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Allelic imbalance at loci containing *FGFR*, *FGF*, *c-Met* and *HGF* candidate genes in non-small cell lung cancer sub-types, implication for progression

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Abstract

Fibroblast growth factors (FGF), hepatocyte growth factor (HGF) and their receptors, FGFR and c-Met, are essential components of the regulatory networks between the epithelium and mesenchyme in embryonic lung, but their respective roles in tumour growth are not clear. We performed allelotyping at loci containing the candidate genes *FGFR-1-2-3-4, FGF-1-2-7-10*, *c-Met* and *HGF* in 36 non-small cell lung cancer (NSCLC) (20 squamous-cell carcinomas (SQC) and 16 adenocarcinomas (ADC)), by surrounding each locus with two microsatellites (MS), as close as possible to the genes of interest. Unexpectedly, SQC and ADC were frequently altered at all of these loci, and SQC showed more simultaneously altered loci. In ADC, alterations at the 15q13-22 locus (*FGF7* candidate gene) were significantly more frequent. Thus, these loci showed different patterns of molecular alterations between SQC and ADC. Finally, alterations at loci containing *FGFR* and *HGF* candidate genes were inversely correlated to the lymph node status in SQC and ADC, respectively.

Keywords: Non-small cell lung cancer; Allelotyping; *FGFR*/*FGF*; *c-Met*/*HGF*; Histological sub-types; Tumour progression

1. Introduction

Lung cancer is one of the most common tumours in the world, classified into four main groups by the World Health Organization (WHO) as small-cell lung cancer (SCLC), squamous-cell carcinoma (SQC), adenocarcinoma (ADC) and large-cell carcinoma (LCC) [1]. For prognostic and therapeutic purposes, SQC, ADC and LCC are pooled together in the non-small cell lung cancer (NSCLC) group, which represents 80% of lung cancers. Histological NSCLC sub-types present different clinical patterns. SQC arise centrally, whereas ADC are often located peripherally, can grow more rapidly and have shown an increased incidence, particularly in females and non-smokers [2,3]. Accumulation of genetic alterations are common in lung cancer and include gene alterations, mutations, allelic gains or losses, allelic instabilities and aberrant gene methylation, targeting oncogenes and tumour suppressor genes (TSG). Specific genomic alterations have been observed between NSCLC sub-groups by Comparative Genomic Hybridisation (CGH) [4–6]. In allelotyping analyses, distinct patterns of chromosomal alterations have been shown for SCLC and NSCLC at 3p, 5q, 6q, 9p, 10q, 11p, 13q, 17p and 19p [7]. A more defined molecular classification of lung carcinomas could help in the prediction of patient outcome, the selection of available therapies,

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and the identification of new molecular targets for chemotherapy.

The fibroblast growth factor (FGF) family represents a group of 21 heparin-binding, multifunctional polypeptides with a mitogenic activity exerted via binding to their high affinity transmembrane tyrosine kinase receptors, the fibroblast-growth-factor-receptors (FGFR) [8]. In embryonic lung, both FGFs and FGFRs are essential components of the regulatory networks between the epithelium and mesenchyme. They are involved in different processes including lung embryonic development, but also in wound healing, tissue regeneration and angiogenesis. They are also essential for postnatal remodelling and repair of alveoli, as well as for tumour growth and tumour vascularisation [9,10]. FGF1 (aFGF) appears to be chemotactic for lung buds, in contrast to FGF7 (or KGF), which appears to promote epithelial growth and modulates the pulmonary alveolar type II phenotype [11]. FGF10 plays an important role in epithelial branching. When overexpressed, it can induce adenomatous malformations in fetal lung and multifocal pulmonary tumours after birth [12]. FGFR-2 appears to be implicated in the branching morphogenesis of lung, whereas FGFR-3 and FGFR-4 seem essential in regulating alveolisation during postnatal life [9,10]. In lung cancer, FGF2 (bFGF) and FGFR-1 expressions have been mostly investigated at the RNA and protein levels, but consequences of their overexpression are controversial [13–18]. Furthermore, the involvement of FGF7, FGF10, FGFR-3 and FGFR-4 in lung cancer has not yet been precisely defined.

Hepatocyte growth factor (HGF) is a multifunctional factor that stimulates epithelial cell motility, invasion and morphogenesis, and acts through a transmembrane tyrosine-kinase receptor, *c-Met* [19]. HGF is mainly expressed in primitive lung mesenchyme and c-Met in primitive lung epithelium [9]. When stimulated in malignant cells, both are able to enhance cell motility, invasion and, eventually, metastasis [19,20]. Overexpression of HGF in lung cancer cells induces alveolar differentiation and expression of c-Met has been linked to the differentiation phenotype of the lung tumour cells, but with an underexpression in SQC and conversely an overexpression in ADC [20].

Up until now, it has not been clear whether the different *FGFR*, *FGF*, *HGF* and *c-Met* genes are activators or inhibitors of tumour growth in lung cancers, even though activating mutations and overexpression of some of these genes have already been described in some carcinomas [21]. Furthermore, the connections between both signalling pathways involving FGFR/FGF and/or c-Met/HGF have not been clearly established in lung cancer. The aim of our study was to analyse simultaneously, by allelotyping, the presence of allelic imbalance (AI) (corresponding either to an amplification or a deletion of the locus) at the 10 loci containing these candidate genes. We used two microsatellites (MS) flanking each locus to evaluate whether some of these regions could be associated specifically to certain histological sub-types of NSCLC and/or involved in tumour progression.

