

Identification of Specific Gene Copy Number Changes in Asbestos-Related Lung Cancer

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Abstract

Asbestos is a well-known lung cancer-causing mineral fiber. *In vitro* and *in vivo* experiments have shown that asbestos can cause chromosomal damage and aberrations. Lung tumors, in general, have several recurrently amplified and deleted chromosomal regions. To investigate whether a distinct chromosomal aberration profile could be detected in the lung tumors of heavily asbestos-exposed patients, we analyzed the copy number profiles of 14 lung tumors from highly asbestos-exposed patients and 14 matched tumors from nonexposed patients using classic comparative genomic hybridization (CGH). A specific profile could lead to identification of the underlying genes that may act as mediators of tumor formation and progression. In addition, array CGH analyses on cDNA microarrays (13,000 clones) were carried out on 20 of the same patients. Classic CGH showed, on average, more aberrations in asbestos-exposed than in nonexposed patients, and an altered region in chromosome 2 seemed to occur more frequently in the asbestos-exposed patients. Array CGH revealed aberrations in 18 regions that were significantly associated with either of the two groups. The most significant regions were 2p21-p16.3, 5q35.3, 9q33.3-q34.11, 9q34.13-q34.3, 11p15.5, 14q11.2, and 19p13.1-p13.3 ($P < 0.005$). Furthermore, 11 fragile sites coincided with the 18 asbestos-associated regions ($P = 0.08$), which may imply preferentially caused DNA damage at these sites. Our findings are the first evidence, indicating that asbestos exposure may produce a specific DNA damage profile. (Cancer Res 2006; 66(11): 5737-43)

Introduction

Several risk factors are assumed to be associated with lung cancer initiation and tumor development. Tobacco smoking is clearly the most important cause of lung cancer, accounting for ~80% to 90% of all cases in males and ~50% to 80% among females (1), whereas the etiologic fraction of asbestos exposure in lung cancer among men ranges from 6% to 23% in different studies (2). Smoking and asbestos exposure act in a synergistic manner in pulmonary carcinogenesis (3).

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Complex patterns of cytogenetic and molecular genetic changes are typical in lung tumors (4), and several chromosomal regions are recurrently amplified or deleted. This poses a challenge to identify the most essential lung carcinogenesis-associated patterns and alterations specific to asbestos exposure. Several yet unidentified genes in the deleted and amplified regions could be the underlying mediators of tumor formation and progression. Array comparative genomic hybridization (CGH) is rapidly becoming the method of choice for high-resolution screening of genetic imbalances (5).

In vitro and *in vivo* experiments have shown that asbestos fibers can cause DNA double-strand breaks (6) as well as chromosomal aberrations and abnormal chromosome segregation (7). The effect of asbestos fibers on gene mutations is not significant. Despite extensive investigation, the mechanisms of fiber-induced genotoxicity are not yet clear, but direct interaction with the genetic material and indirect effects via production of reactive oxygen species (ROS) have been proposed (8). The exact molecular mechanism behind asbestos-related carcinogenesis is, however, thought to be very complex and involves several parallel pathways (9).

Here, we report investigation of gene copy number changes specific to asbestos-exposed lung cancer patients using classic and array CGHs, leading for the first time to identification of a genome-wide profile of aberrations in lung tumors of asbestos-exposed patients.

Materials and Methods

Patients. We analyzed the copy number profiles of 14 malignant lung tumors from highly asbestos-exposed patients and 14 tumors from nonexposed patients that were matched for age, gender, nationality, and smoking history (Table 1). Asbestos exposure was estimated from work history obtained by personal interviews. In addition, the asbestos fiber count was measured in an electron microscopic analysis of lung tissue (10). The exposed group consisted of persons who had both a definite or probable exposure according to the work history and a pulmonary asbestos fiber count >5 million fibers per gram dry weight. The asbestos fiber concentration of 2 to 5 million is thought to represent roughly a 2-fold increase in risk of lung cancer (11, 12).

Tissue samples. Tissue samples were obtained during lung cancer surgery. The frozen tumor samples were cut to 4 μ m sections for DNA isolation. Standard H&E staining was used to verify the tumor cell content (>50% requirement). DNA was isolated from tumor and reference (peripheral blood from two males) samples using QIAamp DNA Mini kit (Qiagen, Valencia, CA).

