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The Horiike/Saijo Article Reviewed

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As described by Horiike and Saijo, small-cell lung cancer (SCLC) is commonly divided into limited and extensive disease. Patients with limited disease are generally treated with combined-modality therapy, whereas those with extensive disease are treated with chemotherapy alone. However, different definitions of limited and extensive disease exist. The widely used Veterans Administration Lung Study Group (VALSG) staging system defines limited disease as disease confined to the ipsilateral hemithorax, excluding contralateral supraclavicular and hilar adenopathy. Alternatively, the International Association for the Study of Lung Cancer (IASCL) includes all patients without distant metastatic disease in this category.

This is a clinically relevant distinction, as treatment options and prognostication may differ. One report comparing the outcome of patients with limited disease by VALSG or IASCL criteria suggests that the IASCL classification may provide more accurate prognostic stratification.[1] Of note, patients with limited disease and contralateral nodal involvement are often excluded from enrollment in clinical trials.

Limited-Stage SCLC

The standard treatment of limited disease in the United States consists of four cycles of EP (etoposide, cisplatin [Platinol]) every 3 weeks with concurrent thoracic radiotherapy. Clinical trials investigating sequential non–cross-resistant chemotherapy, dose intensity, dose-dense administration, and "maintenance" chemotherapy beyond four cycles have not consistently demonstrated benefit. At this time, the routine use of cytokines to maintain or increase dose intensity is not evidence-based and may be detrimental when used with thoracic radiotherapy.[2]

Thoracic radiotherapy is initiated during the first or second cycle of chemotherapy, as controlled clinical trial data indicate early irradiation offers a survival advantage. If radiotherapy is initiated after the first cycle, limited radiation fields to postchemotherapy tumor volume appear to be adequate. Horiike and Saijo suggest that hyperfractionated administration of thoracic radiation may be superior to a conventional daily schedule, based on an Eastern Cooperative Group study comparing the two approaches, which found improved local control and median survival with the accelerated regimen.[3] However, the total dose of radiation used (45 Gy) was the same in both arms; higher doses of radiation are generally recommended with once-daily schedules. Hyperfractionated thoracic radiation to 45 Gy or once-daily treatment to 50–70 Gy with concurrent chemotherapy are both acceptable approaches in the United States.[4] Ongoing clinical tri-

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als are investigating escalating doses of radiation, the role of radioprotectants such as amifostine (Ethyol), and alternating regimens of chemotherapy and thoracic radiation.

Prophylactic cranial irradiation (PCI) is usually offered to patients with limited disease after a complete or near-complete response. Numerous clinical trials support PCI in this setting, with a meta-analysis of seven randomized trials showing an absolute decrease of 25% (from 58.6% to 33.3%) in the cumulative incidence of brain metastases at 3 years and an absolute increase in overall survival of 5%.[5] Limited data from these trials suggest that PCI up to 36 Gy in small fractions is well tolerated with few neuropsychological sequelae if given sequentially rather than concurrently with chemotherapy. Controlled data for the delayed effects of sequential PCI on cognitive function are awaited.

Extensive-Stage SCLC

Presently, the standard first-line treatment of extensive disease in the United States is four to six cycles of etoposide and a platinum-containing compound. Many oncologists substitute carboplatin (Paraplatin) for cisplatin, as it is less toxic and appears to have similar efficacy. The only randomized study comparing EP to etoposide/carboplatin found no significant difference in response or survival and less toxicity with carboplatin, but the trial was underpowered to show equivalence in either limited or extensive disease.[6,7].

An alternative to EP is IP (irinotecan [Camptosar], cisplatin). Horiike and Saijo summarize the Japan Clinical Oncology Group trial comparing EP to IP, which was terminated after an interim analysis of 154 patients found significant improvement in response rate, progression-free survival, and median survival in the IP group. The results of an ongoing confirmatory Southwest Oncology Group phase III trial may change the standard of care in the United States. The use of carboplatin with irinotecan is also being investigated.