2. Materials and methods

2.1. Patients and tumour samples

We prospectively examined surgical specimens obtained from 36 cases of NSCLC (20 SQC and 16 ADC). Patients gave their informed consent to be included in this prospective study. Stage was defined as recommended in Ref. [22]. Clinical patient's characteristics are summarised in Tables 1 and 2. Tumour and paired normal lung peripheral tissue samples collected at the most distal end of the tumour were immediately stored at −80 °C. For 2 patients, normal control DNA was obtained from paired peripheral blood leucocytes, collected into two 5-ml ethylene diamine tetraacetic acid (EDTA) tubes before any treatment.

2.2. Histology

All tumour samples were examined carefully histopathologically on sections stained with haematoxylin and eosin and the percentage of tumour cells was evaluated. Histological diagnosis was performed according to the WHO lung carcinoma classification [1]. Samples were scored for two parameters: differentiation and vascular invasion (Table 1).

2.3. DNA extraction

DNA was extracted from tissue and from blood cells by a phenol-chloroform method as previously described in Refs. [23,24].

2.4. Microsatellite analysis

Normal and paired tumour DNA were amplified by a fluorescent polymerase chain reaction (PCR) [23,24]. The analysis of MS alterations was performed using 19 polymorphic MS listed in Tables 2 and 3. All MS had CA dinucleotide repeats. The nucleotide sequences of primers used for MS analysis are available through the Genome Database (www.ncbi.nlm.nih.gov/Genemap 99). For each of the 10 regions 8p11, 10q26, 4p16, 5q35 loci (*FGFR-1-2-3-4* as candidate genes), 5q31, 4q25-27, 15q13-22, 5p13-12 loci (*FGF1*, *2*, *7*, *10* as candidate genes) and 7q31, 7q25 loci (*c-Met* and *HGF* as candidate genes), two MS were chosen flanking these genes. The closest possible to the gene of interest was chosen, except for the 5q31 locus for which the chosen MS is

localised in intron 1B of *FGF1*. If no alteration was observed with this selected panel of MS, other MS (chosen among the loci known to be frequently rearranged in NSCLC) were used to confirm the presence of tumour cells in the analysed tumour sample [24]. 50 ng of DNA were used for each PCR amplification. The fluorescent PCR protocol was performed as already described except that the hybridisation temperature was set at 58 °C for 35 cycles [24]. A modification of the quantitative allele ratio (estimated by measuring the peak height of both alleles) in tumour DNA versus paired normal lung or blood cell DNA was described as allelic imbalance (AI). A cut-off value (of the intensity of the AI) $\geqslant 20\%$ for each MS indicated the presence of significant AI, allowing a specificity of 100% [23,24]. Our control DNA were prepared from a large cell population of healthy tissue. Since most of micro-alterations in normal tissue have been observed in a few hundred microdissected cells, the eventual presence of such microtumour clones would not significantly affect the sensitivity of our test. In our study, the presence or absence of an AI was confirmed by at least two independent PCRs.

Table 1
Clinical and pathological features of 36 patients with NSCLC, classified by histological sub-type[a]

Features	Number of patients	SQC	ADC
Number of patients	36	20	16
Age[b] (years)	63 (42–80)	63 (42–80)	63 (50–79)
Gender			
Male	31	19	12
Female	5	1	4
T-stage			
T1	5	4	1
T2	22	13	9
T3	6	2	4
T4	3	1	2
N-stage			
N0	18	9	9
N1	8	5	3
N2	8	5	3
N3	2	1	1
Stage			
I	15	9	6
II	7	4	3
III	12	6	6
IV	2	1	1
Differentiation			
1	13	7	6
2	22	13	9
Vascular invasion			
0	24	15	9
1	10	4	6

[a] Samples were scored for two histological parameters: differentiation (poor: 1; moderate and well: 2) and vascular invasion (presence: 1; absence: 0).
[b] Median (range).

2.5. Data analysis

In order to calculate the frequency of alteration at each locus, since two MS surrounding the locus were analysed, we considered a locus altered (amplified or deleted) when AI was observed at both MS, or at one MS when the other one was not informative (homozygous MS or non-amplified MS). Since discordant results (e.g. one AI and one normal informative MS simultaneously observed at the same locus, for 1 patient) were not frequent (less than 10% of all the informative loci), calculations of frequencies of alterations by locus were realised by excluding these discordant results.

To facilitate the comparisons, we used two quantitative values: (a) By locus, we determined the frequencies of alterations by using a Fractional Alteration frequency (FA) as defined below: ((number of alterations for the locus)/(number of informative cases for the locus))×100; (b) By group of loci depending on the family of the candidate genes, we determined the frequencies of alterations by using a Fractional Regional Alteration frequency (FRA) as defined below: ((number of alterations in the group of loci)/(number of informative loci in the group of loci))×100.

Statistical analysis by using the Fisher's exact test was performed to compare qualitative data. A P value of <0.05 was considered significant and Bonferroni's corrections were taken into account when required. The t-test was used for mean comparisons. A cluster analysis was also used. The aggregation criterion is the mean distance between clusters, and the distance is the Sokal-Sneath-1 distance. All statistical calculations were performed with the Statistical Package for the Social Sciences (SPSS) (number 9) statistical software.

3. Results

To evaluate the potential role in lung carcinogenesis of the different regions containing the *FGFR*, *FGF*, *c-Met* and *HGF* candidate genes, we screened, by allelotyping, a population of 36 NSCLC. Unexpectedly, all of the loci were frequently altered (Table 3, Fig. 1). 32 patients (89%) had at least one locus altered (Table 2). For the four non-rearranged tumours, we confirmed the presence of tumour cells by testing additional MS (data not shown).

