http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 10 (23): 4246-4250, 2007
ISSN 1028-8880
© 2007 Asian Network for Scientific Information

Frequent Homozygous Deletion of *p16/CDKN2A* Gene in Malignant Gliomas of Iranian Patients

[1]Mohammad Dilmaghani Zadeh, [1]Reza Amini, [1]Mohsen Firoozray and [2]Pupak Derakhshandeh-Peykar
[1]Department of Biochemistry, Faculty of Medicine, Iran University of Medical Sciences,
P.O. Box 14155-5983, Tehran, Iran
[2]Department of Medical Genetics, Faculty of Medicine, Tehran University of Medical Sciences,
P.O. Box 14155-1595, Tehran, Iran

Abstract: Homozygous deletion is the main mechanism of *CDKN2A* gene inactivation in malignant gliomas. However different frequencies were reported for its deletion. In order to find the homozygous deletion frequency among Iranian patients, we have analyzed the status of *CDKN2A* gene in 40 malignant gliomas and examined their 1α and 2 exons by comparative multiplex Polymerase Chain Reaction (PCR), using D9S171 chromosomal marker as an internal control. We found homozygous deletion in 6 out of 7 cases (85.7%) of anaplastic astrocytomas and 20 out of 33 cases (60.6%) of glioblastoma multiforme, in total 26 out of 40 cases (65%) of malignant gliomas. We also found that *CDKN2A* deleted patients were younger than *CDKN2A* non-deleted patients and that exon 2 was deleted more than exon 1α.

Key words: Glioma, *CDKN2A* deletion, astrocytoma, glioblastoma

INTRODUCTION

Gliomas are the most common brain tumors (Maher *et al*., 2001). Astrocytoma types of gliomas were classified by WHO into three grades in order of their malignancy: Grade II or astrocytoma, grade III or Anaplastic Astrocytoma (AA) and the most malignant grade IV or Glioblastoma Multiforme (GBM) (Louis *et al*., 2007). Progression from low-grade glioma (grade II) to malignant gliomas (grades III and IV) is associated with *CDKN2A* gene alterations which is inactivated mainly via homozygous deletion (Nakamura *et al*., 2001), although hypermethylation (Rocco and Sidransky, 2001) and rare mutations (Ichimura *et al*., 2000) are alternate mechanisms of its inactivation.

It has been shown that the survival rate of patients with malignant gliomas was 40% at one year (Ohgaki *et al*., 2004). Their poor prognosis makes them good targets for new therapeutic methods like gene therapy. In a study by Wang *et al*. (2001), $p16^{INK4A}$ expressing vectors has improved survival in animal models of glioma, even when compared with p53 expressing vectors. The frequency of *CDKN2A* homozygous deletion could be a guide for future gene therapy projects using $p16^{INK4A}$ expressing vectors in malignant gliomas.

Homozygous deletion of *CDKN2A* gene was not found in germline of familial gliomas (Tachibana *et al*., 2000), although germline point mutations or microdeletions of *CDKN2A* gene were reported in about 20% of familial malignant melanoma (Goldstein, 2004). In addition, except for the proven causes of brain tumors which are rare hereditary syndromes and therapeutic radiation, no other significant risk factor has been described for gliomas (Wrensch *et al*., 2002).

The *CDKN2A* gene encodes a tumor suppressor known as $p16^{INK4A}$ that binds to CDK4 and CDK6 and inhibits cyclin D attachment to them, which normally leads to pRb hypophosphorylation and cell cycle arrest (Vermeulen *et al*., 2003). Inactivation of $p16^{INK4A}$ allows cells to escape cell cycle arrest in G_1, thus it seems that it could not be inactivated in normal cells including normal glial cells.

The *CDKN2A* gene is 27.5 kb in length, it is located on 9p21 chromosomal band and consists of three exons, 1α, 2 and 3. It generates three transcripts, two of which encode similar proteins and the third transcript with an alternate first exon (1α), encodes for a different protein known as $p14^{ARF}$. Although homozygous deletion is the main mechanism of *CDKN2A* gene inactivation in malignant gliomas, however different frequencies of

Corresponding Author: Mohammad Dilmaghani Zadeh, Department of Biochemistry, Faculty of Medicine, Iran University of Medical Sciences, P.O. Box 14155-5983, Tehran, Iran
Tel: + 98 21 88058742 Fax: + 98 21 88058742

homozygous deletion have been reported. The highest frequency (82%) has been reported by Barker *et al.* (1997) and the lowest frequency (15%) by Mochizuki *et al.* (1999).