Classic CGH. Classic CGH was done on all 28 tumor samples according to Björkqvist et al. (13). In brief, 1 μ g of digested and labeled reference (Texas red-5-dCTP and Texas red-5-dUTP) and tumor (FITC-5-dCTP and FITC-5-dUTP) DNA was used for the hybridizations (NEN Life Science Products, Inc., Boston, MA). The slides were hybridized overnight at 37°C and washed according to standard protocols. The ISIS CGH program version 3 (MetaSystems GmbH, Altlussheim, Germany) was used for the analysis. Standard cutoff thresholds were set to <0.85 for deletions, >1.17 for

Table 1. Patients

Sample no.	Sex	Age (y)	Asbestos fiber*	Smoking			Diagnosis	
				Cigarette/d [†]	Pack-years (20 cigarettes/d)	Age (y)	Start	Stop
Exposed patients								
1	M	64	72.9	20	33	15	48	Adenocarcinoma
2 [‡]	M	59	12.6	50	105	17	—	Adenocarcinoma
3 [‡]	M	59	35	20	36	19	55	Adenocarcinoma
4 [‡]	M	65	9.4	10	25	16	—	Adenocarcinoma
5 [‡]	M	65	10.8	15	23	20	50	Adenocarcinoma
6 [‡]	M	63	10.8	17	27	16	60	SCC
7 [‡]	M	62	6	30	65	14	57	SCC
8 [‡]	M	65	5.9	20	32	18	—	SCC
9 [‡]	M	57	6.6	20	36	17	53	SCC
10 [‡]	M	67	8.4	20	20	36	56	Large cell lung cancer
11 [‡]	M	66	19	15	35	17	61	Large cell lung cancer
12 [‡]	M	58	90	30	65	15	—	Large cell lung cancer
13	M	64	145	20	22	19	43	Adenosquamous carcinoma
14	M	62	12.8	23	55	14	—	SCLC
Mean		62.6	31.8	22.1	41.4	18.1		
Nonexposed patients								
15 [‡]	M	55	0	20	36	19	—	Adenocarcinoma
16 [‡]	M	69	0	20	47	23	—	Adenocarcinoma
17	M	70	0	15	38	18	68	Adenocarcinoma
18 [‡]	M	65	0	20	52	12	—	Adenocarcinoma
19	M	55	0.1	28	54	16	55	Adenocarcinoma
20 [‡]	M	67	0	25	50	27	—	Adenocarcinoma
21	M	65	0	20	47	13	60	SCC
22	M	65	0	15	25	30	64	SCC
23 [‡]	M	50	0	20	35	15	—	SCC
24	M	64	0	20	45	19	—	SCC
25 ^{§,‡}	M	67	0	20	47	19	66	SCC
26 [‡]	M	71	0.5	35	89	20	—	Large cell lung cancer
27 [‡]	M	72	0.5	22	36	18	49	Large cell lung cancer
28 [‡]	M	41	0	25	31	15	40	Adenosquamous carcinoma
29	M	64	0	30	66	17	—	SCLC
Mean		64.2	0.08	22.1	47.6	19		

*Million fibers per gram dry lung tissue, detection limit = <0.1.

[†]Mean lifetime cigarette consumption.

[‡]Used in array CGH.

[§]Used only in array CGH.

amplifications, and >1.5 for high-level amplifications as described by Björkqvist et al. (13).

Array CGH. Array CGH analyses were conducted on 20 individual samples (11 exposed and 9 nonexposed; Table 1). Commercial cDNA microarrays (Human 1.0; Agilent Technologies, Palo Alto, CA) with 12,814 unique clones (97% map to named human genes) were used as described by Wikman et al. (14). In brief, the hybridizations were done with 5 µg of digested (25 units *Alu*I/25 units *Rsa*I) reference and tumor DNA and labeled [³Cy-dUTP (tumor) and Cy-5-dUTP (reference), Amersham Pharmacia Biotech, Piscataway, NJ] using a random priming method (RadPrime DNA Labeling System, Life Technologies, Gaithersburg, MD). After hybridization at 65°C overnight, the slides were washed, dried in a centrifuge, and scanned using Agilent DNA microarray scanner (G2565AA).