Table 2
Allelic imbalance (AI) in 36 NSCLC[a]

	Stage (TNM staging)	D8S1722/ D8S255	D10S209/ D10S1483	D4S3038/ D4S412	D5S2034/ D5S2030	D7S522/ D7S677	APCR1	D4S427/ D4S430	D15S978/ D15S982	D5S2087/ D5S822	D7S660/ D7S524
SQC											
1	IV (T4N3M1)	■/○	■/□	○/□	□/□	□/□	○	□/□	□/	○/○	□/○
3	IIIA (T3N2M0)	□/■	■/○	□/○	○/○	■/□	○	■/■	□/□	○/■	□/□
4	IIIA (T2N2M0)	○/□	○/□	□/□	□/□	□/□	□		○/	□/■	□/○
5	IIB (T2N1M0)	□/□	□/○	○/□	□/□	■/○	○	□/■	■/□	□/○	□/○
10	IA (T1N0M0)	○/■	■/■	■/○	■/■	□/■	■		○/■	○/■	■/○
12	IB (T2N0M0)	○/○	○/■	■/■	■/	○/○	□	■/■	■/■	○/■	○/□
14	IIIA (T2N2M0)	□/□	□/□	□/○	□/□	□/○	○	□/□	○/□	□/○	□/□
15	IIIB (T2N2M0)	■/□	□/○	■/○	■/○	■/○	■	■/■	■/■	○/○	■/□
16	IIB (T2N1M0)	■/□	○/□	■/□	□/○	□/○	□	■/○	□/□	□/○	■/
17	IIA (T1N1M0)	□/□	□/○	○/○	■/■	□/□	■	○/■	■/○	■/■	□/□
18	IB (T2N0M0)	○/□	■/■	○/□	■/○	○/○	■	○/○	□/□	■/■	■/□
19	IA (T1N0M0)	○/■	■/○	○/■	■/○	○/○		■/■	■/■	■/○	■/□
20	IB (T2N0M0)	□/○	■/■	■/○	/□	□/	□	■/■	□/○	○/□	□/●
21	IIIA (T3N1M0)	□/□	□/□	□/□	□/□	□/□	○	□/□	■/□	□/□	■/○
23	IB (T2N0M0)	□/□	□/○	□/□	□/○	□/○	○	□/□	□/□	□/□	○/○
24	IIA (T1N1M0)	□/□	■/○	□/□	■/□	□/□	□	■/□	□/	□/□	□/□
25	IB (T2N0M0)	○/■	○/■	□/□	□/□	■/○	□	□/□	□/□	○/□	■/□
26	IIIA (T2N2M0)	□/□	□/□	□/□	□/□	○/○	○	□/○	○/□	○/□	□/□
27	IB (T2N0M0)	○/□	○/□	□/□	○/○	/■	○	■/■	□/□	□/■	■/■
29	IB (T2N0M0)	■/○	■/■	○/■	■/○	○/□	■	■/■	■/○	○/○	○/□
ADC											
2	IV (T2N3M1)	■/■	■/○	■/■	○/○	○/□	□	○/■	○/■	■/○	○/○
6	IB (T2N0M0)	□/○	□/□	○/○	■/■	○/□	○	□/□	□/□	□/□	■/□
7	IB (T2N0M0)	□/□	○/■	○/■	■/□	○/○	○	○/□	■/□	○/□	■/□
8	IB (T2N0M0)	□/□	□/□	○/○	■/○	○/■	■	□/□	■/■	□/○	■/■
9	IIB (T3N0M0)	○/○	□/□	■/○	□/○	/■	■	■/○	■/■	○/□	■/○
11	IB (T2N0M0)	○/■	■/■	□/□	□/□	○/□	□	○/	■/■	○/○	□/■
13	IIB (T3N0M0)	○/■	■/■	■/■	○/■	■/○	○	□/□	○/○	■/○	■/■
22	IIIB (T4N0M0)	□/□	■/■	○/○	□/○	■/■	○	□/□	■/■	□/■	○/□
28	IIIA (T2N2M0)	■/□	■/○	□/○	■/○	○/■	■	○/■	○/■	□/□	○/□
30	IIIA (T3N2M0)	□/■	■/□	□/○	■/■	■/■	○	□/○	■/■	□/□	■/■
31	IA (T1N0M0)	□/□	○/■	□/○	□/□	■/○	■	□/□	○/■	○/	■/□
32	IIIA (T3N1M0)	□/□	□/□	○/□	□/□	□/○	○	□/○	○/○	□/□	□/○
33	IIIB (T3N2M0)	□/□	○/□	○/□	■/■	□/○	○	○/○	□/○	□/□	○/○
34	IB (T2N0M0)	□/□	■/□	□/□	■/○	/○	○	/□	■/○	○/○	○/○
35	IIIA (T2N2M0)	■/■	■/	□/○	■/○	■/■	○	■/■	■/■	■/○	○/□
36	IIB (T2N1M0)	□/□	○/■	○/■	□/■	○/□	■	■/■	/■	□/□	○/□

■ = presence of AI; □ = absence of AI; ○ = homozygous. The first symbol corresponds to the centromeric MS and the second one to the telomeric MS surrounding the targeted gene. In dark grey are indicated discordant results of AI between the two MS flanking the locus.

[a] Missing results are related to non-amplified MS.

Table 3
Fractional alteration (FA) frequency per locus[a]

MS	Locus	Candidate gene	Tumors ($n=36$) FA (%)	SQC ($n=20$) FA (%)	ADC ($n=16$) FA (%)	P value
D8S1722	8p11	*FGFR1*	31 (9/29)	31 (5/16)	31 (4/13)	NS
D8S255						
D10S209	10q26	*FGFR2*	55 (18/33)	47 (9/19)	64 (9/14)	NS
D10S1483						
D4S3038	4p16	*FGFR3*	37 (11/30)	33 (6/18)	42 (5/12)	NS
D4S412						
D5S2034	5q35	*FGFR4*	50 (15/30)	41 (7/17)	62 (8/13)	NS
D5S2030						
D7S522	7q31	*c-Met*	43 (12/28)	29 (4/14)	57 (8/14)	NS
D7S677						
APCR1	5q31	*FGF1*	56 (10/18)	45 (5/11)	71 (5/7)	NS
D4S427	4q25-27	*FGF2*	52 (15/29)	60 (9/15)	43 (6/14)	NS
D4S430						
D15S978	15q13-22	*FGF7*	55 (17/31)	33 (6/18)	85 (11/13)	0.006
D15S982						
D5S2087	5p13-12	FGF10	41 (11/27)	44 (7/16)	36 (4/11)	NS
D5S822						
D7S660	7q25	*HGF*	35 (9/26)	31 (5/16)	40 (4/10)	NS
D7S524						

NS, non significant.