In this study, we examined the status of *CDKN2A* gene exons 1α and 2 of 40 Iranian patients by comparative multiplex PCR and found that they are frequently deleted in malignant gliomas of Iranian patients.

MATERIALS AND METHODS

The study was done in department of Medical Genetics of Tehran Medical Sciences University. Tumor samples were collected from patients with no history of familial glioma who undergone tumor resection surgery from 2003 till 2006, in six different hospitals from Tehran. All specimens were formalin-fixed, paraffin-embedded and graded according to the WHO classification. The analyzed samples consisted of 7 anaplastic astrocytomas and 33 glioblastoma multiformes. DNA samples were isolated by deparaffination followed by proteinase K digestion and phenolchloroform extraction as described previously (Shi *et al*., 2002).

Comparative multiplex polymerase chain reaction was used to assess the homozygous deletion of exons 1α and 2 of *CDKN2A* gene. The D9S171 microsatellite marker was selected as the internal control because of its proximity to the target gene, which provides a similar exposure rate for both the target and the control to the DNA polymerase and because it was never found to be homozygously deleted (Xing *et al*., 1999). The sequences of oligonucleotide primers (Isogen Life Sciences) were: 5'-GAGCAGCATGGAGCCTTC-3' (sense) and 5'-AATTCCCCTGCAAACTTCGT-3' (antisense) for exon 1α and 5'-CACTCTCACCCGACCCGT-3' (sense) and 5'-ACCTTCCGCGGCATCTAT-3' (antisense) for exon 2. Primers used for the amplification of internal control D9S171 were: 5'-AGCTAAGTGAACCTCATCTCTGTCT-3' (sense) and 5'-ACCCTAGCACTGATGGTATAGTCT-3' (antisense). The PCR amplification was performed in 25 μL reaction volumes with about 200 ng of template DNA, 1 μM of each primer, 0.02 μ μL^{-1} Taq DNA polymerase (Cinnagen), 200 μM of each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.3), 250 mM KCl and 5% dimethyl sulfoxide. All PCR reactions were done in a programmable thermal cycler (Eppendorf Master Cycler 5330) with the following conditions: initial denaturation at 94°C for 5 min, followed by amplification cycles consisting of denaturation at 94°C for 45 sec, annealing at 54°C for 45 sec, extension at 72°C for 45 sec and final extension at 72°C for 5 min. The cycle number for amplification of exon 1α and D9S171 were 31 and for exon 2 and D9S171 were 32. PCR products were run on a 2% agarose gel, stained with 0.5 μg mL^{-1} ethidium bromide and visualized under ultraviolet illumination. The intensity of the bands was compared using ImageQuant TL software (Amersham Biosciences). All results were analyzed with SPSS statistical software.

RESULTS AND DISCUSSION

Statistical analysis of the age of the patients with glioma grade, by t-test revealed a possible correlation between them (p = 0.018). The mean age of patients with anaplastic astrocytoma and glioblastoma multiforme were 33.0±9.6 and 47.5±14.9 years, respectively (Table 1).

Table 1: Summary of the patients characteristics and PCR product intensity ratios

