ORIGINAL ARTICLE

Comparative genomic hybridisation in malignant deciduoid mesothelioma

A Scattone, A Pennella, M Gentile, M Musti, P Nazzaro, A L Buonadonna, A Marzullo, D Cavone, L Pollice, G Serio

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See end of article for authors' affiliations

Correspondence to:
Dr G Serio, Department of
Pathology, Medical
School, University of Bari,
11 G Cesare Square,
70124 Bari, Italy; g.serio@
anatopat.uniba.it

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Background: Malignant deciduoid mesothelioma is a rare variant of epithelioid mesothelioma. This tumour generally has poor prognosis, and can be asbestos related.

Aim: To identify peculiar genetic changes responsible for critical phases in pathogenesis of malignant deciduoid mesothelioma and their prognostic relevance.

Methods: Comparative genomic hybridisation was carried out in six cases of malignant pleural deciduoid mesothelioma, four sporadic and two familial. All cases were found to be asbestos related. Four patients died during follow-up and the mean survival was 29.5 (SD 14.2, range 12–43) months.

Results: Genetic abnormalities were found in all the tumour tissues, the most frequent being chromosomal gains at 1p, 12q, 17, 8q, 19 and 20 and losses at 13q, 6q and 9p. Survival was found to be longer in those patients who presented a smaller number of losses (≤ 2) in the tumorous chromosomes.

Conclusions: Although numerous genetic changes are presented by deciduoid mesotheliomas, certain chromosomal regions are preferentially affected. The clinical outcome for this mesothelioma subtype is predicted by the number of losses.

Malignant mesothelioma, a tumour of the serous membranes that is generally asbestos related, is highly aggressive and largely unresponsive to current treatments. Survival is related to different mesothelioma subtypes and, in particular, rare variants of malignant mesothelioma have a heterogeneous clinical outcome in terms of disease-free and overall survival.^{1–5}

A deciduoid mesothelioma variant has recently been recognised,⁵ which was originally described in the peritoneum of young women.^{6–7} It has since been observed also in the pleura and pericardium, in both sexes and at all ages, with these forms having a worse prognosis.^{8–24}

Only a few studies have been conducted to identify the molecular changes responsible for the carcinogenesis of mesothelioma.^{25–40} Comparative genomic hybridisation (CGH) is currently the most widely used technique for genetic analysis of mesothelioma because it enables the analysis of wide genomic amplifications, gains and losses (deletions) in the tumour cells in a single experiment.^{41–42}

Molecular cytogenetic studies that used karyotyping and CGH analyses showed frequent losses of specific regions in chromosome arms 1p, 3p, 6q, 9p, 13q, 15q and 22q in some malignant mesothelioma series. Deletions are the most common cytogenetic aberrations. This suggests a recessive mechanism in the oncogenesis of malignant mesothelioma and emphasises its morphological heterogeneity.^{25–40}

CGH analyses have focused on analytical description of chromosomal imbalances, but only a few of them described the relationship between chromosomal imbalances, clinicopathological features and the overall survival data.³⁹

To date, no investigation on the genetics of deciduoid mesothelioma has been reported. We present the results of CGH analysis carried out on six cases of pleural deciduoid malignant mesothelioma that were characterised by longer survival.

MATERIALS AND METHODS

Our study comprised six patients (three men and three women; mean age 54.2 (SD 22.3, range 23–74) years), four

with pleural malignant deciduoid mesothelioma recently diagnosed in our institution and two with malignant deciduoid mesothelioma, reported previously.²⁴ Of the six, two patients (both women) were affected by familial forms and the other four by sporadic forms of the disease.

Thoracoscopic biopsies were carried out in all cases. The tissues obtained were fixed in formalin and embedded in paraffin wax for routine examination.

Slides were stained with haematoxylin–eosin, periodic acid–Schiff with or without diastase digestion and alcian blue with or without hyaluronidase digestion.

In each case, selected blocks were processed for immunohistochemical analysis using the Envision method (Dako, Denmark), and stained with the Dako Techmate automatic stainer.

