Genetic Heterogeneity in Patients with Multiple Neoplastic Lung Lesions: A Report of Three Cases

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Introduction: It is important to determine the relation among the various lesions in patients presenting with multiple malignant lung tumors to define the best treatment approach. A better understanding of the molecular alterations present in the different lesions may help in defining this relation.

Methods: We performed a detailed molecular analysis of several tumor specimens obtained from three patients presenting with multiple lung lesions. Tumor specimens were analyzed for epidermal growth factor receptor (EGFR) and k-ras mutations by direct DNA sequencing. In addition, a genome-wide chromosomal copy number analysis was performed on DNA extracted from the various lesions using array-based comparative genomic hybridization.

Results: In one case, a deletion of 15 base pairs in exon 19 of EGFR was present in all tumor sites analyzed. Furthermore, a similar pattern of chromosomal aberrations was observed among the various lesions, suggesting that they share the same clonal origin. In the other two cases, in contrast, we identified distinct k-ras genotypes among the various lesions from the same patient. These lesions, moreover, showed different chromosomal aberration patterns, indicating that they may have different underlying pathways of tumorigenesis.

Conclusion: Our results show that EGFR and k-ras mutation analysis, combined with chromosomal copy number profiling, can help in defining the relationship among different tumors in one patient.

Key Words: Non-small cell lung cancer, Microarray-based comparative genomic hybridization, Epidermal growth factor receptor, k-ras.

(*J Thorac Oncol.* 2007;2: 12–21)

Non-small cell lung cancer (NSCLC) is the leading cause of cancer death in the United States and in Western Europe. Most patients with NSCLC are candidates for systemic therapy at some point in the course of the disease.

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ISSN: 1556-0864/07/0201-0012

Particular molecular tumor characteristics can be of help in deciding on systemic treatment. For example, certain mutations in the epidermal growth factor receptor (EGFR) and k-ras genes have been shown to correlate with either favorable or unfavorable patient response, respectively, to treatment with the EGFR tyrosine kinase inhibitors gefitinib and erlotinib.[1–3]

Patients with NSCLC are at relatively high risk of developing secondary primary lung tumors, depending on stage at initial diagnosis, with percentages ranging from 3% to 14%.[4–6] Lungs are moreover a major site of metastases from common solid tumors. Among patients with multiple lung tumors, the clonal relation of the various tumors is important in deciding on treatment. Histopathological examinations of the multiple lesions are often not conclusive as to the differential diagnosis of second primary or metastasis, and a more extensive examination of the genomic changes that occur may help in this diagnosis. In this regard, microarray-based comparative genomic hybridization (array-CGH), which measures DNA copy number changes throughout the entire genome, can provide important diagnostic information.[7–9]

We performed a detailed genetic analysis of tumor samples obtained from three patients with multiple malignant lung lesions using EGFR and k-ras mutation analysis combined with array CGH, which allowed identification of clonal relationships among the various lung lesions.

CASE REPORTS

Patient 1

In April 2003, a 29-year-old man presented to his primary care physician with thoracic pain and hemoptysis. He had a performance status of 0 and showed no weight loss. Physical examination showed no abnormalities. Besides leukocytosis ($12{\cdot}4 \times 10^9$/L), laboratory values were normal. The patient had no significant medical history and used no medication. He worked as a professional mason and was a very light smoker (smoking history, 1 pack-year).

A computed tomography (CT) scan of the thorax showed a right lower lobe mass. Subsequently, a diagnosis of pneumonia was made, but no clinical or radiographic improvement was observed, despite treatment with antibiotics. Because of persistent hemoptysis, a bronchoscopy was per-