Data processing. Raw signal intensities from the arrays were measured using the Feature Extraction software (Agilent Technologies). Measurements flagged as unreliable by Feature Extraction were removed from subsequent

analysis. Additionally, measurements defined as faulty by our own image analysis methods were removed (see Supplementary data; ref. 15).⁵

Bioinformatics analysis. To identify exposure-related aberrations, the array CGH data from individual patients were analyzed at group level by comparing gene copy number ratios of the tumors of exposed and nonexposed patients. The identification of exposure-related areas was done using overlapping 0.5 to 1 Mbp segments that were tested for differences in copy numbers. First, the data were ordered according to the chromosomal location of the genes. Next, the genes within each segment were detected, and the number of correctly classified patients was calculated based on the copy number ratios of each gene.

The exposure-related aberrant regions were identified by means of hypothesis testing. In the two-tailed testing, the null hypothesis was set as

⁵ CGH array data available at <http://cgh.bioinfo.helsinki.fi/>.

Table 2. Classic CGH results

Histology	Sample	Gains and amplifications	Deletions	Total	Sample	Gains and amplifications	Deletions	Total
Adenocarcinoma	Exposed 1	≥ 1.17 Normal karyotype	≤ 0.85	73 0	Nonexposed 15	≥ 1.17 1q32.2-q42.1, 5p14-pter, 8q24.3-qter	≤ 0.85	70 3
	2	1q21.2-q43, 2p22-p25.1, 6p, 8q12-q24, 8q24.2-qter, 12p11.2-pter, 16p11.2-p13.3, Xq26-q28		7	16	11q23.1		1
	3	1q21.2-qter, 2p22-p23, 4p13-pter, 5p. 7q, 12q13.2-qter, 20q11.2-qter		7	17	7q32-qter		1
	4	17q12-q21.3		1	18	Normal karyotype		0
	5	1q, 2p23-p24, 5p13.2-pter, 9q, 12p-q21.3, 15q24- 26.1, 19q, 20q	9p21-pter, 10	10	19	1q, 2q14.3-22, 11q23.3-24, 12q23-q24.2		4
	6	8q23-q24.1		1	21	5p		1
	7	1q23-qter, 2p21-p25.1, 3q13.3-q29, 5p14-p15.3, 8q24.1-qter		5	22	3q25.3-q27 1q31-q42.3, 2p14-p24, 3q21-qter, 5q31.1-q34, 6p12-p21.3, 7, 8q22.1-qter		6
	8	3q13.3-qter		1	23	3q22-qter, 5p14-pter, 7pcen-p21, 8q, 9p13-qter, 11q13.3-q22.1, 12p12.1-p13.3, 12q13.3-q21.3, 20q, 22	9p23-pter	11
	9	1q41-q42.1, 20q		2	24	1qcen-q31, 2p-q22, 2q32.1-qter, 3qcen-26.1, 3q26.2-qter, 5p, 6p21.3-pter, 6q24-qter, 7q, 8q23-qter, 11, 12p. 12q, 13, 15q23-qter, 17q, 19p-q11.3, 19q12-q13.2, 19q13.3-qter, 20, 22	3p, 4, 5qcen-q31.3, 7p, 14qcen-q23, 15qcen-q21.1, 18q21.3-qter	24
Large cell lung cancer	10	1q21.1-qter, 2p, 3q21-qter, 5p. 8q21.3-qter, 12p-q21.3		6	26	1q32.1-q42.2		1
	11	2p21-p25.2, 2q14.1-q22, 2q36-37.3, 3q21-qter, 8q24.1-24.3, 15qcen-q22, 15q23-25, 15q26-qter		6	27	12p12.1-p13.3		1
	12	3p21.2-p22, 7q31.3-qter		2				

(Continued on the following page)

Table 2. Classic CGH results (Cont'd)

Histology	Sample	Gains and amplifications	Deletions	Total	Sample	Gains and amplifications	Deletions	Total
Adenosquamous carcinoma	13	1q31-qter, 2p22-p24, 2q21.1-22, 7q22-q35, 8q24.1-q24.3		5	28	1q, 5q31.1-q35.1, 8q23-q24.3, 20q		4
SCLC	14	1q21.3-qter, 2p, 2q14.1-q23, 2q35-q37.3, 3qcen-20, 3q21-q24 , 3q25-qter, 5p13.3-15.1, 6pter-q22.3 , 7q32-qter, 8, 11, 18 , 19p, 19q , 20p, 20q	3p, 5q, 9p, 10p, 14	20	29	1p31.2-32.13, 1p32.2-p36.1 , 1p36.2-pter, 1q, 5p , 6p21.1-pter, 8, 18p-q12.1, 18q12.2-q21.2 , 18q21.3-qter1, 20p, 20q , 21, 22	6qcen-23.1, 6q24-27, 10q22.1-qter	12

NOTE: Chromosomal gains and deletions in lung tumors from 14 asbestos-exposed and 14 matched nonexposed patients. High-level amplifications are in boldface.

