DNA Copy Number Changes in Lung Adenocarcinoma in Younger Patients

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We performed a comparative genomic hybridization study on 25 lung adenocarcinoma samples from younger patients (<41 y of age) and compared the results with a previous comparative genomic hybridization analysis of lung adenocarcinoma samples from older patients (50–81 y of age). Twenty of the 25 tumor samples from younger patients had DNA copy number changes. Gains, losses, and high-level amplifications were seen more frequently in the specimens from the younger group. The most striking difference between the two groups was the high frequency of gains and/or high-level amplifications in the long arm of chromosome 20 in the samples from the younger patients (14/25, 56%) compared with that in the samples from the older patients (2/24, 8%, $P < .001$). Gains in the long arm of chromosome 22 and of the chromosomal band 11q13 were also detected significantly more often in the younger group. No correlation was found between DNA copy number changes and clinical parameters. Our results suggest that amplification of genes in the long arm of chromosome 20 may be important in the tumorigenesis of lung adenocarcinoma in young adults. Several candidate genes have already been described in the long arm of chromosome 20, particularly in breast cancer.

KEY WORDS: 20q, Adenocarcinoma, CGH, Gain, Lung neoplasms, Young.

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The incidence of lung adenocarcinoma has increased throughout the world in recent decades, and adenocarcinoma is now the most common type of lung cancer in North America. In western Europe, squamous cell carcinoma is the most frequent subtype, although the proportion of lung adenocarcinoma has increased in many countries (1–5). Typically, young patients with lung cancer more often have adenocarcinoma, especially the male patients, and the male-to-female ratio is lower. According to many reports, there is no difference in the smoking habits of younger and older lung cancer patients (6–9). In many recent studies, the prognosis for younger lung cancer patients has been similar to that for older patients (8–11).

There is little evidence that a predisposition to lung cancer can be inherited, although epidemiological studies have shown an increased familiar risk (12, 13), especially in younger people (14). A genetic factor may lead to carcinogenesis in younger patients. Increased microsatellite instability has been reported in lung cancer patients of ≤40 years of age (15). No significant differences in *KRAS2* and *P53* gene alterations have been found between older and younger patients (16).

Karyotypes in non–small cell lung cancer are often complex, with multiple numerical and structural changes (17, 18). In lung adenocarcinoma, deletions in 3p, 9p, and 17p and gains in 1q, 7p, 7q, and 11q are recurrent karyotypic alterations (18). Studies in non–small cell lung cancer have verified loss of heterozygosity in chromosomal regions 1q, 3p, 5q, 8q, 9p, 13q, and 17p (19), thus indicating the presence of putative tumor suppressor genes in those areas.

Comparative genomic hybridization (CGH) is a powerful molecular cytogenetic method for revealing DNA copy number changes, such as losses, gains, and amplifications of DNA sequences, in the entire tumor genome in a single hybridization ex-

periment (20–22). CGH studies have already been performed in lung adenocarcinoma (23, 24), but not specifically in tumors from younger patients. In this study, we used CGH to study genomic alterations in lung adenocarcinoma in younger patients (<41 y of age), and compared the results with a previously reported CGH study of older patients (>50 y of age; 23). A special gene alteration might predispose young patients to lung cancer and cause DNA copy number changes, which have not been described in tumors from older patients. We also investigated whether there is a correlation between DNA copy number changes and clinical parameters.

MATERIALS AND METHODS

In this study CGH was performed on 25 tumor samples from young Finnish patients. The results were compared with the CGH analysis of a reference group of tumors (24 specimens from older patients; 23).

Tumor Specimens

The Finnish Cancer Registry and the files of the Department of Pathology, University of Helsinki, recorded a total of 84 patients under 41 years of age who had been diagnosed with primary lung adenocarcinoma in Finland between 1980 and 1998. The group comprised 35 women (42%) and 49 men (58%). Only tumors with a histologically confirmed diagnosis and specimens with sufficient material for successful DNA extraction and CGH analysis were selected for the study. The diagnosis was confirmed by a pathologist using the World Health Organization classification for lung tumors. The specimens were formalin fixed and paraffin embedded. All specimens included in the study were taken from primary tumors before either or both chemotherapy radiotherapy. Twenty-five tumor samples from 25 different patients, 12 men and 13 women, were included in the study.

