Carcinogenesis vol.23 no.7 pp.1127–1130, 2002

Alterations of the p16^{INK4} locus in human malignant mesothelial tumors

Tomoko Hirao, Raphael Bueno[1], Chang-Jie Chen[1], Gavin J.Gordon[1], Elizabeth Heilig and Karl T.Kelsey[2]

Department of Cancer Cell Biology, Harvard School of Public Health, Boston, MA 02115 and [1]Department of Surgery, Division of Thoracic Surgery, Brigham and Women's Hospital, Boston, MA 02115, USA

[2]To whom correspondence should be sent
Email: kelsey@hsph.harvard.edu

The INK4 locus has two promoters and encodes two unique proteins that share exons in different reading frames, p16^{INK4a} and p14^{ARF}. The p16^{INK4a} protein, by inhibiting cyclin-dependent kinase, down regulates Rb-E2F and leads to cell cycle arrest in the G1 phase. The p14^{ARF} protein interacts with the MDM2 protein, neutralizing MDM2-mediated degradation of p53. Since p53/Rb genes are not altered in malignant mesothelioma, additional components of these pathways, such as p16^{INK4a} and p14^{ARF}, are candidates for inactivation. In this study, we have examined p16^{INK4a} and p14^{ARF} alterations (gene deletion, mutation and promoter methylation) in 45 primary malignant mesothelioma specimens. Fourteen patients (31%) had altered *p16*; four tumors had a methylated promoter region (8.8%), 10 tumors showed *p16* to be deleted (22.2%), and one tumor had a point mutation (2%). We did not find any instances of methylation in the p14^{ARF} 5′-CpG island. Patients whose tumors had *p16* deletion were significantly younger than those with methylation, and, in the patients whose lungs were studied for the prevalence of asbestos fibers, those with any *p16* alteration had lower fiber counts than those with no *p16* alteration. Hence, *p16* gene alteration is relatively common in malignant mesothelioma, while p14^{ARF} is rarely, if ever, methylated. Our data suggest that deletion of *p16* occurs in a relatively susceptible subset of the population.

Introduction

Malignant mesothelioma is one of the most aggressive cancers that arise in thoracic cavity. It is well known that exposure to asbestos, occurring as a result of direct exposure from industrial sources or indirect exposure from household or other environmental material, is a main cause of this disease (1). Asbestos is known to induce chromosomal damage in a variety of cellular systems including normal mesothelial cells, releasing reactive oxygen species and perhaps even interacting physically with mitotic spindles (2–4). Unlike many tumors, the alteration of common tumor suppressor genes such as p53 or Rb is infrequent in malignant mesothelioma (1,5). The most frequent alterations reported in malignant mesothelioma are deletions of specific sites in the chromosomes 1p, 3p, 9p, 6q and 22 (3,4).

In malignant mesothelioma cell lines, a high incidence of homozygous deletion of the INK4 locus in 9p21 has been reported (6–8). The INK4 locus in 9p21 has two promoters and encodes two unique proteins that share an exon in different reading frames, p16^{INK4a} and p14^{ARF}. The p16^{INK4a} protein, by inhibiting cyclin-dependent kinase, down regulates the retinoblastoma pathway and leads to cell cycle arrest in the G1 phase of the cell cycle. The p14^{ARF} protein interacts with the MDM2 protein, neutralizing MDM2-mediated degradation of p53. The dysfunction of both proteins may play important roles in disrupting p53 and Rb pathways in various types of malignancies. Besides deletion or mutation of these tumor suppressor genes, several reports have recently shown that these genes can be silenced by hypermethylation of their 5′-CpG islands (9,10).

Cheng *et al.* demonstrated that homozygous deletion of *p16* is a common occurrence in malignant mesothelioma cell lines (85% of 40 lines), but significantly less common in primary tumors (22% of 23 tumors) (8). Kratzke *et al.* reported absence of *p16* protein in 10 of 12 malignant mesotheliomas detected by immunohistochemical staining (11). Thus, there seem to be important differences in the frequency of *p16* deletion between established cell lines and primary tumor tissues. Further, in primary tumor tissue there is also evidence of a high level of *p16* alteration that exceeds the reported frequency of *p16* deletion.

