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Recurrent genomic imbalances affect gene expression in mesothelioma: combined strategies to identify genes involved in tumour development

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ABSTRACT

Human mesothelioma induced by asbestos is a step-by-step accumulation of genetic modifications including chromosomal amplifications and deletions. Three major histologic subtypes have been already identified: epithelioid, sarcomatoid and biphasic. To compare these subtypes, cell lines exhibiting a pure epithelioid, a pure sarcomatoid and mixed phenotypes were studied using array-based comparative genomic hybridization. For the epithelioid lines these studies revealed homogeneous deletions and duplications for several chromosomes ranging from 1 to 97.5 Mb. The abnormalities affected complete or partial chromosomes, and chromosome losses were more common than gains. In contrast, no abnormality was detected using this technique in the sarcomatoid and biphasic cell lines. However, more detailed analysis of the 9p21 region and its gene products by Fluorescence *in situ* hybridization (FISH), quantitative polymerase chain reaction (PCR) and fluorescence activated cell sorting (FACS) revealed the loss of p14ARF expression, a product of cyclin-dependent kinase inhibitor 2A (CDKN2A) gene, in the sarcomatoid cell lines, while expression of p16INK4a, an alternative splicing isoform of the same gene, was retained. In the epithelioid cell lines the opposite situation was present with expression of p14ARF, but loss of p16INK4a. These studies confirm the importance of the tumour suppressor genes encoded at the 9p21 locus in the pathogenesis of mesothelioma. The present results may help in the development of prognostic factors and in better understanding the basic biological processes.

KEYWORDS: malignant mesothelioma, array-CGH, biphasic form, homozygous and heterozygous deletions, tumour heterogeneity

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INTRODUCTION

Malignant mesothelioma (MM) is an asbestos related malignancy arising from cells of mesodermal origin [1]. In humans, this disease is characterized by a silent latency period of up to 40 years between first exposure to asbestos and the development of mesothelioma. It is assumed that during this time, a cumulation of specific alterations is required for genetic transformation [2]. Studies have shown many

complex structural and numerical changes in mesothelioma genetic material [2-5]. However, in primary tumour specimens, neoplastic cells are mixed with normal reactive stromal components, which may lead to underestimation of DNA gains or losses in comparative genomic hybridization (CGH) analysis [6-8]. To address this drawback new approaches are necessary, including analysis of the pure cell lines and their comparison with primary tumour samples.

MM has shown complex karyotypes with common chromosomal numerical changes such as monosomy 22 [6, 9-11] and molecular genetic changes such as deletions of tumour suppressor genes (TSG) [4, 12]. The difference between two characteristic phenotypes of mesothelioma, epithelioid and sarcomatoid forms, have been reported by immunohistochemical staining and by chromosomal analysis [11, 13]. Analysis of altered chromosomes revealed that many changes affected the known TSG CDKN2A in 9p21. This gene encodes two proteins that share an exon in different reading frames, p16INK4a protein and p14ARF [14-16]. Although the p16INK4a and p14ARF proteins are structurally and functionally different, they are both involved in cell cycle progression. The dysfunction of these proteins plays an important role in various types of malignancies. Moreover, few purified mesothelioma cell lines are available in tumour banks and thus, cytogenetic and molecular genetic studies are lacking.

Conventional genetic analysis revealed that cell lines originating from the sarcomatoid form bore fewer genetic alterations than the epithelioid subtype [12] although in other studies, examination of these tumours did reveal the presence of numerous chromosomal alterations [12, 13] . The biphasic form, a mixture of the epithelioid and sarcomatoid types, may constitute a dominant component.

Microarray-based Comparative Genomic Hybridization (array-CGH) identifies copy-number variations (amplifications or deletions) across the entire genome at high resolution [17] allowing a detailed comparison of the genomic content of different cell populations. Array-CGH technologies were used to perform an analysis of genetic homology in synchronously diagnosed parallel tumours [18]. This technique offers great promise for many studies, but the most important factor that may influence the reliability of genetic analysis of tumour biopsies or cell lines is the purity and homogeneity of the investigated cells. To overcome this problem, several methods have been developed to obtain pure tumour cell types from a tissue section or pleural effusion with various cell populations [7, 11, 19].

Frequent genomic alterations in MM, identified by microarray technologies, confirmed previous cytogenetic investigations reporting deletions in 1p21-22, 3p21, 4q31-32, 4p12-13, 6q14-25, 9p21, 13q, 14q, 15q15, 17p13, and monosomy 22. High resolution microarray technologies also unveiled other genomic unbalances such as loss in 1p36, 1p13, 3p21, 3p14, 9q34, 13q12-14, 15q11.1-15, 14q24.2-qter, 17q21, 19p13, 19q13, and 22q12 and gain in 5p14, 17q21-q23, 8q23-q24 and 18q12.1 [13, 19].

Heterozygous and homozygous deletions of the chromosomal region 9p21 are involved in almost all mesotheliomas and many other cancers. The TSG CDKN2A codes for two completely different proteins, p14ARF and p16INK4a, through the usage of two alternative first exons joined to a common exon 2 at the same acceptor site but in different reading frames. Both isoforms play a role in inducing cell cycle arrest and appear to act by different cell cycle inhibition pathways [20-22]. The authors decided to identify genomic aberrations in 5 well-characterized MM tumour lines, one epithelioid (JL1) and its related biphasic form, that shows a distinct epithelioid predominance (GER-8M); two sarcomatoid cell lines (DM3 and RS1) and their related biphasic form with a dominant sarcomatoid phenotype (PJ2). We completed this investigation by FISH analysis of 9p21 and 17p13.1 p53, (official gene name TP53), FACS analysis to assess the expression of 9p21 genes or loci such as CDKN2A, CDKN2B and MTAP and quantitative RT-PCR analysis of CDKN2A using TaqMan. Our results showed the relative importance of these tumour suppressor genes in parallel cell lines and gave new information about immunophenotypic evaluation exclusive to the opposite cell lines.

