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ORIGINAL ARTICLE

Prognostic significance of p16/cdkn2a loss in pleural malignant mesotheliomas

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Abstract Homozygous deletion of p16/CDKN2A is the most common genetic abnormality in malignant mesotheliomas. The aim of this study was to determine prognostic significance of p16/CDKN2A loss in malignant pleural mesotheliomas (MPM) as defined by immunohistochemistry and fluorescence in situ hybridization (FISH). High-density tissue microarrays were constructed from archival formalin-fixed paraffin-embedded samples of 48 MPM. Long survival (LS) was defined as survival greater than 3 years from the time of diagnosis, and short survival was defined as less than 3 years from the time of diagnosis. Both loss of p16 protein expression by immunohistochemistry and homozygous deletion of p16 by FISH were associated with adverse prognosis. Female gender, positive p16 immunoexpression, and lack of p16/CDKN2A deletion significantly predicted the survival for the LS group. Statistical analysis showed a very strong correlation of immunohistochemistry and FISH data. Cases positive for p16 immunoexpression and negative for 9p21 deletion showed the best survival time. Our study is the first to demonstrate decreased frequency of homozygous deletion of 9p21 and loss of p16 immunoreactivity in pleural mesotheliomas from patients

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with long-term survival of greater than 3 years in contrast to patients with rapidly fatal mesotheliomas. A possible implementation of these tests into preoperative prognostication of MPM and therapeutic decisions should be considered.

Keywords Pleural mesothelioma · p16 · FISH · Immunohistochemistry · Prognosis

Introduction

Malignant pleural mesothelioma (MPM) is an aggressive tumor usually associated with a poor prognosis and median survival period of only 6–12 months [1, 2]. Approximately 2,000 new cases are diagnosed each year in the US and there is an increasing incidence worldwide [3]. More than 80% of cases can be attributed to asbestos exposure, but approximately 20% of cases may be related to other factors such as exposures to simian virus 40, thorotrast, and radiation [4, 5]. The incidence of MPM is expected to rise worldwide in the next 10–20 years as a result of widespread exposure to asbestos in past decades and continuing exposure in developing countries [6].

There is no reliable definitive therapy for MPM, and only a tiny minority of patients is eligible for potentially curative treatments. Response to different treatments is variable but there is a small subset of patients that will have longer survival than typically expected. The major clinical issue is to establish early diagnosis and adequate treatment triage of the patients with MPM. Diagnosis of early disease is difficult and most patients are diagnosed at an advanced stage. The TNM staging system is of limited prognostic value in patients with MPM, and two currently accepted staging systems include the International Mesothelioma Interest Group and Brigham systems [7, 8]. A limitation of both staging systems is that the extent of the disease can be assessed only at thoracotomy, which means that a minority of patients can be adequately staged. Radiologic prediction of stage proposed by these two systems is problematic. Therefore, alternative prognostic scoring systems have been proposed by the European Organization for the Research and Treatment of Cancer and the Cancer and Leukemia Group B [9, 10]. These prognostic scoring systems indicate that the most important predictors of poor prognosis in MPM are nonepithelioid histology, male gender, poor performance status, low hemoglobin, high platelet count, high white blood cell count, and high lactate dehydrogenase level. These prognostic factors are proposed to be very important in selecting appropriate treatment. Nonepithelioid histology of MPM argues against surgery as a treatment option [11, 12]. However, it has been shown that up to 40% of malignant mesotheliomas are inadequately histologically classified on small biopsy specimens [11, 13]. This means that some of the patients will not receive adequate therapy because of inadequate histologic subclassification. This also has an impact on survival, variable response to therapy, and poor outcome. It is clear that the search for other prognostic factors is necessary to improve treatment of patients with MPM.

Molecular studies at DNA and RNA levels, as well as cytogenetic studies, have resulted in identification of potential diagnostic and prognostic markers that may be useful in clinical practice [14–17]. One of the most common genetic alterations in MPM is the homozygous deletion of the 9p21 locus harboring genes CDKN2A, CDKN2B, and MTAP [18–21]. Deletion of p16/CDKN2A deletions has been reported in up to 72% of primary mesotheliomas [22, 23]. Recent studies demonstrated the diagnostic usefulness of this abnormality in separation of benign from malignant mesothelial proliferations in body fluid and biopsy specimens [24, 25]. By gene expression analysis using fluorescence in situ hybridization (FISH) as a validation method, Lopez-Rios et al. [19] demonstrated the prognostic significance of p16 deletion in MPM using 1-year survival as a cutoff for long survival.