The sections were subjected to the labelled avidin–biotin–peroxidase complex (ABC) method after pretreatment in a pressure cooker.⁴³ Positive and negative control tissue specimens were used to evaluate antibody specificity.

A wide panel of immunohistochemical markers was used, including AE1/AE3 (Dako, Glostrup, Denmark; 1:50); CAM 5.2 (Becton Dickinson, San Jose, California, USA; prediluted) and 5/6 (Zymed, San Francisco, California, USA; 1:100) cytokeratins; epithelial membrane antigen (Dako; 1:75); human bone marrow endothelial (cell)-1 (Dako; 1:80); calretinin (DBA, Milan, Italy; 1:3000); monoclonal carcinoembryonic antigen (Dako; 1:25); Leu-M1 (Becton Dickinson, San Jose, California, USA; 1:40); vimentin (Dako; 1:300); S-100 protein (Dako; 1:200); desmin (Dako; 1:50); and MIB-1 (Dako; 1:100).

Molecular analysis

DNA extraction

Normal sex-matched DNA was prepared from peripheral blood lymphocytes (Nucleon BACC3; Amersham Pharmacia Biotech, Bucks, UK).

Abbreviation: CGH, comparative genomic hybridisation

Table 1 Clinical data of six patients with deciduoid malignant mesothelioma

Patient	Age (years)	Sex	F/S	Site	Pain	Effusion	Asbestos exposure	Stage (IMIG system)	Treatment (chemotherapy)	Survival (months)
1	23	F	F	Left pleura	Yes	Yes	Environmental	T1NxMx	Palliative (talco pleurodesis)	39*
2	73	M	S	Right pleura	No	Yes	Occupational	T2NxMx	Cisplatin-gemcitabine	43*
3	71	M	S	Right pleura	Yes	Yes	Occupational	T2NxMx	Cisplatin-gemcitabine	38
4	32	F	F	Bilateral and trans-diaphragmatic pleura	Yes	Yes	Environmental	T4NxMx	Palliative	24*
5	52	F	S	Right pleura	No	Yes	Environmental	T2NxMx	Cisplatin-gemcitabine	24
6	74	M	S	Right pleura and chest wall	Yes	Yes	Occupational	T3NxMx	Cisplatin-gemcitabine	12*

F, familial; IMIG, International Mesothelioma Interest Group; S, sporadic.

*Died of disease progression.

For each case, a 5- μ m section was stained with haematoxylin-eosin and used as a guide to identify and manually microdissect, under an inverted microscope, suitable tumour areas from serial sections.

After incubation in xylene and rehydration in ethanol, microdissected cells were collected into an Eppendorf tube, resuspended in 100 μ l of extraction buffer (50 mM TRIS-HCl pH 8.5, 1 mM EDTA, 0.5% Tween 20) and 200 μ g/ml of proteinase K, and incubated overnight at 37°C. The digested sample was then incubated at 95°C for 8 min. To inactivate proteinase K, DNA was precipitated with ethanol.

Degenerate oligonucleotide primer-PCR

Degenerate oligonucleotide primer (DOP)-PCR was carried out in two steps, according to the method of Huang *et al.*⁴⁴ The DOP-PCR products were precipitated with ethanol before labelling and resuspended in distilled water.

CGH

The DOP-PCR products and normal DNA were labelled with a nick translation Vysis kit (Naperville, Illinois, USA) according to the manufacturer's instructions. The tumorous DNA samples were labelled with Spectrum Green dUTP (Vysis, Downers Grove, Illinois, USA) and reference DNA was labelled with Spectrum Red dUTP (Vysis). The nick translation reaction was monitored to obtain a probe size of 600–2000 bp, confirmed by electrophoresis of 5 μ l of the product in a 1% agarose gel.