MATERIALS AND METHODS

Sample collections and cell line culture conditions

Five mesothelioma cell lines, JL1 (epitheliod), DM3 (sarcomatoid), RS5 (another sarcomatoid

form), PJ2 (a mixed form dominated by the sarcomatoid cells) and GER-8M (a new biphasic form dominated by the epithelioid cells) were purified from malignant pleural effusion or patient biopsies, after informed consent through collaboration with clinicians and in full compliance with ethical guidelines. Two normal mesothelial cells from pleural effusions of patients with benign inflammation were also purified under the same conditions. All cultures were performed in NCTC-109 antibiotic free medium [11]. Before utilization, they were cultivated for 5 days in medium free fetal calf serum (FCS) allowing synchronized cells to be obtained which were mostly in G1 phase. Confluent cells were dissociated and identified. Growing normal mesothelial cells in tissue culture is limited to four or five passages. To analyse gene expression and study cell types based on DNA, RNA or protein preparations, pure tumour lines were established following subsequent passages after one year [11]. Identification and cytogenetic data of the above lines were confirmed according to the "Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) Germany - Protocols of Identity".

Array-comparative genomic hybridization analysis

Genomic DNA was extracted from dissociated JL1 and GER-8M, DM3 and PJ2 cells using QIAMP DNA Blood mini kit (Qiagen) according to the supplier's instructions. Array-CGH was performed using the Agilent Human Genome CGH Microarray Kit 44A, Agilent Technologies (Santa Clara, California, USA). This platform is a high-resolution 60-mer oligonucleotide-based microarray that allows analysis of genome-wide molecular profiling of genomic aberrations with a resolution of ~80 kb. Genomic DNA labeling and hybridization were performed following the protocols provided by Agilent. Briefly, 0.5 µg of purified patient DNA and a control (Promega Corporation, Madison, Wisconsin, USA) were double-digested with RsaI and AluI for two hours at 37 °C. After twenty minutes at 65 °C, each digested sample was labeled by the Agilent random primers labeling kit for two hours using Cy5-dUTP for the patient DNA and Cy3-dUTP for the control DNA. Labeled products were column purified and prepared according to the Agilent protocol. After probe denaturation and pre-annealing with 50 µl of Cot-1 DNA, hybridization was performed at 65 °C with rotation for 24 hours. After two washing steps the arrays were analyzed with the Agilent scanner and the Feature Extraction software (v9.5.3.1). Graphical overview was obtained using the CGH analytics software (v3.5.14).

Cytofluorometric analysis

Phenotypic analysis of mesothelioma cell lines, two pure sarcomatoïd forms: DM3 and RS5, two epithelioid forms: JL1 a pure form and one purified from a mixed form and two purified normal mesothelial cells from donors with benign inflammation were performed by immunostaining. The polyclonal antibodies, rabbit anti-Human p14ARF and p15INK4b were obtained from Santa Cruz Biotechnology, INC, and Mouse mAb anti-p16INK4a from Calbiochem, (isotype IgG_1, IgG_2). Monoclonal chicken Anti-MTAP antibody was kindly obtained from Dennis Carson, University of California, USA. Indirect immunofluorescence was performed as described previously [11]. FACS data acquisition and analysis were performed using CELL-Quest software (BD).

FISH analysis

Alterations occurring at the loci 9p21 and the presence of 17p13.1 (p53) were confirmed by using commercially available probes Vysis Urovysion™ (Abbott Laboratories, USA).

The 9p21 LSI p16INK4a Spectrum Yellow probe covers ~190 kb, covering genomic markers D9S1749, D9S1747, D9S1748 and D9S1752 CEP9 covers p16INK4a, p15INK4b (CDKN2B) and p14ARF, and LSIp53 (17p13.1) Spectrum Orange probe.

Fluorescence *in situ* hybridization (FISH) was performed according to the manufacturer's protocol, with slight modifications. Cells were detached from tissue culture, washed and cyto-centrifuged. The slides were fixed for 10 min in fresh 3:1 methanol glacial acetic acid at room temperature. If not immediately used for hybridization, they were dried and stored at -20 °C for up to 6 months.

Hybridization was performed as indicated in the manufacturer's instructions. Before microscopic evaluation, nuclei were counterstained with DAPI (Vysis, Inc.), coverslipped and stored at -20 °C until analysis.

Heterozygous or homozygous deletion in CDKN2A gene is represented by loss of one or two orange p16INK4a signal(s), respectively. Gain in the CDKN2A gene is represented by a complementary signal orange p16INK4a signal. 40 to 50 nuclei were studied per sample and the experiment was performed on each cell type using a Zeiss Axioskop microscope equipped with a selective filter for the detection of Spectrum Yellow, Spectrum Orange and DAPI. Three-color images of p16INK4a, p15INK4b and p14ARF were captured using the ISIS digital imaging analysis system.

Gene expression analysis

Total RNA, prepared from cells using TRIZOL Reagent (Gibco BRL), was treated with RQ1 DNAse (Promega). Primers and probes for the quantification of p16INK4a and cyclophilin mRNA were purchased from Applied Biosystems catalogue no.4453320 (p16INK4a) 4351372 (cyclophilin) with the p16INK4a assay (CDKN2A) spanning exons 2 and 3. Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was performed as described using an *icycler* (Bio-Rad). Results were normalized to cyclophilin and expressed as arbitrary units. Amplification was performed in duplicates and experiments performed twice.

RESULTS

Cell line collection

Cell lines used in this study have been described previously [11]. The production of these lines reflects changes related to the tumourigenic processes with cell line predominance for the epithelioid or the sarcomatoid form. The final diagnosis is made by histological and morphological analysis of biopsy material, assisted by a panel of special stains and immunohistochemistry. The data are reproducible and concord with the clinical and pathological diagnosis for each patient. These lines tested by DSMZ (see Methods) confirm their identity and the cytogenetic analysis revealed no consistent abnormality for the sarcomatoid form as the published karyotype. For the biphasic forms, the dominant phenotype for GER-8M and PJ2, was determined according to the functional markers.