"classification capability of the segment is not deviating" and the alternative hypothesis as "classification capability of the segment is deviating." The average number of correctly classified patients by the genes within each segment was used as a test statistic. The regions likely to be associated with exposure were identified using a permutation test with 10,000 permutations. The empirical *P*s for the regions were calculated by comparing the test statistic to the permutation distribution. Regions containing less than five genes were filtered out.

Results and Discussion

Classic CGH. Typical patterns of aberrations were identified using classic CGH for different histologic types of lung cancer, of which small cell lung cancer (SCLC) had the most aberrations irrespective of exposure (Table 2; online CGH database).⁶ In general, we detected more gains than losses using classic CGH, probably due to nonmicrodissected material. The most frequent changes in all patients were gains at 1q23-q24 (36%), 1q41 (50%), 2p23 (36%), 3q22-q23 (29%), 5p14-p15.1 (36%), 8q24.1 (46%), and 20q13.1-q13.2 (29%) and losses at 9p23-p24 (11%) and 5q (7%).

All histologic types, except squamous cell carcinoma (SCC) showed slightly more aberrations in the exposed group (mean number of aberrations: 6.4 and 2.8 in the exposed and nonexposed groups, respectively). The SCC tumors of nonexposed patients showed more aberrations than those of the exposed patients due to two nonexposed samples with 11 and 24 aberrations. The single amplification that seemed to differ significantly between the asbestos-exposed and the nonexposed groups in classic CGH was a minimal overlapping region at 2p23. This amplification was present in 57% (8 of 14 cases) of the exposed and 14% (2 of 14 cases) of the nonexposed patients (*P* = 0.025). In seven of these eight exposed cases, the amplification affected also 2p22 and, in four cases, 2p21.

Array CGH. As the only clear difference our classic CGH analysis showed between the two groups was the 2p amplification, we chose to analyze the array CGH results at group level by comparing the signal log ratios in segments. This type of analysis does not require *a priori* knowledge of the type of aberrations in individual patients.

Especially in this kind of comparative study, when the aim is to detect changes associated with a certain factor, our choice of statistical method is beneficial due to synergistic reasons. The identification of aberrations from each array data separately is also possible, but small changes may not be detected due to the background noise on the arrays. In addition, when comparing several sets of copy number data simultaneously, small changes common to a group of patients and significant low copy number changes may be detected.

Using the combined statistical analysis on the array CGH data, we found 18 regions in which the DNA copy number between the two groups differed significantly (Table 3; Figs. 1 and 2). As expected from the classic CGH data, none of these regions harbored a high DNA copy number change but either a low-level gain or a low-level deletion. Figure 1 shows the mean log ratios for each significant region (see also Supplementary Figs. S1 and S2). The choice of using combined analysis may not, however, fully compensate for the noise on the arrays caused by normal cell contamination. Thus, there is a chance that, for instance, a gain in one group of patients is misinterpreted as a loss in the other group. In addition, some of these loci seemed to be amplified in one group and deleted in the other.

Most of the asbestos-associated regions were very small (median size 1.74 Mbp), whereas the largest was 19p13.3-p13.1 (18.47 Mbp). Two of the regions, 17p and 19p, were large enough (6.96 and 18.47 Mbp, respectively) to be detected by classic CGH, whereas the rest of the regions spanned 0.9 to 3.25 Mbp, which usually remain undetected in classic CGH (16). However, classic CGH failed to reveal the two large regions of loss, probably because of normal cell contamination, which seemed to affect our classic CGH results more than the array CGH results. Furthermore, these regions as well as 16p (3.13 Mbp) and 9q34 (3.25 Mbp; Supplementary Fig. S2) are problematic areas in classic CGH, producing false-positive or negative results due to hybridization artifacts (17, 18). Indeed, loss of heterozygosity (LOH) analyses of all of these regions have shown that lung tumors often harbor allelic imbalance at these loci (19, 20).

