Original Article

Methylation of *RAR-β2*, *RASSF1A*, and *CDKN2A* Genes Induced by Nickel Subsulfide and Nickel-carcinogenesis in Rats*

ZHANG Jun[1,#,+], ZHANG Jing[+,2], LI MengJie[2], Wu YouJun[2], FAN YingYing[1], ZHOU Yang[1], TAN LiLi[2], SHAO ZhiHua[1], and SHI HongJun[1]

1.Department of Regenerative Medicine, Tongji University School of Medicine, Shanghai 200092, China;
2.School of Life Science and Technology, Tongji University, Shanghai 200092, China

Abstract

Objective To investigate the expression variation of *RAR-β2*, *RASSF1A*, and *CDKN2A* gene in the process of nickel-induced carcinogenesis.

Methods Nickel subsulfide (Ni_3S_2) at dose of 10 mg was given to Wistar rats by intramuscular injection. The mRNA expression of the three genes in induced tumors and their lung metastasis were examined by Real-time PCR. The methylation status of the 5' region of these genes were detected by Quantitative Real-time methylation specific PCR.

Results The mRNA expressions of the three genes both in muscle and lung tumor were decreased distinctly in comparison with normal tissue. But hypermethylation was found only in muscle tumor.

Conclusion These findings suggest that loss of function or decrease of *RAR-β2*, *RASSF1A,* and *CDKN2A*, as well as the hypermethylation of 5' region of these genes, are related with nickel exposure.

Key words: Nickel; DNA methylation; *RAR-β2*; *RASSF1A*; *CDKN2A*; Quantitative Real-time Methylation Specific PCR

Biomed Environ Sci, 2011; 24(2): 163-171 doi: 10.3967/0895-3988.2011.02.011 ISSN:0895-3988

www.besjournal.com (full text) CN: 11-2816/Q

INTRODUCTION

As a natural metallic element in earth's crust, nickel is widely distributed and is industrially applied in many forms. As an important material in a number of processes of modern industry, such as electroplating, welding, and alloy production, nickel compounds may be released into the environment with relatively high amounts at all stages of production, recycling, and disposal[1-2]. The chronic exposure to nickel compounds can lead to asthma, inflammation, lung fibrosis, and kidney disease, but the most serious concerns are related to the carcinogenic activity of nickel. Epidemiological studies have clearly implicated nickel compounds as human carcinogens based upon a higher incidence of lung and nasal cancer among nickel mining, smelting and refinery workers. In various kinds of animal models, nickel compounds induce tumors at virtually any site of administration. Additionally, insoluble nickel compounds like nickel subsulfide efficiently transform rodent and human cells *in vitro*[3]. Based on these observations, the International Agency for Research on Cancer (IARC) evaluated the carcinogenicity of nickel in 1990[4]. And it is also one of the top 50 substances on the 1997 ATSDR priority list (ATSDR 2001)[5]. Although the molecular mechanisms for which nickel compounds cause cancer are still under intense investigation, it is noteworthy that the carcinogenic actions of nickel compounds are thought to involve oxidative stress,

*This research was supported by the Natural Science Foundation of China (30570690 and 81041069) and the Natural Science Foundation of Shanghai (08ZR1420700).

#Correspondence should be addressed to ZHANG Jun. Tel: 86-21-65988785. Fax: 86-21-65988785. E-mail: junzhang@tongji.edu.cn

+These authors contributed equally to this work.

Biographical note of the first authors: ZHANG Jun, male, born in 1965, associate professor, PhD, majoring in Medical Molecular Genetics. ZHANG Jing, female, born in 1968, associate professor, MD, majoring in Environmental toxicology.

Received: July 22, 2010; Accepted: January 4, 2011

genomic DNA damage, epigenetic effects, and the regulation of gene expression by activation of certain transcription factors related to corresponding signal transduction pathways[2]. There is strong evidence that the mechanisms of many toxic effects of nickel are associated with the production of oxidative DNA damage and the inhibition of DNA repair activity. Additionally, nickel can activate sensitive transcription factors or hypoxic signaling pathways by inducing oxidative stress. It is noteworthy that epigenetic changes exerted by nickel compounds such as DNA methylation and histone deacetylation would lead to the inherited inactivation of the tumor suppressor genes and additionally contribute to the carcinogenic mechanism[3].

The low mutagenic and high carcinogenic activity of nickel compounds suggests that epigenetic events might play an important role in nickel carcinogenesis. Alterations of CpG island (DNA hypermethylation) and chromatin modifications have been widely documented in human cancers[6-7]. The hypermethylation of the promoter of the tumor suppressor gene (TSGs), *P16*, was observed in all tumors[8]. Changes in DNA methylation leading to the inactivation of gene expression following the exposure to nickel compounds were initially found using the transgenic *E. coli GPT* gene in Chinese hamster G12 cells as a model[9].

Although these studies *in vitro* display that nickel could induce some TSGs silencing, the detailed mechanism of nickel carcinogenesis needs to be further studied. In consideration of the known mechanisms of nickel carcinogenesis, we hypothesized that the inactivation of hypoxia related genes and tumor suppressor genes which have been found to be frequently hypermethylated in human cancer might play an important role in nickel induced tumor.