Whole-Genome analysis by Array-CGH

Genome-wide array-based comparative genomic hybridization analysis of mesothelioma cell lines JL1 and GER-8M, DM3 and PJ2 was carried out to identify regions that display DNA copy number alterations and to compare the genomic content. The analysis showed many chromosomal imbalanced abnormalities in JL1 and GER-8M epithelioid forms. No chromosomal imbalances were detected in sarcomatoid form DM3 passage 8 (P8) and PJ2 (P9). Table 1 reports changes in the chromosomal content of JL1 (samples 1 and 2 corresponding to p48 and p55, respectively) and GER-8M (p13). Both samples of JL1 revealed many homogeneous deletions and duplications for several chromosomes sizing from 1 to 97.5 Mb. The abnormalities affected complete or partial chromosomes and chromosome losses were more common than gains.

Table 1. Summary of genomic losses and gains detected in JL1 and GER-8M cell lines.
(*) Heterozygous deletion is indicated as loss, and homozygous is specified.

JL-1 genomic imbalances	Chromosome	Region	Mb position	Size Mb
Sample 1 (p48)				
Gain	5	p arm		
Gain	20	entire chromosome		
Gain	X	q arm		
Loss	3	p24.3	20.787 to 27.079	6.3
Loss*	9	p21	20.111 to 26.454	6.3
Loss homozygous	9	p21.3	20.99 to 21.99	1
Loss	14	q31.1qter	79.806 to 106.311	23.5
Loss	22	q arm	11.8 to 49.6	37.8

Table 1 continued..

Sample 2 (p55)				
Gain	8	q23.3-qter	115.965 to 146.201	30.23
Gain	Y	p arm		
Loss	1	p22.2	92.442 to 100.633	8.2
Loss	3	p24.3	20.787 to 27.079	6.3
Loss*	9	p21	20.111 to 26.454	6.3
Loss homozygous	9	p21.3	20.99 to 21.99	1
Loss	10	pter-q21.1	0.138 to 61.335	61.2
Loss	11	q22.1-qter	97.267 to 133.951	36.7
Loss	13	entire chromosome	16.5 to 114	97.5
Loss	14	q31.1-qter	79.806 to 106.311	26.5
Loss	22	q arm	11.8 to 49.6	37.8
GER-8M imbalances p13	**Chromosome**	**Region**	**Mb position**	**Size Mb**
Gain	15	q11.2-q12	22.3 to 23.8	1.5
Gain	19	q12-q13.12	36.4 to 41.2	4.8
Loss	1	p arm	1 to 124	124
Loss	2	q22.1	183.8 to 186.7	2.9
Loss	2	q34	210.3 to 211.4	1.1
Loss	3	p26.3-p14.3	1 to 54.4	54.4
Loss	3	q11.1-q13.13	91.7 to 113.5	12.2
Loss	3	q22.3-q23	140.6 to 143.2	3.4
Loss	3	q26.3-q28	183.2 to 192.5	9.3
Loss	4	entire chromosome	1 to 191	191
Loss	5	q11.1-q14.3	47.7 to 84.4	36.7
Loss	6	q14.1-q27	79.6 to 170.0	90.4
Loss	9	p arm	1 to 51.5	51.5
Loss homozygous	9	p21.3	20.99 to 21.99	1
Loss	10	p15.3-p14	1 to 11.2	11.2
Loss	13	entire chromosome	16.5 to 114	97.5
Loss	14	q22.12-q32.33	91.44 to 106.0	14.5
Loss	17	p arm	1 to 23.1	23.1
Loss	19	q12	34.8 to 36.3	1.5
Loss homozygous	19	q13.12	42.2 to 43.4	0.8
Loss	21	q arm	12.3 to 46.9	34.6
Loss	22	q arm	11.8 to 49.6	37.8

For sample 1 of JL1, we report four partial deletions and 3 duplications while sample 2, representing a longer time culture, shows a higher rate of partial deletions (8 regions concerned) and only 2 partial duplications. Complete deletion of chromosome 13 was observed in sample 2 for JL1 and GER-8M (Table 1). The GER-8M showed more deletions than duplications. Most of the imbalances were partial; only chromosomes 4 and 13 were completely deleted (Table 1, 2).

Table 2. Summary of the genomic losses in the epithelioid JL1 cell line compared with the epithelioid predominant GER-8M cell line. (*) heterozygous deletion.

JL-1 genomic imbalances	**Chromosome**	**Region**	**Mb position**	**Size Mb**
Sample 1 (p48)				
Loss	3	p24.3	20.787 to 27.079	6.3
Loss*	9	p21	20.111 to 26.454	6.3
Loss (homozygous)	9	p21.3	20.99 to 21.99	1
Loss	14	q31.1qter	79.806 to 106.311	23.5
Loss	22	q arm	11.8 to 49.6	37.8
Sample 2 (p55)				
Loss	3	p24.3	20.787 to 27.079	6.3
Loss*	9	p21	20.111 to 26.454	6.3
Loss (homozygous)	9	p21.3	20.99 to 21.99	1
Loss	13	entire chromosome	16.5 to 114	97.5
Loss	14	q31.1-qter	79.806 to 106.311	26.5
Loss	22	q arm	11.8 to 49.6	37.8
GER-8M genomic imbalances (p13)	**Chromosome**	**Region**	**Mb position**	**Size Mb**
Loss	3	p26.3-p14.3	1 to 54.4	54.4
Loss	4	entire chromosome	1 to 191	191
Loss (homozygous)	9	p21.3	20.99 to 21.99	1
Loss	13	entire chromosome	16.5 to 114	97.5
Loss	14	q22.12-q32.33	91.44 to 106.0	14.5
Loss	22	q arm	11.8 to 49.6	37.8

The common losses of JL1 and GER-8M were total chromosome 22 monosomy, small imbalances identified for 14q31.1-qter (size of 26.5 Mb), 3p24.3 (size of 6.29 Mb) and homozygous deletion of 9p21 region (Table 1, 2).