Case	Age	Sex	Grade	Exon 1α to D9S171	Exon 2 to D9S171
1	35	F	GBM	0.359	0.260
2	18	F	AA	0.393	0.143
3	57	M	GBM	0.289	0.156
4	30	M	GBM	0.525	0.188
5	53	M	GBM	0.381	0.207
6	46	F	GBM	1.468	0.465
7	33	M	AA	0.465	0.214
8	35	M	GBM	0.802	0.330
9	54	M	GBM	0.829	0.095
10	55	M	GBM	0.435	0.201
11	51	M	GBM	0.825	0.197
12	65	F	GBM	0.135	0.456
13	30	F	GBM	0.754	0.323
14	31	M	GBM	0.526	0.097
15	29	M	GBM	1.149	0.092
16	73	M	GBM	1.593	0.584
17	30	F	AA	0.138	0.081
18	49	F	AA	0.425	0.113
19	51	M	GBM	1.070	0.557
20	52	M	GBM	0.551	0.175
21	15	F	GBM	0.265	0.132
22	63	M	GBM	1.297	0.736
23	58	M	GBM	0.655	0.591
24	39	F	GBM	0.344	0.286
25	37	M	GBM	0.940	0.502
26	70	M	GBM	0.133	0.193
27	75	M	GBM	0.381	0.514
28	39	M	AA	0.686	0.107
29	54	M	GBM	0.352	0.166
30	60	M	GBM	1.178	0.982
31	22	M	GBM	0.206	0.491
32	53	M	GBM	0.352	0.281
33	30	M	GBM	0.146	0.222
34	34	M	AA	0.331	1.120
35	43	M	GBM	0.289	0.129
36	45	F	GBM	1.097	0.979
37	53	M	GBM	1.540	1.133
38	28	M	AA	0.466	0.113
39	43	M	GBM	0.339	0.101
40	61	M	GBM	0.378	0.541

Table 2: Spectrum of deletions, according to tumor grades and the sex of the patients

Tumor grade	Sex	Exon 1α deletion	Exon 2 deletion	*CDKN2A* gene (exon 1α or 2) deletion (%)
GBM	Male (26)	5	15	16 (61.5)
	Female (7)	2	3	4 (57.1)
	Total (33)	7	18	20 (60.6)
AA	Male (4)	0	3	3 (75.0)
	Female (3)	1	3	3 (100.0)
	Total (7)	1	6	6 (85.7)
Malignant	Male (30)	5	18	19 (63.3)
glioma	Female (10)	3	6	7 (70.0)
(GBM and AA)	Total (40)	8	24	26 (65.0)

Based upon similar studies (Xing *et al*., 1999; Kamiryo *et al*., 2002; Kraus *et al*., 2000) 0.30 was defined as an arbitrary cutoff for determining the deletion of the target gene in examined samples. Patients with PCR products intensity ratios below this threshold were considered to have lost their *CDKN2A* gene. Thus, of the 40 malignant gliomas, exon 1α was deleted in 8 cases (20%), 7 of which were GBM and 1 was AA. Exon 2 was deleted in 24 cases (60%), 18 of which were GBM and 6 were AA. Finally, 26 cases (65%) showed deletion in one of the exons 1α or 2, 20 of which were GBM and 6 were AA (Table 2). The incidence of *CDKN2A* gene deletion for females and males were 70% (7 out of 10) and 63.3% (19 out of 30), respectively. Fisher's exact test showed no correlation between sex and *CDKN2A* deletion ($p>0.05$). In contrast a correlation between *CDKN2A* gene deletion and patient age was found by t-test examination ($p = 0.043$). The mean ages of the *CDKN2A* gene deleted and non-deleted patients were 41.5±14.5 and 51.5±14.4 years, respectively (Fig. 3).

Examples of the multiplex PCRs for exon 1α and 2 (Fig. 1, 2). In Fig. 1, exon 1α is deleted in lanes 3 and 4 which correspond to tumor samples 17 and 21 with bands intensity ratios of 0.138 and 0.265, respectively (Fig. 1). Exon 2 is deleted in lanes 2, 3, 4 and 5 which are correspondent with tumor samples 24, 39, 17 and 21 with bands intensity ratios of 0.286, 0.101, 0.081 and 0.132, respectively (Fig. 2).

GBMs were clinically divided into two entities. Primary or *de novo* GBMs present with no prior history and affect mainly the elderly and secondary GBMs which progress from gliomas of lower grades over a number of years and manifest in younger patients. Although histologically indistinguishable, two distinct genetic pathways are involved in their genesis. Primary GBMs are genetically characterized by LOH 10q, *EGFR* amplification, *CDKN2A* homozygous deletion and *PTEN* mutations (Ohgaki, 2005). Secondary GBMs develop through progression from low-grade astrocytoma or AA.