From institutions in multiple countries, we have assembled a collection of 26 patients with MPM and very unusually long survivals of three or more years after diagnosis, the largest collection of MPM patients with this exceptional outcome. We hypothesized that the tumors from this unique cohort of patients have molecular markers that should correlate with their unusually good prognosis. The aim of our study was to determine the prognostic significance of the loss of p16 as determined by FISH and immunohistochemistry in epithelioid MPM with long-term survival of three or more years.

Material and methods

Forty-eight cases of epithelioid MPM from either open biopsies or pleurectomies diagnosed between 1987 and 2003 were selected by contributing authors. The tumors were classified according to the World Health Organization Classification of tumors of the lung and pleura [26]. The diagnosis was confirmed immunohistochemically by at least three positive and two negative markers. The study was approved by the University of Pittsburgh Institutional Review Board (IRB # 0612074). The patients were divided into two groups using 36 months as cutoff survival. Twenty-six patients were identified as long survivors (LS; one USA, one UK, one Austria, three France, five Turkey, 15 Italy) and 22 as short survivors (SS; five UK, eight Italy, nine Austria) [27].

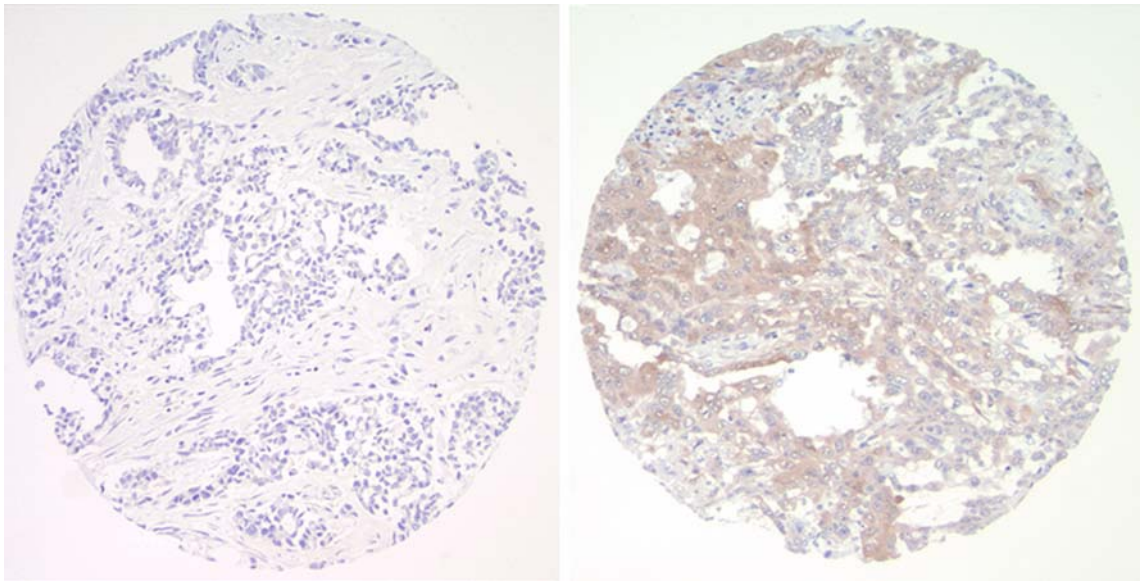

Fig. 1 P16 immunoreactivity was nuclear and cytoplasmic and was scored for intensity and extent. Cases were considered negative if no staining or weak focal staining was present (*left*). Staining was interpreted as positive if weak multifocal–diffuse or strong diffuse staining was observed (*right*)

Tissue microarray constructions

High-density tissue microarrays were constructed from archival formalin-fixed paraffin-embedded samples of 48 cases of epithelioid malignant mesotheliomas collected in USA, UK, Turkey, Italy, France, and Austria. For each sample, five representative areas rich in tumor cells were identified by light microscopic examination and marked on the hematoxylin-and-eosin sections. Five cores measuring 0.6 mm in diameter were taken from the donor paraffin tissue blocks of each case and were arranged in a recipient paraffin tissue array block by using a Manual Tissue Arrayer (MTA-1, Beecher Instruments Inc, Sun Prairie, WI, USA). Each donor block was punched six to ten times for the construction of two recipient blocks, each containing 243 tissue cores. Thirteen cores of morphologically normal-appearing pleura and lung parenchyma were also included [27].