Patient Characteristics

The clinical characteristics of the 25 younger patients were collected retrospectively from the patients' case records and are summarized with those of the reference group of the older patients in Table 1. The mean age of the younger patients was 35.6 years. Eight percent (2/24) of the samples were from patients with clinical Stage I disease, 4% (1/24) from patients with Stage II, 17% (4/24) from patients with Stage IIIa, 25% (6/24) from patients with Stage IIIb, and 42% (10/24) from Stage IV. In one case, the stage was unknown. Staging was based on surgical records where available (in 68% of the cases); otherwise, the stage was determined from radiological examination and from computed tomography scans, which were available for all patients. The old staging classification for lung cancer was used (25). Smoking history was known for 22 of the younger patients; 19 (86%) had been smokers, and 8 of these had smoked >20 pack-years. Only three were nonsmokers. Four of the patients had a history of asbestos exposure. According to the case records, two patients had first-grade relatives with cancer. The history of asbestos exposure was unknown in 18 cases, and the family history of cancer was unknown in 14 cases. The median survival was 8 months (range from <1 mo to >13 y). In three patients, the diagnosis was made at autopsy. Only three patients (13%) had survived 5 years; all three had Stage I or II disease.

TABLE 1. Clinical Characteristics of the Patients

Parameter	Younger Group (n = 25)	Older Group (n = 24)
Average age, y (range)	35.6 (28–40)	63 (50–81)
Gender, *n* (%)		
Male	12 (48)	24 (100)
Female	13 (52)	0 (0)
Smoking habit, *n* (%)		
Smoker	19 (86)	22 (92)
Nonsmoker	3 (14)	2 (8)
Not known	3	
Stage, *n* (%)		
I	2 (8)	11 (46)
II	1 (4)	4 (17)
IIIa	4 (17)	8 (29)
IIIb	6 (25)	
IV	10 (42)	2 (8)
Not known	1	
Median survival	8 mo	>2 y

Reference Group

The reference group comprised 25 older adenocarcinoma patients, whose fresh-frozen tumor samples had previously undergone CGH analysis in our laboratory (23). All patients in the reference group were men who underwent surgery for suspected operable lung cancer. One patient was excluded from the comparison because he was too young (35 y). The mean age of the reference group was 63 years (range, 50–81 y). Ninety-two percent of the patients were smokers, and 44% had a history of asbestos exposure. Forty-six percent of the samples were from patients with clinical Stage I disease, 17% from patients with Stage II, 29% from patients with Stage IIIa, none from patients with Stage IIIb, and 8% from patients with Stage IV. In every case, the stage classification was made at surgery. The 2-year survival rate was 56%.

DNA Extraction

Sections were examined and the tumor area was marked. A new paraffin block was made of tissue

that contained ≥60% malignant cells, and DNA extraction was performed as described elsewhere (26, 27). DNA in peripheral blood specimens from healthy donors (male and female) was extracted according to standard procedures and used as a reference in the CGH analyses.

Comparative Genomic Hybridization

The CGH experiments and digital image analyses were performed as described by Kallioniemi *et al.* (28), with slight modifications for labeling tumor and reference DNA (29). In brief, tumor DNA was labeled green by nick translation with fluorescein-isothiocyanate-conjugated dCTP and dUTP (DuPont, Boston, MA), and normal reference DNA was labeled red with Texas red-conjugated dCTP and dUTP (DuPont). Labeled tumor DNA (1 μg) and labeled reference DNA (1 μg) were hybridized onto normal metaphase chromosomes, together with unlabeled Cot-1 DNA (20 μg). Hybridization and washes were performed as described elsewhere (30). The slides were counterstained with 4, 6-diamidino-2-phenyl-indole-dihydrochloride (DAPI; Sigma, St. Louis, MO) and covered with an antifade solution (Vector Laboratories, Burlingame, CA).