The 9p21.3 common deletion was identified for both JL1 and GER-8M (Fig.1-2, Table 1-2) with a similar 1 Mb homozygous deletion (between position 20.999 and 21.999 Mb) located close to the MTAP that encodes methylthioadenosine phosphorylase [(Fig. 1 & 2), black arrow]. In both samples of the cell lines, this homozygous deletion (9p21.3) was flanked by a 6.3 Mb heterozygous deleted region (between positions 20.111 and 26.454 Mb) (Fig. 1 & 2). However, the array-CGH and FISH results suggest that in GER-8M, some homozygous deletions affect putative tumour suppressor gene(s) on chromosome 4 that have been implicated in the pathogenesis of MM and many other tumours. Previous cytogenetic studies have reported partial or complete loss of chromosome 4 in MMs as one of the frequent karyotypic changes in tumour cell lines [2, 6, 23, 24]. The DM3 cell line, a sarcomatoid form and the mixed preponderant form PJ2, were analyzed by the same approach and gave, according to the resolution of the array, a normal genomic content.

The other DNA copy number gains reported for the pure and mixed epithelioid cells were detected by the array-CGH technology as mosaicism with additional trisomies and duplications in JL1-samples 1 and 2 (not shown). The most frequent chromosomal losses in the sarcomatoid MM have been reported in [12]. Unfortunately, only a small number of sarcomatoid deletions have been documented in the fibroblastic form studied here.

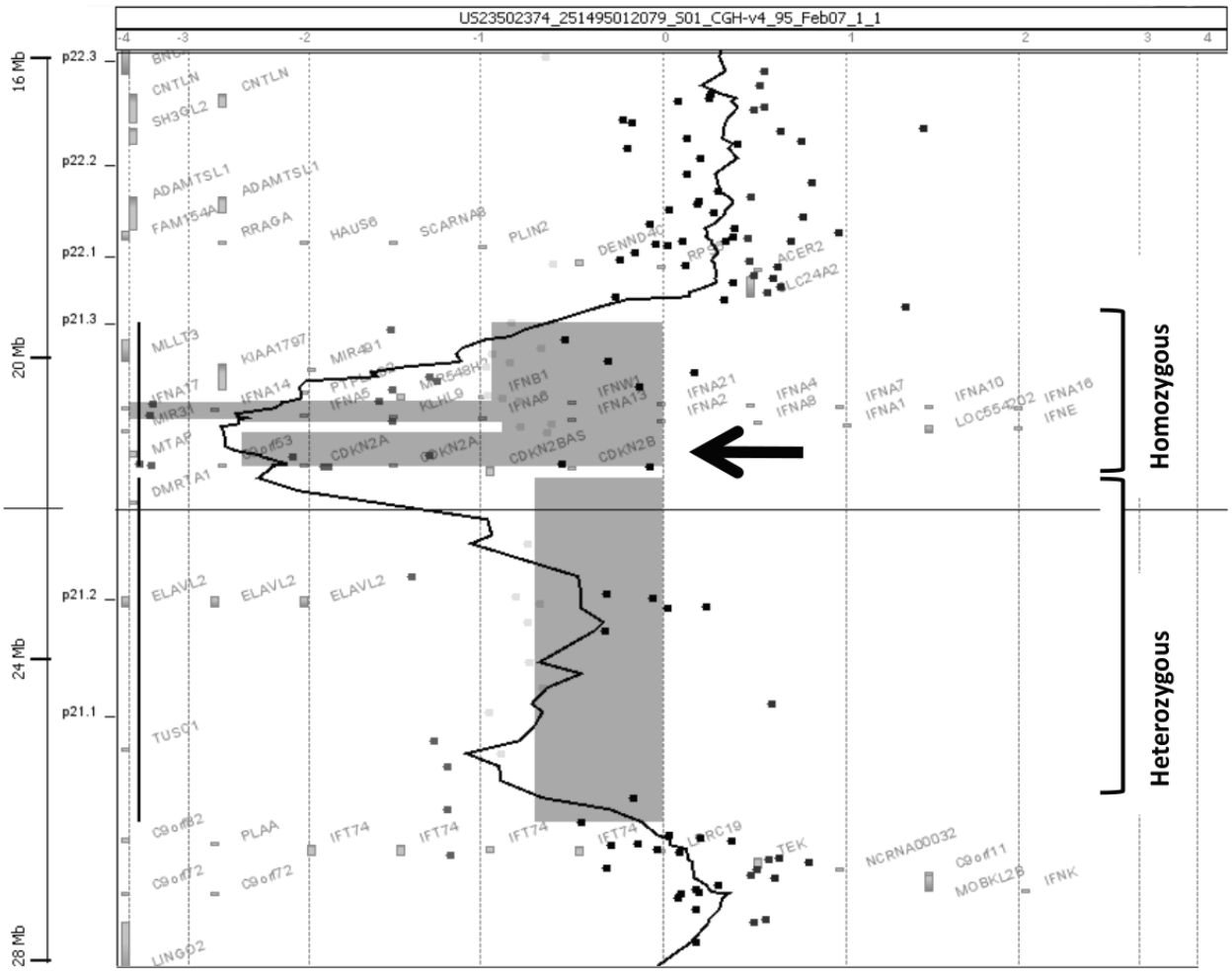

Fig. 1. Array CGH profile of the deleted region on chromosomal band 9p21.3 for JL1 cell lines. The deletions are indicated by the shaded grey region which reflect a deviation from the log2 ratio of zero. Two contiguous regions are visible: a homozygous deletion (see black arrow) flanked by an extended heterozygous region.

Differentiating between the two cell types can be difficult according to the number of acquired genetic events, especially deletions which lead to the inactivation of multiple tumour suppressor genes.

Given the commercial availability of a FISH probe to 9p21, we decided first, to test this marker for potential utility in order to evaluate the frequency of eventual TSG 9p21 abnormalities in each cell population compared to the normal counterpart. Secondly, to determine how p16INK4a, p15INK4b (CDKN2B) and p14ARF expression is correlated, we analyzed protein expression by FACS analysis and mRNA levels using TaqMan real-time quantitative polymerase chain reaction (RT-PCR).