Hence, in an effort to characterize more completely the frequency and mechanism of inactivation of the Rb and p53 pathways in primary tumors, we have examined p16^{INK4a} and p14^{ARF} alteration status including deletion, mutation and methylation in 45 primary malignant mesothelioma specimens.

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; RT, reverse transcriptase.

Materials and methods

Study population

Forty-five malignant mesothelioma specimens were obtained after surgical resection at Brigham and Women's Hospital. Tumor DNA was extracted from the frozen tissue using QIAamp DNA mini kit according to the manufacturer's instructions (Qiagen, Valencia, CA). Each patient's history of exposure to asbestos was assessed either by medical and occupational history or by asbestos fiber counting (34 cases) using the method reported by Churg and Warnock (12). If the tissue showed >200 fibers/g the exposure history was recorded as heavy (versus light).

Methylation analysis

Tumor DNA was modified by sodium bisulfite for methylation-specific PCR as described previously (13). Briefly, 1 µg of DNA was treated with 3.6 M sodium bisulfite and 10 mM hydroquinone, followed by column purification (Wizard DNA clean up system, Promega, Madison, WI) and ethanol precipitation. The modified DNA was resuspended in 40 µl of distilled water, used as a template for methylation-specific PCR. The primer sequence and annealing temperatures for both unmethylated and methylated sets for p16^{INK4a} and p14^{ARF} can be obtained from a previous report (13). PCR was performed with the reaction mix of 50 ng modified DNA, 1× PCR buffer (Applied Biosystems, Foster City, CA), 0.2 mM dNTPs, 0.5 µM primers and 1.25 units of Ampli Taq Gold (Applied Biosystems) in a total volume of 50 µl. Twenty microliters of PCR products were analyzed by electrophoresis in 2.5% agarose gel. The SW 480 colon cancer cell line and NCI-H 209 lung cancer cell line were used as a positive control for the *p16* methylated and unmethylated PCR, respectively. In the study of p14 methylation, p14 hypermethylated DLD colon cancer cell line and the unmethylated Caco colon cancer cell line were used as controls (kindly provided from Drs Wiencke and Zheng, Laboratory for Molecular Epidemiology, UCSF).

© Oxford University Press

Deletion analysis

Since primary tumor generally contains normal stromal tissues, it is difficult to ascertain its deletion status by conventional methods. We used a real-time quantitative PCR method based on Taqman chemistry (Applied Biosystems) (14,15). In brief, real-time quantitative PCR was carried out using the ABI Prism 5500 (Perkin-Elmer Applied Biosystems, LOCATION?) with 50 ng DNA, 400 nM primers and 200 nM Taqman probe in 50 µl Taqman buffer according to the manufacturer's procedure (Applied Biosystems). The primers and probe sequence of *p16* exon 2 and control gene ψ*x4* were identical to that used by M'soka *et al.* (14). For each sample, PCR was performed in triplicate and threshold cycle was normalized to detect homozygous deletion as previously described (14).

Mutation analysis

SSCP analysis of *p16* exons 1–3 was performed on all samples without homozygous deletion. Each exon was amplified by PCR containing fluorescence dye labeled primers (16) and analyzed by DNA autosequencer ABI 310 (Applied Biosystems). Samples with a variant SSCP band were directly sequenced by DNA autosequencer ABI 377 using BigDye Terminator v3.0 (Applied Biosystems).

Reverse transcriptase–PCR

p16 and *p14* expression were analyzed by RT–PCR. Total RNA was extracted from microdissected frozen tissue using Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. RT–PCR was carried out on 150 ng total RNA using OneStep RT–PCR (Qiagen), and the following primers were used: *p16* 5′ primer (5′-GCTGCCCAACGCACCGAATA) and 3′ primer (5′-ACCACCAGCGTGTCCAGGAA), *p14* 5′ primer (5′-TGCTCACCTCTGGTGCCAAAG) and 3′ primer (5′-TGGTCTTCTAGGAAGCGGCTG), and GAPDH 5′ primer (5′-GAGTCAACGGATTTGGTCGT) and 3′ primer (5′-TGACAAAGTGGTCGTTGAGG). The primers corresponded to the sequences in exon 1α-exon 2 for *p16* (180 bp), exon 1β (258 bp) for *p14*. The PCR cycle conditions were 28 cycles of 94°C for 30 s, 64°C for 30 s and 72°C for 50 s. Seven microlitres of PCR products were analyzed by electrophoresis in 2.5% agarose gel, and GAPDH amplified band was used as a standardized control for *p16* and *p14*.