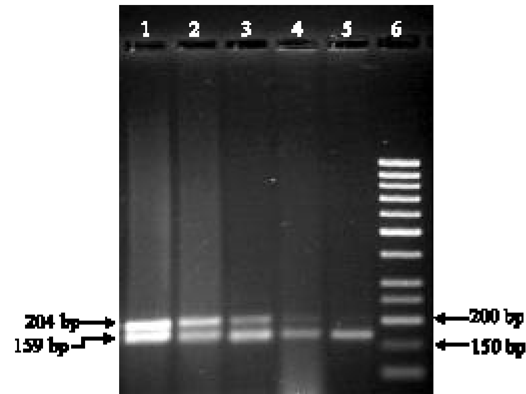

Fig. 1: The gel image of exon 1α and D9S171 multiplex PCR. Lane numbers correspond to samples as follow: 1, 31; 2, 11; 3, 4; 4, 21; 5, 17 and 6, molecular weigh marker (Lf 50). Exon 1α is deleted in samples 17 and 21 (Lanes 4 and 5). PCR products were run on a 2% agarose gel (at 100 volt for 45 min)

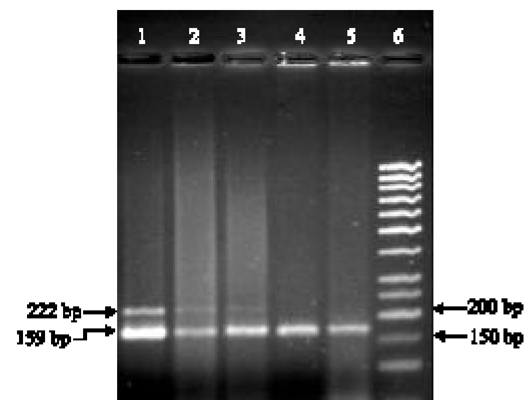

Fig. 2: The gel image of exon 2 and D9S171 multiplex PCR. Lane numbers correspond to samples as follow: 1, 19; 2, 24; 3, 39; 4, 17; 5, 21 and 6, molecular weigh marker (Lf 50). Exon 2 is deleted in samples 24, 39, 17 and 21 (Lanes 2, 3, 4 and 5). PCR products were run on a 2% agarose gel (at 100 volt for 45 min)

In the pathway to secondary GBM, *TP53* mutations are the most frequent and earliest detectable genetic alteration, although LOH 10q, *CDKN2A*, *p14* and *RB1* inactivation through deletion or methylation are frequently observed (Ohgaki, 2005).

Different studies were done on 9p21 locus which contains this gene in low and high-grade gliomas. Bigner *et al*. (1988) showed a high incidence of chromosomal 9p structural rearrangement in high-grade gliomas. Olopade *et al*. (1992), reported

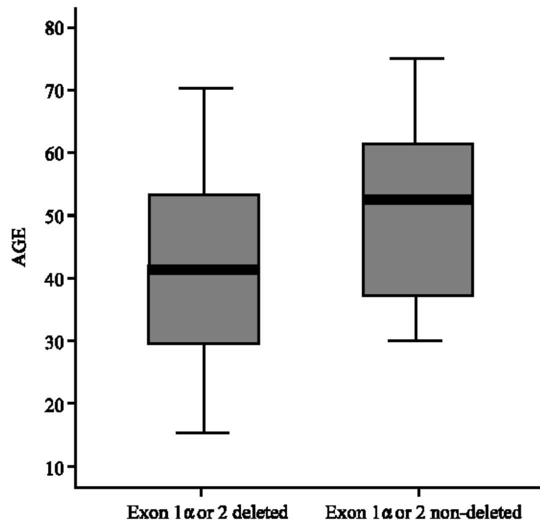

Fig. 3: Possible correlation between age and *CDKN2A* gene deletion (p = 0.043). Patients with exons 1α or 2 deletions were younger (mean age 41.5±14.5 years) than patients without deletions (mean age 51.5±14.4 years)

molecular evidence of deletion in 9p in 10 of 15 glioma-derived cell lines and 13 of 35 primary gliomas. Nobori *et al*. (1994) were the first who reported *CDKN2A* homozygous deletion in 61.5% of their analyzed glioma specimens. The frequency reported by Barker *et al*. (1997) was 82% among Caucasians. By contrast, such a frequency was 34.6% in Chinese, reported by Guang and Xianhou (1998) and the lowest frequency (15%) was found among Japanese by Mochizuki *et al*. (1999).

