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Recurrent DNA copy number changes in 1q, 4q, 6q, 9p, 13q, 14q and 22q detected by comparative genomic hybridization in malignant mesothelioma

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Summary Comparative genomic hybridization (CGH) analyses were performed on 27 human pleural mesothelioma tumour specimens, consisting of 18 frozen tumours and nine paraffin-embedded tumours, to screen for gains and losses of DNA sequences. Copy number changes were detected in 15 of the 27 specimens with a range from one to eight per specimen. On average, more losses than gains of genetic material were observed. The loss of DNA sequences occurred most commonly in the short arm of chromosome 9 (p21–pter), in 60% of the abnormal specimens. Other losses among the abnormal specimens were frequently detected in the long arms of chromosomes 4 (q31.1–qter, 20%), 6 (q22–q24, 33%), 13 (33%), 14 (q24–qter, 33%) and 22 (q13, 20%). A gain in DNA sequences was found in the long arm of chromosome 1 (cen–qter) in 33% of the abnormal specimens. Our analysis is the first genome-wide screening for gains and losses of DNA sequences using comparative genomic hybridization in malignant pleural mesothelioma tumours. The recurrent DNA sequence changes detected in this study suggest that the corresponding chromosomal areas most probably contain genes important for the initiation and progression of mesothelioma.

Keywords: mesothelioma; comparative genomic hybridization, gain, loss, DNA sequence

Malignant mesothelioma is a rare tumour of mesodermal origin. Exposure to asbestos and possibly genetic susceptibility are considered to be contributing factors in the development of this cancer (Wagner et al, 1960; Hirvonen et al, 1995). The biology of this cancer is, however, poorly understood. The latent period between the first exposure to asbestos and the diagnosis of mesothelioma is very long and ranges from 20 to 40 years (Lynch et al, 1985). This suggests that multiple genetic hits are required for the development of the malignancy (Fearon and Vogelstein, 1990).

No specific chromosomal abnormalities have been found in mesothelioma. However, cytogenetic analyses have demonstrated that most mesotheliomas have numerical and structural changes. Several recurrent abnormalities have been found, particularly losses or structural rearrangements of 1p, 3p, 4, 6q, 9p, 14, 22 and gains of chromosomes 5, 7 and 20 (Popescu et al, 1988; Flejter et al, 1989; Tiainen et al, 1989; Hagemeijer et al, 1990; Taguchi et al, 1993).

The prognosis of patients with mesothelioma is poor because the tumour is resistant to treatment. The median survival time after diagnosis is 15 months (Antman et al, 1988). Flow cytometry studies have revealed a better prognosis for patients with diploid tumours and low S-phase fraction (Pyrhönen et al, 1991; Isobe et al, 1995), whereas polysomy 7 and a hyperdiploid chromosomal number correlate with a poorer survival (Tiainen et al, 1989).

The genes most commonly altered in other human malignancies, such as *p53* the retinoblastoma gene, and *ras*, are not frequently mutated in mesothelioma (Metcalf et al, 1992; Van der Meeren et al, 1993*a*). Studies on loss of heterozygosity, which are thought to reveal chromosomal sites harbouring mutated tumour-suppressor genes, have implicated 3p and 9p (Center et al, 1993; Cheng et al, 1993; Mead et al, 1994; Zeiger et al, 1994). Alterations of the newly described tumour-suppressor genes *p16* (MTS1) and *p15* (MTS2) at 9p21 have been detected in mesothelioma tumours and cell lines (Cheng et al, 1994; Kamb et al, 1994; Xiao et al, 1995). Platelet-derived growth factor (PDGF) has been postulated to act as an autocrine growth factor in mesothelioma (Gerwin et al, 1987). Furthermore, a report by Van der Meeren et al (1993*b*) showed that overexpression of the PDGF-A chain is associated with tumorigenic conversion of human mesothelial cells. Even although some specific genes have been found that might be important in the development and progression of mesothelioma, little is known about amplification of genes (oncogene activation) or inactivation of tumour-suppressor genes, which have been shown to play an important role in tumorigenesis (Knudson, 1985). The CGH technique, which we have used in this study, is a method expressly for the detection of losses, gains and amplifications of genetic material, which may be significant in the initiation, progression and drug resistance of mesothelioma. CGH is a method that does not require the tumour specimens to be cultured and makes it possible to screen for losses and gains of DNA sequences along all the chromosomes in a single hybridization (Kallioniemi et al, 1992).