Table I. Patient and tumor traits stratified by asbestos and tobacco smoke exposure

	Known asbestos exposure[a]		Smoking status[b]	
	Light (n = 16)	Heavy (n = 28)	Non-smoker (n = 11)	Smoker (n =32)
Age	52.3 ± 12.7	57.2 ± 9.4	50.2 ± 12.9	57.2 ± 9.8
Gender				
Female	6	5	6	5
Male	10	23	5	27
Histology[c]				
Epithelial	10	14	7	17
Mixed	6	11	4	12
Sarcomatoid	0	2	0	2

[a]Asbestos exposure information missing for one case.
[b]Smoking information missing for two cases.
[c]One case was recorded as advanced mesothelioma without typing.

Results

Clinicopathological characteristics of the 45 cases studied are shown in Table I. Twenty-eight patients had a known history of heavy asbestos exposure (62%) and 32 were smokers (71%). There were three times more male than female patients (34 male, 11 female). The most common histological type was epithelial malignant mesothelioma, and 15 out of 34 (44%) male patients had epithelial type malignant mesothelioma whereas 9 out of 11 (82%) female patients had this histology. No significant association was observed between asbestos exposure status or smoking status and age, or histology.

Table II summarizes the age, gender, smoking and asbestos exposure history of patients whose tumors had an altered *p16* gene, by the type of change observed. Fourteen out of 45 patients (31%) had altered *p16*. There were four cases whose gene was methylated (8.8%), 10 cases had the *p16* gene deleted (22.2%), and one tumor revealed a point mutation (2%). The point mutation was a C→A /His→Gln change at codon 75. No hypermethylation was observed in the promoter region of p14. The methylation of the CpG site in the *p16* promoter region was seen in four cases; half had a known heavy asbestos exposure history and all of them were smokers.

The alterations of *p16* and p14 were assessed together with RNA expression by RT–PCR. Ten cases with *p16* gene deletion and four cases with *p16* promoter hypermethylation showed negative expression of *p16* transcript when normalized with GAPDH, whereas one case with *p16* mutation showed positive expression of *p16* and p14 (Figure 1). All the cases which showed deletion in *p16* exon 2 revealed negative expression in both *p16* and p14 transcript. We did not detect any specific loss of only p14 (i.e. no cases were found where p14 was not expressed and *p16* was expressed).

We examined next *p16* deletion and methylation status with clinical features and carcinogen exposures (Table III). The mean age of *p16* deleted cases was significantly younger than *p16* methylated cases, and both *p16* deleted cases and methylation positive cases were younger than the respective negative cases; however, the later differences were not statistically significant. The deletion of *p16* was more common in

Table II. Type of *p16* alteration, age, histology, gender and carcinogen exposure history in patient's tumors with an altered *p16* gene

Gender	Age	Histology	*p16* alteration	Ever smoked	Asbestos exposure[a]
Female	35	Epithelial	Deletion	No	Light or none
Male	40	Epithelial	Deletion	Yes	Light or none
Female	42	Epithelial	Deletion	No	Light or none
Male	46	Epithelial	Deletion	Yes	Heavy
Male	48	Epithelial	Deletion	No	Heavy
Male	53	Epithelial	Deletion	Yes	Heavy
Male	55	Mixed	Methylation	Yes	Heavy
Male	56	Mixed	Deletion	Yes	Heavy
Male	57	Mixed	Deletion + methylation	Yes	Heavy
Male	59	Mixed	Deletion	Yes	Heavy
Male	61	Epithelial	Methylation	Yes	Light or none
Male	62	Mixed	Point mutation	Unknown	Unknown
Female	68	Epithelial	Methylation	Yes	Light or none
Male	72	Mixed	Deletion	Yes	Heavy

[a]Asbestos exposure was called heavy if fiber counts were in excess of 200; it was light if non-occupational.

heavy asbestos exposed cases (7/28, 25%) than in patients with light (or unknown) asbestos exposure (3/16, 19%). Interestingly, when lung asbestos burdens, measured by asbestos fiber counts, were compared, those with either *p16* deletion or methylation had markedly fewer asbestos fibers present (Table IV).