Here, we report the first study on the *CDKN2A* gene homozygous deletion frequency in malignant glioma of Iranian patients. In this study, we examined 40 malignant gliomas and we found that 26 of them (65%) had lost their *CDKN2A* gene exons 1α or 2. We also found that exon 2 was more deleted than exon 1α in both tumor grades (p = 0.000).

We analyzed the band intensity ratios by cutoffs of 0.25 and 0.35 and found the frequency of *CDKN2A* deletion as 57.5 and 72.5%, respectively. In fact, with both thresholds, *CDKN2A* gene is frequently lost in malignant gliomas among Iranian patients. Although these frequencies are greater than the frequency reported for the Japanese patients, it is lower than that reported for the Caucasian patients. The obtained results are based on the assumption that the reduced intensity of the *CDKN2A* band compared to the internal control, corresponds to a reduced amount of template DNA containing the *CDKN2A* gene.

Analyzing malignant glioma samples we found that patients with *CDKN2A* gene deletion were younger (p = 0.043). It has been shown that the majority of GBM cases (>90%) are primary glioblastomas that develop rapidly *de novo* (Ohgaki, 2005) and that *CDKN2A* gene homozygous deletion occurs in primary GBM more frequently than secondary GBM (Biernat *et al*., 1997). Although two entities of GBM were not separated in current study, high frequency of the *CDKN2A* gene homozygous deletion could result from the high number of primary GBMs. Here we suggest that $p16^{INK4A}$ inactivity could be an important mechanism in primary GBM gliomagenesis in young patients.

Present study shows that the rate of exon 2 deletion is higher than exon 1α deletion and a significant difference exists between them, analyzed by the McNemar's test (p = 0.000). This could be due to an alteration in $p14^{ARF}$ gene which shares exon 2 with *CDKN2A* gene. Further studies are underway to investigate other mechanisms responsible for $p16^{INK4A}$ inactivity in gliomas that do not have homozygous deletion.

ACKNOWLEDGMENTS

We thank Dr. Alireza Sadeghipoor for helping with specimen collection and classification of tumor grades. We also thank Dr. Mehrdad Pedram (Tehran University of Medical Sciences (TUMS), Tehran, Iran) for his careful proofreading and critical reviews of manuscript.

REFERENCES

Barker, F.G., P. Chen, F. Furman, K.D. Aldape, M.S. Edwards and M.A. Israel, 1997. *p16* deletion and mutation analysis in human brain tumors. J. Neurooncol., 3: 17-23.

Biernat, W., Y. Tohma, Y. Yonekawa, P. Kleihues and H. Ohgaki, 1997. Alterations of cell cycle regulatory genes in primary (*de novo*) and secondary glioblastomas. Acta Neuropathol., 94: 303-309.

Bigner, S.H., J. Mark, P.C. Burger, M.S. Jr. Mahaley, D.E. Bullard, L.H. Muhlbaier and D.D. Bigner, 1988. Specific chromosomal abnormalities in malignant human gliomas. Cancer Res., 48: 405-411.

Goldstein, A.M., 2004. Familial melanoma, pancreatic cancer and germline *CDKN2A* mutations. Hum. Mutat., 23: 630.

Guang, Z. and Y. Xianhou, 1998. Study of deletion of *p16* gene in the progression of brain astrocytomas. Chin. J. Cancer Res., 10: 412-417.

Ichimura, K., M.B. Bolin, H.M. Goike, E.E. Schmidt, A. Moshref and V.P. Collins, 2000. Deregulation of the p14^{ARF}/MDM2/p53 pathway is a prerequisite for human astrocytic gliomas with G1-S transition control gene abnormalities. Cancer Res., 60: 417-424.

Kamiryo, T., K. Tada, S. Shiraishi, N. Shinojima, H. Nakamaura, M. Kochi, J. Kuratsu, H. Saya and Y. Ushio, 2002. Analysis of homozygous deletion of the *p16* gene and correlation with survival in patients with glioblastoma multiforme. J. Neurosurg., 96: 815-822.

Kraus, J.A., N. Glesmann, M. Beck, D. Krex, T. Klockgether, G. Schackert and U. Schlegel, 2000. Molecular analysis of the PTEN, TP53 and *CDKN2A* tumor suppressor genes in long-term survivors of glioblastoma multiforme. J. Neurooncol., 48: 89-94.