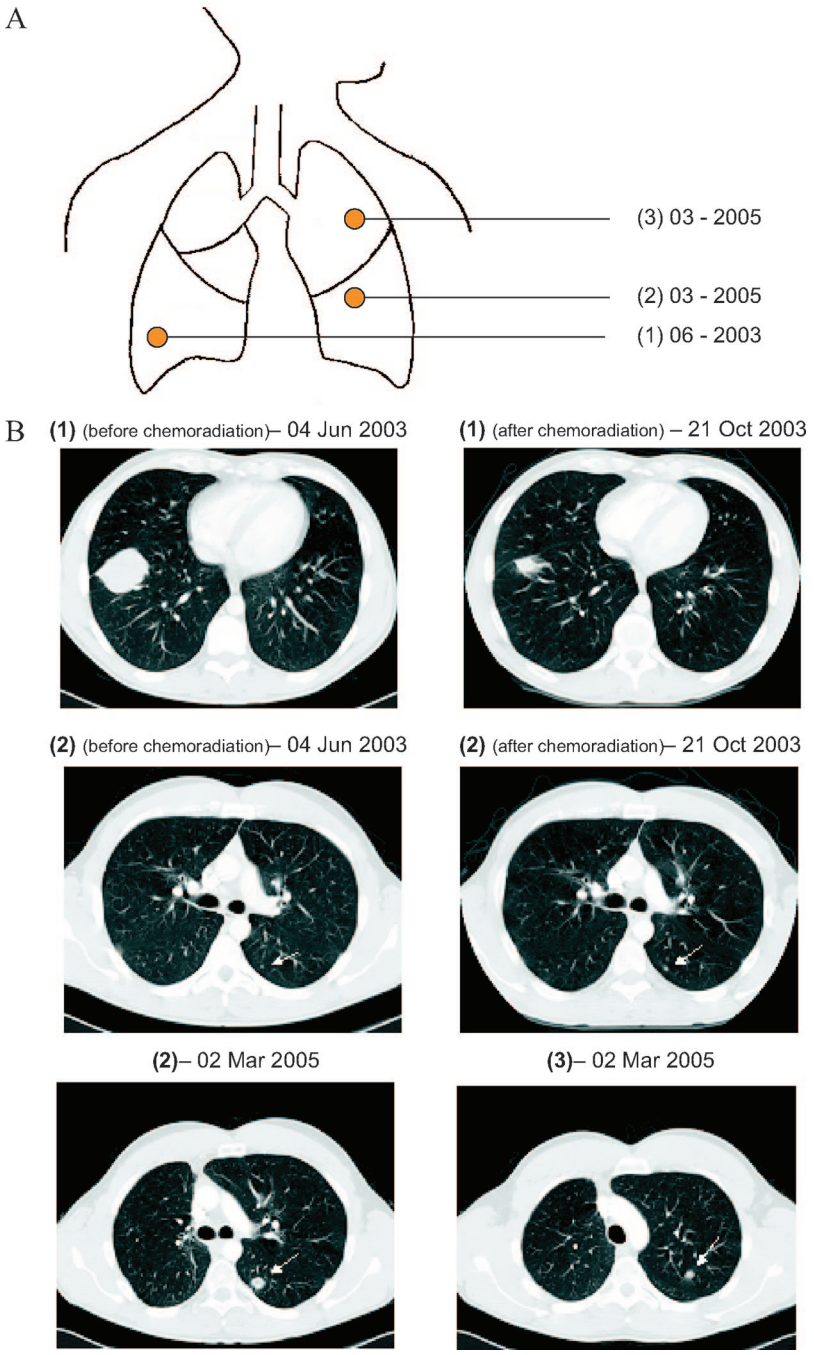

FIGURE 1. Localization and molecular analysis of tumors from patient 1. (*A*) A schematic illustration of localization and time of diagnosis of multiple malignant lung lesions in patient 1. (*B*) Computed tomographic scans of the thorax show lesion 1 in the right lung before and after chemoradiation, and lesion 2 and 3 in the left lung. (*C*) Genome wide profiles of tumor sites 2 and 3. Tumor DNA of patient 1 was hybridized with normal female reference DNA on a 30K human oligonucleotide array. On the X axis, array elements are ordered according to their position on the genome. Odd and even chromosomes are indicated in *light* and *dark blue*, respectively. On the Y axis, ratios are plotted as $\log^2$ ratio of the fluorescent intensities of tumor (Cy3) divided by reference (Cy5) signal for each element on the array. Smoothed values are indicated in *red*. A smoothed $\log^2$ ratio close to zero indicates no difference in fluorescence intensity between tumor and reference DNA and, hence, no chromosomal copy number aberrations. Log^2 ratios greater or lower than zero indicate gains or losses of chromosomal elements, respectively. For patient 1, similar patterns of chromosomal aberrations can be observed for tumor sites 2 and 3, indicating that these lesions share the same clonal origin.