FISH results are shown in Fig. 3. Normal mesothelial cells are indicated in Fig. 3A and 3B. Homozygous deletion was observed using an epithelioid form (purified from the mixed form, GER-8M) where no signal could be observed in the nucleus (not shown). Heterozygous deletion was identified in JL-1 as shown by the presence of one fluorescence signal (Fig. 3C). Analysis of the sarcomatoid form revealed multiple abnormalities with zero to three signals being detected in different cells (Fig. 3D). TP53 was present in both cell types (not shown).

FACS analysis of two different cell lines (DM3 and RS5) confirmed the loss of p14ARF protein expression in sarcomatoid cells with complete or

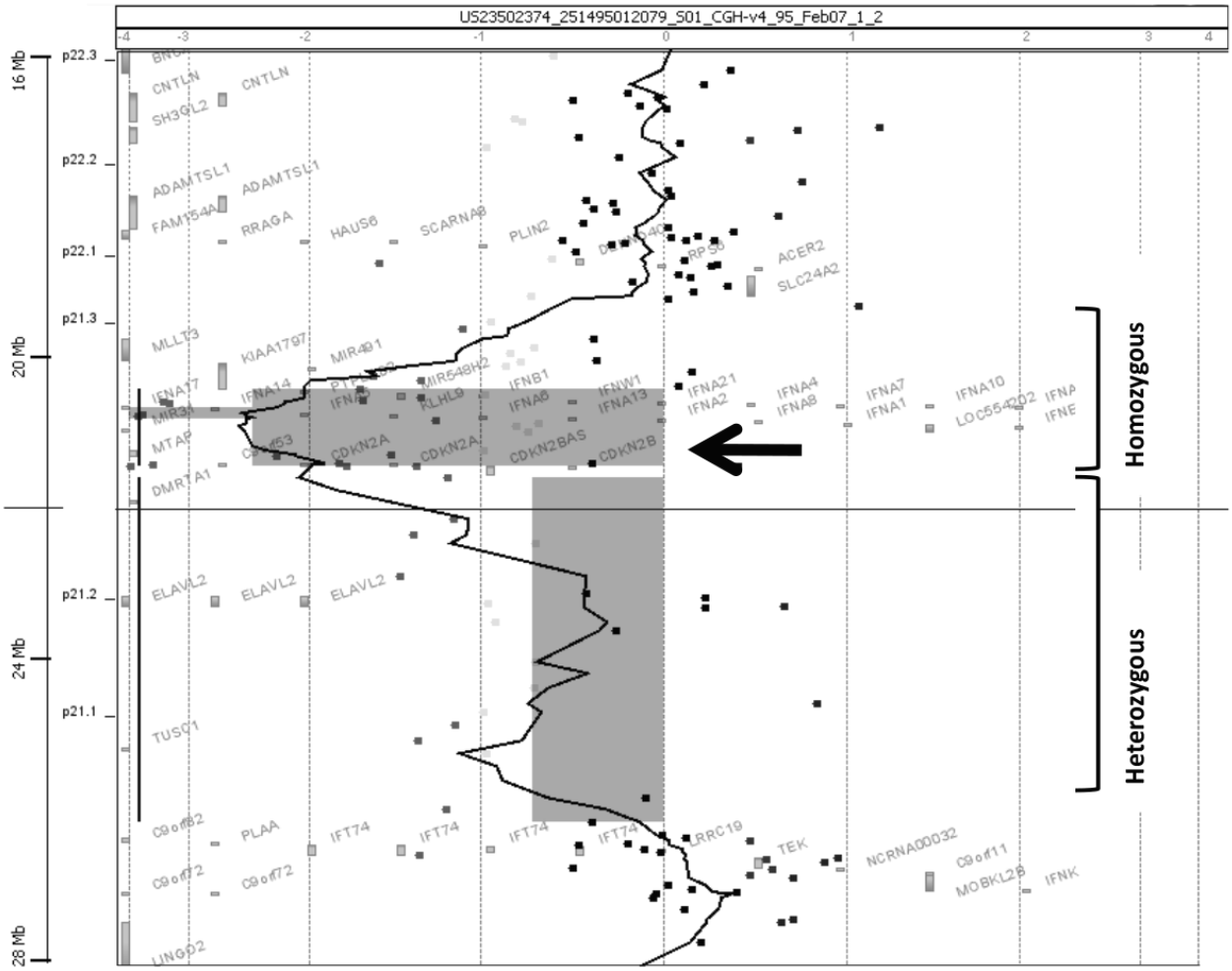

Fig. 2. Array-CGH profile of chromosome 9 showing the deletions in 9p21.3 in GER-8M purified from a mixed epithelioid dominant cell line. The deletions are indicated by the shaded grey regions as for the Fig. 1. As seen in JL1, two different deletions are detected with one homozygous indicated by the black arrow flanked by an extended heterozygous deleted region.

partial loss depending of the cell lines, not visible with other techniques used (Fig. 4A and 4B). Epithelioid lines express this protein but were negative for p16INK4a (Fig. 5A) as also observed in Taqman by RT-PCR (not shown). p15INK4b and methylation (MTAP) analysis did not reveal any abnormality in either cell types (Fig. 4 and 5). Positive expression was obtained for normal mesothelial cells compared to tumour cells (Fig. 5B).