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Salvage Therapy

Although most patients respond to chemotherapy, the median duration of response is only 6 to 8 months. Salvage therapy can provide palliation with improved quality of life and possibly longer survival, depending on performance status, tumor extent, and interval between first-line therapy and relapse/progression. However, the toxicities of chemotherapy can be substantial and responses short. An open discussion with shared medical decision-making, including consideration of a patient's realistic assessment and physician recommendation, is essential.

Based on data from a phase III trial involving 211 patients, topotecan (Hycamtin) has largely replaced the CAV regimen (cyclophosphamide [Cytoxan, Neosar], doxorubicin, vincristine) in the second-line treatment of progressive SCLC.[8] Although both regimens had similar response rates and median survivals, topotecan resulted in better control of disease-related symptoms. Topotecan has also led to regression of brain metastases in some patients with extensive disease.[9] Other regimens for progressive disease incorporate paclitaxel/docetaxel (Taxotere), gemcitabine (Gemzar), vinorelbine (Navelbine), ifosfamide (Ifex), and most recently, premetrexed (Alimta). Although some of these regimens have resulted in minimal improvements in survival, associated toxicities do not support their routine use. Horiike and Saijo describe the promising activity of amrubicin, a new anthracycline developed in Japan. A phase III trial comparing amrubicin and cisplatin to IP is planned.[10]

Targeted Therapy

Novel targeted therapies for SCLC are actively being explored. Numerous targets have been identified, including the c-kit receptor, vascular endothelial growth factor receptor, neural cell adhesion molecule (NCAM), gastrin releasing peptide (GRP), matrix metalloproteinases (MMPs), retinoid signaling pathway, bcl-2, and various gangliosides. Initial approaches using the c-kit inhibitor imatinib (Gleevec); the antisense oligonucleotide against bcl-2, oblimersen (Genasense); the MMP inhibitor marimastat; the synthetic retinoid fenretinide; and the BEC2/BCG vaccine against the G3 ganglioside have yet to show benefit in phase II and III clinical trials. Other agents being developed include toxin-conjugated antibodies against the neural cell adhesion molecule (NCAM), gastrin-releasing peptide (GRP) antibodies, and replacement of lost retinoblastoma protein or p53 function with gene therapy.

Conclusions

Although there has been progress in treating SCLC over the past 25 years, overall prognosis is still poor. Current trials are exploring innovative chemoradiation schedules with dose escalation of both chemotherapy and radiation in limited disease. Irinotecan and other newer agents are actively being evaluated in extensive disease. Targeted therapy to date has been unsuccessful but holds promise for the future.

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—Ronald H. Blum, MD

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The Horiike/Saijo Article Reviewed

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Lung cancer is the most common cause of cancer death in the United States, with a projected 160,000 or more Americans dying of the disease in 2004. Small-cell lung cancer (SCLC) comprises approximately 14% of lung cancer cases. In the past 5 years, as outlined by Horiike and Saijo, significant advances have been made both in defining new combination chemotherapy options for extensive-stage SCLC and in optimizing radiotherapy for limited-stage SCLC. Despite these advances, and despite the exquisite sensitivity of SCLC to radiation and chemotherapy, the majority of patients with SCLC still die within 2 years of diagnosis. Clearly new approaches are needed.

Characteristic molecular alterations are present in the large majority of SCLC cases. These include upregulation of bcl-2, overexpression of IGF-1R, activating mutation of Rb, and inactivation of p53. These oncogenic alterations in key regulators of cell survival and proliferation are each present in 80% to 95% of patients with SCLC and together offer opportunities for selective targeted anticancer therapy. To date, only a small subset of these possible targets have been explored therapeutically.

Apoptotic Pathways

Overexpression of bcl-2, a central regulator of apoptotic induction, is a key molecular alteration associated with chemotherapeutic resistance and poor outcome in SCLC. Preclinical models suggest that suppression of bcl-2 in SCLC augments the sensitivity of SCLC to standard chemotherapeutic agents. We have conducted phase I studies in SCLC patients evaluating the combination of oblimersen (Genasense)—an antisense oligonucleotide directed against bcl-2 mRNA—either with paclitaxel or with etoposide and carboplatin (Paraplatin).[1] These studies have supported an ongoing randomized trial by the Cancer and Leukemia Group B assessing carboplatin and etoposide with or without oblimersen in untreated extensive-stage SCLC.