C Site (1) - EGFR mt Del 746-750, K-ras wildtype

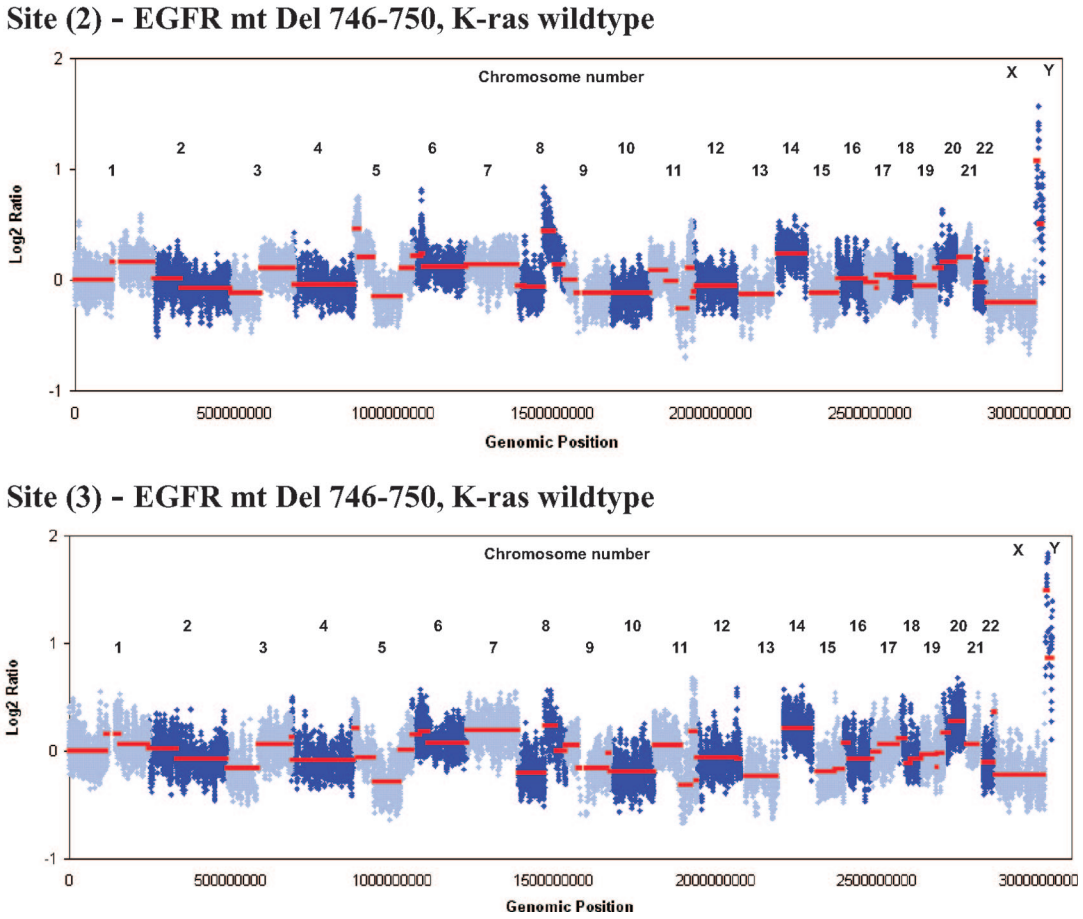

FIGURE 1. *(Continued).*

formed and showed no endobronchial lesions. The CT scan of the thorax was repeated and revealed the known lesion in the right lower lobe and two enlarged mediastinal lymph nodes; i.e., mediastinal nodal station 4R and 7. The lesion in the right lower lobe and mediastinal lymph nodes had uptake on a [^{18}FDG] positron emission tomography (PET) scan. Cytological analysis of the subcarinal lymph node, obtained by an Endoscopic Ultrasound-Fine Needle Aspiration, showed adenocarcinoma. The tumor was staged as IIIaN2 non-small cell lung carcinoma (T2N2M0). The patient was treated using a chemoradiation protocol of platinum-based chemotherapy and definite thoracic radiotherapy. After treatment, a CT scan and a [^{18}FDG] PET scan were repeated. CT scan of the thorax showed a partial response of the lesion (Figure 1B). [^{18}FDG] PET scan showed no uptake in the primary lesion and minimal uptake in a subcarinal lymph node. Biopsies of mediastinal lymph nodes obtained by mediastinoscopy showed no malignant cells, and the patient underwent an R0 bilobectomy. Histological analysis revealed residual adenocarcinoma at the primary tumor site and no detectable tumor in hilar and mediastinal lymph nodes. The patient recovered well after surgery. No adjuvant therapy was administered.

In February 2005, the patient again reported slight hemoptysis. A CT scan showed two small lesions in the left lung: one in the left lower lobe and one in the left upper lobe. These two lesions, as well as two left hilar lymph nodes, were positive on an [^{18}FDG] PET scan. The lesion in the left lower lobe appeared radiologically compatible with a second primary tumor, and was actually already visible on the CT scans performed before the operation in 2003. At that time this was not considered to be a tumor because of the lack of FDG avidity and the lack of change after induction chemotherapy (Figure 1B). The patient underwent a segmental resection of the left upper lobe, a segmental resection of the left lower lobe, and mediastinal lymph node dissection (stations 4, 5, 8, and 10) in May 2005. Histological analysis showed poorly differentiated adenocarcinoma in both lung nodules and two mediastinal lymph nodes (5 and 10) contained metastatic adenocarcinoma. Postoperatively, he underwent three cycles of cisplatin and vinorelbine. A tyrosine kinase inhibitor of EGFR was administered after chemotherapy. The patient is well without evidence of disease more than 1 year after the operation.

Patient 2

In May 2005, a 48-year-old woman was referred by her primary care physician because of persisting cough. A radiograph of the chest showed two contralateral pulmonary lesions. She was in a good clinical condition and had no weight loss. Physical examination revealed no abnormalities. Besides a mild increase in the erythrosedimentation rate (27 mm) and leukocytosis (13.3×10^9/L), laboratory values were normal. The patient had a smoking history of 35 pack-year and used fluoxetine for depression. Her medical and family histories were negative for malignancy.

A CT scan of the thorax showed a cavitating lesion in the right upper lobe and a solid lesion in the left lower lobe (Figure 2B). There were no mediastinal lymphadenopathies. Pulmonary function tests and diffusion capacity were normal. Bronchoscopy showed no endobronchial lesions; cytologic evaluation of a broncho-alveolar lavage of the left lower lobe identified adenocarcinoma cells. A [^{18}FDG] PET-scan showed the two pulmonary lesions and a hot spot in the right lobe of the thyroid gland. Histological analysis of the thyroid lesion revealed a papillary thyroid carcinoma.

In June 2005, the patient underwent complete resection of the thyroid gland and resection of the cervical lymph nodes. On histopathological evaluation of the resection specimens, a papillary thyroid carcinoma of 8 mm was found, whereas all resected lymph nodes were negative for malignancy.

Because the postoperative ^{131}I scanning showed no uptake in the pulmonary nodules and the early stage of the papillary thyroid carcinoma, a tentative diagnosis of double tumor (adenocarcinoma of the lung and papillary thyroid carcinoma) was made.

In August 2005, patient 2 was referred to our hospital and a [^{18}FDG] PET scan showed only uptake in both pulmonary lesions. In September 2005, a wedge resection of the tumor in the right upper lobe was performed. Pathological analysis showed moderately differentiated adenocarcinoma of the lung. Immunohistochemical staining for thyroglobulin was negative. In October 2005, the lower lobe of the left lung