DISCUSSION

The present study revealed a clear relationship between two histological groups of mesothelioma, i.e. the pure epithelioid cells and the mixed form with epithelioid predominance. Both cell lines share common aberrations such as monosomy of chromosome 13 and 22, as well as sub-regional imbalances for 3p24.3, 14q31.1-qter and homozygous deletion of the 9p21.3 region detected in primary cell lines. These aberrations were specifically associated with the transformation of normal cells to the epithelioid form of mesothelioma indicating that multiple abnormalities may contribute to the dysfunction of TSG in MM. No changes were observed in the sarcomatoid specimens by Array-CGH and Taqman, whereas surprisingly FISH and FACS analysis revealed heterozygote deletion of the 9p21.3 region, detected in cell lines selectively

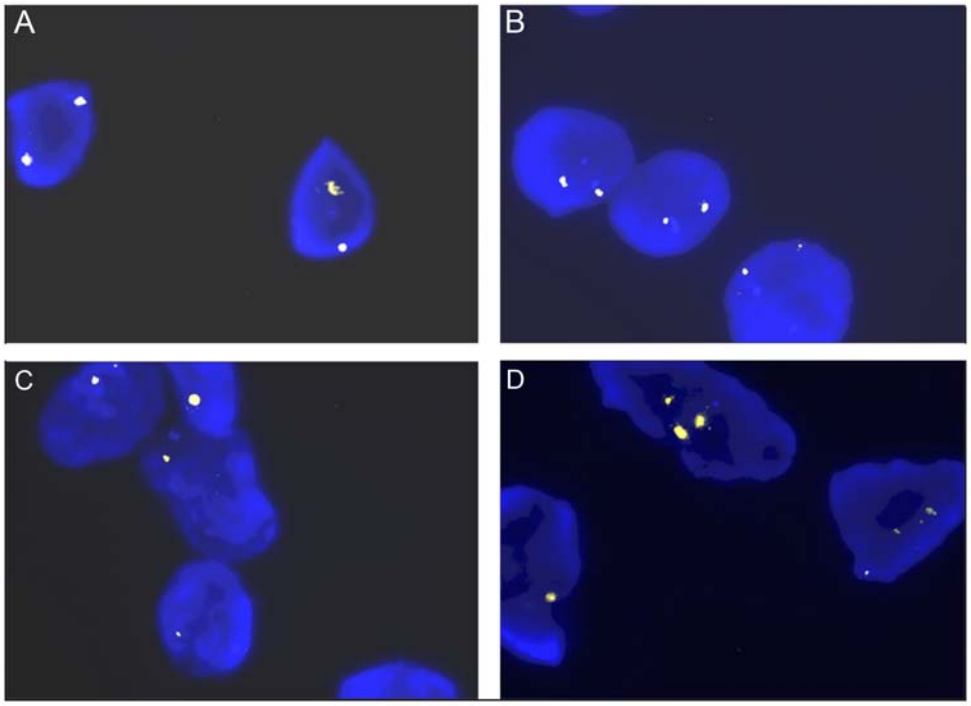

Fig. 3. Fluorescence *in situ* hybridization (FISH) using a gene-specific probe for 9p21 (P16). Control normal mesothelial cells shows two visible yellow gene-specific signals in two different cell populations (3A, B). Using the same probe 9p21, FISH reveals a deletion showing one yellow gene-specific signal in the epithelioid cells (3C); and one to three signals of yellow gene-specific signals in the sarcomatoid form of mesothelioma (3D), indicating tumor heterogeneity.

associated with the transformation of fibroblastic cells to the sarcomatoid forms of mesothelioma.

Many abnormalities may affect TSG such as loss of p16INK4a in the epithelioid cells and p14ARF in the sarcomatoid forms. Several groups have suggested that accumulation of multiple genetic alterations might be required before a mesothelial cell is definitively converted into a neoplastic cell [4, 9, 15]. Recurrent deletion in the CDKN2A gene affecting p16INK4a isoform production has been observed. Evidence for the susceptibility to the presence of $SV_{40}T$ antigen was not detected in our cell lines [11] and FISH analysis express a functional TP53 locus. MM is usually wild type for the TP53 gene, but contain homozygous deletions in the CDKN2A locus that encodes p14ARF, an inhibitor of p53-MDM2 interaction [25]. The TP53 gene, which encodes p53, is one of the most mutated genes in human cancer [26]. In this study, p53 is functional in MM in the absence of p14ARF. The role of TSG is to maintain the normal cell cycle and genomic integrity [12]. Aberrations in TSG CDKN2A, leading to loss of p14ARF isoform, is common in MM. The deletion of p14ARF interrupts the p53/MDM2 pathways which is important in cancer. Others [14] suggest the possibility of inducing the loss of p14ARF using an adenoviral vector in order to restore the genetic alteration of TP53. Hopkins-Donaldson *et al.* demonstrated that p53 can induce the transcription of target genes, and contribute to the apoptotic response in the presence of cisplatine (cis-diammine-dichloro-platinum) (CDDP) [25]. A difference was shown in the response to DNA damaging agents such as CDDP that induce apoptosis by way of the p53-specific death in mesothelioma [25]. Moreover, spontaneous inactivation of p16INK4a, or inactivation in conjunction with other therapeutic modalities, could be potentially used as an effective treatment for MM [27]. A recent study has shown that p16INK4a inactivation leads to increased levels of p53 in human mammary epithelial cells, indicating that p16INK4a simultaneously plays an anti-apoptotic role [27]. In our study, FISH confirms