Immunohistochemistry

The immunohistochemical study was performed using anti-p16 mouse monoclonal antibody at a dilution of 1:200 (BD PharMingen, San Diego, CA, USA) according to the standard avidin–biotin–peroxidase complex method. The p16 immunostain for each case was interpreted as previously described [28]. Briefly, nuclear and cytoplasmic p16 immunoreactivity was scored for intensity and extent. Intensity of immunoreactivity was scored as 0—no staining; 1+—weak staining; 2+—strong staining. The extent of immunoreactivity was assessed as focal (<5%) or multifocal–diffuse.

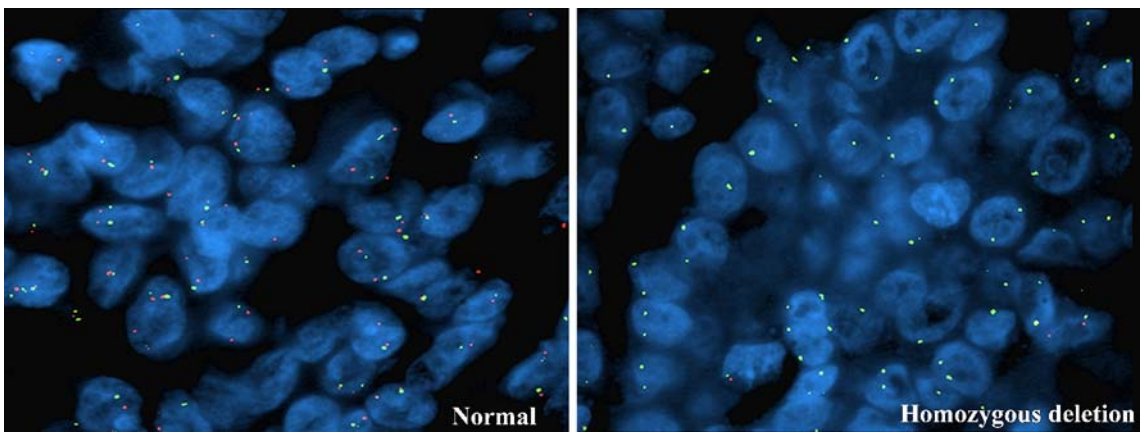

Fig. 2 Homozygous deletion by FISH was defined if both 9p21 signals were lost in at least 20% of nuclei. Normal cells are expected to show two green (chromosome 9 centromeric probe) and two red signals (locus-specific CDKN2A (p16) probe; Vysis, Downers Grove, IL, USA)

Cases were considered negative if 0 or 1+ focal, positive if 1+, multifocal–diffuse if 2+ (Fig. 1). Sections of cervical biopsy previously identified as immunoreactive for p16 protein served as a positive control. Negative controls included omitting the primary antibody and its substitution with normal serum.

Fluorescence in situ hybridization

Dual-color FISH analysis was performed using a Spectrum-Green-labeled chromosome 9 centromeric probe and a Spectrum-Orange-labeled locus-specific CDKN2A (p16) probe (Vysis, Downers Grove, IL, USA), as previously described [24]. In brief, paraffin sections were deparaffinized, dehydrated in ethanol, and air-dried. Sections were digested with protease K (0.5 mg/ml) at 37°C for 28 min. The slides were denatured at 75°C for 5 min in 70% formamide (Chemicon, Billerica, MA, USA) and dehydrated in ethanol. The probes were denatured for 5 min at 75°C prior to hybridization. Slides were hybridized overnight at 37°C and washed in 2XSSC/0.3% Igepal (Sigma, St. Louis, MO USA) at 72°C for 2 min. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI)–antifade (Vysis, Inc., Downers Grove, IL, USA). Each FISH assay included normal lung tissue sections as a negative control, and sections of malignant mesothelioma previously identified as carrying p16 deletion as a positive control. Analyses were performed using a fluorescence microscope (Nikon Eclipse E600) and Cytovysion Workstation (Applied Imaging, Santa Clara, CA, USA) equipped with filter sets with single- and dual-band excitors for Spectrum Green, Spectrum Orange, and DAPI (UV 360 nm). The histological areas previously identified on the hematoxylin-and-eosin-stained sections were analyzed on the FISH-treated slides. Only individual and well-delineated cells were scored. Overlapping cells were excluded from the analysis. At least 60 cells were scored for each case and control.