We also compared smoking history and *p16* deletion (Table IV). The mean pack years smoked was 32.8 ± 25.7 in cases showing no *p16* deletion and 10.1 ± 10.8 in cases where the gene was deleted ($P < 0.01$). When the median pack years smoked in each group was compared the difference remained significant ($P < 0.04$).

Discussion

It is well known that the primary cause of malignant mesothelioma is exposure to asbestos fibers. Interestingly, the disease has a very long latent period, and once diagnosed, the prognosis for long-term survival is very poor. The onset of the disease is usually 20–40 years after the initial exposure to asbestos and, after it becomes clinically appreciable, the cancer grows aggressively with the median survival of the disease ordinarily ranging from 4 to 18 months (1). Several investigators have demonstrated common chromosomal aberrations in their search for the genes that are in the causal pathway for this disease. The major tumor suppressor genes such as p53 and the *Retinoblastoma* gene are infrequently modified in malignant mesothelioma (1–8). The most frequent alterations include deletions of specific sites on several chromosomes, specifically including: 1p21-22, 3p21, 6q15-21, and 9p21 (3,4). Hence, several groups have suggested that the accumulation of multiple genetic alterations might be required before a mesothelial cell is fully converted into a neoplastic cell (3,4).

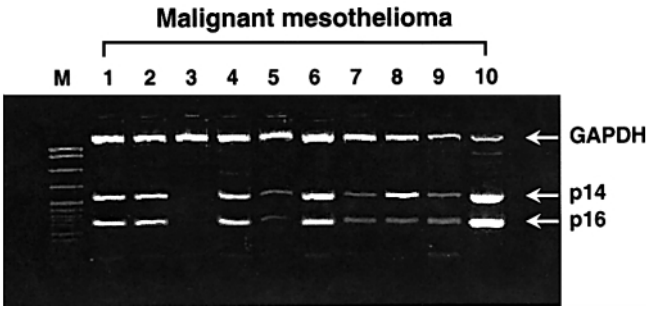

Fig. 1. Representative results of RT–PCR analysis. M, marker (pBR322–MSP1 digest). Cases 1, 2, 4 and 6, malignant mesothelioma without alteration. Cases 3, 5, 7 and 9, malignant mesothelioma with deletion in INK4 locus. Case 8, malignant mesothelioma with *p16* promoter hypermethylation. Case 10, malignant mesothelioma with point mutation.

Table III. Carcinogen exposure status and demographics of the patient population stratified by *p16* deletion and methylation

	p16 Deletion		*p16* Methylation	
	Absent ($n = 35$)	Present ($n = 10$)	Absent ($n = 41$)	Present ($n = 4$)
Age[a]	56.9 ± 10.5	50.8 ± 10.9	55.1 ± 11.1	60.3 ± 5.7
Gender				
Female	9	2	10	1
Male	26	8	31	3
Histology				
Epithelial	18	6	22	2
Mixed	14	4	16	2
Sarcomatoid	2	0	2	0
Known asbestos exposure[b]				
Light	13	3	14	2
Heavy	21	7	26	2
Smoking status[c]				
Non smoker	8	3	11	0
Smoker	25	7	28	4

[a]$P < 0.04$ comparing *p16* deletion present with methylation present.
[b]Asbestos exposure information missing for one case.
[c]Smoking information missing for two cases.