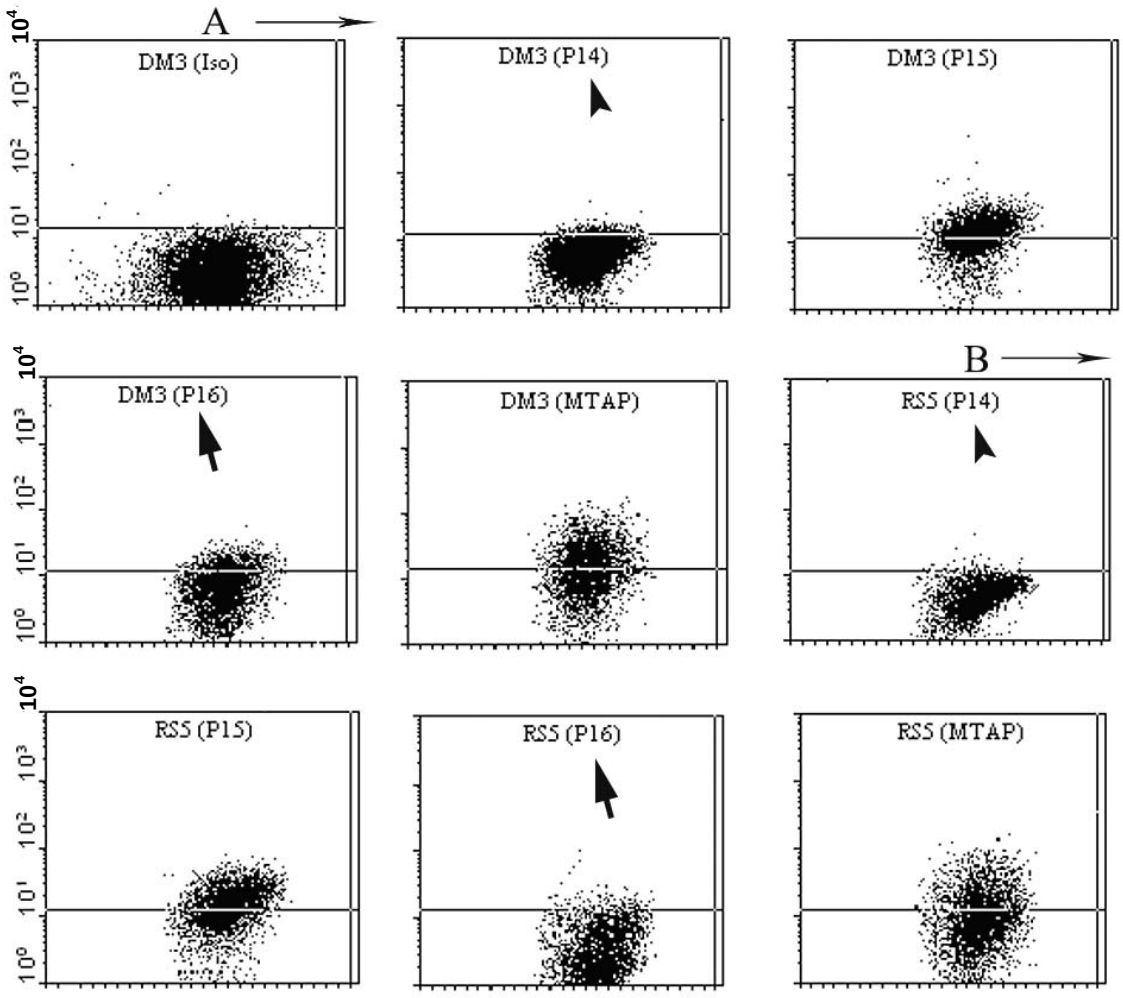

Fig. 4. Flow cytometric analysis of two different sarcomatoid cell lines (A and B) reveals a deletion at the level of p14 which was effective on both cell types (DM3 and RS5) of mesothelioma (see arrowheads), whereas p16 expression shows a concomitant expression of these lines (small arrow). No abnormality was observed in the two cell types for p15 and MTAP expression.

the presence of an intact TP53 locus in the absence of p14ARF or p16INK4a. These observations reflect a combined expression of p14ARF and p16INK4a that may together modulate tumour suppression, dependent of the cell type.

In human mesothelioma, frequent losses of chromosome 22 indicate that this chromosome includes genes potentially implicated in the control of tumour progression [12, 13, 28]. Others hypothesized that co-deletion of two tumour suppressor genes NF2 (Neurofibromatosis type 2) and OSM (Oncostatin M) located on chromosome 22 may predispose patients to develop malignant tumours. NF2 disease does not usually occur with MM, but the risk for MM development may increase if an NF2 patient exposed to asbestos loses the NF2 function [29, 30]. Both partial loss and monosomy of chromosome 22 have been previously observed in mesothelioma [10]. Monosomy of chromosome 13 observed in both of our epithelioid cell lines and other types of tumours raise the possibility that genomic instability itself may be independently predictive of tumour progression [12, 28].

Sub-regional 3p24.3 heterozygous deletion encompasses 13 genes among which several are potential tumour suppressors. The gene NKIRAS1 (NM 020345.3) belongs to a subclass of