To test this hypothesis, we first examined the mRNA level of a set of genes including *RAR-β2*, *RASSF1A*, *CDKN2A*, *DAPK1*, *HIF1-α*, *VEGF-α*, *DICER*, *TXN2 DNMT1*, and *MGMT* in primary muscle tumors of nickel-injected rats. Interestingly, despite robust expression in normal tissues, we found that *RAR-β2*, *RASSF1A*, and *CDKN2A* expression was down-regulated significantly in tumor tissue. In order to determine the potential cause of the silencing of these three genes, we used a new highly specific, sensitive, and quantitative method, methylation specific quantitative PCR (MSQP)[10] to detect the methylation status of the 5' region of the three TSGs in primary and lung metastasis tumors of nickel-injected rats.

MATERIALS AND METHODS

Animals

A total of 40 male Wistar rats were used in this study, of which 30 rats in experimental group received intramuscular injection of 10 mg of Ni_3S_2 (Sigma-Aldrich, U.S.A.) suspended in 0.2 mL of Chloromycetin solution at 10 weeks of age and the rest 10 rats in the control groups were given Chloromycetin solution only, as a solvent, in the right hind leg (the triceps surae muscle). The rats were then observed for an additional 32 weeks[11]. A necropsy was performed on all tumor-bearing or moribund and deceased animals, or rats at terminal sacrifice. The injection site muscle tissues and lung tissues were excised and a portion of the samples were fixed in 10% neutral buffered formalin for 48 h, embedded in paraffin, sectioned at 5 nm and stained with hematoxylin and eosin for histological analysis. The remaining tissues were stored properly for subsequent analyses. At the termination of experiment, muscle and lung samples of normal controls, muscle tumor and lung metastasis tumor samples of experimental groups were collected and used to assess the expressions of genes and the methylation status of the 5' region of *RAR-β2*, *RASSF1A,* and *CDKN2A*.

Isolation of RNA and Real-time PCR Quantification

Total RNA from tissue was extracted by Trizol (Invitrogen, U.S.A). cDNA was generated from 1 μg total RNA per sample by RNA reverse transcription kit (Fermentas, Canada), following manufacturer's protocol. The primer sequences and PCR product sizes are showed in Table 1. The relative $2^{(\triangle\triangle CT)}$ quantification approach was used[12]. β-actin was used as an internal reference gene and PCR cycling and analysis were performed on the Rotor-Gene 3 000 TM (Corbett Research, Mortlake, Australia), 100 ng of cDNA in a total volume of 25 μL. The reactions were heated at 94 °C for 10 s, followed by 35 cycles of 5 s at 94 °C, 20 s at 60 °C, 15 s at 72 °C.

Sodium Bisulfite Conversion

Genomic DNA was prepared from tissue and blood using Wizard® Genomic DNA Purification Kit (Promega, U.S.A), according to manufacturer's instructions. Genomic DNA (1 μg) was bisulfite

modified by the CpGenome™ DNA Modification Kit (Chemicon® International, U.S.A), following manufacturer's protocol. Modified DNA was purified, and then re-suspended in 80 µL TE buffer (pH 8).

Quantitative Real-time Methylation Specific PCR

After sodium bisulfite conversion, methylation-specific quantitative PCR was performed to analyze methylation status of the three genes (*RAR-β2*, *RASSF1A* and *CDKN2A*) using the EASY Dilution (for Real Time PCR) SYBR green PCR Kit (TaKaRa, China). For each gene, two sets of primers, designed specifically according to the CpG island prediction (Figure 1) for bisulfite converted DNA, were used: a methylated set and unmethylated set (i.e. *RAR-β2*-M, *RAR-β2*-U). The primer sequences and PCR product sizes are showed in Table 2. PCR cycling and analysis were performed on the Rotor-Gene 3000TM (Corbett Research, Mortlake, Australia). 20 ng of bisulfite modified DNA in a total volume of 25 µL. The reactions were heated at 94 °C for 10 s, followed by 40 cycles of 5 s at 94 °C, 20 s at 57 °C, 15 s at 72 °C. The PCR products were subcloned and then sequenced.

DNA that was completely methylated by SssI methylase (New England Biolab, Beverly, MA) was mixed with that of samples where no methylation was detected (from a healthy donor) with ratios (0%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%) to represent the linear relationship between the percentage of methylated DNA and the threshold cycle (CT). Then, CT of each tumor and normal samples were measured and substituted into the liner relationship obtained above which can give a semi-quantitative idea of the amount of methylated-unmethylated DNA.

Statistical Analysis

Statistical significance of data presented as mean±SE was analyzed by *t*-test.