[a] By locus, the frequencies of alterations were determined by using a Fractional Alteration frequency (FA) as defined below: ((number of alterations for the locus)/(number of informative cases for the locus))×100.

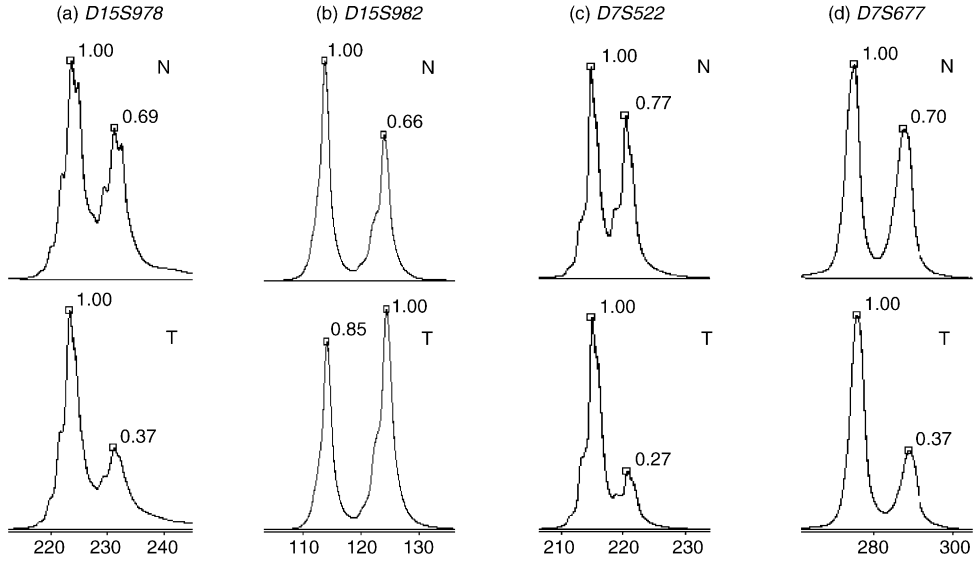

Fig. 1. Profiles obtained from fluorescent MS analysis of normal lung tissue and paired tumour lung DNA. Fluorescent PCR amplifications were quantified after separation by electrophoresis in a semi-automated sequencer. A variation of the allele ratio (estimated by measuring the peak height of both alleles) in normal lung tissue (N) versus paired tumour lung DNA (T) will be described as allelic imbalance (AI). A cut-off value (of the intensity of the AI) $\geqslant 20\%$ for each MS indicates the presence of a significant AI: (a) and (b) represent examples of AI at *D15S978* and *D15S982*, respectively, in patient number 22; (c) and (d) represent examples of AI at *D7S522* and *D7S677*, respectively, in patient number 30.

Since it was previously reported that the FGFR/FGF and c-Met/HGF signalling pathways could be involved in different steps of lung development, we asked if our allelotyping could distinguish histological sub-groups of NSCLC, since 17/20 (85%) of SQC and 15/16 (94%) of ADC patients presented at least one locus altered. Considering the groups of loci containing either *FGFR* (*-1-2-3-4*) or *FGF* (*1*, *2*, *7*, *10*) candidate genes, even though they often appeared simultaneously altered in all of our NSCLC tumours, ADC more frequently showed only one locus altered in each group, whereas SQC frequently presented simultaneously several alterations per

group (data not shown). ADC presented a higher global FRA frequency (see Materials and methods) than the SQC patients (50% versus 39%), although this difference was not significant (Table 4). Only the 15q13-22 locus (*FGF7* candidate gene) had a higher FA frequency in ADC than in SQC patients (85% versus 33%, $P=0.006$) (Table 3). Combined alterations (FRA) at loci containing *c-Met* and group of loci containing *FGFR* or *FGF* candidate genes were significantly higher in ADC (Table 4). Interestingly, hierarchical cluster analysis using locus 15q13-22 (*FGF7* candidate gene) and locus 7q31 (*c-Met* candidate gene) correctly classified 79% of our NSCLC into SQC or ADC (Fig. 2). Furthermore, significant associations of alterations between two loci were mainly observed between loci 15q13-22 and 4p16 ($P=0.001$) and 5q35 ($P=0.001$), in SQC only (data not shown). Altogether, our allelotyping results at the 10 loci suggest a different pattern of alterations for SQC and ADC.

Furthermore, as expected, our study showed that vascular invasion of the primary lung tumour was correlated with a N1–N3 lymph node status ($n=34$, $P=0.01$). We observed an overall FRA frequency that was significantly higher in N0 (54%) than in N1–N3 patients (34%) ($P=0.04$) (Table 5). Such a difference was conserved in SQC ($P=0.02$), mainly due to the presence of alterations at loci containing *FGFR* candidate genes, notably at the 8p11 locus (*FGFR1* candidate gene) ($P=0.02$). In ADC, a trend towards a higher FA frequency at the 7q25 locus (*HGF* candidate gene) was observed in N0 patients ($P=0.07$), such a correlation becoming significant when taking into account the discordant results at this locus ($P=0.016$) (Tables 2 and 5).