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MATERIALS AND METHODS

Tumour specimens

The study was carried out on 27 tumour specimens from patients with malignant pleural mesothelioma. The patients were all from the catchment area of the Helsinki University Central Hospital. The

Table 1 Clinical characteristics, sample data and CGH findings from 27 patients with malignant pleural mesothelioma

Case	Sex/age at diagnosis	Histological subtype	Clinical stage	Treatment before study	Exposure to asbestos	Survival (months from diagnosis)	F/P	Tumour tissue in the sample (%)	CGH result
1	M/56	Sarcomatous	IIIB	–	–	3	P	>75, necrosis	Normal
2	M/54	Epithelial	IIA	–	+	6	F	NK	–1p, +1q –9p21–pter, –9q, –17p, –22q
3	F/61	Epithelial	IIA	–	–	34	P	50–75	+1q, +6p, –14q21–qter
4	M/47	Epithelial	IIA	–	+	5+	F	NK	–3q, –4q31.1–qter, –6q, –9p, –13cen–q21, +19q
5	F/43	Epithelial	IIA	–	–	42	F	NK	+6p, –6q22–qter
6	M/51	Epithelial	IIA	–	+	5	F	NK	–6q16–q24, –9p13–pter
7	F/71	Epithelial	IIA	–	–	7	F	NK	–9p21–pter
8	M/74	Epithelial	IIA	–	+	14	P	50–75	Normal
9	M/71	Epithelial	IIA	–	–	17	F	NK	Normal
10	M/56	Mixed	I	–	+	15	F	NK	+1q, –4q, –6q21–qter, –9p, –10p13–pter, –10cen–q23, –14q
11	M/39	Mixed	IIA	–	+	20	F	NK	+1q, –4q, –14q
12	M/63	Mixed	IIA	–	–	30	P	>75	–9p, +17q21–qter
13	M/65	Mixed	IIA	–	+	6	P	>75	–9p21–pter, –13q22–qter
14	M/71	Mixed	IIA	–	+	8+	F	NK	–9p, –13q, –14q24–qter
15	M/67	Mixed	IIA	–	?+	4	F	NK	–20p12–pter
16	M/62	Mixed	IIA	–	+	17	P	50	–20p, –22q13
17	M/51	Mixed	IIIB	+	+	3.5	F	NK	+1, –2q33–qter, –10p12–pter, –13q, –14q, +15q22–qter, +17q22–qter, –22q
18	M/60	Mixed	IIIB	–	–	26	F	NK	–6q, –9p, +10q, –13q
19	M/41	Mixed	IIA	–	+	18	F	NK	Normal
20	M/61	Mixed	IIA	–	+	12	P	<25	Normal
21	M/65	Mixed	IIA	–	–	78	F	NK	Normal
22	M/71	Mixed	IIA	–	+	6	F	NK	Normal
23	M/65	Mixed	IIA	–	+	6	P	<25	Normal
24	M/55	Mixed	IIA	–	+	13	F	NK	Normal
25	M/59	Mixed	IIA	–	+	4	F	NK	Normal
26	F/57	Mixed	IIA	–	–	13	F	NK	Normal
27	M/60	Mixed	IIIB	–	+	4	P	50	Normal

+, Alive; F, frozen sample; P, paraffin-embedded sample; NK, not known.