Digital Image Analysis

An Olympus fluorescence microscope and the *isis* digital image analysis system (MetaSystems GmbH, Altlussheim, Germany), based on an integrated high-sensitivity monochrome charge-coupled device (CCD) camera and an automated CGH camera, were used to analyze the hybridization. Eight to twelve metaphase images were acquired from each sample, and only nonoverlapping chromosomes with good morphology and uniformly intense colors were included in the analysis. Heterochromatic regions, p arms of acrocentric chromosomes, and the entire Y chromosome were excluded from the analysis. Chromosomal regions were considered overrepresented (gains) when the green-to-red fluorescence ratio was ≥1.17 and were considered underrepresented (losses) when the ratio was <0.85. The cutoff level for a high-level amplification was 1.5.

Cutoff values were based on negative hybridization experiments, in other words, hybridization of two normal DNAs. Only ratio changes that exceeded the fluctuation seen in negative-control experiments were interpreted as evidence of a real gain or loss. Furthermore, positive-control experiments with tumor DNA of known DNA copy number changes (both losses and gains) were performed to confirm the cutoff values mentioned above. DNA for the positive and negative control experiments was extracted from paraffin-embedded samples.

At least 10 copies of each chromosome were analyzed. All results were confirmed using a 99% confidence interval. Briefly, intraexperiment standard deviations for every position in the CGH ratio profiles were calculated from the variation of the ratio values of all homologous chromosomes within the experiment. Confidence intervals for the ratio profiles were then computed by combining them with an empirical interexperiment standard deviation and by estimating the error probability based on the *t* distribution.

Statistical Analysis

For statistical analysis, we applied Fisher's exact two-tailed test, because of the small number of cases, using the SAS statistical package. *P* values of <.05 were considered significant. The CGH results from the younger group were compared with those from the older group and with the clinical characteristics (stage and survival). Survival was measured from the date of surgery or from the date of diagnosis. Cumulative survival rates were calculated using the product limit method, and differences were analyzed using the log-rank test.

RESULTS

Table 2 shows the CGH results for all patients. In the younger group, 20 of the 25 (80%) tumor samples had DNA copy number changes. Gains dominated losses at a ratio of 4.6:1 (116 gains and 25 losses). High-level amplifications were detected 20 times: three times each in chromosomes 7 and 5p, twice each in 16p and 20q, and once each in 1q, 8q, 9q, 11q, 12q, 14q, 15q, 16q, 17q, and 22q. The most frequent changes were gains in the long arms of chromosomes 1, 8, 14, 20, and 22 and gains in the short arms of chromosomes 5, 7, and 16. Chromosomal band 11q13 was also commonly gained. All of these alterations were present in >20% of the cases. Losses in chromosomal arms 6q and 9p were detected in at least four tumors.

The comparison of DNA copy number changes between the younger and the older groups is summarized in Figure 1. In this study, all types of DNA copy number changes were detected more frequently in the tumors from the younger patients. In contrast to 116 gains seen in the younger group, only 62 gains were observed in the older group. High-level amplifications were seen 20 times in the younger group and 8 times in the older group. There were 25 losses of chromosomal material in the tumors from the younger patients, compared with 9 losses in the tumors from the older patients.

The most striking difference between the younger group and the older group was the high frequency of gains and/or high-level amplifications in the long

TABLE 2. Comparative Genomic Hybridization Results of the 25 Young Adenocarcinoma Patients