Many of the regions that significantly separated the two groups have previously been generally implicated in lung carcinogenesis (i.e., 1p36.1, 1q21.2, 3p21.31, 4q31.21, 5q35.2-q35.3, 9q34, 11p15.5, 17p13.3, 19p13, and 22q13).⁶ Yet, one of these regions, 3p21, has

⁶ http://www.helsinki.fi/cmg/cgh_data.html.

Table 3. Regions differing in copy number between the asbestos-exposed and nonexposed lung cancer patients achieved with array CGH

Chromosomal region	Chromosomal position (bp)*		Size (Mbp) University of California at Santa Cruz (UCSC)	No. genes in region [†]	P	Type of aberration	Fragile sites [‡]	Genes
	Start	Stop						
1p36.12-p36.11	23154144	24426681	1.27	11/18	0.022	Deletion in nonexposed	FRA1A, fra(1)(p36)	
1q21.2	146635105	147599667	0.96	12/19	0.0125	Amplification in exposed	FRA1F, fra(1)(q21)	
2p21-p16.3	45527471	47945059	2.42	12/14	0.001	Amplification in exposed + deletion in nonexposed		
3p21.31	48530239	49428484	0.9	7/14	0.0085	Deletion in exposed + amplification in nonexposed		
4q31.21	145931413	147128135	1.2	6/7	0.008	Deletion in nonexposed	FRA4C, fra(4)(q31.1)	
5q35.2-q35.3 [§]	175775917	178511817	2.74	14/27	0.001	Deletion in exposed	FRA5G, fra(5)(q35)	
9q32 [§]	112313201	114085370	1.77	10/13	0.021	Amplification in nonexposed + deletion in exposed	FRA9E, fra(9)(q32) and FRA9B, fra(9)(q32)	AKNA, ZFP37, HPRP4P, ALAD, POLE3, AMBP, ATP6V1G1
9q33.3-q34.11 [§]	127249351	128990540	1.74	15/25	0.0055	Amplification in nonexposed + deletion in exposed		
9q34.13-q34.3 [§]	132796807	136051087	3.25	18/29	0.0005	Amplification in nonexposed + deletion in exposed		
11p15.5	753634	2547429	1.79	13/21	0.003	Amplification in nonexposed		
11q12.3-q13.1	62517311	64095160	1.58	11/22	0.0285	Amplification in nonexposed	FRA11H, fra(11)(q13)	
11q13.2	65886587	67191050	1.3	9/18	0.025	Amplification in nonexposed	FRA11A, fra(11)(q13)	SLC29A2, MRPL11, DPP3, ACTN3
14q11.2	22004517	23479883	1.48	12/21	0.0015	Amplification in nonexposed		
16p13.3	258759	3387671	3.13	27/51	0.013	Amplification in nonexposed		
17p13.3-p13.1	1194933	8156236	6.96	44/84	0.012	Deletion in exposed + amplification in nonexposed		
19p13.3-p13.11	367881	18839479	18.47	133/233	0.001	Deletion in exposed + amplification in nonexposed	FRA19B, fra(19)(p13)	
22q12.3-q13.1	34861229	36289912	1.43	10/22	0.0455	Amplification in nonexposed	FRA22A, fra(22)(q13)	
Xq28	148268137	149603500	1.34	9/15	0.034	Amplification in exposed	FRA(X), fra(X)(q28)	FMR2

^{*}Base pair obtained by blasting array probe sequence in UCSC Blat.[†]Number of genes within the region (present on the array) with different DNA copy number between the exposed and nonexposed patients samples.[‡]Obtained from Entrez Gene.[§]Supplementary Figs. S1 and S2.^{||}(31–35).

