USA).

Results

To identify genes which expression was altered in response to nickel(II) acetate, cultured immortalized NRK-52E cells, with feature of epithelial cells derived from normal rat kidney, were exposed to 160 uM nickel acetate. For nonradioactive mRNA differential display analysis, a total arbitrary 10-mer primers were used in combination with all $T_{11}VV$ primers. Utilizing the application of different primer sets (Table 1), seventy-one differentially expressed cDNA fragments with 2 fold change were detected and excised from the gels. To ensure reproducibility for specific bands, duplicate reactions using RNAs prepared from identically treated NRK-52E cells in separate experiment were also performed. These results yielded 15 up-regulated and 17 down-regulated cDNA fragments. Following 32 cDNAs were cloned into the pGEM-T vector, their DNA sequences were obtained. Using the NCBI BLAST homology search program, 19 cDNA fragments were found to show significant homology with the nucleotide sequences in GenBank and EMBL databases (Table 2). Out of these, three cDNAs with interest in particular were finally selected for further evaluation.

Two of them (NT-2 and NT-19) were down-regulated and one (NT-70) was up-regulated by nickel(II) treatment (Fig. 1). Nucleotide sequence analysis showed that NT-2 shared 90% sequence identity with the mouse SH3 binding glutamic acid-rich protein 3 (SH3BGRL3, GenBank accession no. XM_344007), a redox-controlling protein protecting cells against oxidant-induced apoptosis. NT-19 was identified as rat fragile histidine triad (FHIT, GenBank accession no. AF170064), a candidate human tumor suppressor. NT-70 was identified as rat metallothionein

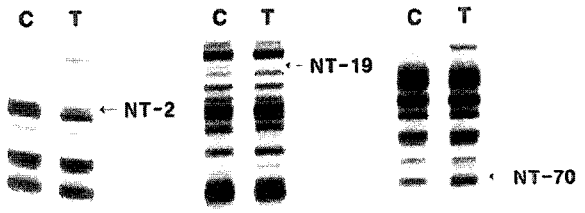

Fig. 1. Three representative differential display analyses using a nonradioactive method and 10% polyacrylamide mini-gels. C: untreated cells; T: nickel(II) acetate-treated cells.

Table 1. Oligonucleotide primers used in mRNA differential display

Downstream primers		Upstream primers	
1, 5'TTTTTTTTTTTAA3'	1, 5'GATCATAGCC3'	10, 5'TACCTAAGCG3'	19, 5'TACAACGAGG3'
2, 5'TTTTTTTTTTTAC3'	2, 5'CTGCTTGATG3'	11, 5'GATCTGACAC3'	20, 5'GATCAAGTCC3'
3, 5'TTTTTTTTTTTAG3'	3, 5'GATCCAGTAC3'	12, 5'GATCTAACCG3'	21, 5'GATCTCAGAC3'
4, 5'TTTTTTTTTTTCA3'	4, 5'GATCGCATTG3'	13, 5'TGGATTGGTC3'	22, 5'GGTACTAAGG3'
5, 5'TTTTTTTTTTTCC3'	5, 5'AAACTCCGTC3'	14, 5'GGAACCAATC3'	23, 5'GATCACGTAC3'
6, 5'TTTTTTTTTTTCG3'	6,5'TGGTAAAGGG3'	15, 5'GATCAATCGC3'	24, 5'CTTTCTACCC3'
7, 5'TTTTTTTTTTTGA3'	7, 5'GATCATGGTC3'	16, 5'TCGGTCATAG3'	
8, 5'TTTTTTTTTTTGC3'	8, 5'TTTTGGCTCC3'	17, 5'GATCTGACTG3'	
9, 5'TTTTTTTTTTTGG3'	9, 5'GTTTTCGCAG3'	18, 5'TCGATACAGG3	