Spectrum Green-labelled test DNA (400 ng), Spectrum Red-labelled reference DNA (200 ng) and 8 μ g Cot-1 DNA (Roche Diagnostics GmbH, Mannheim, Germany) were combined and precipitated in the presence of 0.1 volumes of 3 M sodium acetate and 2.5 volumes of absolute ethanol. The hybridisation mix was centrifuged and the supernatant removed and air dried. The pellet was redissolved in 3 μ l purified water and 7 μ l CGH hybridisation buffer (Vysis) at 37°C for 5 h. Before hybridisation, normal male metaphase slides (Vysis) were denatured in 70% formamide/2 $\times$ standard saline citrate at 68°C for 2 min and dehydrated in ice-cold 70%, 85% and 100% ethanol. Probes were denatured at 73°C for 5 min. Each probe was applied to the slide and covered with a 20 $\times$ 20 mm coverslip, sealed with rubber cement and hybridised in a moist chamber at 37°C for 3–4 days. After hybridisation, the slide was washed twice in 0.4 $\times$ standard saline citrate or 0.3% non-ionic detergent (Igepal CA-630, Sigma, St Louis, California, USA) at 73°C for 2 min, in 2 $\times$ standard saline citrate or 0.1% non-ionic detergent at room temperature for 5 min, and in water at room temperature for 5 min. The slide was counterstained with 0.4 μ g/ml 4',6-diamidino-2-phenylindole (Sigma, Milan, Italy) in an antifade Vectashield solution (Vector Laboratories, Burlingame, California, USA).

Digital image analysis

Metaphases that showed uniform and intense hybridisation and contained well-separated chromosomes were captured with a cooled charge-coupled device camera (Photometrics, Tucson, Arizona, USA) mounted on a Leitz DM RBE (Leica, Wetzlar, Germany) fluorescence microscope. A 100 $\times$ Plan fluotar objective (numerical aperture 1.30, oil) was used to capture the images. Three-coloured digital images (green for the green spectrum, red for the red spectrum and blue for 4',6-diamidino-2-phenylindole) were acquired from at least 10–15 metaphases for hybridisation with a filter wheel containing appropriate excitation filters for Spectrum Green, Spectrum Red and 4',6-diamidino-2-phenylindole fluorochromes. The PSI digital imaging system (software MacProbe V.4.1, Perceptive Scientific Imaging, League City, Texas, USA) was used for calculation of the green-to-red fluorescence ratio for each chromosome. Chromosomes were identified on the reverse 4',6-diamidino-2-phenylindole-banding images. The calculated average ratio was plotted along with the ideogram of each corresponding chromosome. To identify chromosomal imbalances, the ratios 1.25 and 0.75 were used as cut-off values for gains and losses, respectively. The Cot-1 DNA included in the hybridisation inhibited the binding of labelled DNA to the centromeric and heterochromatic regions, and thus these regions were not analysed. Chromosomes that were heavily bent or overlapping or had overlying artefacts were excluded from the analysis. A careful interpretation was made, as recommended by Kallioniemi *et al.*^{41, 42} in the telomeric and heterochromatic regions. False-positive DNA gains at chromosomes 1p32-pter, 16q21 and 19 have been reported.^{42–45} Therefore, direct labelling of probes, which seems to diminish these problems considerably, was used in our protocol.⁴⁶

Chromosome 19 gain has been reported in malignant mesothelioma.⁴⁷ It was therefore decided that these regions should not be excluded from our analysis, even though such gains should be interpreted with caution.

RESULTS

Table 1 shows clinical data of patients with deciduoid malignant mesothelioma. Exposure to asbestos was documented in all cases registered at the Apulia Regional Operative Centre of the Italian National Register of Mesotheliomas,⁴⁸ and was recognised as occupational in men and environmental in women. At the time of retrospective analysis, four patients had died (mean survival 29.5 (SD 14.2) months) and two survived (mean survival 31 (SD 9.9) months). Four patients received adjuvant chemotherapy (six courses of cisplatin and gemcitabine in combination). No patient underwent any type of resection (ie, extrapleural pneumonectomy or a pleurectomy/decortication). Staging was carried out by preoperative computed tomography,

Figure 1 Histology of the deciduoid mesothelioma (case 3, haematoxylin-eosin staining, 250 $\times$).