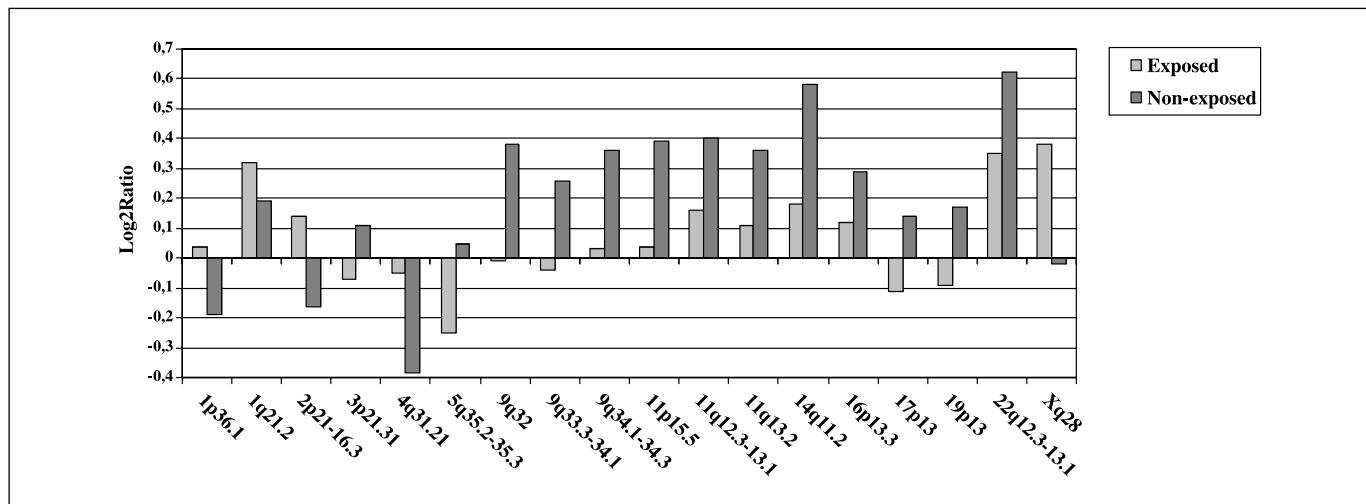


Figure 1. Array CGH results. Average \log_2 ratios (Y axis) of all probes in all samples in each chromosomal region (X axis) show differences in copy number between asbestos-exposed (light columns) and nonexposed (dark columns) patients.

recently been shown by LOH analysis to be significantly associated with asbestos exposure (21). Moreover, *in vitro*, asbestos fibers are mainly involved in causing breaks in chromosomes 1 and 9 (22, 23).

The regions on the chromosomal arms 4q and 22q have been reported to be commonly lost also in mesothelioma (13, 24), a cancer type very closely linked to asbestos exposure. Similarly, 11q13.1 contains the *FOSL1* (*Fra-1*) gene, which has been reported to be up-regulated in transformed mesothelial cells after asbestos exposure (25).

Interestingly, the gain at 2p seemed to be specific to the exposed group in both array CGH and classic CGH. A bit surprisingly, though, the minimal overlapping region in classic CGH was 2p23, whereas 2p21 was altered in array CGH. However, by classic CGH, the 2p23 gain in most cases was larger and contained 2p21 in half of the cases. This quite large region could thus be a target for further investigation because a region homologous to the human 2p21-25 has previously been reported to be amplified in radon-induced rat

lung tumors (26). Otherwise 2p amplifications have rarely been described in non-SCLC. Neither has the region at 14q11.2, to our knowledge, been reported to be altered in lung cancer, but it has been assumed to be involved in chromosomal aberrations (inversions and translocations) in the blood samples of a population exposed to prolonged low dose-rate ^{60}Co γ -irradiation (27). This could be interesting, considering that radiation might cause similar aberrations as asbestos through the production of ROS (28).

Eleven of the 125 known fragile sites coincide with the 18 potentially asbestos-associated regions ($P = 0.08$; Table 3). Fragile sites are predetermined chromosomal breakage regions, which experimentally can be shown as site-specific gaps or breaks on metaphase chromosomes under conditions of replicative stress. As chromosomal expression of genetic instability, they have been suggested to have a role in carcinogenesis. As an example, the *FHT* gene at FRA3B (3p14.2) is often damaged in tumors and presumably acts as a tumor suppressor (29). In addition, FRA16D