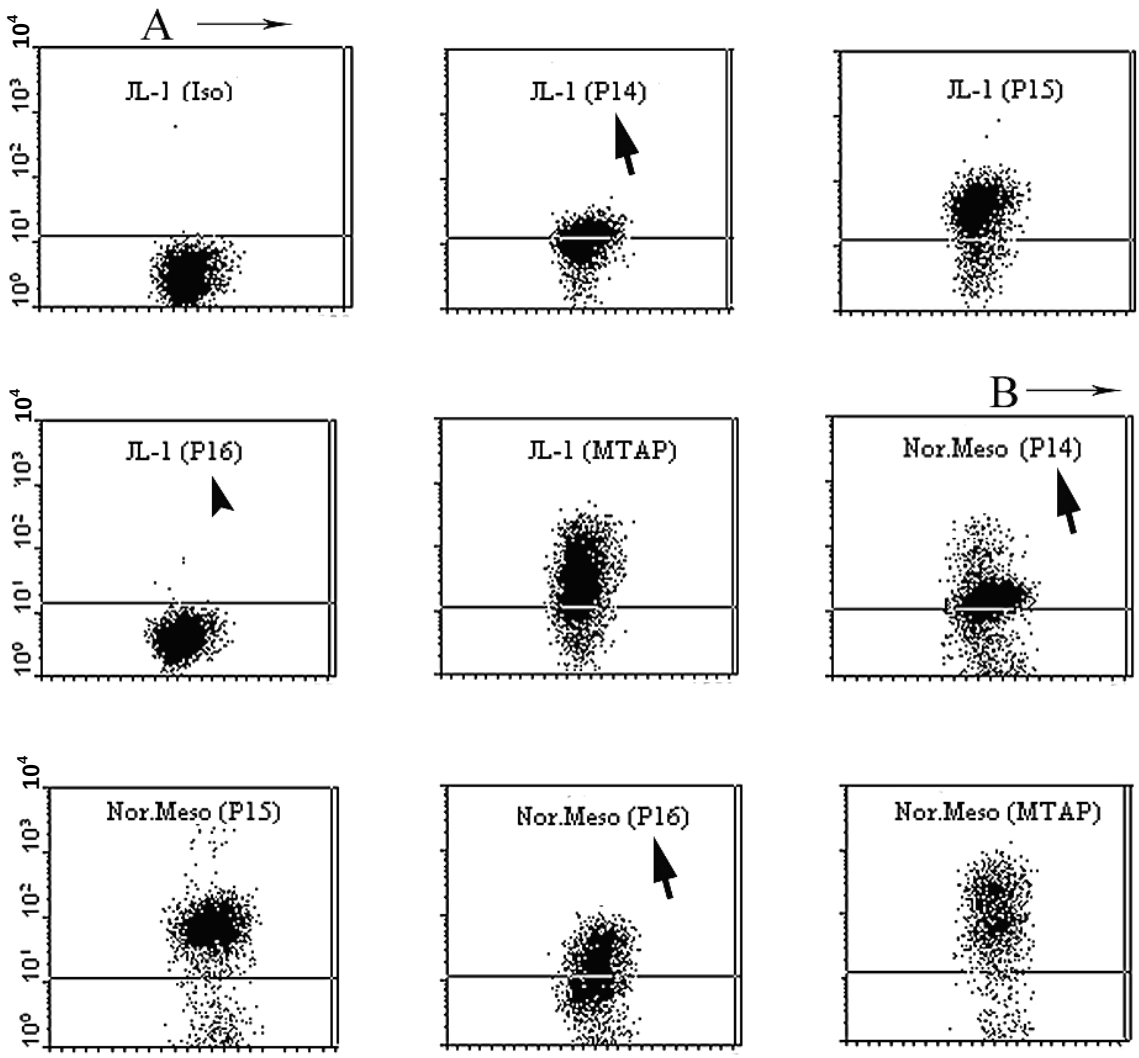

Fig. 5. Flow cytometric analysis of epithelioid form of mesothelioma was performed comparing their normal counterpart mesothelial cells for their expression of the p16, p14 and p15 proteins. (A) Epithelioid cell lines present a complete p16 deletion (arrowheads) with a positive expression for p14 (small arrows). No abnormality was observed in these cells for p15 and MTAP expression. (B) Normal epithelial cells were not affected by any abnormality. This experiment is a representative data from three similar results.

evolutionarily conserved Ras-like proteins which differ from other Ras proteins in that they contain amino acids at positions 12 and 61, which are identical to those present in the oncogenic forms of Ras. The mammalian Ras proteins are important in the regulation of numerous cellular pathways [28, 31]. The second interesting gene in the 3p24.3 deletion is RARB (Retinoic acid receptor beta) (NM 001290216.1), a member of the nuclear receptor superfamily, first identified in a hepatocellular carcinoma where it surrounds a hepatitis B virus integration site [32]. The heterozygous loss of 14q31.1-qter implicates a large segment of 26.5 Mb encompassing more than 150 genes. The region 14q32.13 was previously reported as a recurrent deletion detected in 11 of the 22 human mesothelioma [24]. Homozygous deletions in 9p21.3 were detected in various cancers [33] and the CDKN2A locus is known to induce inactivation of TSG residing in these deleted chromosomal regions [34].

The two heterozygous deletions in JL1 (Fig. 1) were contiguous to the homozygous deletion of 9p21.3. The first segment deleted, includes the

gene MLLT3 (Homo sapiens myeloid/lymphoid or mixed-lineage leukemia, (MLLT) 001286691,1) the most common fusion partner for the MLL gene in translocations t(9;11)(p22;q23) and p14ARF (CDKN2A) in the sarcomatoid forms associated with acute myeloid leukemia and acute lymphocytic leukemia [33-35]. The second heterozygous deletion includes the gene TUSC1 (NM 0011004125.2) that is described as down-regulated in non-small-cell lung cancer and small-cell lung cancer cell lines, suggesting that it plays a role in lung tumourigenesis [20, 33]. Recently the gene FUS1/TUSC2 located on chromosome 3p21.3 was described as a major key gene in mesothelioma showing a tumouregenic transcriptional effect with 42 TUSC2 targets which proved to be concordantly modulated in MM [19, 36].

With regard to the sarcomatoid lines and their mixed predominant form, no genomic abnormality was revealed but according to previous studies [13], we cannot exclude the smallest regions of genomic imbalances (< 80 kb) whatever the method used. FACS analysis confirmed a deletion at the level of p14ARF but not of the p16INK4a present on these cells suggesting not only a common phenotype for both sarcomatoid cell lines but a dependence on an eventual alternative cooperation and regulation, based on the inactivation of p16INK4a for the epithelioid form in which two important products encoded by CDKN2A, are essential for controlling tumourigenesis. More detailed analysis of p14ARF will be needed to better understand the contribution of this protein in the development of the sarcomatoid form in mesothelioma.

As anticancer agents are rarely selective for tumour cells, utilization of specific modalities in comparative investigations may help in standardizing a prognostic scoring system, which would assist in selecting patients for appropriate clinical trials and for some degree of comparison between concerned populations for the same treatment.

CONCLUSION

These results confirmed two potential genetic alterations in MM: p16INK4a in the epithelioid cells and p14ARF for the sarcomatoid form. They are probably the most sensitive targets in the 9p21 regions and these alterations are probably dependent on parental sensitivity to induce a common disease. Furthermore, expression of this analysis assumes independent markers, which are focused on interesting regions of the genome. Identification of such alterations that contribute to susceptibility to the same disease in two different cell types may help in the development of precise diagnostic and therapeutic approaches for mesothelioma.

Authors' contributions

MMP designed, performed and supervised the research; LB proposed the array-CGH method; FB performed, supervised this analysis and wrote a part of the genetic version; TAM supervised FISH and Taqman analysis and critically reviewed the manuscript; SG, FM performed the microarray analysis; MMP supervised, performed FACS and FISH analysis; CI reviewed the manuscript; MMP wrote, critically reviewed the manuscript and gave the final approval to the version to be published.

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CONFLICT OF INTEREST STATEMENT

There is no potential conflict of interests with any company/organization whose products or services may have been discussed in this article.

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