Each tumor was assessed by the average and the maximum numbers of copies of p16 gene per cell and the average ratio of p16 gene to chromosome 9 copy numbers (CEP9). Homozygous deletion was defined if both 9p21 signals were lost in at least 20% of nuclei (Fig. 2).

Statistical analysis

The Kaplan–Meier method was used to estimate the survival function. The agreement of immunohistochemistry and FISH was tested by the simple kappa coefficient. Logistic regression was performed to determine whether all available explanatory variables can be used to predict survival status (LS versus SS).

Results

Clinical characteristics

The patients were divided into two groups using the overall median survival for all patients in this study of 36 months. Twenty-six patients were identified as LS and 22 as SS. There

Table 1 Immunoexpression of p16 protein and homozygous deletion of p16 determined by FISH in epithelioid type of malignant pleural mesotheliomas (39 analyzed cases)

Study group	Case #	Gender	Age (years)	Survival (months)	IHC	FISH
LS	1	F	63	47	POS	DEL
	2	F	65	37	NEG	ND
	3	F	63	68	NEG	NA
	4	F	55	37	POS	DEL
	5	M	61	57	POS	ND
	6	M	45	39	NEG	DEL
	7	M	60	44	POS	ND
	8	M	53	39	NEG	DEL
	9	F	56	42	POS	ND
	10	F	71	72	POS	ND
	11	F	56	41	POS	ND
	12	M	64	37	NEG	ND
	13	M	63	76	POS	ND
	14	F	NA	61	POS	ND
	15	F	67	48	NEG	ND
	16	F	30	51	POS	ND
	17	M	72	45	NEG	NA
	18	M	63	55	POS	ND
	19	F	71	36	NEG	DEL
	20	F	41	37	NEG	DEL
SS	1	M	69	8	NEG	DEL
	2	M	64	5	NEG	DEL
	3	M	63	<1	NEG	NA
	4	F	NA	12	NEG	NA
	5	M	78	6	NEG	DEL
	6	M	81	2	POS	DEL
	7	M	67	18	NEG	DEL
	8	M	59	12	POS	DEL
	9	M	54	8	POS	ND
	10	M	47	12	NEG	ND
	11	M	69	12	NEG	DEL
	12	M	62	12	POS	DEL
	13	M	72	3	NEG	DEL
	14	M	73	<1	NEG	DEL
	15	F	63	1	NEG	DEL
	16	M	50	2	NEG	DEL
	17	M	66	18	NEG	DEL
	18	F	41	13	NEG	DEL
	19	M	59	31	NEG	DEL

LS long survival (>3 years), *SS* short survival (<3 years), *IHC* immunohistochemistry, *FISH* fluorescence in situ hybridization, *POS* positive, *NEG* negative, *DEL* deletion, *ND* not deleted, *NA* not applicable