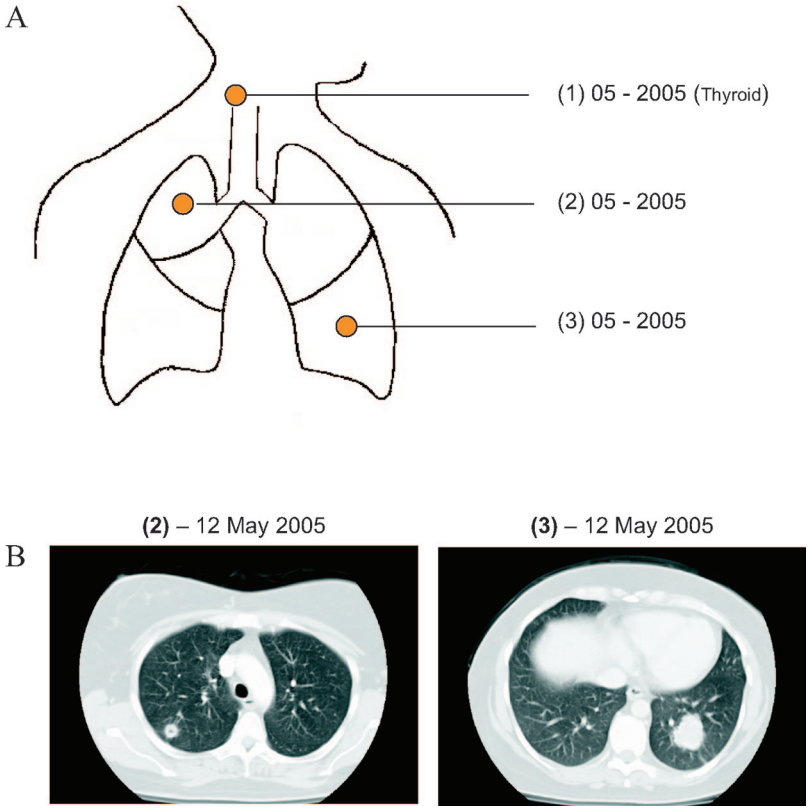

FIGURE 2. Localization and molecular analysis of tumors from patient 2. (*A*) Schematic illustration of localization and time of diagnosis of the various malignant lung lesions of patient 2. (*B*) Computed tomographic scans of the thorax. Lesion 2 can be observed in the right lung and lesion 3 in the left lung. (*C*) Chromosomal aberration plots are depicted of lesion 2 and 3. Tumor DNA of patient 2 was hybridized with normal male DNA on a 6K human BAC array. Log^2 ratios are displayed against their position on the genome. Odd and even chromosomes are indicated in *light* and *dark blue*, respectively. Smoothed values are indicated in *red*. The chromosomal aberration patterns differ between tumor site 2 and 3. In particular, site 3 shows a $\log^2$ ratio of –0.35 at chromosome 3, 6, 9, 10, and 14q (*arrows*), these areas have a lower intensity of fluorescence in tumor DNA versus reference DNA, indicating that these chromosomal regions are deleted. The smoothed $\log^2$ ratio of +1.4 on chromosome 14p (*arrow*) indicates that this chromosomal area has a high copy number in comparison to the reference DNA. These narrow, high-level gains are referred to as amplification.[21] Thus, in patient 2, less chromosomal aberrations are present in site 2 versus site 3, indicating they arose from different clones.

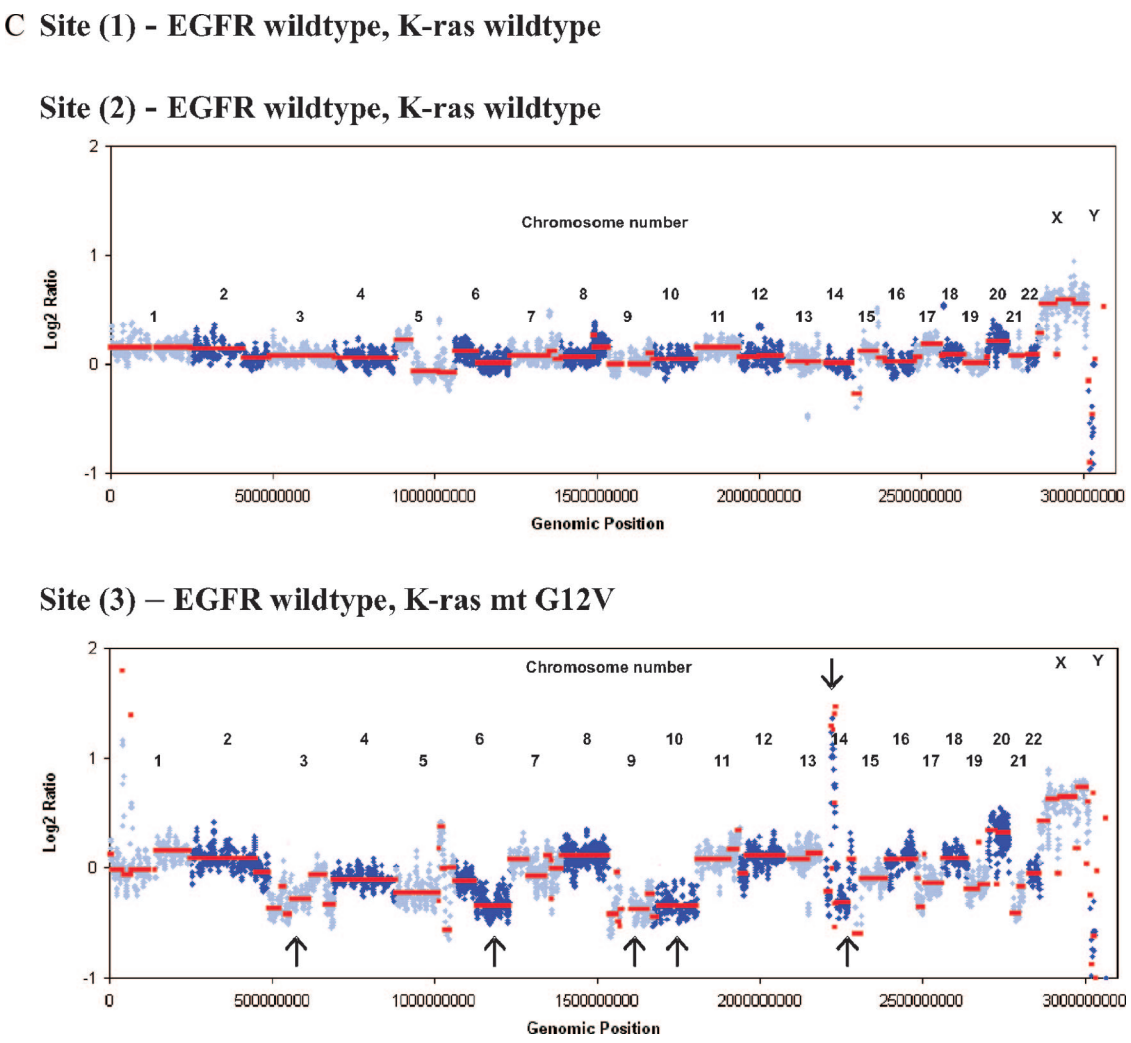

FIGURE 2. *(Continued).*

and lymph nodes 7 to 11 were also resected. Pathology again showed a moderately differentiated adenocarcinoma of the lung with no positive staining for thyroglobulin. All resected lymph nodes appeared free of malignancy. The patient recovered well from both resections. Five months after the latter operation, the patient presented with neurological symptoms, and brain metastases were diagnosed. She underwent whole-brain radiotherapy and eventually died of her disease.