specimens comprised eight with epithelial histology, 18 with mixed histology and one with sarcomatous histology. The histological subtyping was performed by two mesothelioma panels, the Finnish National Mesothelioma Panel and the European Organisation for Research and Treatment of Cancer Mesothelioma Panel. Four of the patients were women and 23 were men, with a median age of 59 years (range 39–74). Seventeen of the patients had a known history of asbestos exposure. One of the patients had received treatment before surgery. Eighteen samples were from frozen tumours and nine from paraffin-embedded tumours, and they all originated from the same sample on which the histological analyses were performed (Table 1). High-molecular-weight DNA was extracted from the frozen tumours and from peripheral blood samples from two healthy donors, one man and one woman (reference DNA), according to standard procedures. The DNA from the paraffin-embedded tumours was isolated as described by Isola et al (1994).

The proportion of tumour tissue in the paraffin-embedded samples, estimated by a pathologist from the Finnish National Mesothelioma Panel after staining the tissue slides with haematoxylin and eosin, ranged from less than 25% to more than 75% (Table 1).

Comparative genomic hybridization

The hybridizations were performed according to the method of Kallioniemi et al (1992), with some modifications. Briefly, tumour DNA was labelled with biotin-14-dATP (Gibco BRL, Gaithersburg, MD, USA) and the reference DNA, from the healthy blood donors, with digoxigenin-11-dUTP (Boehringer Mannheim, Germany) in a standard nick-translation reaction. Equal amounts (400–800 ng) of the two DNAs together with 10–20 μg of human Cot-1 DNA (Gibco BRL) in 10 μl of hybridization buffer [50% formamide, 10% dextran sulphate, 2 × SSC (1 × SSC is 0.15 M sodium chloride–0.015 M sodium citrate, pH 7)] were denaturated at 70°C for 5 min and applied to normal lymphocyte preparations. Before hybridization, the preparations were dehydrated in a series of 70, 85 and 100% ethanols (to achieve better morphology) and denatured at 69–71°C for 2–2.5 min in a formamide solution (70% formamide/2 × SSC). The slides were then dehydrated on ice as described above and treated with proteinase K (0.1 μg ml^{-1} in 20 mM Tris-HCl, 2 mM calcium chloride, pH 7) in 37°C for 7.5 min and dehydrated once again on ice. The hybridization was performed in a moist chamber at 37°C during 2–3 days. After hybridization the slides were washed

three times in 50% formamide/2 × SSC, twice in 2 × SSC, three times in 0.1 × SSC at 45°C for 10 min each and once in 4 × SSC/0.2% Tween at room temperature for 5 min, in order to remove unbound and non-specifically bound probe fragments. Tumour and reference DNA were detected with tetrarhodamine isothiocyanate (TRITC)-conjugated avidin and fluorescein isothiocyanate (FITC)-conjugated antibodies respectively. Finally, the slides were counterstained with 4′, 6-diamidino-2-phenyl-indole-dihydrochloride (DAPI; Sigma, St Louis, MO, USA) and covered with an antifade solution (Vector Laboratories, Burlingame, CA, USA).

Digital image analysis

The hybridizations were analysed using an Olympus fluorescence microscope and the *isis* digital image analysis system (MetaSystems GmbH, Altlussheim, Germany) based on a high-sensitivity integrated monochrome charge-coupled device (CCD) camera and an automated CGH analysis software package (for details see Kivipensas et al, 1996). Briefly, three-colour images, red (TRITC) for the tumour hybridization, green (FITC) for the normal reference DNA hybridization and blue (DAPI) for the DNA counterstain, were acquired from 5–8 metaphase spreads per hybridization. The chromosomes were identified based on the DAPI banding pattern.