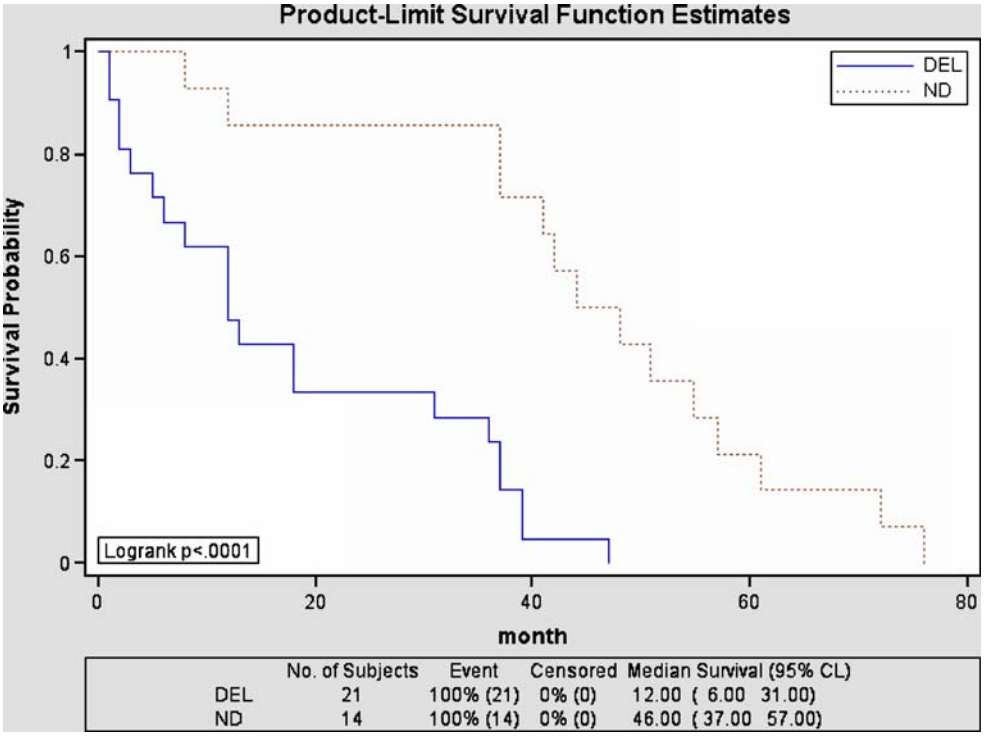

Fig. 3 Survival probability in respect to presence or absence of p16 deletion as determined by FISH in patients with epithelioid MPM

were 11 men and 15 women in LS group and 17 men and five women in SS group. Mean age in LS group was 63.7 years (range 41–78) and 57.4 years (range 30–71) in SS group. Mean survival in LS group was 50.7 months (range 36–116 months) and 9.1 months (range <1–31 months) in SS group.

Fluorescence in situ hybridization

The results of p16 expression assessed by immunohistochemistry and p16 deletion assessed by FISH are shown in Table 1.

Fluorescence in situ hybridization was successful in 35 (18 LS; 17 SS) of 48 cases (73%). Homozygous deletions of p16/CDKN2A was seen in 21 (60%) of cases, more frequently in cases with shorter survival. The estimated median survival time was 46 months for the subjects negative for deletion. For the subjects positive for deletion, the estimated median survival time was 12 months. Subjects negative for deletion as determined by FISH lived significantly longer than those subjects positive for deletion (p value <0.0001 for log-rank test; Fig. 3).

Of 18 cases with LS, homozygous deletion of p16/CDKN2A was identified in six cases (33%), while 12 cases (67%) were negative for deletion (Fig. 4a). Of 17 cases with SS, homozygous deletion of p16/CDKN2A was identified in 15 cases (88%) and two cases (12%) were negative for deletion (Fig. 4a). The difference in frequency of homozygous deletion of gene p16 was statistically significant between the two groups of malignant mesotheliomas (p=0.001). In addition to homozygous loss, two cases in LS group and one case in SS group showed hemizygous loss. Polyploidy of chromosome 9 was observed in three SS cases. Age was not a significant predictor of survival status in respect to presence or absence of deletion determined by FISH. However, gender and FISH results could significantly predict the survival. Subjects negative for deletion were more frequently women in LS group. The estimated odds ratio, female versus male,

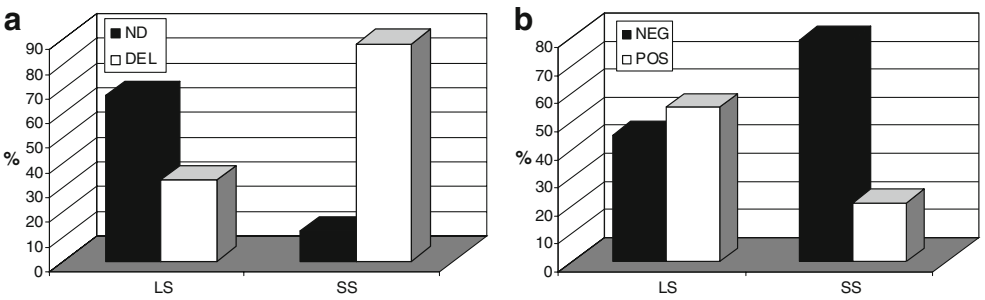

Fig. 4 **a** Frequencies of 9p21 deletion determined by FISH in patients with epithelioid type of MPM with long (*LS*) and short (*SS*) survival. **b** Immunoexpression of p16 protein in patients with epithelioid-type MPM with long (*LS*) and short (*SS*) survival