Table 2. Summary of nickel(II)-responsive differential display clones in NRK-52E cells

cDNA fragments	Primers used in PCR	Effect of Ni(II)	Size of PCR products (bp)	GenBank search
NT-2	D1U13	Down	210	SH3 glutamic acid-rich protein
NT-6	D1U22	Up	164	RNA binding motif protein 5
NT-11	D2U8	Up	250	Sprouty 2
NT-14	D2U18	Down	240	Integrin alpha 6
NT-15	D2U19	Up	200	Thrombospondin 1
NT-19	D3U1	Down	290	Fragile histidine triad
NT-23	D3U4	Down	260	Heat shock protein 75
NT-28	D3U18	Up	240	Mitochondrial elongation factor G1
NT-35	D4U11	Up	190	Metalloprotease
NT-41	D4U19	Up	250	Rattus norvegius CDK 109
NT-48	D5U9	Down	370	CD68 protein
NT-50	D5U10	Up	210	Splicing factor 3b, subunit 1
NT-55	D5U13	Up	210	Mitochondrial ribosomal protein S14
NT-56	D5U19	Up	440	NADH oxidoreductase
NT-59	D6U11	Up	360	H ferritin
NT-62	D6U17	Down	180	Parathyroid hormone
NT-66	D7U22	Down	190	Ribosomal protein LB
NT-67	D9U17	Up	150	Nucleolar phosphoprotein p130
NT-70	D9U20	Up	180	Metallothionein

(GenBank accession no. BC058442), a metal-binding protein protecting cells against oxidative stress.

To assess the specificity of differential expression of mRNAs, we performed studies using 160 and 240 uM nickel(II) acetate and collected sample of cells exposed to the nickel(II) for 3 days. RT-PCR analysis indicated that SH3BGRL3 and FHIT expression were remained down-regulated with 160 and 240 uM nickel(II) acetate (Fig. 2). Also, down-regulation of FHIT protein was consistent with the results for mRNA. The metallothionein mRNA was up-regulated by nickel(II) acetate treatment. The increase of metallothionein protein level correlated with the concentration of nickel(II) acetate (Fig. 3).

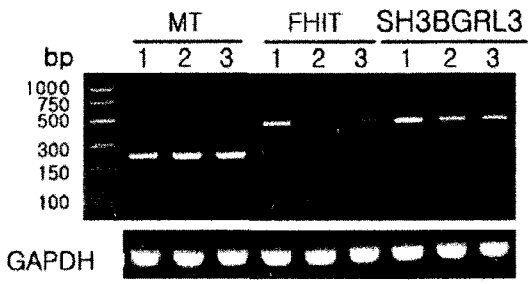

Fig. 2. Reverse transcription-polymerase chain reaction (RT-PCR) for metallothionein (MT), FHIT, and SH3BGRL3 genes in untreated and nickel(II) acetate-treated NRK-52E cells for 3 days. 1: untreated cells; 2: 160 uM nickel(II)-treated cells; 240 uM nickel(II)-treated cells.

Discussion

The molecular mechanisms of nickel(II) toxicity and carcinogenicity have been the focus of numerous studies, since the identification of their specific markers will shed light on early detection, treatment, and prevention of the disease. In search of new molecular pathways toward understanding the mechanism(s) of nickel(II)-induced carcinogenesis, we compared the mRNA expression patterns of 160 uM nickel(II)-treated NRK-52E cells with those of untreated cells by a nonradioactive mRNA differential display analysis. In

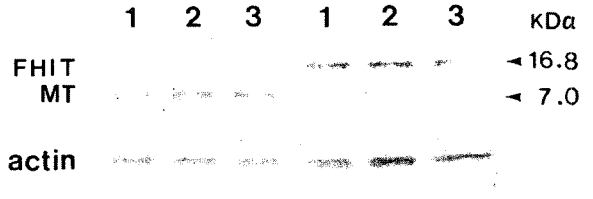

Fig. 3. Immunoblotting analyses of metallothionein (MT) and FHIT proteins in untreated and nickel(II) acetate-treated NRK-52E cells for 3 days. 1: untreated cells; 2: 160 uM nickel(II)-treated cells; 240 uM nickel(II)-treated cells.

present study, we detected several genes whose mRNA levels were altered under nickel(II) acetate treatment for 3 days. Of these, up-regulation of metallothionein and down-regulation of both SH3BGRL3 and FHIT mRNA were of interest because they might be expected to be involved in nickel(II) toxicity or carcinogenicity.