Inhibition of nuclear factor (NF)-kappaB, another key survival factor, also promotes tumor cell apoptosis. Bortezomib (Velcade) is a novel proteasome inhibitor that, among other effects, suppresses NF-kappaB activation by stabilizing the inhibitory regulator of NF-kappaB, I-kappaB kinase.[2] A phase II trial of bortezomib in previously treated SCLC patients is currently being conducted by the Southwest Oncology Group.

Failure to proceed through the G1 cell-cycle checkpoint triggers an apoptotic response in cancer cells. CCI-779 is a novel agent that inhibits the translation of multiple proteins, including critical factors that regulate progression through the G1 checkpoint.[3] A phase II trial of CCI-779 as maintenance therapy for patients with extensive SCLC after response to initial therapy has completed accrual. The results of this study should be available in the next year.

Signal Transduction

Activation of the receptor tyrosine kinase c-kit has been found in multiple malignancies including SCLC. Trials of imatinib (Gleevec)—a high-affinity inhibitor of c-kit—in SCLC

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have shown no benefit either as a single agent or in combination with chemotherapy.[4] Recent data suggesting that c-kit exon 9 and 11 mutations (as opposed to overexpression) are present in about 10% of SCLC cases suggest that reevaluation of imatinib specifically in this subset of patients may be warranted.[5]

Other receptor tyrosine kinases that have been strongly implicated in SCLC survival and proliferative signaling include c-MET and IGF-1R. Agents specifically targeting these alternative receptor tyrosine kinases are in preclinical and very early-phase clinical evaluation.

Angiogenesis

Serum levels of the critical angiogenic regulator vascular endothelial growth factor (VEGF) have been correlated with vessel density, advanced disease stage, and poor outcome in SCLC.[6-8] The Eastern Cooperative Oncology Group is currently conducting a phase II study of the anti-VEGF monoclonal antibody bevacizumab (Avastin) with cisplatin and etoposide. Analysis of an oral small molecule inhibitor of VEGF (ZD6474) in SCLC patients after response to first-line therapy has been initiated by the National Cancer Institute of Canada.

Thalidomide (Thalomid) functions in part as an angiogenesis inhibitor and appears to be well tolerated in combination with carboplatin and etoposide in patients with SCLC.[9] A phase III trial of this combination is also under way.

Immunotherapy

The consistency of molecular alterations associated with SCLC, serving as potential tumor-specific antigens, has made SCLC an attractive target for immunotherapy. Results to date however have been disappointing. Two recent large randomized phase III trials, including evaluation of the anti-idiotype antibody BEC2, have had clearly negative results.[10]

Conclusions

Small-cell lung cancer is an aggressive disease despite initial sensitivity to chemotherapy and radiation. The recent elucidation of key biologic determinants of SCLC carcinogenesis has suggested targets for novel therapeutic development that may translate into improved outcome for patients with this disease. Further efforts should focus on exploitation of these key oncogenic determinants.

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A third review, by Drs. Dubey and Schiller, appears on the following page.

The Horiike/Saijo Article Reviewed

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In this article, Drs. Horiike and Saijo provide a comprehensive review of the treatment of small-cell lung cancer (SCLC). We concur with the authors and would like to highlight certain areas of interest.

The incidence of SCLC has declined over the past several years, the etiology for which is unclear. Nevertheless, it remains a lethal malignancy: More than half of those diagnosed will have distant disease with a 5-year survival rate of approximately 2%.

Extensive Disease

Small-cell lung cancer is an exquisitely chemosensitive disease. Clinical trials have demonstrated the survival benefit of combination chemotherapy over single-agent therapy.[1,2] The authors have addressed the benefit of cisplatin-based chemotherapy in comparison to other regimens. Even though higher doses of etoposide are associated with improved response rates, they have had no impact on survival, and increasing doses of cisplatin have affected neither response rates nor survival.[3,4] Further evidence for the lack of benefit of high-dose chemotherapy in SCLC can be drawn from the negative results obtained after dose-intense chemotherapy using granulocyte colony-stimulating factor (G-CSF, Neupogen) and autologous bone marrow transplant.[5,6] The implication is that higher doses offer no added benefit, at the expense of increased toxicity.