Next, we analysed how the groups of loci targeting the FGFR/FGF and c-Met/HGF signalling pathways could be involved together in loco-regional metastatic disease. Combinations including the 7q25 locus (*HGF* candidate gene) had a higher FRA frequency in N0 SQC, but not in ADC (Table 5). Furthermore, a FA frequency at the 4q25-27 locus was significantly higher in well differentiated SQC ($P=0.04$) (data not shown). Reciprocally, FA frequencies at the 5p13-12 or 5q35 loci were significantly higher in less differentiated ADC ($P=0.02$ and $P=0.04$, respectively). Thus, in SQC patients, it appeared that alterations at loci containing *FGFR*/*FGF* candidate genes alone or combined with alterations at the 7q25 locus, could be associated with lymph node-negative tumours. By contrast, alterations at 7q25 alone (*HGF* candidate gene) could be involved in lymph node-negative tumours in ADC patients.

4. Discussion

As the *FGF* (*1, 2, 7, 10*), *FGFR* (*-1-2-3-4*), *HGF* and *c-Met* genes appear to be implicated in lung development and cell differentiation, we asked whether they could play a role in the emergence of different histological sub-groups and in tumour progression in NSCLC patients. Some of these genes have already been shown to be overexpressed in previous studies. We chose to study the different chromosomal regions containing these genes by allelotyping, since this allowed us to analyse all these sites simultaneously, even though it did not indicate the real status (either amplified or deleted) of the alleles. Our results showed that NSCLC patients

Table 4
Fractional regional alteration (FRA) frequency per group of loci[a]

Locus	Candidate genes	All patients ($n=36$) FRA (%)	SQC ($n=20$) FRA (%)	ADC ($n=16$) FRA (%)	*P* value
All loci	*FGFR*+*FGF*+*c-Met*+*HGF*	44	39	50	NS
8p11, 10q26, 4p16, 5q35	*FGFR*	44	38	52	NS
5q31, 4q25-27, 15q13-22, 5p13-12	*FGF*	46	42	52	NS
8p11, 10q26, 4p16, 5q35, 5q31, 4q25-27, 15q13-22, 5p13-12	*FGFR*+*FGF*	45	40	51	NS
7q31, 7q25	*c-Met*+*HGF*	32	29	37	NS
8p11, 10q26, 4p16, 5q35, 7q31	*FGFR*+*c-Met*	40	26	53	0.01
8p11, 10q26, 4p16, 5q35, 7q25	*FGFR*+*HGF*	44	38	56	NS
5q31, 4q25-27, 15q13-22, 5p13-12, 7q31	*FGF*+*c-Met*	76	54	98	0.04
5q31, 4q25-27, 15q13-22, 5p13-12, 7q25	*FGF*+*HGF*	46	41	54	NS
8p11, 10q26, 4p16, 5q35, 5q31, 4q25-27, 15q13-22, 5p13-12, 7q35	*FGFR*+*FGF*+*c-Met*	41	29	53	0.03
8p11, 10q26, 4p16, 5q35, 5q31, 4q25-27, 15q13-22, 5p13-12, 7q25	*FGFR*+*FGF*+*HGF*	46	40	57	NS

NS, non significant.

[a] The frequencies of alterations by group of loci, depending on the family of candidate genes, were determined by using a Fractional Regional Alteration frequency (FRA) as defined below: ((number of alterations in the group of loci)/(number of informative loci in the group of loci))×100.

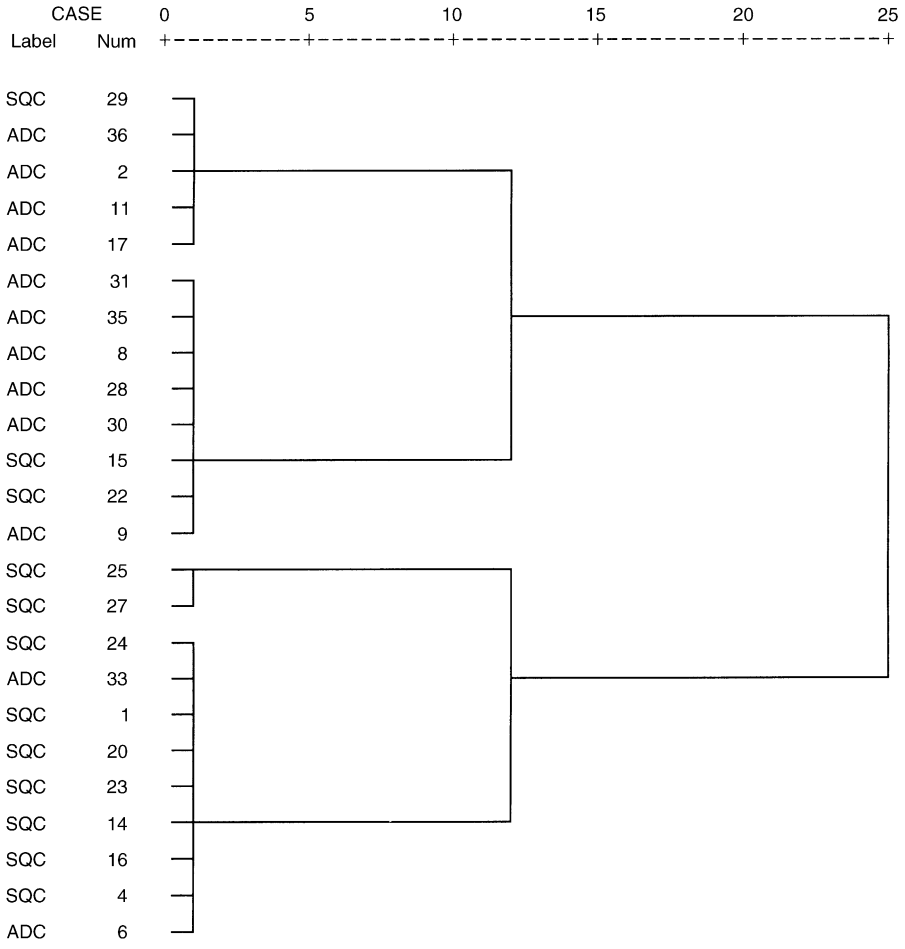

Fig. 2. Hierarchical cluster analysis of using locus 15q13-12 (*FGF7* candidate gene) and locus 7q31 (*c-Met* candidate gene). "The aggregation criterion is the mean distance between clusters, and the distance is the Sokal-Sneath-1 distance." 19/24 (79%) of the patients appeared correctly classified into SQC or ADC. Label, corresponding the histological sub-type of NSCLC patients; Num, corresponding to the anonymous number of the patients (described in Table 2).

had frequent rearrangements at all of the 10 loci analysed.