Table 1. Real-time PCR Primer Sequences

Target Gene	Primer Sequence	Product Size(bp)	Target Gene	Primer Sequence	Product Size (bp)
β-actin	F 5'-gccactgccgcatcctctt-3'	91	*RASSF1A*	F 5'-gagacacctgatctttccca-3'	161
	R 5'-ggcatcggaaccgctca-3'			R 5'-ctggaaggcactgaaaca-3'	
RAR-β2	F 5'- tggccttaccctaaatcgaac-3'	198	*CDKN2A*	R 5'-tagagcggggacatca-3'	134
	R 5'-ggctcttggagcttgtctac-3'			F 5'-gggttggcctcgaagt-3'	

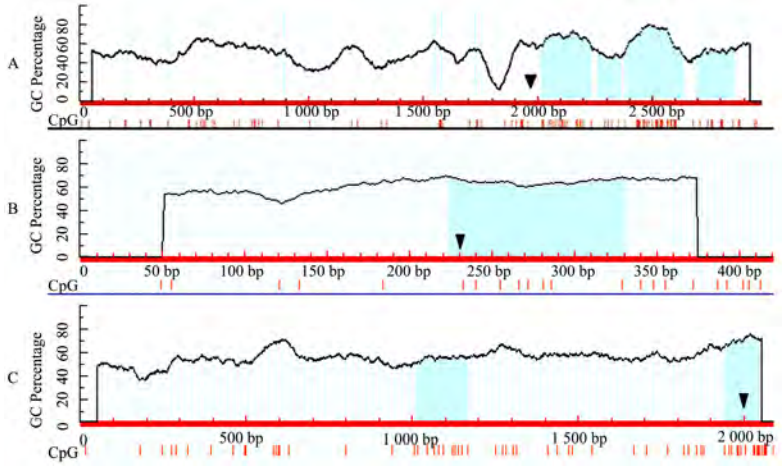


Figure 1. The Distribution of CpG Island in the Promoter Region. (A) There are four CpG islands from transcription initiation site downstream to the first exon of *RAR-β2*. Island 1 (49-226, 178 bp); Island 2 (262-361, 100 bp); Island 3 (383-640, 258 bp) and Island 4 (694-857, 164 bp). Island 4 selected for QRMSP. (B) There is only one CpG island in the rage from 2000 bp upstream of transcription initiation site to the first exon of *CDKN2A* gene (-30-78, 108 bp). (C) Island 1 (-1087--933, 155 bp), island 2 (-159-211, 371 bp) of *RASSF1A* gene. Island 2 analyzed by QRMSP. ▼Transcriptional start site.

Table 2. Quantitative Real-time Methylation Specific PCR Primers Sequences

Target Gene	Primer Sequence	Product Size (bp)
RAR-β2- M	F 5′- ggttgggaaaaagattaatagtttac -3′	222
	R 5′- ctcctacaacatacaaaaaaacgaa -3′	
RAR-β2- U	F 5′- tgggaaaaagattaatagtttatgt -3′	219
	R 5′- ctcctacaacatacaaaaaaacaaa -3′	
RASSF1A-M	F 5′- gcggagttagaatttattgaattac -3′	164
	R 5′- acaaactaaaaacgataaccacgac -3′	
RASSF1A-U	F 5′- tggagttagaatttattgaattatga -3′	163
	R 5′- acaaactaaaaacaataaccacaac -3′	
CDKN2A-M	F 5′-tgtagatagattagttagggtagcgg-3′	102
	R 5′-gaccgaaaatattcgaaacgtt-3′	
CDKN2A-U	F 5′-tgtagatagattagttagggtagtgg-3′	104
	R 5′-tacaaccaaaaatattcaaaacatt-3′	

RESULT

Pathological Examination

A complete necropsy was performed on 3 moribund animals from the experiment group and 37 at terminal sacrifice. None of the rats from the control group had tumor formation at injection site or in lung metastases. In the experiment group, 17 tumor-bearing animals, with tumors formed at the injection sites, and 8 of the 17, including the 3 moribund rats mentioned above, were also inspected for tumor formation in lungs. But the rest 13 rats were examined for tumor formation neither at injection sites nor in lung metasistases.

