Comparison of Ki-67 antigen expression and K-ras mutation in lung tumours induced by urethane in mice

Koohdani F, Sasani F, Mohammad K, Mehdipour P

ABSTRACT

Introduction: This study aims to compare Ki-67 antigen expression and K-ras mutation in lung tumours induced by the interfering effects of urethane followed by sodium nitrite, sodium chloride and vitamin D3.

Methods: The samples were classified into six groups: control (C) group; urethane only (U) group; urethane and vitamin D (U+D) group which received 3.5 mg/kg vitamin D3 for four weeks; urethane and sodium nitrite (U+NS) group which was given sodium nitrite (50mg/L); urethane and physiological serum (U+NaCl) group; and sodium nitrite and physiological serum (NS+NaCl) group which was given 50 mg/L sodium nitrite and physiological serum, instead of water. The four carcinogen groups receiving urethane were injected intraperitoneally with 600 mg/kg of urethane three times. After 20 weeks of intervention, the mice were killed; the tissues were removed and examined for histopathological changes and comparison of Ki-67 antigen expression and mutations in the exon 1 of the K-ras gene in lung tumours.

Results: There were significant differences in the Ki-67 index between the C group and the U (p-value is less than 0.006, 95 percent confidence interval [CI] −432.9 to −55.6), U+D (p-value is less than 0.05, 95 percent CI −408.3 to −4.6), U+NS (p-value less than 0.02, 95 percent CI −415.7 to −27.2), U+NaCl (p-value less than 0.002, 95 percent CI −478.8 to −90.3) groups. There was no difference between the C and NS+NaCl groups. There was no mutation in the exon 1 of K-ras gene of the lung tumours.

Conclusion: The expression of Ki-67 antigen was found to be increased by urethane in the present study. However, a study on a larger sample size may show anti-tumourogenic effect of vitamin D3. However, the K-ras exon 1 mutations do not play any role in the interfering effects of urethane followed by sodium nitrite and sodium chloride.

Keywords: carcinogens, K-ras genes, Ki-67 antigen, lung tumour, vitamin D, urethane

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INTRODUCTION

Lung cancer is the most important cause of cancer-related death all over the world. Unfortunately, 75% of patients with lung cancer have symptoms caused by advanced disease that is incurable. Furthermore, despite progresses in therapy, the five-year survival rate for all stages combined is nearly 16%.[1] It was estimated that in 2007, the incidence and mortality of lung cancer in the United States would be more than breast, colon and prostate cancers combined during the same period.[1]

It has been estimated that a correct lifestyle and diet can prevent 30%–40% of all tumours. Factors, such as retinoid, vitamins E, D_3 and C, poliphenols, fibres, calcium, soya, selenium and polyunsaturated fatty acids such as Omega-3, can inhibit cancerogenesis, which is the loss of cellular differentiation that causes cancer. It can be supported by other factors such as proteins, lipids, sodium chloride, aflatoxin, nitrites and nitrates, and some processes such as salting, smoking and broiling.[2] Urethane is a known animal carcinogen and has been classified as reasonably anticipated to be a human carcinogen. Urethane is a fermentation by-product and is found at considerable levels in alcoholic beverages as well as foods such as bread and cheese.[3] Among the many kinetic parameters, Ki-67 protein is an excellent marker for determining proliferating cells in human and animal neoplasm.[4] Ras genes have GTPase activity and oncogenic mutations in ras impair GTP hydrolysis. Point mutations in ras oncogenes are detected in 20%–30% of human primary lung adenocarcinoma. These mutations are found most frequently in codon 12, followed by mutations in codons 13 and 61. 90% of the mutations are found in K-ras in primary lung adenocarcinoma.[5]

In this investigation, we compared Ki-67 antigen

Table I. Description of the intervention received by the six groups.