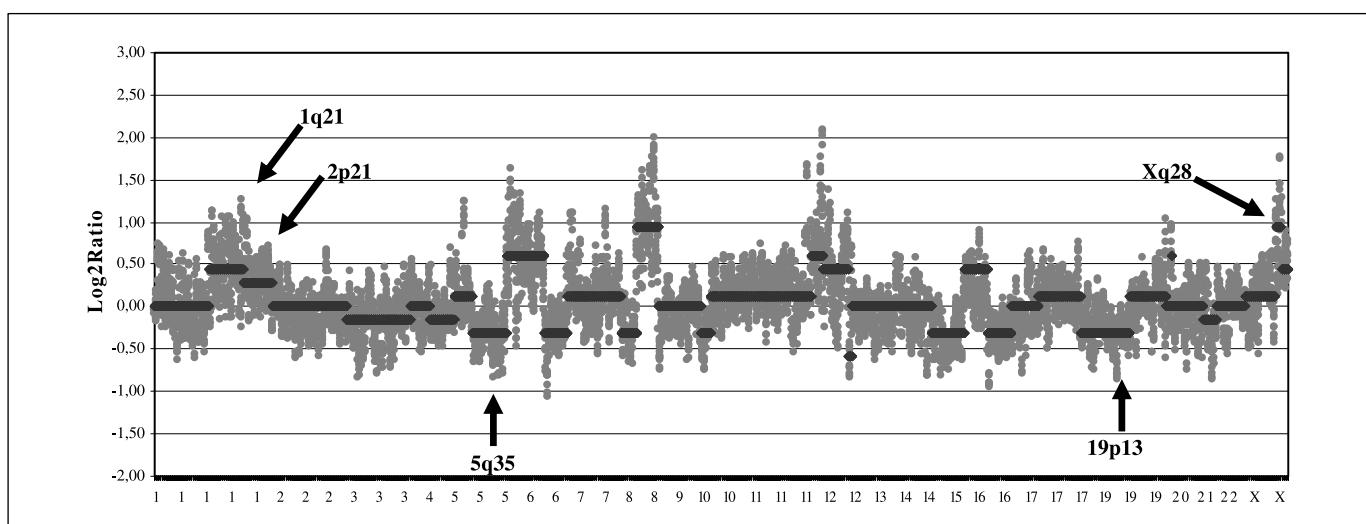


Figure 2. Array-based CGH copy number profile of an asbestos-exposed lung tumor with five asbestos-specific aberrations. The copy number ratios (Y axis) between the tumor and the reference are plotted against the cumulative base-pair locations from the short arm to the long arm of the chromosomes (X axis). Gray spots, moving average ratio of 10 adjacent clones and a smoothing algorithm (black vertical lines). Gains and losses are marked using the aCGH-Smooth software (<http://www.few.vu.nl/~vumarray/>) with default settings, apart from the λ -variable set to 5.

has been found to contain mutations in cancer (30). Furthermore, in 11q13.2, a 700-kb deletion has recently been identified in cervical cancer, containing the fragile site FRA11A. This 700-kb region lies almost completely within the asbestos-associated regions (chromosome 11: 65,886,587-67,191,050 bp; ref. 31). All together, 12 genes that lie within the asbestos-related regions have previously been associated with fragile sites (Table 3; refs. 31–34). Interestingly, all except one of the fragile sites that coincide with the asbestos-associated regions have been implicated in bladder cancer (35) that is also associated with asbestos exposure (36).

In conclusion, to reveal the possible aberrations related to asbestos exposure in the array data, we chose to carry out the data analysis using a combined array data set. We detected for the first time several, mostly small chromosomal regions that differed in DNA copy number between two groups of patients with asbestos-exposed and nonexposed lung tumors. The aberrations were either low copy number gains or losses with no high copy number amplifications. Previous studies have implied that smoking makes the genetic system of the cells more vulnerable to the deleterious effects of asbestos (22, 23). This evidence agrees with our classic CGH results, in which the same complex patterns of aberrations were generally found in both groups with just slightly more

aberrations in the exposed group. Furthermore, our array CGH results showed that many of these sites coincided with fragile sites, implying that smoking and asbestos fibers may preferentially cause aberrations at fragile sites. To conclude, we report for the first time a gene copy number aberration profile related to asbestos exposure. A larger series of patients is needed for further verifying analysis, using for example expression data and analysis of allelic imbalance to determine whether these regions are specific and harbor putative target genes. Knowledge of the target genes could be important for the development of methods for early diagnosis of asbestos-related lung cancer. These findings could also benefit the research of mesothelioma and other asbestos-related cancers.

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