In our study, we used a fluorescence-based protocol in order to increase the sensitivity of the results [23,24]. In addition, two MS flanking the gene of interest were, for the most part, chosen. This allowed a more precise targeting of alterations at these loci. Discordant results were observed in less than 10% of the informative loci at both MS. These results were excluded from our statistical analysis as, since the length of the alteration was not known in these cases, we could not conclude on the presence of an alteration at the considered locus. Nevertheless, calculations were not significantly different when including these discordant results (Table 5 and data not shown). Remarkably, 20 out of the 29 discordant results (six out of the seven discordant results at the *HGF* locus), corresponded to an AI at the centromeric MS, generally the nearest one to the targeted gene.

Up until now, CGH studies have shown some discrepancies in the results obtained in NSCLC, although amplification of chromosome arms 7q, 5p and 15q has generally been observed as well as deletion of 8p [4–6]. Allelotyping appears to be a complementary and more precise method for loci analyses and our FA frequencies are in agreement with the expression studies that have been published in the literature [13–18,20,25–28]. Up until now, it is also not clear whether the *FGFR*, *FGF*, *HGF* and *c-Met* genes function as activators or inhibitors of tumour growth. In fact, their role appears to depend on the organs or histological type of tumours. Indeed, even though high expression of *FGF2* is sufficient to transform normal cells into a malignant phenotype, a study in breast cancer reported that higher protein levels of FGF2 were associated with an improved survival [29]. Likewise, the biological effect on carcinoma cells (proliferation and/or differentiation) of the c-Met/HGF autocrine loop over activation may also be cell-dependent and the prognosis associated with c-Met/HGF expression is variable [20,30,31]. Obviously, following this allelotyping, further studies would be required to determine the exact status of the alterations

Table 5
Correlations between FRA frequency observed at each group of loci and lymph node status[a]

Locus	Candidate genes	All patients ($n=36$) FRA (%)			SQC ($n=20$) FRA (%)			ADC ($n=16$) FRA (%)		
		N0	N1–N3	*P* value	N0	N1–N3	*P* value	N0	N1–N3	*P* value
All loci	*FGFR+FGF+c-Met+HGF*	54	34	0.04	57	24	0.02	50	50	NS
8p11, 10q26, 4p16, 5q35	*FGFR*	53	36	NS	61	20	0.02[b]	44	62	NS
5q31, 4q25-27, 15q13-22, 5p13-12	*FGF*	56	36	0.09	60	27	0.08	53	51	NS
8p11, 10q26, 4p16, 5q35, 5q31, 4q25-27, 15q13-22, 5p13-12	*FGFR+FGF*	54	36	0.09	60	24	0.04	49	55	NS
7q31	*c-Met*	58	31	NS	40	22	NS	71	43	NS
7q25	*HGF*	60	19	0.03	50	20	NS	75	17	0.07[c]
7q31, 7q25	*c-Met+HGF*	40	25	NS	38	23	NS	44	29	NS
8p11, 10q26, 4p16, 5q35, 7q31	*FGFR+c-Met*	47	36	NS	41	18	NS	23	35	NS
8p11, 10q26, 4p16, 5q35, 7q25	*FGFR+HGF*	65	32	0.01	70	17	0.001	31	33	NS
5q31, 4q25-27, 15q13-22, 5p13-12, 7q31	*FGF+c-Met*	92	64	NS	76	42	NS	46	56	NS
5q31, 4q25-27, 15q13-22, 5p13-12, 7q25	*FGF+HGF*	68	33	0.003	74	22	0.004	19	26	NS
8p11, 10q26, 4p16, 5q35, 5q31, 4q25-27, 15q13-22, 5p13-12, 7q35	*FGFR+FGF+c-Met*	47	36	NS	40	23	NS	53	54	NS
8p11, 10q26, 4p16, 5q35, 5q31, 4q25-27, 15q13-22, 5p13-12, 7q25	*FGFR+FGF+HGF*	67	33	0.008	73	20	0.004	58	56	NS

NS, non significant.

[a] Patients were classified depending on their N-stage, either without lymph node invasion (N0) or with (N1–N3). The frequencies of alterations by group of loci, depending on the family of candidate genes, were determined by using a Fractional Regional Alteration frequency (FRA) as defined below: ((number of alterations in the group of loci)/(number of informative loci in the group of loci))×100.

[b] 8p11 ($P=0.02$), 4p16 ($P=0.06$).

[c] When the seven discordant results for alterations at the same MS of locus 7q25 were included in the calculation (with results ■/□ interpreted as AI and □/■ as non-rearranged as shown in Table 2), $P=0.016$ in ADC, with FA frequencies at 88% in N0 patients and at 17% in N1–N3 patients were observed, and remained non-significant for SQC patients.

(deletion or amplification) of these different candidate genes, in particular *FGF7, FGF10*, *c-Met* and *HGF*.