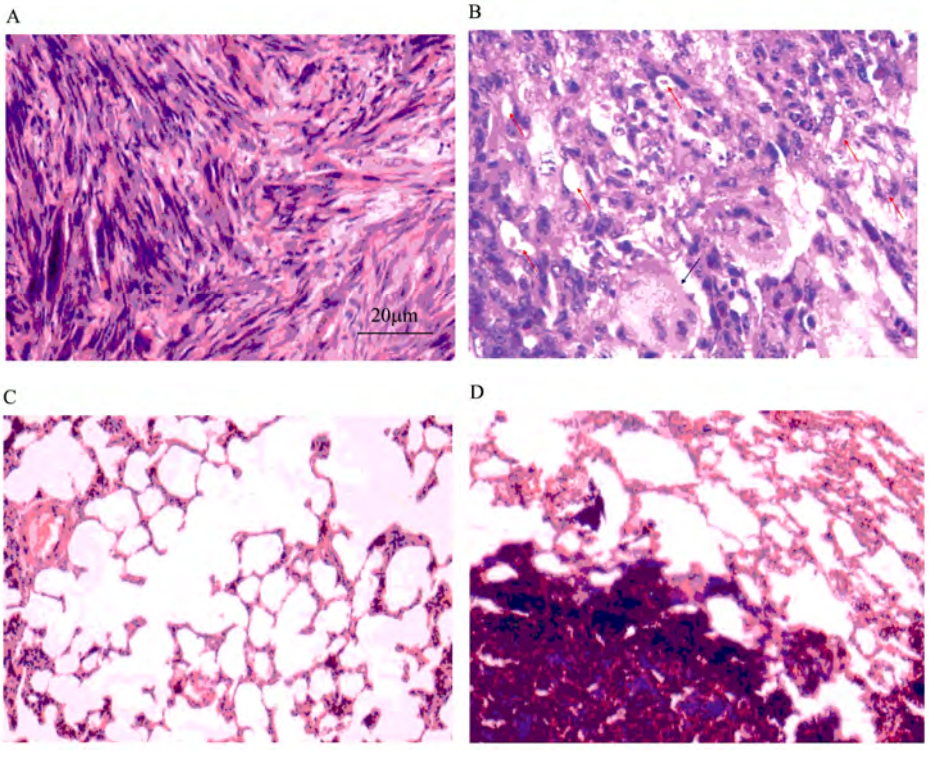


Figure 2. The Histological Section of the Normal or Tumor Muscle and Lung Tissues. Paraffin embedded tissue sections were counter stained with hematoxylin. The photomicrograph shows (A) Normal striated muscle tissue in control group; (B) Primary rhabdomyosarcoma from the triceps surae muscle which was injected by nickel compounds (Ni_3S_2); (C) Normal lung tissue in control group; (D) Rhabdomyosarcoma lung metastases from the striated muscle of the rat which was injected by nickel compounds (Ni_3S_2) (A- D, original magnific ation ×200).

Histopathological examination revealed that the majority of tumors after injections were rhabdomyosarcomas. It showed that the muscle tumors were primary rhabdomyosarcomas and the lung lesions were the rhabdomyosarcoma metastases (Figure 2). Figure 2(A) showed the normal striated muscle cells. Rhabdomyosarcoma cells, giant cells (blue arrow) and more intensive nutrition blood vessels (red arrow) were found in Figure 2(B). Figure 2C showed the normal lung tissue, while the metastasis tumor cells could be found in Figure 2(D) (blue arrow). They were disorder and honeycomb-like with a pathological karyokinesis.

Expression Studies of RAR-β2, RASSF1A, and CDKN2A

In order to determine whether CpG methylation caused down-regulation of *RAR-β2*, *RASSF1A,* and *CDKN2A* expression, mRNA was evaluated by Real-time PCR quantification (the relative $^{2(\triangle\triangle CT)}$ quantification)[13]. The mRNA expressions of these genes were down regulated in muscle and lung tumors corresponding to the morphologically normal tissues (Figure 3). We observed that the expression of *RAR-β2* was 0.108±0.093 and 0.23±0.15 fold in muscle tumor and lung tumor respectively compared with their corresponding normal tissues (n=8, P<0.01). Similarly, compared with expression levels in normal tissues, *RASSF1A* were also down-regulated (0.483±0.318 in muscle tumor and 0.23±0.08 fold in lung tumor, respectively). As to *CDKN2A*, it was expressed 0.073±0.108 and 0.38±0.2 fold in muscle tumor and lung tumor respectively than in their corresponding normal samples.

The differential express of the three genes were detected by Real-time PCR. The graph shows a significantly higher expression of the three genes in tumor samples than in corresponding normal samples (P< 0.01).

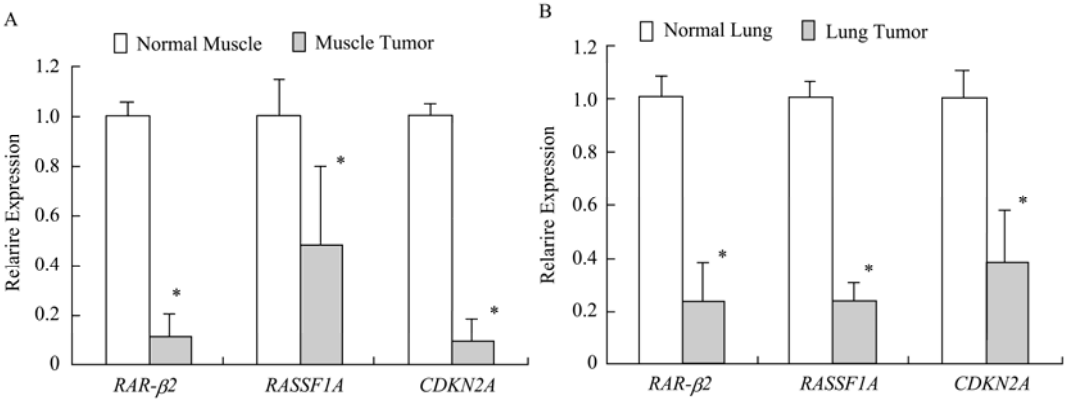


Figure 3. mRNA Expression of *RAR-β2*, *RASSF1A,* and *CDKN2A* in muscle or lung tumor and normal tissues.