Group	Intervention
C	No intervention
U	Injected intraperitoneally with 600 mg/kg of urethane ($C_3H_7NO_2$, MW 89, lot 125 H03/8, Sigma Chemical Company, Saint Louis, MO, USA) in 0.9% sodium chloride (NaCl) three times at two-day intervals.
U+D	3.5 mg/kg vitamin D_3 ($C_{27}H_{44}D$, MW 384.65, code no. 500936, Merck KGaA, Darmstadt, Germany) orally in drinking water for four weeks starting simultaneously with the injection of urethane.
U+NS	Sodium nitrite ($NaNO_2$, MW 69 g/mol, code no. 1/06549/0500 from Merck KGaA, Darmstadt, Germany) in drinking water (50 mg/L) during 20 weeks of intervention starting simultaneously with the injection of urethane.
U+NaCl	Physiological serum (instead of drinking water) during 20 weeks of intervention starting simultaneously with the injection of urethane.
NS+NaCl	50 mg/L sodium nitrite added to physiological serum for 20 weeks.

C: control; U: urethane; U+D: urethane & vitamin D; U+NS: urethane & sodium nitrite; U+NaCl: urethane & sodium chloride; NS+NaCl: sodium nitrite & sodium chloride

expression in lung tumours of BALB/c mice, induced by the interfering effects of urethane followed by sodium nitrite, sodium chloride and vitamin D_3. In our previous study, it had been reported that mutation in the exon 1 of the K-ras gene was not one of the main factors for tumours induced by urethane and urethane followed by vitamin D_3 groups.[6] In this study, we assessed whether K-ras mutation in the exon 1 of K-ras gene varied in lung tumours of mice induced by the interfering effects of urethane followed by sodium nitrite and sodium chloride.

METHODS

The survey was performed from 2006 to 2007 at the Unit of Cancer Genetics, Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Iran. This study was conducted only on animals and did not involve human subjects. It was approved by the Animal Ethics Committee at Tehran University of Medical Sciences. A total number of 60 inbred BALB/c mice (30 males and 30 females), with an age range of 9–11 weeks and weighing 12–15 g (female) and 17–20 g (male) at the commencement, were housed in plastic cages with stainless steel wire lids (five mice per cage). The animals were classified into six groups, as listed in Table I, with ten mice in each group and five mice per gender, and matched for age and weight within each group.

The animals received standard food pellets and drinking water *ad libitum*. The consumption of water was measured in each group. After a two-week acclimatisation, oral intake per animal was measured according to the average animal's weight and water consumed per cage. All the surviving mice were killed 20 weeks following intraperitoneal injection of urethane 1% (0.5–1.2 ml). Their lungs were removed, a portion of each lung was fixed in 10% neutral buffered formalin for histopathological diagnosis and the remaining lungs were stored at –70°C for detection of K-ras mutation. The fixed tissues were then embedded in paraffin. The paraffin blocks were sectioned at approximately equal thickness (5 µ) by a Leica RM 2135 microscope (Leica Microsystems, Wetzlar, Germany) and subjected to routine haematoxylin and eosin staining.

The slides were then examined by light microscopy. 4-µm slices of formalin-fixed paraffin-embedded mice tissue, 21 test (tumour) and ten control (without tumour) samples, were prepared on coated glass slides. The Ki-67 monoclonal antibody can be detected on formalin-fixed paraffin-embedded section immunohistochemistry.[4] After deparaffinisation and antigen retrieval in 0.01 mM citrate buffer, we used monoclonal primary antibody Ki-67 (ready-to-use, Novocastra Company, Milton Keynes, UK) for 60 minutes and then they were sequentially treated with secondary antibody (biotinylated link), streptavidine-horseradish peroxidase, and finally diaminobenzidine (DAB-substrate) each for ten minutes. Between the above stages, the smears were rinsed in phosphate-buffered saline. Haematoxylin was used for background staining. Nuclear brown staining with any intensity was considered positive. Published statistical data from the Novocastra Company showed that previous users also achieved a positive result using the above antibody in mouse tissues. Proliferation was quantified under a 40× light objective and was expressed as a proliferative index (PI) score, which was determined as the mean percentage of nuclei staining positive for Ki-67 antibody in 1,000 cells at 400× magnification. 3–4 fields of view for each animal were included in the calculation of PI score. All counts were completed by a blinded investigator.