Case	Sex	Gains	Losses
1	M	5p14-pter, 11q13, 16p, 20q, 22	
2	F	1q22-qter, 16, **16p13.1**	9p21-pter
3	M	1q, 5p, **5p15.1-pter**, 7p15-pter, 7q32-qter, 11q13-qter, 14q12-qter, 17p, 20q, 22	6q11-22, 9p21-pter
4	F	1q12-qter, 2p21-pter, 5p13-pter, 6p12-pter, 7q32-qter, 11q13-14, 15q21-qter, 20q11.2-qter, **20q13.1**, 22, **22q11.1-13**	1p21-31, 4p15.1-q31.3, 5q15-23 X, 6q11-22, 13q14-31
5	M	1q21-qter, 7p, 14q23-qter, 20, **20q13.1**	
6	F	5p14-pter, 8q21-22, **8q22-qter**, 12q12-22, **12q13-21**, 14, 20	
7	F		
8	M	5, 7, 8q21.3-qter, 9p, **9p32-qter**, 11q13, 14q23-qter, 16, **16p12-pter**, **16q23, 19, 20**	11p11-22, Xp, 9p, 13q14-qter, 18
9	M	1q12-qter, **1q21-24**, 2p22-qter, 3p13.3-qter, 5p, 6p21.3-pter, 7p12-pter, 8, 11q13, 12p11.2-pter, 14q21-qter, **14q31-qter**, 15q23-qter, **15q25-qter** 16p, 17, **17q24-qter**, 20	
10	M	5q13-qter, 11q, **11q23**, 16p	
11	F	2, 5p, 6, 7q11.2-31, 8q, 20	4, 5q11.1-23, 8p, 18q
12	M	1q12-31, 6p, 10, 17, 20q11.2-qter	9p
13	F	1q32-qter, 7p13-21, 8q24.1, 11q13, 20q, 22	
14	F	22q11.2-13	
15	M		
16	F	22	
17	F	1q12-qter, 8q	6q
18	M	1q12-25, 1q32-qter, 5p, **5p14-pter**, 7p14-21, 17q12-22, 20q11.2-qter, 21q11.2-22	
19	F		
20	M		
21	M	4p15.2-pter, 5p14-pter, **5p15.2-pter**, 5q31-qter, 7p21-q34, **7q11.1-32**, 8q23-qter, 11, 12q22-qter, 16p11.2-pter, 17, 20q, **20q12-qter**, 21q21-22, 22	
22	F	1q, 5, Xp11.2-qter, 6p, 7, **7pter-q11.2**, 20q	3p11-21, 6q, 8pter-q23, 13, 4q
23	F	1p31-pter, 1q12-qter, 7p, 17q, 19q, 22	
24	F		
25	M	8q22-qter, 12q22-24.2, 14q22-31, 20q11.2-qter	

High-level amplifications are in bold.

arm of chromosome 20 in the adenocarcinoma samples from the younger patients (14/25, 56%) compared with that in the older patient group (2/24, 8%, $P < .001$). Other changes in which significant differences between the younger and the older groups were observed were the gain in the long arm of chromosome 22 (younger group: 7/25, 28%; older group: 0/24, 0%; $P < .01$) and the gain in the chromosomal band 11q13 (8/25: 32%; 1/24: 4%, $P < .05$). Gain in the short arm of chromosome 12 was more frequent in the older group (6/24, 25%) than in the younger group (1/25, 4%, $P < .05$). Gains in the long arm of chromosome 14 and in the short arm of chromosome 16 were more frequent in the