CpG Island DNA Methylation Analysis

To find out whether the promoter hypermethylation is the main cause of *RAR-β2*, *RASSF1A*, and *CDKN2A* repression in cancer caused by nickel compounds, the normal and tumor samples were subjected to the Quantitative Real-time Methylation Specific PCR .This assay allows detection of methylated DNA after the modification of DNA by sodium bisulfite[10]. We measured the CT of each tumor and normal samples and substituted those values into the graphs obtained with the percentages above, and got a semiquantitative idea of the amount of methylated-unmethylated DNA. From the Figure 4, *RAR-β2*, *RASSF1A,* and *CDKN2A* showed a higher methylation status in muscle tumors (83.27%±9.29%, 73.63%±10.88%, and 66.75%±8.5%, respectively) than in normal samples (49.27%±8.37%, 40.41%±8.84%, and 27.26%±4.72%, respectively, n=8, P<0.01). But there are no significant differences in lung samples. All the three genes had the similar methylation status between normal lung tissues and lung metastases tumors (Figure 5). In normal lung tissues, the methylated DNA of *RAR-β2*, *RASSF1A*, and *CDKN2A* was 47.78%±6.97%, 77.2%±11.24%, and 46.17%±6.97% respectively, which was 53.56%±15.68%, 77.8%±14.47%, and 50.56%±8.72% in corresponding meta stases tumors. The same result was obtained by MSP assay either.

The percentages of average methylated DNA present of the three genes were detected by Quantitative Real-time Methylation Specific PCR. The

graph shows a highly % methylated DNA in muscle tumor (P<0.01) but not a significant difference in lung tumor.

To verify these results, we performed a MSP assay[14] and bisulfite sequencing for analysis of DNA methylation (*RAR-β2*) which were consistent to the QMSP (Figures 5 and 6).

These findings suggest that methylation of the 5' region of *RAR*-β*2*, *RASSF1A*, and *CDKN2A* gene by nickel-induced may contribute to gene silencing and that methylation of this region may be an important event in primary tumorigenesis. And other factors or pathways may exist leading to the silence of these three genes expression in tumor metastasis process.

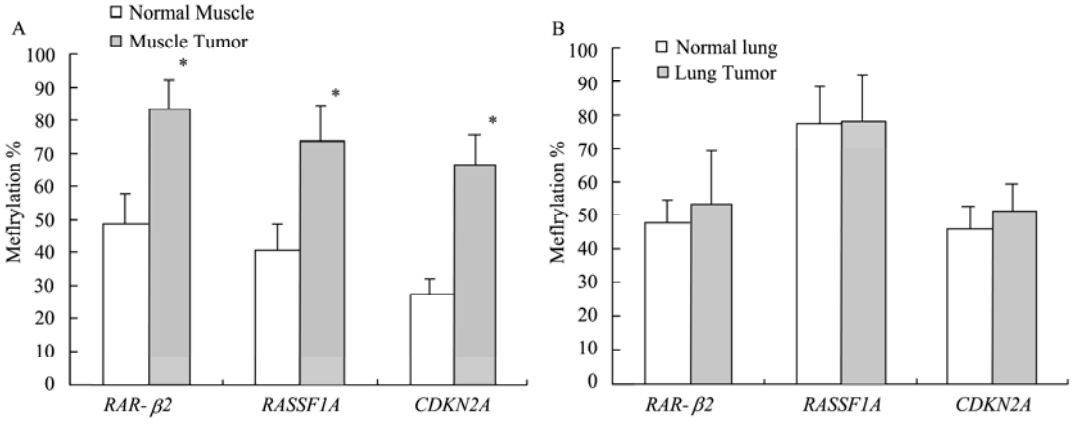


Figure 4. Percentages of Average Methylated DNA Present of *RAR-β2*, *RASSF1A,* and *CDKN2A* Genes in Samples.