The most compelling evidence of advances in chemotherapeutic management in recent years has been the combination of cisplatin and irinotecan (Camptosar), to which the authors allude. In the study by the Japanese Clinical Oncology Group (JCOG), the cisplatin/irinotecan combination had a median survival advantage of approximately 3 months and a 2-year survival benefit of approximately 15% over the cisplatin/etoposide regimen. An ongoing North American trial using the same drug combination will attempt to confirm these results in the American population. Interim analyses indicate an improved toxicity profile in the North American study. However, the North American study excluded patients with a performance status of 2, whereas 16% of patients in the JCOG study had a performance status of 2. In addition, the North American regimen differed from the JCOG regimen, with both drugs being administered on days 1 and 8 of a 21-day cycle.

The elderly remain a special population. Surveillance, Epidemiology, and End Results (SEER) data indicate that the incidence of SCLC peaks at age 60 to 80 years. The medical comorbidities that frequently accompany patients in this age range necessitate special considerations in treatment. Moderately aggressive regimens using carboplatin (Paraplatin) and etoposide have been well tolerated and have resulted in efficacy comparable to that in younger cohorts. Functional status, and not age alone, should guide the choice of chemotherapy in this subgroup.

Even in the general SCLC population, direct comparisons of cisplatin/etoposide and carboplatin/etoposide have demonstrated similar efficacy and an improved toxicity profile in the carboplatin arm.[7] Thus, while the cisplatin vs carboplatin debate in non–small-cell lung cancer is ongoing, carboplatin-based regimens have become an attractive option in small-cell lung cancer, particularly for those with poor performance status and medical comorbidities.

In the United States, topotecan (Hycamtin) is the Food and Drug Administration (FDA)-approved drug for the treatment of relapsed SCLC. Other regimens that are effective in this setting include combinations of cyclophosphamide (Cytoxan, Neosar), doxorubicin (Adriamycin), and vincristine (the CAV regimen), as well as etoposide, gemcitabine (Gemzar), and paclitaxel. The prognosis of sensitive disease is better than that of refractory disease but continues to be poor.

Brain metastases from SCLC are distinct from those associated with other malignancies. The underlying chemosensitivity coupled with disruption of the blood-brain barrier due to the presence of malignant deposits in the brain make chemotherapy without radiation therapy an appealing therapeutic option. In fact, the response rate of initial brain metastases to chemotherapy is approximately 70%. However, the response rates to chemotherapy alone at relapse decrease to 40%.[8]

Limited Disease

Cure can be achieved in a small number of patients with aggressive combined-modality chemoradiation. Concurrent chemoradiation offers improved benefit over sequential treatment. As discussed by the authors, twice-daily hyperfractionated radiation concurrent with chemotherapy offers the greatest survival benefit.[9] However, patients must be cautiously selected for concurrent and accelerated treatment regimens, given the higher toxicities accompanying these modalities.

Since it appears that, in SCLC, "more is better," trials have been designed to incorporate consolidation chemotherapy after concurrent chemoradiation.[10-13] Consolidation therapies in these trials have included older and newer agents, such as carboplatin/paclitaxel, single-agent cy-

clophosphamide, vincristine/methotrexate/etoposide alternating with doxorubicin/cyclophosphamide, and interferon. Median survival has ranged from 11 to 18 months without evidence of survival benefit. Even the addition of BEC2/BCG vaccination offered no benefit after response to chemotherapy and radiation.[14]

In SCLC patients, the brain is a sanctuary for relapse. The 2- to 3-year cumulative risk of brain metastases after treatment of limited disease is approximately 50%.[15,16] A meta-analysis demonstrated that prophylactic cranial irradiation (PCI) not only decreased the incidence of brain metastases, but also improved survival. On careful examination, neurocognitive deficits have been found in patients prior to PCI, and no significant deterioration was found after PCI.[17,18] In randomized trials, patients who underwent PCI experienced the same neurocognitive difficulties as those who did not, and these difficulties did not interfere with day-to-day functioning. Thus, cognitive decline may be more a reflection of the actual disease process than of the treatment. That said, PCI should be offered to those who have achieved a complete response to chemoradiation.