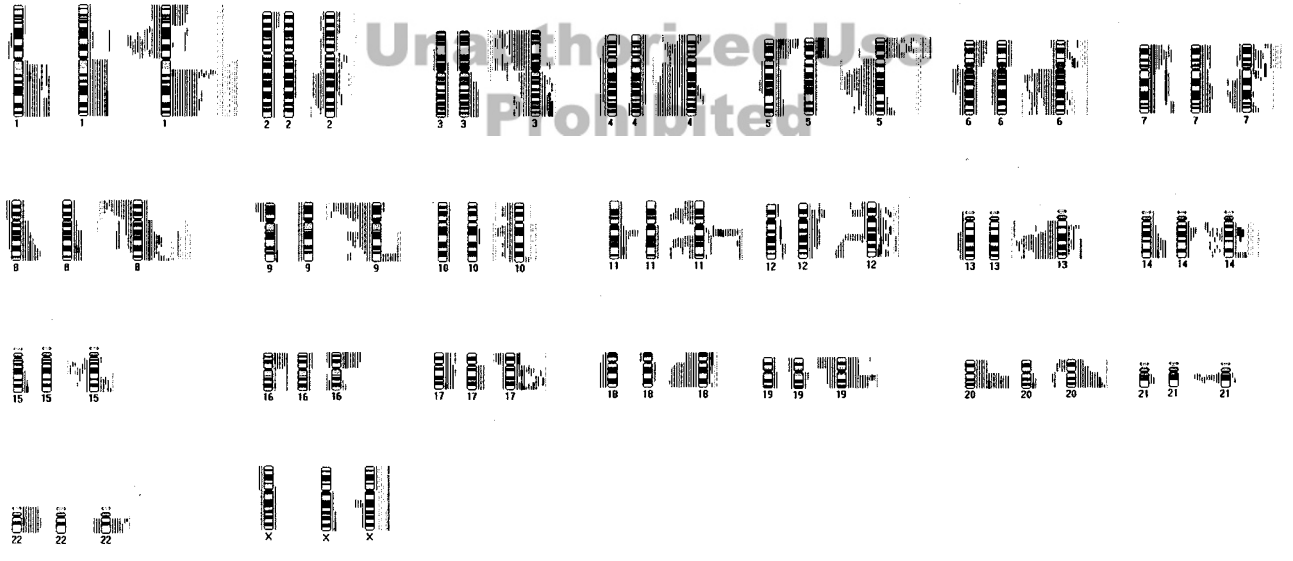

FIGURE 1. The first chromosome in the triplet represents our comparative genomic hybridization (CGH) results in the younger patient group; the second one in the older reference group; and the third, CGH results from lung adenocarcinoma tumor samples reported in the literature. Black lines in the third chromosome represent the study of 25 lung adenocarcinomas (31); gray lines, the study of 10 adenocarcinomas (23); and broken lines, the study of 3 lung adenocarcinomas (32).

tumors from the younger patients. No high-level amplification in the short arm of chromosome 7 was observed in tumors from the older group, whereas this was observed twice in the tumors from the younger group.

There was no correlation between DNA copy number changes and stage of the disease or survival. In the younger patients, DNA copy number changes were as frequent in tumors from patients with localized disease (Stage I or Stage II) as in tumors from patients with Stage IIIa, Stage IIIb, or Stage IV disease. The gain in the long arm of chromosome 20 was detected in two of four younger patients with Stage I or Stage II disease.

DISCUSSION

In this study, we used CGH to analyze tumor samples from young lung adenocarcinoma patients and compared the results with a previous CGH analysis of the older patients. We found that gains, high-level amplifications, and losses are more frequent in the younger patients. The most striking differences between these two groups were high frequencies of gains in the long arms of chromosomes 20 and 22 and the gain in chromosomal band 11q13 in the younger patients.

In this study, the risk factors for lung cancer in the younger adults were similar to those in the older patients. Tobacco smoking was as common in the younger group as it is generally in lung cancer patients. Only two patients had a family history of cancer. Women were more represented in the younger group, which may suggest that women are more susceptible to lung carcinogens. In the whole group of young lung adenocarcinoma patients, 42% (35/84) were women, and 52% (13/25) of the samples selected for CGH analysis were from women.

Most patients in the older group had local disease (Stage I or Stage II), whereas in the younger group, the majority of patients had Stage IIIb or Stage IV disease. Although difference in stage distribution can affect CGH results, an equal number of DNA copy number changes were detected in the young patients with Stage I or Stage II disease as in the young patients with more advanced disease. DNA gain in 20q was demonstrated in 50% (2/4) of the young patients with localized disease. The CGH study of the older patients was performed on fresh-frozen tumor samples, whereas the samples from the younger patients were formalin fixed and paraffin embedded. Because positive and negative controls have been used in both studies, the results are comparable.

Ours is the only study to compare DNA copy number changes in lung adenocarcinoma according to age. The present study and the CGH experiments in the reference group were both carried out in the same laboratory. In the literature, the majority of lung adenocarcinoma cases (25/38) that have been studied using CGH are from the work of Petersen *et al.* (31). Those investigators did not describe the clinical characteristics of the patients, such as age and stage of the disease, and they used both postmortem and fresh-frozen tumor samples. Furthermore, Petersen *et al.* (31) performed CGH using the indirect method, whereas we used the direct method in both the present study and the reference study.