The fresh tissues were stored at –70°C. Genomic DNA was extracted by phenol-chloroform. It was obtained by proteinase K (20 mg/ml), digestion of samples in a lysis buffer (pH 7.5 EDTA 10 mM, NaCl 100 mM, Tris-HCl 100 mM) and phenol-chloroform extraction. 100 ng of

Fig. 1 Ki-67 immunostaining of one lung tomour viewed in high-power (× 400). The black nuclei are Ki-67 antigen positive. Haematoxylin was used for background staining.

Table II. Incidence of tumour found in the different groups.

Group	No. (%) of animals with normal tissues	No. (%) of animals with tumourous tissues	Total no. of animals examined
C	9 (100)	0	9
U	3 (30)	7 (70)	10
U+D	5 (71.4)	2 (28.4)	7
U+NS	2 (22.2)	7 (77.8)	9
U+NaCl	4 (50)	4 (50)	8
NS+NaCl	8 (88.9)	1 (11.1)	9

C: control; U: urethane; U+D: urethane & vitamin D; U+NS: urethane & sodium nitrite; U+NaCl: urethane & sodium chloride; NS+NaCl: sodium nitrite & sodium chloride

Table III. Comparison of the mean difference in the Ki-67 index between the control and interventional groups.

Group*	Mean difference	Standard error	p-value	95% confidence interval
U	−244.3	52.3	0.005	−432.9 to −55.6
U+D	−206.5	55.9	0.043	−408.3 to −4.6
U+NS	−221.4	53.8	0.018	−415.7 to −27.2
U+NaCl	−284.6	53.8	0.001	−478.8 to −90.3
NS+NaCl	−88.2	63.7	0.855	−318 to 141.6

*compared with the C group
C: control; U: urethane; U+D: urethane & vitamin D; U+NS: urethane & sodium nitrite; U+NaCl: urethane & sodium chloride; NS+NaCl: sodium nitrite & sodium chloride

genomic DNA was mixed in polymerase chain reaction (PCR) buffer (10 mM Tris-Hcl, pH 8.4, 50 mM KCl, and 1.5 mM $MgCl_2$) with 200 mM dNTPs each, 0.2 µM of each 5' and 3' primer and 2.5 U AmpliTaq bp product of K-ras (exon 1), FPK primer 5'-TGATAATCTTGTGTGAGACA-3' and RPK primer 5'-CTCTATCGTAGGGTCGTACT-3' were used. The samples were amplified by PCR for 50 cycles. Each cycle consisted of 30 s denaturation at 95°C, 30 s annealing at 55°C, and 30 s extension at 72°C. The final extension step was performed at 72°C for 7 minutes.[7]

A negative control (1 µL of double-distilled water) was included in each PCR to exclude the possibility of PCR contamination. Amplified products were subjected to electrophoresis in 2% agarose gels and were visualised with ethidium bromide. The PCR products were purified and then sequenced. Sequencing primers were the same as described for PCR reactions. Results of Ki-67 index are expressed as mean ± SD. Statistical significance was determined by one-way analysis of variance followed by the Scheffe's test.[8] The mean difference was significant at the 0.05 level. All the statistical analysis was performed using the Statistical Package for Social Sciences version 13 (SPSS Inc, Chiacgo, IL, USA).

RESULTS

One mouse belonging to the C group died during the study. In the treated groups U+D, U+NS, U+NaCl and NS+NaCl, three, one, two, and one mice died, respectively. No mortality was observed in the U group. There was no proliferative lesion in the dead mice. The results of the incidence of lung tumours are shown in Table II. The mean of Ki-67 positive index was 103.7 ± 72 in the C group as compared to 388.3 ± 88.5 in the U+NaCl group. There were significant differences in the Ki-67 index between the C and U groups ($p < 0.006$, 95% confidence interval [CI] −432.9 to −55.6), U+D ($p < 0.05$, 95% CI −408.3 to −4.6), U+NS (Fig. 1) ($p < 0.02$, 95% CI −415.7 to −27.2), U+NaCl ($p < 0.002$, 95% CI −478.8 to −90.3) groups, but there was no difference between the C and NS+NaCl groups (Table III). In this study, the relationship between other groups as compared with each other (except the C group) has not been observed and will be addressed in another study in the future. There was no mutation in the exon 1 of K-ras gene of the lung tumours.