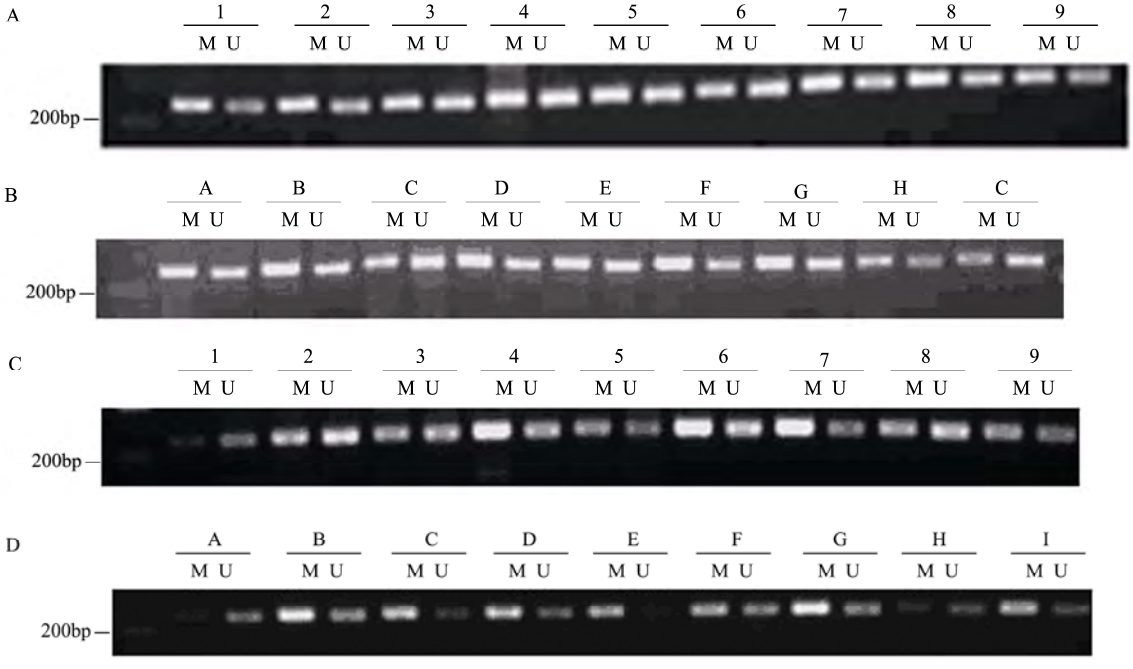
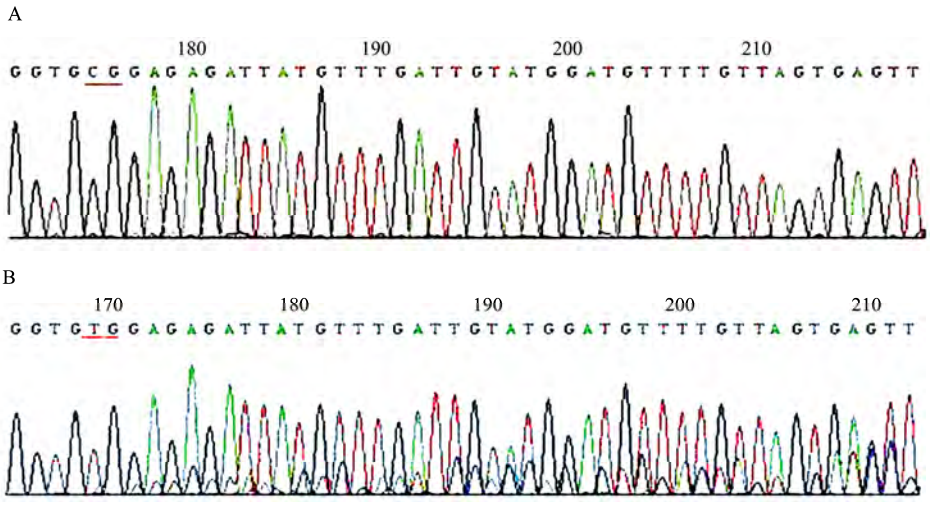


Figure 5. Hypermethylation Analysis by Methylation Special PCR, MSP. (A)-(D) MSP results of *RAR-β2* in normal tissue of lung, tumor of lung, normal tissue of muscle and tumor of muscle, respectively; U: PCR with primers specific for the unmethylated sequence; M: PCR with primers specific for the methylated sequence.1-9: nine parallel samples of normal tissues from nine animals respectively; A-I: nine parallel samples of tumor from nine animals respectively.



Figue 6. Bisulfite Genomic Sequencing Analyse. The PCR products were subcloned and then sequenced. (A)the sequence for the methylated product. (B) the sequence for the unmethylated product.

DISCUSSION

Nickel compounds have been confirmed as human carcinogen with the growing evidence from epidemiological studies and laboratory investigation in the last several decades. Many experiments *in vivo* and *in vitro* have confirmed that gene silencing is an important event in nickel-induced carcinogenesis. The loss of expression of tumor suppressor and senescence genes is a critical event in chemical carcinogenesis[15]. We have found in our study that the hypermethylation of *RAR-β2*, *RASSF1A*, and *CDKN2A* promoter may be related to nickel exposure.

Promoter hypermethylated mediated gene silencing has been reported for several genes in cancers. These genes play an important role in regulation of DNA repair, cell cycle, apoptosis, cell-cell adhesion, metastasis, tissue and organ architecture and various signaling pathways[16].

RAR-β2, *RASSF1A*, and *CDKN2A* are all known to be widely hypermethylated in various human cancers[17].

RAR-β is a potent tumor suppressor gene, and is also a member of the nuclear receptor superfamily with the main function to convey retinoid signaling into target gene transcription in the nucleus. Loss of expression of *RAR-β*, primarily mediated by promoter methylation induced gene silencing has been reported in breast, cervical, lung, thyroid, and other carcinomas[18-22].

RASSF1A is one of the most frequently inactivated proteins ever identified in human cancer which lacks apparent enzymatic activity but contains a Ras association (RA) domain and is potentially an effecter of the RAS oncoprotein. RASSF1A modulates multiple apoptotic and cell cycle checkpoint pathways. Because the gene remains intact but dormant in most tumors, reactivation by promoter demethylation would present a novel approach to therapy[22].