Patient 3

A 65-year-old woman with a 50 pack-year history of smoking was seen by her family physician in February 1997 because of mild dyspnea on exertion, asthenia, and fatigue.

A radiograph of the chest revealed a 3-cm lesion in the right lower lobe, which was confirmed on CT scan of the thorax. The lesion was not radiologically typical for malignancy, and there were no lymphadenopathies. A CT scan after 5 months showed no increase in the dimension of the lesion. A [^{18}FDG] PET scan showed minimal uptake in the lesion in the right lower lobe but increased uptake in several mediastinal lymph nodes. No endobronchial lesions were seen on bronchoscopy, and cytology of broncho-alveolar lavage revealed no malignant cells. Lymph node biopsies obtained by mediastinoscopy showed no malignant cells. A thoracotomy was performed in March 1998, and the lesion in the right lower lobe was resected. Intraoperative pathological analysis revealed papillary adenocarcinoma, and the surgical procedure was limited to a lobectomy of the right lower lobe (pT2N0M0).

Postoperatively, the patient experienced continuous thoracic pain, which was resistant to several pain medications. Entrapment of an intercostal nerve as a result of callus formation after the surgical procedure, which might benefit of a partial resection of ribs 6 and 7, was considered a potential cause for her pain. A new CT scan of the thorax showed two subpleural lesions in the right lung in November 2002. One of these two lesions was positive on a [^{18}FDG] PET scan. A partial resection of ribs 6 and 7 was performed, and a wedge resection of the two aforementioned lesions in the right lung was performed in March 2003. Pathological analysis showed papillary adenocarcinoma of the lung, which was radically

resected. No adjuvant therapy was undertaken. Unfortunately, the resection of ribs 6 and 7 did not have the desired palliative effect on the patient's thoracic pain.

A CT scan performed in January 2006 showed multiple intrapulmonary lesions in the right and left lungs. Pathological examination of a CT-guided biopsy confirmed papillary adenocarcinoma. The patient consented to an investigational treatment with bevacizumab and erlotinib. After four courses of therapy, she had signs of disease progression, and protocol treatment was halted. She is currently alive with disease.

MATERIALS AND METHODS

Samples

Tumor specimens obtained before therapy were collected, and informed consent was obtained from all patients. Paraffin-embedded tissue blocks were cut into 4 μm-thick sections and placed onto glass slides. Slides were stained with hematoxylin and eosin, and a pathologist (GAM) verified the presence of tumor cells.

Mutation Analysis of EGFR and k-ras Genes

Isolation of genomic DNA and nested polymerase chain reactions (PCR) were performed to amplify exon 18-21 of EGFR and exon 1-2 of k-ras, as previously described.[10] Sequencing of PCR products was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), with the ABI PRISM 3100 Genetic analyzer (Applied Biosystems). Mutations were confirmed by sequencing independent PCR products. In doubtful cases, the PCR product was cloned into the pGEM-T easy Vector (Promega Benelux BV, Leiden, The Netherlands), and several independent clones were sequenced.

Array Comparative Genomic Hybridization

DNA isolation from paraffin-embedded material was performed as previously described.[11] DNA labeling and hybridization on CGH BAC and oligonucleotide microarrays was performed as described by van den Ijssel et al.[12] In short, genomic DNA (300 ng) was labeled with Cy3-dCTP (tumor sample) or Cy5-dCTP (reference sample of the opposite sex) (Perkin Elmer, Boston, MA). Labeled, precipitated DNA was dissolved in a hybridization mixture and hybridized to the 30K human oligo (patient 1 and 3) or to a 6K human BAC (patient 2) array using a GeneTAC/HybArray12 hybstation (Genomic Solutions; Perkin Elmer). Slides were scanned using a Microarray Scanner G2505B (Agilent Technologies, Palo Alto, CA). Log^2 ratios were calculated with moving average and are displayed against their position on the genome. Smoothed values were calculated using the January 2005 version of array CGH smooth.[13]

RESULTS

Case 1

Tumor localizations and CT scans of patient 1 are shown in Figure 1A and B. In March 2005, two lesions were observed in the left lung (2 and 3). In retrospect, lesion 2 was already visible at diagnosis in 2003 and did not respond to chemotherapy. EGFR and k-ras mutation analyses were performed on tumor samples from sites 1, 2, and 3, as well as on tumor cells from lymph nodes number 5 and 10. All tumor sites analyzed contained the same heterozygous, in-frame deletion of 15 base pairs in exon 19 of the EGFR gene, leading to the deletion of amino acids E746 to A750. This deletion affects the ATP-binding pocket and is related to favorable response to treatment with EGFR tyrosine kinase inhibitors.[1,2] All sites analyzed were wild type for k-ras. Although pathological examination suggested metastatic adenocarcinoma, the tumors in the left lung were radiologically compatible with being second primaries. To gain better insight into the relation of the various lesions, array CGH was performed. Because of the small number of tumor cells left in site 1, this analysis was performed on tumor sites 2 and 3 only. Profiles of the genetic aberrations are depicted in Figure 1*C* . Log^2 tumor to normal DNA copy number ratios are plotted in genomic order. The aberrations are spread over the whole genome and follow a nearly identical pattern of gains and losses in both sites, indicating that these tumors share the same clonal origin.