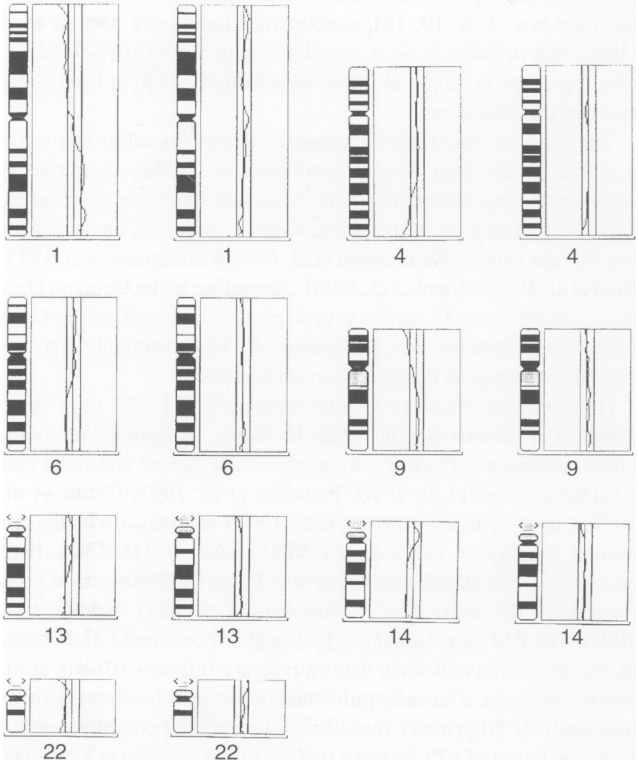

Figure 1 Mean red-to-green ratio profiles from pter to qter, obtained from CGH analysis of malignant mesothelioma. Pictured profiles are those of chromosomes 1, 4, 6, 9, 13, 14 and 22, which showed the most frequent genetic changes. Chromosome ideograms are presented for approximate visual reference only. The line in the middle of the profile indicates the base line ratio (1.0), the left and the right lines indicate ratio values of 0.85 and 1.17 respectively. Left: The profiles represent the following aberrations: loss of 1p and gain of 1q (case no. 2), loss of 4q31.1–qter (no. 4), loss of 6q21–qter (no. 10), loss of 9p (no. 4), loss of 13q22–qter (no. 13), loss of 14q (no. 10) and loss of 22q (no. 2). Right: The profiles of chromosomes with no aberrations obtained from various negative control experiments

The red and green fluorescence intensities were calculated and the red-to-green ratio profiles along the chromosome axis were displayed. For normalization of the ratio profiles the modal value of the red-to-green ratio for the entire metaphase was set to 1.0. Finally the individual ratio profiles were combined using separate p- and q-arm length normalization to yield the average ratio profiles, which were displayed next to ideograms together with significance intervals of 0.85 and 1.17 (see below) (Figure 1).

Interpretation of CGH results and quality control

The regions in the chromosomes where the ratio exceeded 1.17 or was less than 0.85 were considered overrepresented (gained) or underrepresented (lost), respectively. These cut-off values were based on hybridizations with DNA from the healthy donors (negative controls). Only ratio changes that exceeded the fluctuation seen in the negative control experiments were interpreted as evidence of a real gain or loss of DNA sequences. The chromosomal regions where the ratio changes exceeded the value 1.5 were considered highly amplified. A positive control, with known DNA copy number changes (both gains and losses), and a negative control were included in each hybridization as quality controls. Only the metaphases with a homogenous hybridization were analysed. The heterochromatic regions at 1q12, 9q12 and 16q11, the p-arms of the acrocentric chromosomes and the Y chromosome were excluded from the analysis. The profiles of the chromosomes 1p32–pter, 16p, 19 and 22 were interpreted with special caution, because these areas have been known to give false positive results (A Kallioniemi et al, 1994).

Statistical analysis

The DNA sequence copy number changes detected by CGH were correlated with the parameters mentioned in Table 1. *P*-values were analysed using the Fisher's exact test owing to the small number of cases.