The tumor suppressor protein CDKN2A regulates the G1 to S phase transition of the cerll cycle through its inhibition of the interaction between CDK 4/6 and CYCLIN D1. Other studies have detected that the inactivation of the *CDKN2A* gene creates a hyperproliferative process as the result of loss of cell cycle control[23].

It was shown in our study that *RAR-β2*, *RASSF1A*, and *CDKN2A* were repressed in both primary and metastases cancer tissues examined. The expression rates of the three genes were much lower in tumors compared with the normal samples in muscle and lung, respectively. However, an interesting phenomenon we have found is that the repression was associated with promoter hypermethylation only in muscle primary tumors but lung metastases tumors. These strongly suggest that promoter methylation be the epigenetic cause of *RAR-β2*, *RASSF1A*, and *CDKN2A* repression in the early stage of nickel exposure, rather than in the late period. It is also reported by D J Smiraglia that in head and neck squamous cell carcinoma, many loci methylated in a patient's primary tumor are no longer methylated in the metastatic tumor of the same patient[24]. We infer that DNA methylation might be an early event in nickel–induced carcinogenesis.

As we speculate, other factors or pathways

might lead to the silence of the three genes' expression in both primary and metastasis tumor.

DNA methylation and histone modifications are dynamically linked in the epigenetic control of gene expression and it play an important role in tumorigenesis[25]. Observations of others have shown that the decrease of *RAR-β2* is more often because of the methylation of affecting the *RAR-β* P2 promoter of one or more *RAR-β* alleles[26-28]. However, in the presence of RA, a normal *RAR-β* is activated first by RARα/RXR heterodimers and cofactors and subsequently by RARβ2/RXR heterodimers via dynamic histone acetylation[29]. Moreover, Sirchia et al. showed that by inducing an appropriate level of *RAR-β* P2 acetylation they could restore *RAR-β2* transcription from both unmethylated and methylated *RAR-β* P2 promoters in *RAR-β2*-negative carcinoma cells of breast[30].

As the study of Lan et al.[31] has shown, *CDKN2A* can be regulated indirectly by has-let-7, which can directly regulate C-MYC. let-7 is underexpressed in various cancers, and its restoration can limit cancer growth by targeting oncogenes such as *C-MYC*, multiple cell-cycle- and proliferation-associated genes such as *CDK6*. It could be inferred that the down-regulation of let-7 in cancers can trigger the lower expression of *CDKN2A* in metastatic tumors. Meanwhile, hypoxic signaling pathways activated by nickel may also be concerned with the repression of this gene. Nickel is a potent inducer of hypoxia inducible factor-1 (HIF-1) activity, which may stimulate the cell proliferation[3]. And the weak genotoxic effects of nickel that primarily originate from oxidative stress may be amplified by both epigenetic modifications and HIF-1 signaling. Meanwhile, Box and Demetrick reported that *CDKN2A* mRNA expression is reduced under hypoxic condition in WI-38, HMECs, and HTB-30 cells[32].

The elucidation of mechanisms of nickel-induced carcinogenesis is essential for the risk assessment of nickel compounds to protect human health as well as the design of chemotherapies for nickel induced carcinogenic effects.