SH3BGRL3 has been predicted to belong to thioredoxin-like protein superfamily (Mazzocco *et al.*, 2001). The proteins of this family, including thioredoxin, glutaredoxin and NrdH-redoxin, are small disulfide reducing enzyme, which act as hydrogen donor and are thought to be involved in regenerating glutathionylated proteins. The control of redox-dependent processes is also important for the regulation of basic cellular functions as proliferation, apoptosis and senescence. Overexpression of human thioredoxin has been shown to play important roles in protection against oxidant-induced apoptosis (Tsutsui *et al.*, 2003). Conversely, down-regulation of thioredoxin could promote it. In our previous studies, we observed cell cycle arrest and apoptosis after nickel(II) acetate treatment of CHO cells and murine T cell hybridoma cells (Lee *et al.*, 1998; Shiao *et al.*, 1998; Kim *et al.*, 2002). These studies, in conjunction with the present finding of SH3BGRL3 down-regulation, indicate that a molecular pathway mediated by the SH3BGRL3 may be linked to the apoptotic death of NRK-52E cells induced by nickel(II) acetate.

Metallothionein has been proposed to be involved in the regulation of the metabolism of physiologically important trace metals, such as copper and zinc, detoxification of toxic metals and free radical scavenging (Sato and Kondoh, 2002). Metallothionein is highly inducible and accumulates intracellularly in response to a variety of stimulants, such as heavy metals. The kidney has long been recognized as a critical target organ for nickel(II) toxicity because nickel(II) enters the blood through pulmonary and enteral pathways and rapidly clears from the circulation, depositing mainly kidney. In concordance with the known effect of nickel(II) and other metals in metallothionein expression (Bauman *et al.*, 1993), nickel(II)-induced up-regulation of metallothionein in our study appears to be mediated by oxidative stress and involved in protection against nickel(II) nephrotoxicity. Indeed, up-regulation of metallothionein has been reported to be closely related to oxidative stress (Reus *et al.*, 2003), a key cellular effect of nickel(II) acetate treatment.

Our studies clearly demonstrate that nickel(II) decreases simultaneously FHIT, a candidate tumor suppressor, at both mRNA and protein level, suggesting that the protein level reflects the suppression of FHIT mRNA induced by nickel(II) treatment. This protein of uncertain function may be of particular interest, since it is down-regulated in several types of primary tumors and cell lines, including kidney cancer (Sukosd *et al.*, 2003; Simon *et al.*, 1998; Lee *et al.*, 2001). Reduced expression of FHIT gene has been reported to relate to oxidative damage in Merkel and skin squamous cell carcinomas (Popp *et al.*, 2002). In addition, Kowara *et al* have reported that nickel(II) inhibits the enzymatic activity of FHIT protein (Kowara *et al.*, 2002). The concordance of down-regulation of FHIT gene products in the current study and previous findings of altered expression of FHIT gene in various carcinomas implies that this protein may be involved in nickel(II)-mediated carcinogenicity. Further studies are needed to clarify the mechanism of action of FHIT and cellular physiological consequences of altered FHIT expression in nickel(II)-treated cells. We are currently investigating the activities of this gene in chronic exposure with low doses of nickel(II), in correlation with the process of transformation.

In summary, our data reveal differential expression of three nickel(II) toxicity-related genes, metallothionein, FHIT, and SH3BGRL3. Although dysregulation of the identified genes indicates their involvement in the cellular response to nickel(II), their role in nickel(II) carcinogenicity needs further study.

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