Although differential genotypes and phenotypes are well characterised between SCLC and NSCLC, only a few studies have looked for differences in the histological subtypes of NSCLC. In our study, a higher global FRA frequency was shown in ADC, in agreement with the overexpression of FGFR/FGF and c-Met/HGF reported in ADC studies [13–18,20,25]. Remarkably, one alteration in each group of loci containing either *FGFR* or *FGF* candidate genes seemed to be sufficient for ADC development. This was in contrast to SQC, where several simultaneous alterations in each group were observed. Accordingly, different patterns of correlations of FA were observed when comparing the SQC and ADC samples. Our results were in agreement with the observation that the ADC population is more heterogeneous, probably presenting several more sub-classes than SQC, and we can speculate that alterations at loci containing *FGFR*/*FGF* candidate genes could help in classifying and understanding the carcinogenesis of ADC. *FGF7* is one of the candidate genes at the 15q13-22 locus. It has been shown that FGF7 produced by fibroblasts obtained from a fetal lung could influence patterning by promoting epithelial growth, but could also induce cystic adenomatoid malformation *in vivo*, and FGF7 is a differentiation factor for the developing embryonic lung inducing an alveolar type II cell-like phenotype [9–11]. Interestingly, the FA frequency at the 15q13-22 locus was significantly higher in the ADC than in the SQC samples. This suggests a specific role for this locus in ADC lung cancer development, according to both the spatio-temporal localisation of FGF7 during lung embryogenesis and to the formation of ADC from either type II pneumocytes in the alveolar sac or Clara cells in the bronchiole. Only combinations of alterations including the 7q31 locus (*c-Met* candidate gene) had a higher significantly FRA frequency in ADC. Remarkably, hierarchical cluster analysis using only alterations at the 7q31 and 15q13-22 loci correctly classified 79% of our NSCLC as SQC or ADC. However, more investigations would be necessary to understand the role of the potential candidate genes of these two loci, *FGF7* and *c-Met,* in the phenotypic differentiation of NSCLC.

Since half of our patients showed lymph node metastasis, we looked for frequencies of alterations at our loci depending on the lymph node status of the histological sub-type, to identify molecular markers for locoregional metastasis. The FRA frequency at loci containing *FGFR* appeared higher in the N0-SQC patients.

Interestingly, a higher FA frequency at specific loci containing some of the *FGFR*/*FGF* candidate genes was correlated with well differentiated SQC but with poorly differentiated ADC. Furthermore, we observed that the FA frequency at the locus 7q25 was significantly higher in N0-ADC patients, and that the FRA frequency at the 7q31 locus combined with loci containing *FGFR*/*FGF* candidate genes could classify SQC and ADC. Accordingly, it was shown that FGF2 and FGFR overexpression seemed to correspond with less aggressive tumours in SQC [13,14,16–18,26,27]. Furthermore, it has been reported that overexpression of c-Met in ADC was correlated with a higher degree of differentiation, appeared more frequently in the N0-stage compared with N1-stage patients, and it was recently reported that overexpression of *c-Met* cDNA in lung carcinoma cells suppressed tumorigenicity [20,30]. Besides its classical oncogenic role, HGF overexpression could, by contrast, consistently inhibit carcinoma cell proliferation, such as in hepatocellular and breast carcinomas [30]. In addition, the fact that the FA frequency at the 7q25 locus was higher in our N0-ADC patients could be linked to a high glandular differentiation, in agreement with the fact that HGF cell growth inhibition in monolayer cultures was reported in human lung carcinoma cell lines [30]. Altogether, these results allow us to speculate on the potential role of the FGFR/FGF signalling pathway in the differentiation of SQC, and propose that the *c-Met*/*HGF* loop could be involved in the phenotypic differentiation of ADC.

In conclusion, our results allowed us to determine a differential pattern of molecular alterations between SQC and ADC samples, confirmed by distinct correlations with lymph node metastasis. In SQC, loci containing *FGFR*/*FGF* candidate genes seemed to be more involved in differentiation, while in ADC, loci containing *c-Met*/*HGF* seemed to be implicated in phenotypic differentiation. These results suggest that ADC and SQC do not have the same developmental pattern and should be separated into different groups in prognostic analyses. An evaluation of the exact status of the candidate genes at the analysed loci (especially *FGF7*, *FGF10*, *FGFR-1*, *c-Met* and *HGF*) should be performed in order to define more precisely their role in lung cancer sub-types.