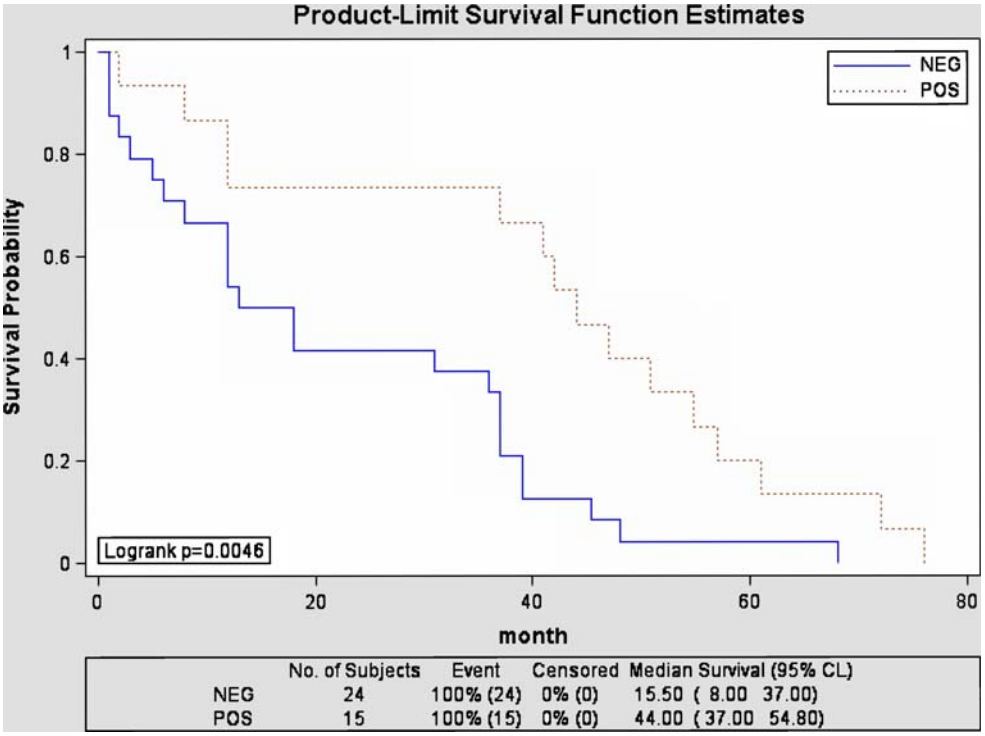

Fig. 5 Survival probability in respect to p16 protein expression determined by immunohistochemistry in patients with epithelioid type of MPM

was 16.014. The estimated odd ratio for negative and positive for deletion was 19.87.

Immunohistochemistry

P16 protein expression by immunohistochemistry was successfully determined in 39 cases. Discrepancies in number of cases analyzed by immunohistochemistry and FISH assay reflect unsuccessful hybridization. Of 20 cases with LS, positive p16 immunoexpression was identified in 11 cases (55%), while nine cases (45%) showed loss of expression (Fig. 4b). Of 19 cases with SS, only four cases (21%) showed positive p16 immunoexpression and 15 cases (79%) were negative (Fig. 4b). The difference in p16 protein expression was statistically significant between the two study groups (p=0.04). Similar to FISH results, age was not a significant predictor of survival status in respect to presence or absence of p16 immunoexpression. Female gender and positive p16 immunoexpression could significantly predict the survival for LS. As for female versus male, the estimated odds ratio was 12.476. For positive versus negative p16 immunoexpression, the estimated odds ratio was 5.495.

The estimated median survival time was 44 months for cases showing positive p16 immunoexpression. For the negative cases, the estimated median survival time was 15.5 months. Subjects with positive p16 immunoexpression lived significantly longer than those subjects with negative results (p value=0.0046 for log-rank test; Fig. 5).

Table 2 summarizes the results of 9p21 homozygous deletion determined by FISH in comparison with p16 expression determined by immunohistochemistry. Statistical analysis showed a very strong correlation of immunohistochemistry and FISH data (kappa coefficient=0.47), with only eight discrepant cases out of 34. Cases with the 9p21 deletion showed loss of p16 protein expression by immunohistochemistry, while cases without a 9p21 deletion maintained intact p16 protein expression (p value=0.003). Five cases were negative for deletion but showed positive p16 expression by immunohistochemistry. There were also three cases that showed loss of p16 immunoexpression, but were negative for deletion. Survival analysis showed that cases with positive p16 protein immunoexpression and negative for deletion of 9p21 as determined by FISH have the best survival time (Fig. 6).