DISCUSSION

Previous studies demonstrate that Ki-67 is a reliable marker for measurement of proliferative activity[9-11] and can be a prognostic value to determine the biological potential of non-small cell lung cancer.[12] Ki-67 is expressed in all cell cycle phases except G0 and early G1, and expression is strongly associated with the percentage of growth fraction.[9,13-15] We evaluated the expression of the nuclear antigen Ki-67 through immunohistochemical methods to assess the proliferative rates in the different groups. In our study, Ki-67 antigen immunostaining in the lung tumours was significantly increased in all groups that

were injected with urethane and its various combinations as compared with the control group. This study is the first known investigation on the relationship between urethane and Ki-67 antigen expression and the authors suggest further studies are required to confirm this result.

Previous investigations showed the anti-tumourigenic effect of vitamin D_3 in lung tumours induced by carcinogenesis in mice.[6,16] The mechanisms by which 1α,25-dihydroxyvitamin [$(OH)_2$] D_3 inhibits tumour growth were not fully understood. 1α,25$(OH)_2$ D_3 induces a growth arrest in G0/G1 and was shown to modulate the expression of cell cycle-associated genes, including p21, p27, cyclin D and cyclin E in breast and prostate cancer and leukaemia cells.[17-19] The previous *in vitro* study had shown that 1α,25$(OH)_2D_3$ inhibits cell proliferation and induces growth arrest at G0/G1 in Lewis lung carcinoma after an intravenous injection of green fluorescent protein cells as well as breast and prostate cancer cells.[20]

Our results also showed a significant increase of the proliferation index in the U+D group, compared with the C group, and because the Scheffe's test is a conservative test[8] and with due attention given to the CI range in the U+D group (−408.3 to −4.6), this difference does not seem remarkable. Further studies with a larger sample size might show an anti-tumourogenic effect of 1α,25 $(OH)_2D_3$ in lung tumours induced by urethane. We also demonstrated that the K-ras exon 1 mutations did not exist in the tumours induced by the interfering effects of urethane followed by $NaNO_2$ and NaCl. Also in our previous study, it has been reported that mutation in the exon 1 of the K-ras gene was not shown in tumours induced in the urethane and U+D groups.[6]

In human lung adenocarcinoma, activating K-ras mutations predominately occur at codon 12; however, mutations at codon 12 and 61 elicit similar inhibition of GTPase activity to constitutively activate K-ras and downstream signalling.[21] We could not find any mutations in the exon 1 of the K-ras gene; however, urethane can be associated with the K-ras codon 61 mutation.[22-24] We suggest investigating mutations in both exons 1 and 2 of the K-ras gene in future studies.

In conclusion, the Ki-67 antigen expression proved to be increased by urethane. Obviously, the mechanisms underlying the anti-tumour effects of vitamin D are complex. Further investigations with a larger sample size are required in order to obtain a better understanding. However, the K-ras exon 1 mutations do not play any role in the interfering effects of urethane followed by $NaNO_2$ and NACl. Other K-ras mutations or genetic changes are therefore associated with the tumourogenic process.