In our study, 56% of the young patients' tumors had gained DNA material in 20q, including two high-level amplifications, whereas only 8% of the older patients' tumors showed an increase of these DNA sequences. This 20q gain has been reported earlier in lung adenocarcinoma in 13/38, 34% of the cases (24, 31, 32; Fig. 1). Invasive adenocarcinoma of the breast also shows an increased frequency of amplifications within the chromosomal band 20q13, which is implicated in tumor progression and poor prognosis (33). In epithelial cancers, especially in breast cancer, the long arm of chromosome 20 is known to harbor specific genes. The *AIBC1* gene (amplified in breast cancer 1) at 20q12 is amplified and overexpressed in breast and ovarian cancer cell lines and in breast cancer biopsies. *AIBC1* expression may contribute to the development of steroid-dependent cancers (34).

One established oncogene and several putative oncogenes are located in chromosomal band 20q13, in which we detected two high-level amplifications and gains in more than half of the adenocarcinoma cases in the younger group. *SRC* oncogene (avian sarcoma) is mapped to locus 20q12-q13. It has been found to be mutated in 12% of cases of advanced human colon cancer, but not in primary early-stage human colon cancer specimens or in normal genomic DNA. The mutation has been demonstrated to be activating, transforming, tumorigenic, and metastasis promoting (35). The *BTAK* gene encodes a centrosome-associated kinase, and it is involved in the induction of centrosome duplication–distribution abnormalities leading to the chromosome segregation abnormalities and the aneuploidy seen in many types of cancer cells. The *BTAK* gene is responsible for chromosomal instability, and its expression in the younger group could hypothetically explain why all types of DNA copy number alteration were observed in the younger group more frequently than in the older group. The *BTAK* gene has been reported to be overexpressed in human breast cancer cell lines. Cellular apoptosis susceptibility, the *CAS* gene, has been observed to be amplified in breast and co-

lon cancer cell lines and in a leukemia cell line (36). *CAS* may have a dual function in mammalian cells, one in apoptosis and another in cell proliferation. It has been detected that *CAS* antisense RNA can interfere with apoptosis mediated by tumor necrosis factors α and β and by ADP-ribosylating toxins, suggesting that CAS may play a role in selected pathways of apoptosis (37). In breast cancer, three putative oncogenes have been reported at 20q13.2: *CYP24 (*cytochrome P450), *ZNF217* (zinc finger protein 217), and *NABC1* (novel-1 amplified in breast cancer; 38). The overexpression of *CYP24* is likely to lead to abrogation of growth control mediated by vitamin D (39). Decoy receptor 3, the *DCR3* gene, is located at 20q13, and it has been observed to be amplified in about half of primary lung and colon tumors studied. The *DCR3* gene belongs to the tumor necrosis factor receptor family members that function as secreted decoys to modulate ligands that induce apoptosis (40).

In our analysis, 11q13 was among the most frequently amplified bands in the younger patients (in 8/25, 32% of the cases). This gain was rarely seen in our reference group of older patients, but it has been reported in lung adenocarcinoma, in 15/38 (39%) of the cases studied (24, 31, 32). Chromosomal band 11q13 contains several known oncogenes. Among the major regulators of the G1 restriction point is cyclin D1 gene (*CCND1*), which is located at 11q13 and has been reported to be amplified or overexpressed in almost 50% of non–small cell lung cancer tumors. Marchetti *et al.* (41) observed that the cyclin D1 gene was amplified in 25% (4/12) and overexpressed in 44% (7/16) of lung adenocarcinoma tumors. Chromosomal band 11q13.3 harbors the *HSTF1* and *INT2* genes, fibroblast growth factors, and two putative oncogenes: *ESM1*, the human analogue of cortactin, and the *SEA* gene (42, 43).

The long arm of chromosome 22 was frequently amplified in young adults. It was described as gained in an earlier study, in 8/38 (21%) of the cases (24, 31, 32; Fig. 1). The BCR gene on chromosome 22 is the breakpoint for the Philadelphia chromosome in >95% of chronic myeloid leukemia cases. No well-known oncogenes have been found on chromosome 22 in carcinoma tumors.

In summary, using CGH analysis, we have been able to demonstrate that DNA gains in the long arm of chromosome 20 are frequent in tumor samples from young lung adenocarcinoma patients. This chromosomal arm contains several candidate oncogenes. We conclude that amplification in 20q may be particularly important in the pathogenesis of lung adenocarcinoma in young adults. However, further investigations are needed to determine the role of this chromosomal aberration in lung cancer generally.

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