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References

1. Travis WD, Colby TV, Corrin B, Shimosato Y, Brambilla E. In Sobin LH, ed. *World Health Organization International Classification of Tumors. Histological Typing of Lung and Pleura*. New York, Springer Verlag, 1999.
2. Konata C, Ikeda N, Hiyoshi T, *et al*. Peripheral non-small cell lung cancers 2.0 cm or less in diameter: proposed criteria for limited pulmonary resection based upon clinicopathological presentation. *Lung Cancer* 1998, **21**, 185–191.
3. Charloux A, Quoix E, Wolkowe N, Small D, Pauli G, Kreisman H. The increasing incidence of lung adenocarcinoma: reality or artefact, a review of the epidemiology of lung adenocarcinoma. *Intern J Epidemiol* 1997, **26**, 14–23.
4. Petersen S, Aninat-Meyer M, Schlüns K, Gellert K, Dietel M, Petersen I. Chromosomal alterations in the clonal evolution to the metastatic stage of squamous cell carcinomas of the lung. *Br J Cancer* 2000, **82**, 65–73.
5. Pei J, Balsara B, Li W, *et al*. Genomic imbalances in human lung adenocarcinomas and squamous cell carcinomas. *Gene Chromosome Cancer* 2001, **31**, 282–287.
6. Goeze A, Schluns K, Wolf G, Thasler Z, Petersen S, Petersen I. Chromosomal imbalances of primary and metastatic lung adenocarcinomas. *J Pathol* 2002, **196**, 8–16.
7. Virmani AK, Fong KW, Kodagoda D, *et al*. Allelotyping demonstrates common and distinct patterns of chromosomal loss in human lung cancer types. *Genes Chromosome Cancer* 1998, **21**, 308–319.
8. Basilico C, Moscatelli D. The FGF family of growth factors and oncogenes. *Adv Cancer Res* 1992, **59**, 115–165.
9. Warbuton D, Schwarz M, Tefft D, Lores-Delgado G, Abderson KD, Cardoso WV. The molecular basis of lung morphogenesis. *Mech Dev* 2000, **92**, 55–81.
10. Warbuton D, Tefft D, Mailleux A, *et al*. Do lung remodeling, repair, and regeneration recapitulate respiratory ontogeny? *Am J Respir Crit Care Med* 2001, **164**, S59–S62.
11. Isakson BE, Luman RL, Seedorf GJ, Boitano S. Modulation of pulmonary alveolar type II cell phenotype and communication by extracellular matrix and KGF. *Am J Physiol Cell Physiol* 2001, **281**, C1291–C1299.
12. Clark JC, Tichelaar JW, Wert SE, *et al*. FGF-10 disrupts lung morphogenesis and causes pulmonary adenomas in vivo. *Am J Physiol Lung Cell Mol Physiol* 2001, **280**, L705–L715.
13. Takanami I, Imamuma T, Hashizume Y, *et al*. Expression of PDGF, IGF-II, bFGF and TGF-B1 in pulmonary adenocarcinoma. *Path Res Pract* 1996, **192**, 1113–1120.
14. Takanami I, Tanaka F, Hashizume T, *et al*. The basic fibroblast growth factor and its receptor in pulmonary adenocarcinomas: an investigation of their expression as prognostic markers. *Eur J Cancer* 1996, **32A**, 1504–1509.
15. Takanami I, Tanaka F, Hashizume T, Kodaira S. Tumor angiogenesis in pulmonary adenocarcinomas: relationship with basic fibroblast growth factor, its receptor, and survival. *Neoplasma* 1997, **44**, 295–298.
16. Volm M, Koomägi R, Mattern J, Stammler G. Angiogenic growth factors and theirs receptors in non-small cell lung carcinomas and their relationship to drug response in vitro. *Anticancer Res* 1997, **17**, 99–104.
17. Volm M, Koomägi R, Mattern J, Stammler G. Prognostic value of basic fibroblast growth factor and its receptor (FGFR-1) in patients with non-small cell lung carcinoma. *Eur J Cancer* 1997, **33**, 691–693.
18. Guddo F, Fontanini G, Reina C, Vignola AM, Angeletti A, Bonsignore G. The expression of basic-fibroblast growth factor (bFGF) in tumor-associated stromal cells and vessels is inversely correlated with non-small cell lung cancer progression. *Hum Pathol* 1999, **30**, 788–794.

19. Maulik G, Shrikhande A, Kijima T, Ma PC, Morrison PT, Salgia R. Role of hepatocyte growth factor receptor, c-Met, in oncogenesis and potential for therapeutic inhibition. *Cytokine Growth Factor Rev* 2002, **13**, 41–59.
20. Tsao M-S, Liu N, Chen J-M, *et al*. Differential expression of Met/hepatocyte growth factor receptor in sub-types of non-small cell lung cancers. *Lung Cancer* 1998, **20**, 1–16.
21. Karoui M, Hofmann-Radvanyi H, Zimmermann Ute, *et al*. No evidence of somatic FGFR3 mutation in various types of carcinoma. *Oncogene* 2001, **20**, 5059–5061.
22. Mountain CF. Revision in the international system for staging lung cancer. *Chest* 1997, **111**, 1710–1717.
23. Schneider A, Borgnat S, Lang H, *et al*. Evaluation of microsatellite analysis in urine sediment for diagnosis of bladder cancer. *Cancer Res* 2000, **60**, 4617–4622.
24. Cave-Riant F, Cuillerier B, Beau-Faller M, *et al*. Association of genetic defects in primary resected lung adenocarcinoma revealed by targeted allelic imbalance analysis. *Am J Respir Cell Mol Biol* 2002, **27**, 495–502.
25. Berger W, Setinek U, Mohr T, *et al*. Evidence of a role of FGF-2 and FGF receptors in the proliferation of non-small cell lung cancer cells. *Int J Cancer* 1999, **83**, 415–423.
26. Slodkowska J, Sikora J, Roszkowski-Sliz K, Radomyski A, Androsiuk W. Expression of vascular endothelial growth factor and basic fibroblast growth factor receptors in lung cancer. *Analyt Quant Cytol Histol* 2000, **22**, 398–402.
27. Mattern J, Koomägi R, Volm M. Biological characterization of sub-groups of squamous cell lung carcinoma. *Clin Cancer Res* 1999, **5**, 1459–1463.
28. Lund EL, Thorsen C, Pedersen MWB, Junker N, Kristjansen PEG. Relationship between vessel density and expression of vascular endothelial growth factor and basic fibroblast growth factor in small cell lung cancer in vivo and in vitro. *Clin Cancer Res* 2000, **6**, 4287–4291.
29. Yiangou C, Gomm JJ, Coope RC, *et al*. Fibroblast Growth Factor 2 in breast cancer: occurrence and prognostic significance. *Br J Cancer* 1997, **75**, 28–33.
30. To C, Seiden I, Liu N, Wigle D, Tsao M-S. High expression of Met/Hepatocyte Growth factor receptor suppresses tumorigenicity in NCI-H1264 lung carcinoma cells. *Exp Cell Res* 2002, **273**, 45–53.
31. Yi S, Chen JR, Viallet J, Schwall RH, Nakamura T, Schmidt EV. Paracrine effects of hepatocyte growth factor/scatter factor on non-small-cell lung carcinoma cell lines. *Br J Cancer* 1998, **77**, 2162–2170.