Table 2 Comparison of p16 immunoexpression by IHC and p16 homozygous deletion by FISH

	IHC	
	NEGATIVE	POSITIVE
ND	4	10
DEL	16	5

Kappa coefficient=0.47
ND not deleted by FISH, *DEL* deleted by FISH, *IHC* immunohistochemistry

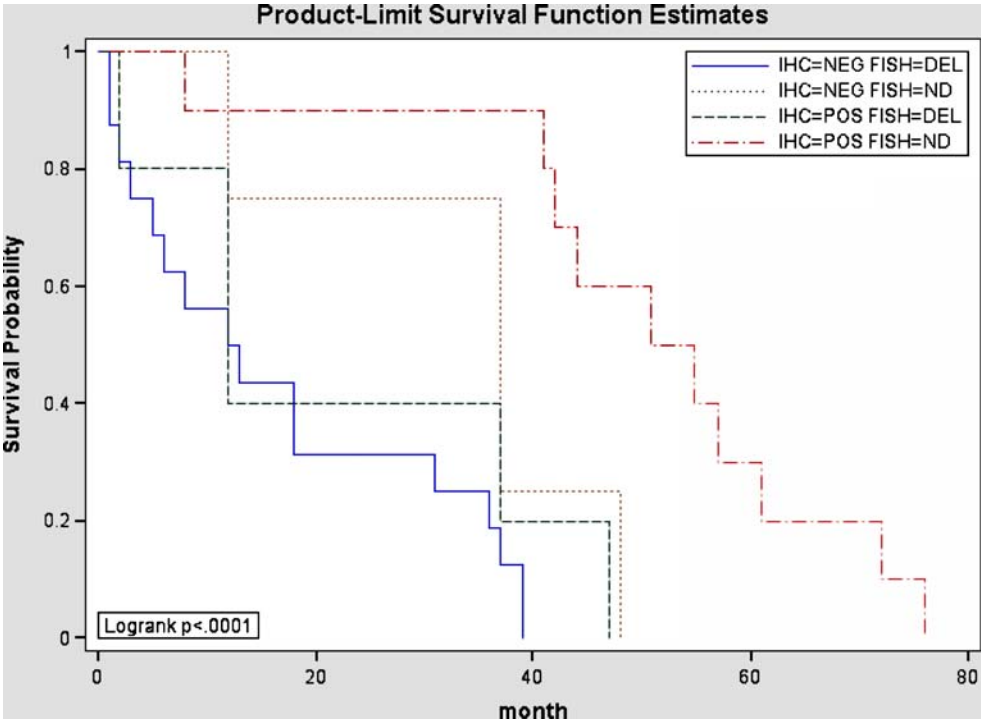

Fig. 6 Survival probability in respect to status of p16 loss determined by FISH and IHC in epithelioid type of MPM

Discussion

Several prognostic factors in pleural mesotheliomas have been reported previously, one of which is histology. Preoperative histologic classification of mesothelioma is very important in treatment decision because in cases of nonepithelioid morphology surgery is not a choice and other therapeutic options are considered. Although epithelioid mesotheliomas are usually surgically treated, there is still variability in survival despite similar treatment. Therefore, it is important to improve prognostic prediction before definitive surgery. For that reason, our study focused on epithelioid mesotheliomas only.

Loss of p16 gene is one of the most frequent genetic alterations in malignant mesotheliomas. Differences in reported frequency of loss of p16 usually reflect different methodology. It is known that loss of p16 may be a result of homozygous deletion, methylation, or point mutation, with homozygous deletion being the most common [14]. Homozygous deletion could be detected either by FISH or polymerase chain reaction (PCR)-based assays. FISH assay has certain advantages of being able to identify homozygous and hemizygous deletions. In contrast to PCR-based assays, contamination with normal cells is not an issue since those could be easily excluded from FISH analysis.

There are several clinical applications for detection of p16 deletion. One is diagnostic, particularly in differentiation between benign and malignant mesothelial proliferations, which is a common diagnostic problem for pathologists [24, 25]. Illei et al. [25] demonstrated that detection of p16/CDKN2A deletion by FISH is a very powerful ancillary test in the body cavity effusion specimens. Our recent study demonstrated that the same diagnostic assay could be used on formalin-fixed paraffin-embedded surgical specimens [24]. This approach has certainly improved the accuracy of diagnosis of malignant mesotheliomas.