according to the International Mesothelioma Interest Group-system criteria.⁴⁹

Pathology

Histologically, tumour cells were large, polygonal and ovoid, with an abundant eosinophilic cytoplasm, arranged in solid nests or in trabeculae. Occasionally, a tumour showed glandular and papillary structures. The cytoplasm of tumour cells contained diastase-digested granules positive to periodic acid-Schiff. The nuclei were large, round and vesicular with prominent nucleoli (fig 1). All tumours were positive for cytokeratins, calretinin, vimentin, epithelial membrane antigen and human bone marrow endothelial (cell)-1 antibodies. Moderate mitoses (2–5/10 HPF) were observed and the mean value of the Ki-67 labelling index, using the MIB-1 monoclonal antibody, was 26.5% (case 1, 18%; case 2, 19%; case 3, 18%; case 4, 31%; case 5, 33%; case 6, 40%).

Deciduoid mesotheliomas were diagnosed on the basis of the morphological appearance and immunoreactive profile.

Genetic findings

CGH analysis showed chromosomal abnormalities in all the cases. Table 2 lists the results for the mesotheliomas. The mean number of chromosomal aberrations was 21.3 (SD 8.8, range 11–37); the mean number of gains was 18.3 (SD 10.1, range 6–36) and mean number of losses was 3 (SD 1.7, range 1–5). Figure 2 shows the chromosomal aberrations in the four cases we diagnosed recently and in the two previously reported cases.

The most frequent gains were at 1p (83.3%), 12q (83.3%), 17 (83.3%), 8q (66.6%), 19 (66.6%) and 20 (66.6%). Frequent losses were observed at 13q (66.6%), 6q (33.3%) and 9p (33.3%). The abnormalities affected entire chromosomes or chromosomal arms. Subregional imbalances were detected at 1p (gains at 1p31.3-pter), 12q (gains at 12q24.1-qter, 12q21.3, 12q21.1–q21.3, 12q21.3–q22), 17 (gains at 17q24-qter, 17q21.3–q22, 17q, 17q21.3-qter), 20 (gains at 20q12-qter, 20q), 13q (losses at 13q21.1–q22, 13q12.2–q14.1), 6q (losses at 6q12–q21, 6q12–q14) and 9p (losses at 9p22-pter, 9p21–p24).

Survival was longer (more than 3 years) in those cases that presented a smaller number of losses (≤ 2), although the sample size was insufficient for statistical analysis.

DISCUSSION

Conventional mesothelioma is an aggressive malignancy that can be caused by environmental carcinogens (asbestos and erionite), a virus (SV40) and a genetic predisposition.⁵⁰

Although this cancer is not common, an incidence has been noted in the past three decades that is distressing. Also, an accurate diagnosis is needed in view of the medicolegal aspects and prognostic implications. The World Health Organization classification⁵ lists the deciduoid variant among the epithelioid mesothelioma subtypes.

Of the 32 cases of deciduoid mesothelioma recognised up to now, 28 cases have been reported in the past 4 years.^{9–24} Initially, this variant was described by Nascimento *et al*,⁷ Talerman *et al*⁶ and Orosz *et al*⁸ in the peritoneum of younger women, as mimicking a florid ectopic decidual reaction. The tumour mainly affected women (female:male ratio 20:12) and exposure to asbestos was well documented in 12 cases. Ages ranged between 13 and 78 years (mean 49.3 years); 19 patients died (survival was 10.3 months; range 1–39) and 7 patients were alive at the end of follow-up (mean survival 22.7 months, range 8–60). Data are not available for six patients. Subsequently, the tumour was recognised in all the serosa, independent of sex, age and site. Only in one case was the pericardium found to be affected.¹⁹ A review of the literature does not show a worse prognosis in patients affected by the deciduoid mesothelioma variant.

We identified a total of six cases of pleural mesothelioma with deciduoid morphology, characterised by both longer survival and asbestos exposure. We used CGH to study whether deciduoid mesothelioma showed a specific genetic profile that could be useful for diagnosis, classification and prognosis.