REFERENCES

1. Costa M. Mechanisms of nickel genotoxicity and carcinogenicity. In: Chang LW, Magos L, Suzuki T (eds). Toxicology of Metals, vol 1. Lewis Publishers, New York, 1996; pp. 245-51.
2. Lu HT, Shi XL, Costa M, et al. Carcinogenic effect of nickel compounds Nelson Institute of Environmental Medicine. Molecular and Cellular Biochemistry, 2005; 279(1-2), 45-67.
3. Denkhaus E, Salnikow K. Nickel essentiality, toxicity, and carcinogenicity. Crit Rev in Oncol Hematol, 2002; 42(1), 35-56.
4. IARC. Chromium, nickel and welding. Monogr Eval Carcinog Risks Hum, 1990; 49, 1-648.
5. Andrew AS, Warren AJ, Barchowsky A, et al. Genomic and Proteomic Profiling of Responses to Toxic Metals in Human Lung Cells. Environ Health Perspect, 2003; 111(77), 825-38.
6. Jones PA, Baylin SB. The epigenomics of cancer. Cell, 2007; 128(4), 683-92.
7. Laird P W. Cancer epigenetics. Hum Mol Genet, 2005; 14(suppl 1), R65-R76.
8. Salnikow K, Zhitkovich A. Genetic and Epigenetic Mechanisms in Metal Carcinogenesis and Cocarcinogenesis: Nickel, Arsenic, and Chromium. Chem Res Toxicol, 2008; 21(1), 28-44.
9. Lee YW, Klein CB, Kargacin B, et al. Carcinogenic nickel silences gene expression by chromatin condensation and DNA methylation: a new model for epigenetic carcinogens. Mol Cell Biol, 1995; 15(5), 2547-57.
10. Hattermann K, Mehdorn HM, Mentlein R, et al. A methylation-specific and SYBR-green-based quantitative polymerase chain reaction technique for O6-methylguanine DNA methyltransferase promoter methylation analysis. Anal Biochem, 2008; 377(1), 62-71.
11. Ohmori T, Okada K, Terada M, et al. Low susceptibility of specific inbred colonies of rats to nickel tumorigenesis in soft tissue. Cancer Lett, 1999; 136(1), 53-8.
12. Livak K J, Schmittgen T D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods, 2001; 25(4), 402-8.
13. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc, 2008; 3(6), 1101-8.
14. Herman JG, Graff JR, Myohanen S, et al. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA, 1996; 93(18), 9821-6.
15. Costa M, Sutherland JE, Wu P. Molecular biology of nickel carcinogenesis. Molecular and Cellular Biochemistry, 2001; 222(1-2), 205-11.
16. Shukla S, Mirza S, Sharma G. Detection of RASSF1A and RARbeta Hypermethylation in Serum DNA from Breast Cancer Patients. Epigenetics, 2006; 1(2), 88-93.
17. Juhlin CC, Kiss NB, Villablanca A, et al. Frequent Promoter Hypermethylation of the APC and RASSF1A Tumour Suppressors in Parathyroid Tumours. PLoS ONE, 2010; 5(3), e9472.
18. Zhang ZM, Keiichiro Joh, Yatsuki H. Retinoic acid receptor b2 is epigenetically silenced either by DNA methylation or repressive histone modifications at the promoter in cervical cancer cells. Cancer Letters, 2007; 247(2), 318-27.
19. Fackler MJ, McVeigh M, Evron E, et al. DNA methylation of RASSF1A, HIN-1, RAR-beta, Cyclin D2 and Twist in in situ and invasive lobular breast carcinoma. Int J Cancer, 2003; 107(6), 970-5.
20. Virmani AK, Rathi A, Zochbauer-Muller S, et al. Promoter methylation and silencing of the retinoic acid receptor-beta gene in lung carcinomas. J Natl Cancer Inst, 2000; 92(16), 1303-7.
21. Cras A, Darsin-Bettinger D, Balitrand N, et al. Epigenetic patterns of the retinoic acid receptor b2 promoter in retinoic acid-resistant thyroid cancer cells. Oncogene, 2007; 26(27), 4018-24.
22. De-Castro AJ, Gockel-Krzikalla E, Rosl F. Retinoic Acid Receptor β Silences Human Papillomavirus-18 Oncogene Expression by Induction of de Novo Methylationand Heterochromatinization of the Viral Control Region. J Biol Chem, 2007; 282(39), 28520-29.
23. Smiraglia DJ, Smith LT, Lang JC, et al. Differential targets of CpG island hypermethylation in primary and metastatic head and neck squamous cell carcinoma (HNSCC). J Med Genet, 2003; 40(1), 25-33.

24. Donninger H, Vos MD, Clark GJ. The RASSF1A tumor suppressor. J Cell Sci, 2007; 120(Pt 18), 3163-72.
25. Egger G, Liang G, Aparicio A, et al. Epigenetics in human disease and prospects for epigenetic therapy. Nature, 2004; 429(6990),457-63.
26. Bovenzi V, Lê NL, Côte S, et al. DNA methylation of retinoic acid receptor beta in breast cancer and possible therapeutic role of 5-aza-2'-deoxycytidine. Anticancer Drugs, 1999; 10(5), 471-6.
27. Sirchia SM, Ferguson AT, Sironi E, et al. Evidence of epigenetic changes affecting the chromatin state of the retinoic acid receptor beta2 promoter in breast cancer cells. Oncogene, 2000; 9(12), 1556-63.
28. Widschwendter M, Berger J, Hermann M, et al. Methylation and silencing of the retinoic acid receptor-beta2 gene in breast cancer. J Natl Cancer Inst, 2000; 92(10), 826-32.
29. Kruyt FA, van den Brink CE, Defize LH, et al. Transcriptional regulation of retinoic acid receptor beta in retinoic acid-sensitive and -resistant P19 embryocarcinoma cells. Mech Dev, 1991; 33(3), 171-8.
30. Sirchia SM, Ren M, Pili R, et al. Endogenous reactivation of the RARbeta2 tumor suppressor gene epigenetically silenced in breast cancer. Cancer Res, 2002; 62(9), 2455-61.
31. Lan FF, Wang H, Chen YC, et al. Hsa-let-7g Inhibits Proliferation of Hepatocellular carcinoma Cells by Down-regulation of c-Myc and Up-regulation of p16INK4A. In: Int J Cancer, 2010; Available via DIALOG: http://www3.interscience.wiley.com/journal/123326244/abstract?CRETRY=1&SRETRY=0 Accessed 22 Mar 2010.
32. Box AH, Demetrick DJ. Cell cycle kinase inhibitor expression and hypoxia-induced cell cycle arrest in human cancer cell lines. Carcinogenesis, 2004; 25(12), 2325-35.