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REFERENCES

1. Gomez M, Silvestri GA. Lung Cancer Screening. Am J Med Sci 2008; 335:46-50.
2. Divisi D, Di Tommaso S, Salvemini S, Garramone M, Crisci R. Diet and cancer. Acta Biomed 2006; 77:118-23.
3. Hoffler U, Dixon D, Peddada S, Ghanayem BI. Inhibition of urethane-induced genotoxicity and cell proliferation in CYP2E1-null mice. Mutat Res 2005; 572:58-72.
4. Ozaki K, Yamagami T, Nomura K, Narama I. Prognostic significance of surgical margin, Ki-67 and cyclin D1 protein expression in grade II canine cutaneous mast cell tumor. J Vet Med Sci 2007; 69:1117-21.
5. Hisamoto A, Kondo E, Kiura K, et al. Point mutation of K-ras gene in cisplatin-induced lung tumours in A/J mice. Lung Cancer 2007; 58:15-20.
6. Koohdani F, Baghdadchi J, Sasani F, Mohammad K, Mehdipour P. Effects of vitamin D in lung, stomach, esophagus and testis tissues following administration of urethane in balb/c mice. Saudi Med J 2008; 29:340-4.
7. Sithanandam G, Ramakrishna G, Diwan BA, Anderson LM. Selective mutation of K-ras by N-ethylnitrosourea shifts from codon 12 to codon 61 during fetal mouse lung maturation. Oncogene 1998; 17:493-502.
8. Dawson B, Trapp RG. Basic and Clinical Biostatistics. 3rd ed. New York: Lange Medical Books-McGraw-Hill, 2001.
9. Petrowsky H, Sturm I, Graubitz O, et al. Relevance of Ki-67 antigen expression and K-ras mutation in colorectal liver metastases. Eur J Surg Oncol 2001; 27:80-7.
10. Yu CC, Filipe MI. Update on proliferation-associated antibodies applicable to formalin-fixed paraffin-embedded tissue and their clinical applications. Histochem J 1993; 25:843-53.
11. McCormick D, Chong H, Hobbs C, Datta C, Hall PA. Detection of the Ki-67 antigen in fixed and wax-embedded sections with the monoclonal antibody MIB1. Histopathology 1993; 22:355-60.
12. Radović S, Babić M, Dorić M, et al. Non-small cell lung carcinoma: cyclin D1, bcl-2, p53, Ki-67 and HER-2 proteins expression in resected tumors. Bosn J Basic Med Sci 2007; 7:205-11.
13. Brown DC, Gatter KC. Monoclonal antibody Ki-67: its use in histopathology Histopathology 1990; 17:489-503.
14. Zölzer F, Speer A, Pelzer T, Streffer C. Evidence for quiescent S- and G2-phase cells in human colorectal carcinomas: a flow cytometric study with the Ki-67 antibody. Cell Prolif 1995; 28:313-27.
15. Gerdes J, Schwab U, Lemke H, Stein H. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int J Cancer 1983; 31:13-20.
16. Wang QM, Jones JB, Studzinski GP. Cyclin-dependent kinase inhibitor p27 as a mediator of the G1-S phase block induced by 1,25-dihydroxyvitamin D3 in HL60 cells. Cancer Res 1996; 56:264-7.
17. Munker R, Kobayashi T, Elstner E, et al. A new series of vitamin D analogs in highly active for clonal inhibition, differentiation, and induction of WAF1 in myeloid leukemia. Blood 1996; 88:2201-9.
18. Kumagai T, O`Kelly J, Said JW, Koeffler P. Vitamin D2 analog 19-nor-1,25-dihydroxyvitamin D2: Antitumor activity against leukemia, myeloma, and colon cancer cells. J Natl Cancer Inst 2003; 95:896-905.

19. Nakagawa K, Sasaki Y, Kato S, Kubodera N, Okano T. 22-Oxa-1alpha,25-dihydroxyvitamin D3 inhibits metastasis and angiogenesis in lung cancer. Carcinogenesis 2005; 26:1044-54.
20. Mernitz H, Smith DE, Wood RJ, Russell RM, Wang XD. Inhibition of lung carcinogenesis by 1alpha,25-dihydroxyvitamin D3 and 9-cis retinoic acid in the A/J mouse model: evidence of retinoid mitigation of vitamin D toxicity. Int J Cancer 2007; 120:1402-9.
21. Malumbres M, Barbacid M. RAS oncogenes: the first 30 years. Nat Rev Cancer 2003; 3:459-65.
22. You M, Candrian U, Maronpot RR, Stoner GD, Anderson MW. Activation of the Ki-ras protooncogene in spontaneously occurring and chemically induced lung tumors of the strain A mouse. Proc Natl Acad Sci U S A 1989; 86:3070-4.
23. Nuzum EO, Malkinson AM, Beer DG. Specific Ki-ras codon 61 mutations may determine the development of urethane-induced mouse lung adenomas or adenocarcinomas. Mol Carcinog 1990; 3:287-95.
24. Zerbe LK, Dwyer-Nield LD, Fritz JM, et al. Inhibition by erlotinib of primary lung adenocarcinoma at an early stage in male mice. Cancer Chemother Pharmacol 2008; 62:605-20.

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