In conventional mesothelioma, karyotypic and CGH analyses have shown frequent deletions of specific chromosomal regions in 1p, 3p, 6q, 9p, 13q, 15q and 22q.³⁷

In our study, the main chromosomal aberrations were located at 1p31.3-pter, 8q, 12q, 17q (gains) and at 6q, 9p, 13q, 15q (losses). Gains were more frequent than deletions. Many of these changes (gains and losses) coincide with those found in conventional mesothelioma and in other tumours, such as those of the breast, prostate or lung.^{24 27–30 37 39–41 46 51–55}

The chromosome most often associated with losses of DNA sequences was 13q, with two minimal regions identified at 13q12.2–q14.1 (sporadic cases) and 13q21.2–q22 (familial cases). Losses on chromosome 13q at region 13q21 have frequently been reported in the literature in a variety of cancers in humans, suggesting the existence of tumour-suppressor genes at this locus.⁵⁴ Molecular genetic approaches have already identified two tumour-suppressor genes in chromosome 13q: RB1 at 13q14 and BRCA2 at 13q12–13.^{36 37}

Losses on chromosome 6q were found only in our familial cases. They were at chromosome regions 6q12–q21 and 6q12–q14. Losses on 6q may be ascribed to a common event in both conventional mesothelioma and other tumours (breast, ovary, etc).^{28 34 40} Recently, three independent regions of common deletion have been found on chromosome 6q (6q14, 6q22 and 6q24). This finding suggests that three putative tumour-suppressor genes, mapped to chromosome 6q, may frequently be associated with the pathogenesis of mesothelioma.

A loss at 9p is another chromosomal aberration commonly found in sporadic and familial malignant mesothelioma.^{37 55} Molecular genetic approaches have already identified tumour-suppressor genes in this target region at 9p21: CDKN2A (p16INK4a and p14ARF).⁵⁶ In a familial cluster affected by malignant mesothelioma, Musti *et al*⁵⁵ found that the loss at 9p was the only change. Interestingly, these patients died after 2 and 4 years, which shows a longer survival.

A loss at 15q12 was reported by Balsara *et al*⁵² (15q11.1–15 minimal region) in 10 of 13 malignant mesothelioma cell lines. As reported in table 2, case 2 showed losses at chromosome 15 as the only genetic change and the patient survived for 43 months after diagnosis.

Figure 2 Profile analysis by comparative genomic hybridisation; green bars, gains; red bars, losses (cases 2, 3, 5, 6; for cases 1 and 4, see Serio *et al*²⁴).

Deletions at 9q and Yq were both found in case 3, and this finding is considered to be rare in conventional mesothelioma.³⁹

Although some CGH studies have been conducted on patients with malignant mesothelioma, no correlation has been reported between chromosomal changes and prognosis.

Table 2 Deciduoid malignant mesothelioma: detailed results of comparative genomic hybridisation in four new and two previously reported cases