RESULTS

DNA sequence copy number changes were detected in 15 of the 27 specimens evaluated (Table 1). Changes were detected in 75%

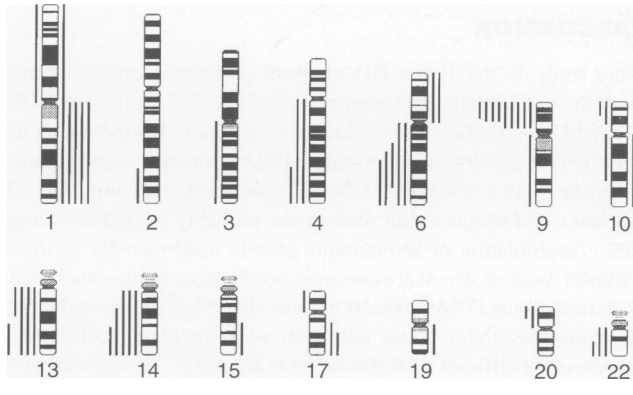

Figure 2 Summary of all losses and gains of DNA sequences observed in 27 mesothelioma specimens using CGH. Losses are shown on the *left* side of the chromosomes and gains on the *right* side. Only those chromosomes where changes in the genetic material were detected are shown

(6/8) and in 50% (9/18) of the epithelial and mixed specimens respectively. No changes were observed in the sarcomatous specimen. DNA copy number changes were detected in 14 chromosomes altogether. On average, there were 3.5 changes per specimen (range from 1 to 8). Losses predominated over gains with a ratio of 3.3:1 (40 losses and 12 gains; Figure 2). No highly amplified chromosomal regions were detected.

Loss of DNA sequences was observed in 12 different chromosomes altogether (Table 1 and Figure 2). The chromosome arm most frequently involved was the short arm of chromosome 9, lost in 9 of the 15 abnormal tumours (60%). The minimal common region of loss extended from band 9p21 to the p-telomere of chromosome 9. Other regions commonly lost among the abnormal tumours were 4q31.1–qter (three cases, 20%), 6q22–q24 (five cases; 33%), 13q (five cases; 33%), 14q24–qter (five cases; 33%) and 22q13 (three cases; 20%). DNA sequences in the short arm of chromosome 9 and the long arm of chromosome 6 were simultaneously lost in four cases (27%; case nos 4, 6, 10, 18) as were DNA sequences in chromosomes 9p and 13q in four cases (27%; case nos 4, 13, 14, 18).

Gain in DNA sequences was detected in six chromosomes and in nine of the 15 abnormal tumours (Table 1 and Figure 2). A gain of DNA sequences was most commonly observed in the long arm of chromosome 1, in five of the abnormal cases (33%). The long arms of chromosomes 10, 15, 17 and 19 were the other locations where a gain in DNA sequences was observed.

A simultaneous gain in the long arm of chromosome 1 and a loss in the long arm of chromosome 14 was detected in four cases (27%; case nos. 3, 10, 11, 17). In cases 5, 7 and 15, copy number changes were observed in only one of the chromosomes, namely a simultaneous gain of 6p and loss of 6q22–qter, loss of 9p21–qter and loss of 20p12–pter respectively.

The statistical analyses showed a higher probability that the loss of genetic material in chromosome 6q would occur in epithelial tumours when the epithelial group was tested against the combined group of mixed and sarcomatous tumours. However, this result was not statistically significant [odds ratio (OR) = 5.1, 90% confidence interval (CI) = 0.9–20.5, $P = 0.28$]. There were however statistically significant correlations for the simultaneous loss of chromosome 9p and either 6q or 13q and for the simultaneous gain of 1q and loss of 14q (OR = 13.6, 95% CI = 1.2–151, $P = 0.03$). None of the aberrations detected in our CGH analysis showed any statistical correlation with the survival data of the patients or their exposure to asbestos.

DISCUSSION

This study is the first CGH analysis performed on uncultured tumour cells of malignant pleural mesothelioma. A previous CGH study of mesothelioma was based on cell lines (Kivipensas et al, 1996). The present study revealed DNA sequence copy number changes in 15 (56%) out of the 27 specimens analysed. The 12 normal CGH results in our series were probably related to normal cell contamination or intratumour genetic heterogeneity in these samples, both of which are common occurrences in mesothelioma. If normal tissue DNA amounts to more than 50% of the total DNA in a sample, the reliable detection of ratio changes becomes increasingly difficult (A Kallioniemi et al, 1994). This statement is in agreement with our findings, because we were able to detect changes in the genetic material in most of the paraffin-embedded specimens, in which the malignant cells comprised more than 50% of the sample tissue.