Louis, D.N., H. Ohgaki, O.D. Wiestler, W.K. Cavenee, P.C. Burger, A. Jouvet, B.W. Scheithauer and P. Kleihues, 2007. The 2007 WHO classifcation of tumours of the central nervous system. Acta Neuropathol., 114: 97-109.

Maher, E.A., F.B. Furnari, R.M. Bachoo, D.H. Rowitch, D.N. Louis, W.K. Cavenee and R.A. DePinho, 2001. Malignant glioma: Genetics and biology of a grave matter. Genes Dev., 15: 1311-1333.

Mochizuki, S., Y. Iwadate, H. Namba, Y. Yoshida, A. Yamaura, S. Sakiyama and M. Taqawa, 1999. Homozygous deletion of the *p16/MTS-1/CDKN2A* gene in malignant gliomas is infrequent among Japanese patients. Int. J. Oncol., 15: 983-989.

Nakamura, M., T. Watanabe, U. Klangby, C. Asker, K. Wiman, Y. Yonekawa, P. Kleihues and H. Ohgaki, 2001. p14^{ARF} deletion and methylation in genetic pathways to glioblastomas. Brain Pathol., 11: 159-168.

Nobori, T., K. Miura, D.J. Wu, A. Lois, K. Takabayashi and D.A. Carson, 1994. Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. Nature, 368: 753-756.

Ohgaki, H., P. Dessen, B. Jourde, S. Horstmann, T. Nishikawa, P.L. Di Patre, C. Burkhard, D. Schüler, N.M. Probst-Hensch, P.C. Maiorka, N. Baeza, P. Pisani, Y. Yonekawa, M.G. Yasargil, U.M. Lütolf and P. Kleihues, 2004. Genetic pathways to glioblastoma: A population-based study. Cancer Res., 64: 6892-6899.

Ohgaki, H., 2005. Genetic pathways to glioblastomas. Neuropathology, 25: 1-7.

Olopade, O.I., R.B. Jenkins, D.T. Ransom, K. Malik, H. Pomykala, T. Nobori, J.M. Cowan, J.D. Rowley and M.O. Diaz, 1992. Molecular analysis of deletions of the short arm of chromosome 9 in human gliomas. Cancer Res., 52: 2523-2529.

Rocco, J.W. and D. Sidransky, 2001. p16(MTS-1/CDKN2A/INK4A) in cancer progression. Exp. Cell Res., 264: 42-55.

Shi, S.R., R.J. Cote, L. Wu, C. Liu, R. Datar, Y. Shi, D. Liu, H. Lim and C.R. Taylor, 2002. DNA extraction from archival formalin-fixed, paraffin-embedded tissue sections based on the antigen retrieval principle: Heating under the influence of pH. J. Histochem. Cytochem., 50: 1005-1011.

Tachibana, I., J.S. Smith, K. Sato, S.M. Hosek, D.W. Kimmel and R.B. Jenkins, 2000. Investigation of germline PTEN, p53, p16^{INK4A}/p14^{ARF} and *CDK4* alterations in familial glioma. Am. J. Med. Genet. A, 92: 136-141.

Vermeulen, K., D.R. Van Bockstaele and Z.N. Berneman, 2003. The cell cycle: A review of regulation, deregulation and therapeutic targets in cancer. Cell Prolif., 36: 131-149.

Wang, T.J., M.S. Huang, C.Y. Hong, V. Tse, G.D. Silverberg and M. Hsiao, 2001. Comparisons of tumor suppressor *p53*, *p21* and *p16* gene therapy effects on glioblastoma tumorigenicity *in situ*. Biochem. Biophys. Res. Commun., 287: 173-180.

Wrensch, M., Y. Minn, T. Chew, M. Bondy and M.S. Berger, 2002. Epidemiology of primary brain tumors: Current concepts and review of the literature. Neuro. Oncol., 4: 278-299.

Xing, E.P., Y. Nie, L.D. Wang, G.Y. Yang and C.S. Yang, 1999. Aberrant methylation of p16^{INK4A} and deletion of p15^{INK4B} are frequent events in human esophageal cancer in Linxian, China. Carcinogenesis, 20: 77-84.