Case	Losses	Gains	Total losses	Total gains	Total defects
1, Serio <i>et al</i> ²⁴	6q12-q21 , 13q21.1-q22	1p31.3-pter, 2q36-qter, 4p15.3-pter, 5p14-pter, 5q31.3-qter, 7q33-qter, 8q24.1-qter , 9q33-qter, 10q25.1-qter, 11q12-q13.3, 11q22.1-qter, 12q24.1-qter , 14q31-qter, 15q13-qter, 16q23-qter, 17, 19, 20, 22q	2	19	21
2	15p11.2-q12	1p32.2-pter, 1q22-q25, 1q31, 1q41-q43, 2p12-p13, 2p21-p23, 2qter, 3p13-p14.1, 3p23-p24.3, 3q22-q24, 4p13-pter, 5q33.1-qter, 6p11.1-p22.3, 7p11.2-pter, 7q31.3-q35, 8p11.2-pter, 8q23-qter , 9p11-p13, 9q22.1-q34.1, 10q23.1-q23.3, 11p11.1-q12, 12p11.2-pter, 12q21.3 , 14q23-qter, 15q21.3-qter, 16q23-qter, 17p11.2-pter , 17q24-qter, 18q21.1-qter, 19, 20, 21q21-qter, 22, Xq13-q21.3, Xq23-q27, Yq11.21-q11.22	1	36	37
3	9q12-q21.1, Yq11.22-q11.23	1pter, 1p21-p25, 1p31.3-p32.1, 2pter, 3q11.2-q13.1, 3q22-qter, 5p11.2-p14, 7q31.1-q31.3, 8q13-qter , 10pter, 11p15.3-pter, 12q21.1-q21.3 , 14q21-q22, 16pter, 16qter, 17q21.3-q22 , 18q12.2-q21.3, 19, 20q12-qter , Xp11.1-q21.1, Xq25-q26	2	21	23
4, Serio <i>et al</i> ²⁴	2q32.1-q33, 4p13-q21.1, 6q12-q14 , 9p22-pter , 13q21.2-q22 , 9p21-p24 , 13q12.2-q14.1 , 20p12-q13.2	1p31.3-pter, 4p15.3-pter, 5q33.2-qter, 8q24.1-qter , 10q25.1-qter, 11q12-q13.5, 11q22.2-qter, 12q24.1-qter , 15q22.2-q25, 16p11.2-p13.1, 17p11.2-p13, 17q , 19, 20q , 22q	5	15	20
5		1pter, 1q22, 1q25-q31, 1q43-qter, 3p12-q13.1, 3q27-qter, 6p11.1-q14, 7q21.3-q31.3, 9q12-q21.1, 12q21.3-q22 , 16p11.1-q11.2, 17q21.3-qter , Yp12-q11.2	3	13	16
6	1p35-pter, 13q12.3-q14.1 , 18p11.2-pter, 20q, 22q12.2-q12.3	1p11-q21.3, 7p13-q11.1, 7p15.1-p21, Xq13, Xq26-qter, Yq11.2-q12	5	6	11

The most frequent losses and gains are depicted in bold type.

Take-home messages

- Malignant deciduoid mesothelioma is a rare variant of epithelioid mesothelioma. This tumour generally has poor prognosis, and it can be asbestos related.
- Comparative genomic hybridisation was carried out in six cases of malignant pleural deciduoid mesothelioma, four sporadic and two familial.
- Genetic abnormalities were found in all the tumour tissues, being most often chromosomal gains at 1p, 12q, 17, 8q, 19 and 20. Losses occurred at 13q, 6q and 9p.
- Survival was longer in those patients who presented a smaller number of losses in the tumorous chromosomes (≤ 2).
- The number of losses is predictive of the clinical outcome for this mesothelioma subtype.

In our study, the specific genetic events and the number of genetic aberrations support the histological diagnosis of mesothelioma. The number and extent of genomic changes found in the examined cases show a strong correlation with stage and survival. The total number of aberrations and the total number of gains per sample were not associated with outcome. In contrast, the total number of losses per sample was associated with outcome, stage (T1–T2) and Ki-67-labelling index. In fact, in our series, the cases with a lower total number of losses survived for considerably longer periods (more than 3 years).

In agreement with Shia *et al.*²¹ our results and survival analysis do not tend to confirm a poor prognosis of this rare neoplastic subtype. In the literature, prognosis does not considerably differ from conventional mesotheliomas, and most patients with follow-up data died of disease progression in 3 years, mean survival being 13.2 months.^{8–24}

In conclusion, our results show the power of CGH as a means of screening for changes in DNA copy number in tumours with complex karyotypic abnormalities. Finally, the deciduoid variant of mesothelioma does not show distinctive biological, genetic and clinical differences compared with the conventional epithelioid-type mesothelioma. Further analysis of the prognostic and genetic correlations will be needed, in a larger number of cases, to confirm the initial hypothesis that the deciduoid variant is a distinctive clinical–pathological entity.

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Authors' affiliations

A Scattone, A Marzullo, L Pollice, G Serio, Department of Pathology, Medical School, University of Bari, Bari, Italy

A Pennella, Department of Surgery and Pathology, Medical School, University of Foggia, Foggia, Italy

M Gentile, A L Buonadonna, Medical Genetics, De Bellis IRCCS Hospital, Castellana Grotte (Bari), Bari

M Musti, D Cavone, Department of Internal Medicine and Public Medicine, Industrial Medicine, Medical School, University of Bari

P Nazzaro, Department of Clinical Methodology, Medical School, University of Bari

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