In tumours that show intratumour genetic heterogeneity, the different genetic aberrations present in individual clones may sometimes balance one another or exist at too low a frequency to be detected by CGH (Kallioniemi et al, 1994). The fact that no gains in chromosomes 5, 7 and 20 or losses in the short arm of chromosome 3, which are common aberrations found in cytogenetic studies (Popescu et al, 1988; Flejter et al, 1989; Tiainen et al, 1989; Hagemeijer et al, 1990; Taguchi et al, 1993), were seen in our CGH analyses, suggests that these changes possibly were present in a small proportion of the cells and therefore not detected by CGH. Furthermore, using in situ hybridization with centromere-specific probes on mesothelioma paraffin sections, Tiainen et al (1992) and Segers et al (1995) have shown a heterogeneous pattern in chromosome copy numbers of chromosomes 1 and 7.

Our study revealed more losses than gains of DNA sequences. The losses of DNA sequences in chromosomes 4q (minimal common region between bands q31.1 and qter), 6q (q22–q24), 9p (p21–pter), 13q, 14q (q24–qter) and 22q (q13) observed in this study, correlate well with previous cytogenetic studies of mesothelioma (Popescu et al, 1988; Flejter et al, 1989; Tiainen et al, 1989; Pelin-Enlund et al, 1990; Hagemeijer et al, 1990; Taguchi et al, 1993). Losses in DNA sequences in the above mentioned chromosomes were also detected in our previous CGH analysis of mesothelioma (Kivipensas et al, 1996). One of our cases (no. 5) did not show any changes other than a loss in 6q (q22–qter) and a gain in 6p (cen–pter). This case and the other cases with losses in 6q (case nos. 4, 6, 10, 18), support the findings of Meloni et al (1992) that deletion in 6q is associated with the loss of key genes, which may be involved in initial transformations in at least some cases of mesothelioma.

The most recurrent gain in genetic material found in this study occurred in the long arm of chromosome 1. This aberration is common among different tumour types and has been detected by CGH for example in diffuse large B-cell lymphoma, breast cancer and bladder cancer (Kallioniemi et al, 1994; Kallioniemi et al, 1995; Ried et al, 1995; Monni et al, 1996). According to the Genome Data Base, chromosome 1q carries several proto-oncogenes, which highlights the probability that imbalances at chromosome 1q may be critical for oncogene dosage in certain neoplasias.

Homozygous deletions of chromosome 9p21–p22 have been detected in various tumour types including leukaemia, mesothelioma, melanoma, bladder carcinomas, lung cancer and renal cell carcinoma (Diaz et al, 1990; Fountain et al, 1992; Cairns et al, 1993; Cheng et al, 1993; Mead et al, 1994). Recently, two putative tumour-suppressor genes *p16* (MTS1) and *p15* (MTS2), both encoding cyclin-dependent kinase 4 (CDK4) inhibitors, have been mapped to the short arm of chromosome 9 (p21) (Kamb et al, 1994). The *p16* gene was initially thought to be altered more often in mesothelioma cell lines than in primary tumours (Cheng et al, 1994). However, a recently published fluorescent in situ hybridization study of 50 primary mesotheliomas showed complete and/or partial deletion of *p15* and *p16* in 72% of cases (Xiao et al, 1995). Whether the genes *p15* and *p16* are lost in our nine specimens with a deletion in 9p, is so far unknown.

In conclusion, our report is the first genome-wide screening of losses and gains of DNA sequences in human malignant pleural mesothelioma tumour specimens. The detected DNA copy number changes were clearly clustered on chromosomes 1q, 4q, 6q, 9p, 13q, 14q and 22q, suggesting that these chromosomal areas, which could be the sites of currently unknown genes, may be involved in the development and progression of this tumour.

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