Case 2

In Figure 2A and B, a schematic diagram of the localization and CT scans of the tumors are presented. The clinical presentation of this patient suggested two independent lung lesions. However, pathological and immunohistochemical examination could not distinguish between second primary and metastasis. Mutation analysis was performed on tumor material of the thyroid (site 1), the right upper lobe (site 2), and the left lower lobe (site 3). No mutations in the EGFR gene were found in any of the sites analyzed. Tumor cells of sites 1 and 2 were wild type for k-ras. In contrast, a point mutation in codon 12 of k-ras leading to an amino acid change from glycine to valine (G12V) was detected in the tumor cells of site 3.

Array CGH analysis was performed on tumor material from the two lung lesions (sites 2 and 3). As shown in Figure 2 *C*, the array CGH profiles of these two lesions differ for almost all of the 22 chromosomes. In site 3, losses on chromosomes 3, 6, 9, 10, and 14 and an amplification on chromosomes 14p can be observed, whereas in tumor 2, these aberrations are not present.

Case 3

A schematic representation of the tumor sites and CT scans of patient 3 are shown in Figure 3A and B. Pathological examination revealed papillary adenocarcinoma with the same morphology in all tumor sites, which suggests that this is most probably a metastasizing tumor. Mutational analysis of EGFR and k-ras was performed on tumor cells from site 1, 2, and 4. All three lesions were wild type for EGFR. Sites 1 and 2 were also wild type for k-ras, whereas a point mutation (G12V) in this gene was detected in site 4. Because k-ras mutations may be an early event in tumorigenesis,[14,15] the k-ras mutation present only in this site suggests that site 4 could be a new primary tumor. Array CGH was performed on tumor sites 1, 2, and 4. As shown by the smoothed patterns in

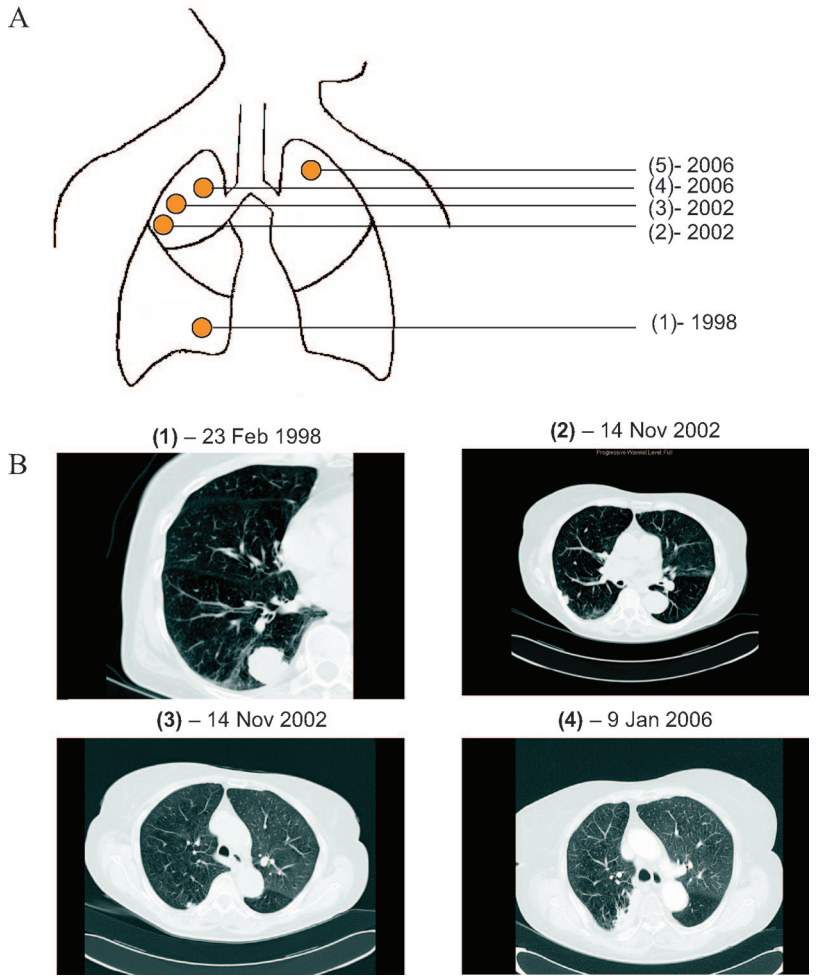

FIGURE 3. Localization and molecular analysis of tumors from patient 3. (*A*) Localization and time of diagnosis of the various malignant lung lesions of patient 3. (*B*) Computed tomography scans of the thorax in which lesions 1, 2, 3, and 4 can be observed. (*C*) Chromosomal aberration patterns of site 1, 2, and 4. Tumor DNA of patient 3 was hybridized with normal male reference DNA on a 30K human oligonucleotide array. Log^2 ratios are displayed against their position on the genome. Odd and even chromosomes are indicated in *light* and *dark blue*, respectively. Smoothed values are indicated in *red*. The chromosomal aberrations of tumor 1 and 2 follow a similar pattern, suggesting they share the same clonal origin. The pattern of chromosomal aberrations of tumor site 4 differs at various sites compared with tumor site 1 and 2, most obviously at chromosome 6 and 7 (*arrows*). (*D*) In site 1, the log^2 ratios of chromosome 6p, 6q, and 7p are +0.08, −0.22, and +0.15, respectively. In site 2, the log^2 ratios of chromosome 6p, 6q, and 7p are +0.13, −0.18, and +0.12, respectively. Site 4, however, shows a log^2 ratio of −0.01 for chromosome 6p-q, and the log^2 ratio for chromosome 7p is zero. Because tumor site 4 has a much less altered chromosomal aberration pattern, it is likely that tumor 4 originated from a different clone than tumors 1 and 2.

Figure 3C, very few chromosomal aberrations are present in site 4, whereas in sites 1 and 2, similar patterns of gains and losses can be observed. In particular, gains of 6p and 7p and loss of 6q present in sites 1 and 2 are absent in tumor site 4 (Figure 3D).