Negative prognostic implications of p16/CDKN2A deletion have been reported in a prior study. Illei et al. clearly demonstrated that p16 deletion is associated with shorter survival using 1-year survival as a cutoff for long survival [19]. Similarly, our study confirmed the same observation, but we used 3 years as a cutoff survival demonstrating that the absence of deletion is a predictor of a long survival. These results indicate that FISH analysis of p16 in pleural mesothelioma can be used as an ancillary prognostic test that may guide treatment decisions.

Adverse prognostic significance of p16 loss determined by immunohistochemistry has been reported in peritoneal mesothelioma [28]. However, the results of this approach have not been reported in pleural mesothelioma. To our knowledge, our study is the first demonstration of loss of p16 immunoreactivity as a negative prognostic factor in pleural mesothelioma. Despite the same scoring method, our study demonstrated loss of p16 immunoreactivity in 62% of cases, in contrast to the reported 48% in peritoneal mesotheliomas. This may reflect differences in histologic types analyzed in two studies. Study on peritoneal mesotheliomas focused mainly on biphasic type, while our study was focused on epithelioid type. The second possible explanation would be biologic and genetic differences

between peritoneal and pleural tumors. It is possible that different genetic mechanisms and environmental factors play a role in the development of these two types of mesotheliomas with subsequent impact on different expression of p16. It is known that chromosome 9p represents a chromosomal region that is more prone to DNA damage by asbestos [11]. The level of exposure may be another explanation for lower frequency of detected p16 loss in peritoneal mesotheliomas. This is a speculation since detailed exposure histories are uncertain in our study and were not reported in the study by Borczuk et al. [28]. Hence, it would be interesting to know whether loss of p16 immunoreactivity correlates with the type of asbestos fibers to which patients were exposed.

Another important observation in our study is correlation between the presence of deletion of p16 determined by FISH and loss of p16 immunoreactivity. Our prior study demonstrated the only trend for such correlation that was statistically insignificant [24]. One possible explanation is that this correlation between the two methods depends on the histologic type of mesothelioma. As mentioned earlier, this study included epithelioid mesothelioma only, while the prior study in addition to epithelioid type also included biphasic and sarcomatoid types. The second most likely explanation is a difference in scoring methods. Based on the current study, loss of p16 immunoreactivity has the same prognostic significance as homozygous deletion of p16. Therefore, one may think that, because of availability and cost, p16 immunoreactivity would be a better clinical prognostic test in malignant mesotheliomas. Our survival analysis would argue that probably both tests should be used since the best survival was observed in patients who demonstrated positive p16 immunoreactivity and lack of p16 deletion. Furthermore, about 23% of our cases showed discrepancy between FISH and immunohistochemistry data, indicating that for clinical applications probably both tests should be used. Loss of p16 immunoexpression in the absence of 9p21 deletion that was seen in three cases may be a result of p16 point mutation or methylation. On the other hand, it is somewhat difficult to explain positive p16 immunoexpression in the presence of homozygous deletion as seen in five cases in our study. One possibility would be that we are dealing with FISH artifact and false-positive results. The other explanation would be the size of commercially available FISH probe used in this study. This probe is rather large (190 kb) and also covers p14, p15, and a portion of the MTAP gene which is frequently codeleted with p16 gene. In our study, we did not investigate the status of MTAP gene, but we believe that deletion of this gene is most likely the underlying reason for the above-described discrepancy.

A codeletion of p16 with MTAP, a gene approximately 100 kb telomeric to p16, has potential therapeutic implications [18]. MTAP encodes methylthioadenosine phosphorylase, an enzyme of the salvage pathway that leads to production of adenine for synthesis of adenosine monophosphate. As demonstrated in hematologic malignancies, L-alanosine (inhibitor of de novo purine synthesis) therapy has toxic effect on MTAP-deficient cells [29, 30]. The frequent codeletion of p16 and MTAP suggests that mesotheliomas with p16 deletion may have greater sensitivity to L-alanosine therapy.

In summary, our study is the first to demonstrate decreased frequency of homozygous deletion of 9p21 and loss of p16 immunoreactivity in pleural mesotheliomas from patients with long-term survival of greater than 3 years in contrast to more typical patients with rapidly fatal mesotheliomas. Implementation of these tests into clinical practice may improve preoperative prognostication of malignant mesotheliomas and inclusion of mesothelioma patients into new targeted chemotherapeutic protocols.

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Conflict of interest statement We declare that we have no conflict of interest.

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