The *p16* gene, localized on chromosome 9p21, is an important tumor suppressor gene involved in the genesis of a variety of cancer types. In malignant mesothelioma cell lines a high frequency of *p16* gene deletion has been observed (7,8). Based upon our data, and that of others, it is clear that primary tumors have less frequent *p16* gene deletions than the cell lines studied to date. This suggests that *p16* deletion may confer an advantage for growth *in vitro* and that the distribution of gene alterations in cell lines may not accurately reflect the situation *in vivo*. This could occur either because *p16* gene deletion selects for the initial ability of cells to grow in culture, or because tumors grown *in vitro* undergo *p16* deletion during clonal expansion in culture. It is, of course, possible that gene deletion is not detected in primary tumors as a result of heterogeneity of the tissue studied. Tissue samples obtained from surgical procedure almost always contain normal stromal cells, which will make the detection of gene deletion difficult. To minimize this, we have dissected tumors and subsequently used a real-time quantitative PCR method introduced by M'soka *et al.* to detect *p16* deletion. This will minimize contamination of tumor tissue with normal stroma, enhancing our ability to detect *p16* deletion. *p16* deletion in exon 2 was observed in 10 (22%) of the 45 tumor samples, consistent with prior reports examining primary tumors (8). We have also confirmed this deletion analysis by examining RNA expression. Ten deleted cases detected by real-time quantitative PCR showed negative expression of both *p16* and p14 transcript. In addition, a point mutation in exon 2 was found only in one sample that revealed positive expression of *p16*/p14 RNA. There is evidence of *p16* point mutations in cell lines, but few reports of mutations in primary tumors (8). The single point mutation that we observed has not been previously reported in primary malignant mesothelioma or in malignant mesothelioma cell lines. In addition, the fact that we did not detect either methylation or deletion of p14 alone suggests that inactivation of this gene alone does not provide a selective advantage for clonal expansion *in vivo*.

While no significant association was observed between the relative level of asbestos exposure and *p16* alteration, there was the suggestion that *p16* alteration occurred in patients with a relatively light exposure history. It is difficult to evaluate accurately relative asbestos exposure, either by questionnaire or fiber counting, but our data suggest that additional efforts need to be directed towards a better understanding of the relationship between the level of asbestos exposure and the precise nature of somatic alterations in mesothelial tumors.

Our data revealed significant associations of *p16* alteration and tobacco smoking history. This observation is curious, given that smoking is not an accepted risk factor for mesothelioma. Hence, either the effect of smoking on the disease exists only in a subpopulation of patients small enough to be obscured in the overall studies of association, or smoking is acting as a confounder in the analysis, or both. All patients

Table IV. Asbestos fiber counts and smoking history stratified by *p16* deletion/methylation

	p16 Deletion		*p16* Methylation	
	Absent (n =25)	Present (n = 7)	Absent (n = 28)	Present (n = 4)
Pack years[a]				
Mean (±SD)	32.8 ± 25.7	10.1 ± 10.8	29.0 ± 26.3	20.0 ± 12.2
Median	26.0	5.0	22.5	22.5
Asbestos fiber count				
Mean (±SD)	5139.9 ± 7786.3[b]	1323.6 ±1176.2[c]	4603.67 ± 7184.3[d]	425.0 ± 224.9[e]
Median	2919	642	2594	425

[a]P = 0.006, comparing *p16* deletion absent with *p16* deletion present.
[b,d]Asbestos count information missing for 10 cases.
[c,e]Asbestos count information missing for 2 cases.

whose tumors showed hypermethylation of the *p16* promoter region were smokers. However, the number of patients whose tumors were methylated at the *p16* promoter was small. The observation of a smoking–*p16* methylation association is consistent with the results of studies of alteration of *p16* and smoking in lung cancer. Prior work has reported that the prevalence of *p16* methylation increases with the duration of smoking in a dose-dependent fashion in non-small cell lung cancers (13). Further, only 9% of patients with mesothelioma had methylated *p16* promoters; thus it is possible that this is a causal association that has gone unnoted in epidemiologic studies. In patients whose tumors had *p16* deleted, the mean pack years smoked was significantly smaller than deletion negative cases ($P < 0.01$). Since over 20% of the patient's tumors showed *p16* deletions and smoking is not a risk factor for this disease, this association most likely represents confounding by another factor rather than a causal association. Possible confounders could include age, age at first exposure to asbestos, and gender. Further study is indicated to understand more precisely the possible relationship of asbestos exposure and both smoking and smoking-related factors that might contribute to the genesis of mesothelioma and, specifically, *p16* alteration in these tumors.

Acknowledgement

We thank Dr John Godleski for assistance with pathology, supported by ES00002, CA78609, ES06717.