DISCUSSION

To identify the optimal treatment in patients with multiple lung tumors, the correct diagnosis of primary versus secondary tumors is essential. Routine clinical and pathological evaluation to determine the relationship between the different lesions often is not fully conclusive and a detailed genetic analysis of tumor samples (e.g., for detection of gene mutations, amplifications, or deletions) may provide important additional information.

In this article, we describe three patients with NSCLC who presented with multiple lesions and the results of genetic analysis of their tumor samples. The analysis included direct sequencing to detect EGFR and k-ras gene mutations and array CGH to obtain tumor-specific DNA copy number signatures. Previous groups have established that CGH is a valuable tool in determining clonal relations between primary tumors and their metastases of the breast,[16] bladder,[17] colon,[18] and lung.[19] Other such studies used loss of heterozygosity-

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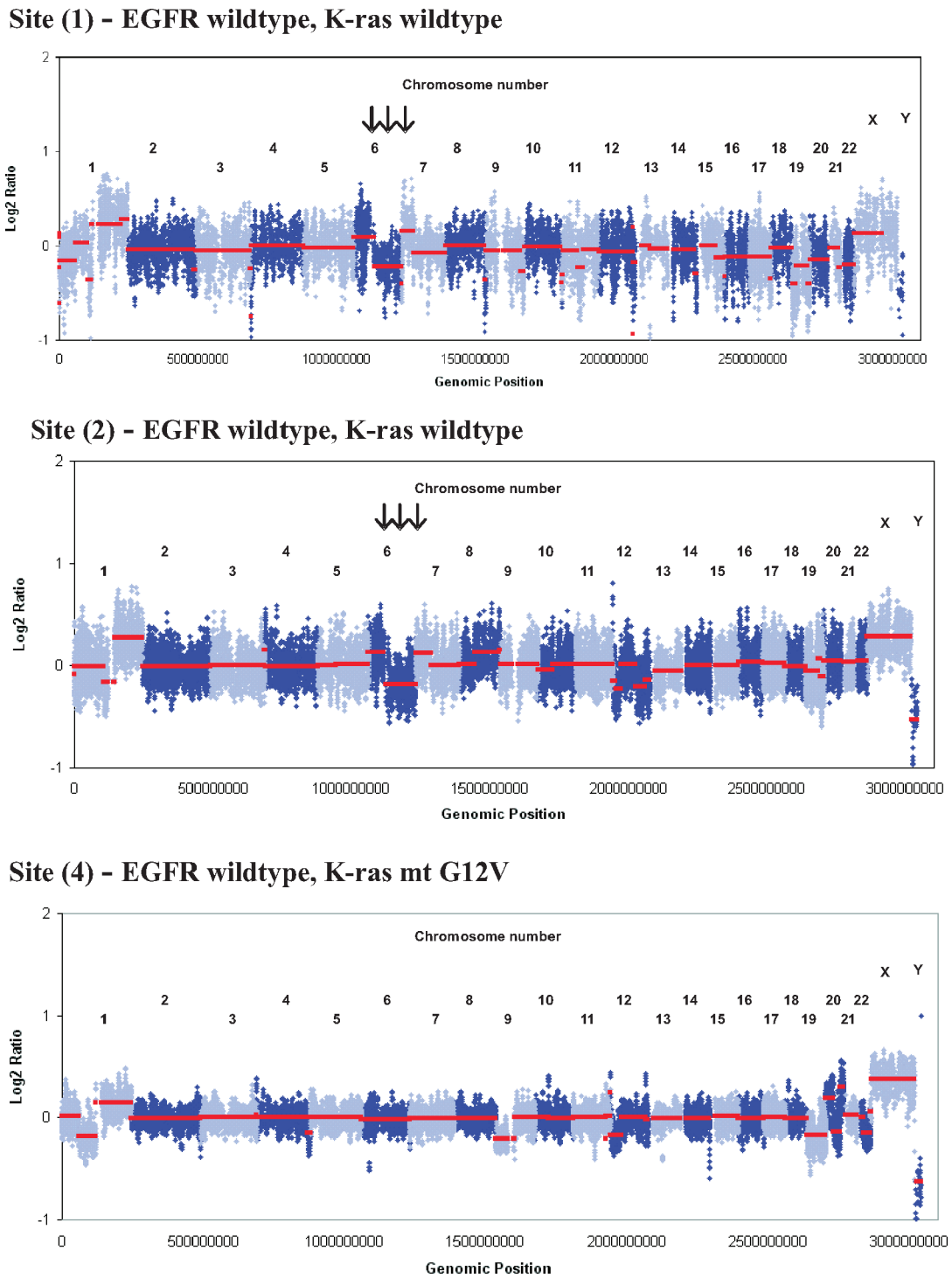
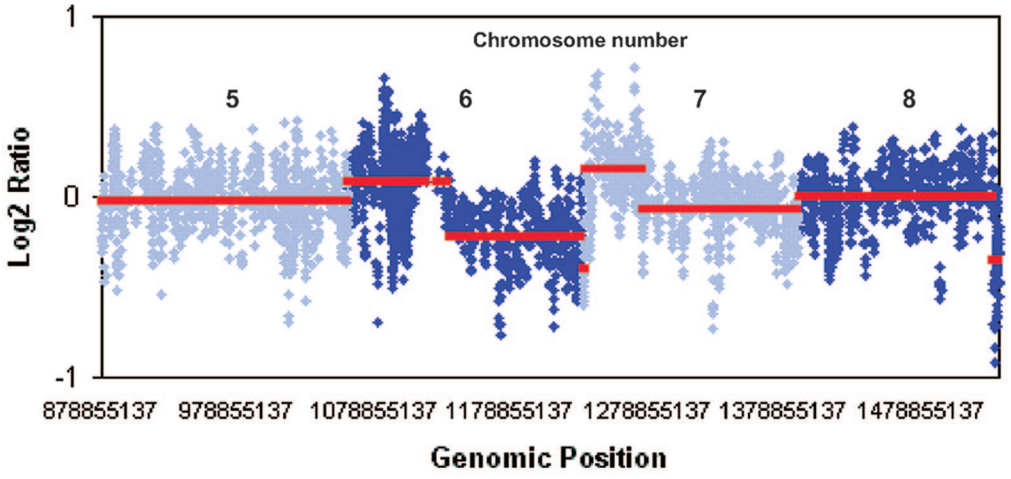