References

1. Antman,K.H., Schiff,P.B. and Pass,H.I. (1997) *Cancer: Principles and Practice of Oncology.* Philadelphia, Lippincott, 5th edn, pp. 1853–1878.
2. Kelsey,T.K., Yano,E., Liber,H.L. and Little,J.B. (1986) The in vitro genetic effects of fibrous erionite and crocidolite asbestos. *Br. J. Cancer*, **54**, 107–114.
3. Murthy,S.S. and Testa,J.R. (1999) Asbestos, chromosomal deletions and tumor suppressor gene alterations in human malignant mesothelioma. *J. Cell Physiol.*, **180,** 150–157.
4. Lee,W.C. and Testa,J.R. (1999) Somatic genetic alterations in human malignant mesothelioma. *Int. J. Oncol.*, **14,** 181–188.
5. Xu,L., Flynn,B.J., Ungar,S., Pass,H.I., Linnainmaa,K., Mattson,K. and Gerwin,B.I. (1999) Asbestos induction of extended lifespan in normal human mesothelioma cells: interindividual susceptibility and SV 40 T antigen. *Carcinogenesis*, **20**, 773–783.
6. Prins,J.B., Williamson,K.T., Kamp,M.M.K., Hezik,E.J., Kwast,T.H., Hagemeijer,A. and Versnel,M.A. (1998) The gene for the cyclin-dependent-kinase-4 inhibitor, CDKN2A, is preferentially deleted in malignant mesothelioma. *Int. J. Cancer*, **75,** 649–653.
7. Xiao,S., Li,D., Vijg,J., Sugarbaker,D.J., Corson,J.M. and Fletcher,J.A. (1995) Codeletion of p15 and *p16* in primary malignant mesothelioma. *Oncogene*, **11**, 511–515.
8. Cheng,J.Q., Jhanwar,S.C., Klein,W.M., Bell,D.W., Lee,W.C., Altomare,D.A., Nobori,T., Olopade,O.I., Buckler,A.J. and Testa,J.R. (1994) *p16* alteration and deletion mapping of 9p21-p22 in malignant mesothelioma. *Cancer Res.*, **54,** 5547–5551.
9. Ahuja,N. and Issa,J.P. (2000) Aging, methylation and cancer. *Histol. Histopathol.*, **15**, 835–842.
10. Herman,J.G., Graff,J.R., Myohanen,S., Nelkin,B.D. and Baylin,S.B. (1996) Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc. Natl Acad. Sci.*, **93**, 9821–9826.
11. Kratzke,R.A., Otterson,G.A., Lincoln,C.E., Ewing,S., Oie,H., Geradts,J. and Kaye,F.J. (1995) Immunohistochemical analysis of the *p16* cyclin-dependent kinase inhibitor in malignant mesothelioma. *J. Natl Cancer Inst.*, **87,** 1870–1875.
12. Churg,A.M. and Warnock,M.L. (1981) Asbestos and other ferruginous bodies: their formation and clinical significance. *Am. J. Pathol.*, **102**, 447–456.
13. Kim,D.K., Nelson,H.H., Wiencke,J.K., Zheng,S., Christiani,D.C., Wain,J.C., Mark,E.J. and Kelsey,K.T. (2001) *P16* and histology-specific methylation of CpG island by exposure to tobacco smoke in non-small cell lung cancer. *Cancer Res.*, **61**, 3419–3424.
14. M'soka,T.J., Nishioka,J., Taga,A., Kata,K., Kawasaki,H., Yamada,Y., Yu,A., Komada,Y. and Nobori,T. (2000) Detection of methylthioadenosine phosphorylase and *p16* gene deletion in T cell acute lymphoblastic leukemia by real-time quantitative PCR assay. *Leukemia*, **14**, 935–940.
15. Tran,P.T., Hori,H., Hori,Y., Okumura,K., Kagotani,K., Taguchi,H., Carson,D.A. and Nobori,T. (1997) Molecular cloning of the human methylthioadenosine phosphorylase processed pseudogene and localization to 3q28. *Gene*, **186**, 263–269.
16. Iwato,M., Tachibana,O., Tohma,Y., Arakawa,Y., Nitta,H., Hasegawa,M., Yamashita,J. and Hayashi,Y. (2000) Alterations of the INK4a/ARF locus in human intracranial germ cell tumors. *Cancer Res.*, **60**, 2113–2115.

Received October 5, 2001; revised February 6, 2002; accepted April 5, 2002