FIGURE 3. *(Continued)*

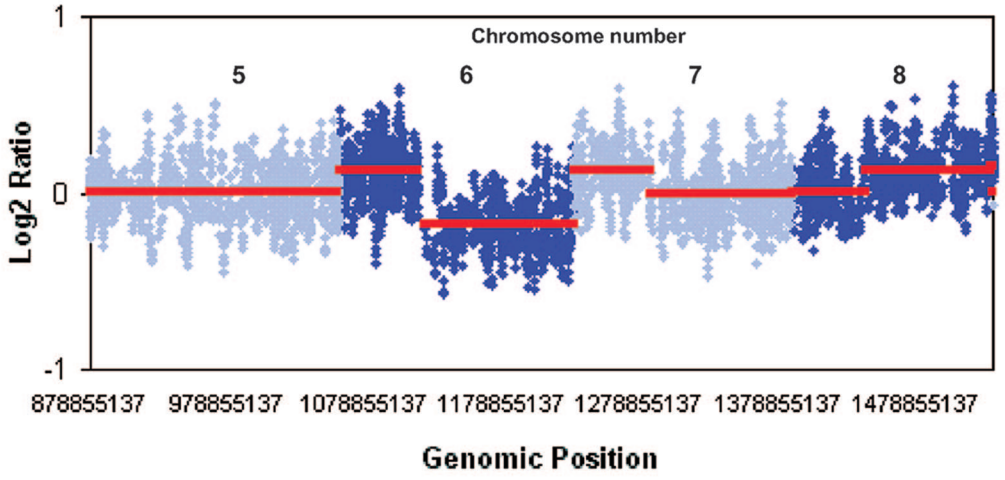
based techniques.[8] Using this technique, only limited regions of the genome are compared with identify a clonal relation. We applied array-based CGH, capable of a genome-wide screening for copy number aberrations, with high resolution and high sensitivity.[7]

The same EGFR mutation was found in all lesions in patient 1. In addition, nearly identical patterns of gains and losses could be observed in lesions 2 and 3. The results of the mutation analysis and the array CGH are consistent with tumors 2 and 3 being metastatic lesions arising from tumor 1. This would also support the view that the occurrence of EGFR mutations is an early event in NSCLC tumorigenesis.[20] In patient 2, clinical diagnosis suggested the presence of two independent tumors. This hypothesis is strengthened by the

D Site (1) - EGFR wildtype, K-ras wildtype



Site (2) - EGFR wildtype, K-ras wildtype



Site (4) - EGFR wildtype, K-ras mt G12V

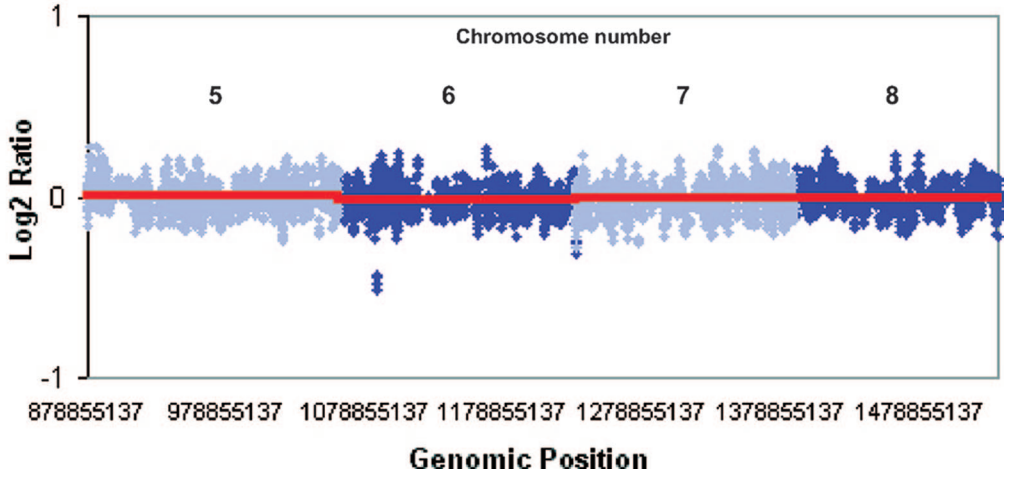


FIGURE 3. *(Continued).*

results of the molecular analysis. The dissimilar chromosomal aberration pattern and the different k-ras mutational status suggest that lesions 2 and 3 are most probably not clonally related and may have genetically different underlying pathways of tumor development. For patient 3, the results of the array CGH analysis suggest that lesions 1 and 2, but not 4, are clonally related. Thus, whereas lesion 2 (2002) could be a metastasis from lesion 1 (1998), it is most likely that lesion 4 (2006) arose independently. As described in patient 2, the possibility of a different tumorigenic pathway driving the development of tumor 4 is further supported by the fact that a k-ras mutation was only detected in this lesion.

In summary, we show that, by using mutation analysis of EGFR and k-ras combined with array CGH, we were able to identify clonal relations among multiple tumors in one patient. Our findings suggest that, in patients presenting with multiple lung lesions, various underlying molecular mechanisms can be responsible for tumor development. The predictive value of specific mutations in EGFR and k-ras genes has already brought us one step closer to more individualized treatment approaches. The cases described herein show that a detailed molecular analysis consisting of both EGFR and k-ras mutation analysis, together with array CGH, can help clinicians to determine the appropriate stage and treatment for patients with lung cancer presenting with multiple pulmonary nodules.

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