Conclusions

Although SCLC is chemosensitive, with higher response rates than most other solid tumors, it remains an elusive disease with a propensity to recur and develop resistance. A ceiling has been reached in terms of combination and intensification of chemotherapeutic regimens. Nevertheless, there has been some progress against SCLC.[19]

Future approaches include exploitation of novel targeted agents such as Bcl-2 antisense oligonucleotide, tyrosine-kinase inhibitors, vascular endothelial growth factor (VEGF) inhibitors, and proteasome inhibitors. We agree with the authors that more patients need to be enrolled in clinical trials. The major challenge with targeted agents is that their cytostatic rather than cytotoxic nature makes it difficult to use conventional criteria to assess response. Surrogate markers are needed to evaluate efficacy. The fact that patients with SCLC represent a minority of the lung cancer cohort may provide an additional challenge in accrual to larger trials.

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Original article

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Phase I–II study of amrubicin and cisplatin in previously untreated patients with extensive-stage small-cell lung cancer

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Background: Amrubicin, a totally synthetic 9-amino-anthracycline, demonstrated excellent single-agent activity for extensive-stage small-cell lung cancer (ED-SCLC). The aims of this trial were to determine the maximum-tolerated doses (MTD) of combination therapy with amrubicin and cisplatin, and to assess the efficacy and safety at their recommended doses (RD).

Patients and methods: Eligibility criteria were patients having histologically or cytologically proven measurable ED-SCLC, no previous systemic therapy, an Eastern Cooperative Oncology Group performance status of 0–2 and adequate organ function. Amrubicin was administered on days 1–3 and cisplatin on day 1, every 3 weeks.

Results: Four patients were enrolled at dose level 1 (amrubicin 40 mg/m^2/day and cisplatin 60 mg/m^2) and three patients at level 2 (amrubicin 45 mg/m^2/day and cisplatin 60 mg/m^2). Consequently, the MTD and RD were determined to be at level 2 and level 1, respectively. The response rate at the RD was 87.8% (36/41). The median survival time (MST) was 13.6 months and the 1-year survival rate was 56.1%. Grade 3/4 neutropenia and leukopenia occurred in 95.1% and 65.9% of patients, respectively.

Conclusions: The combination of amrubicin and cisplatin has demonstrated an impressive response rate and MST in patients with previously untreated ED-SCLC.

Key words: anthracycline, cisplatin, phase I–II, small-cell lung cancer

Introduction

Small-cell lung cancer (SCLC) is one of the most chemosensitive solid tumors, and the outcome of SCLC patients is slowly but surely improving. Combination chemotherapy consisting of cisplatin plus etoposide and concurrent twice-daily thoracic radiotherapy has yielded a 26% 5-year survival rate in limited-stage (LD) patients [1]. Despite the high response rate to combination chemotherapy, however, local and distant failure is very common, especially in extensive-stage (ED) patients. Moreover, resistance to chemotherapeutic agents develops easily after failure of initial treatment. Thus, long-term survivors are still very rare among patients with ED-SCLC. To improve the outcome of SCLC patients, several strategies, such as high-dose chemotherapy, dose-intensive chemotherapy, alternating chemotherapy and introduction of new drugs, have been investigated [2–6]. However, only the introduction of new agents has improved the outcome of SCLC patients. Combination chemotherapy with etoposide plus cisplatin or etoposide plus cisplatin alternating cyclophosphamide, doxorubicin and vincristine had been mainly used for SCLC in North America. Recently, a Japanese trial [Japan Clinical Oncology Group (JCOG) 9511] demonstrated the superiority of the combination of irinotecan and cisplatin for ED-SCLC patients over the combination of etoposide and cisplatin [6]. The development of more active chemotherapy, and especially the introduction of effective new drugs, is therefore essential to improve the survival of SCLC patients.

Amrubicin (SM-5887) is a totally synthetic anthracycline and a potent topoisomerase II inhibitor [7–14]. It has antitumor activity, and is more potent than doxorubicin against various mouse experimental tumors and human tumor

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xenografts. Amrubicin and its 13-hydroxy metabolite, amrubicinol, inhibit purified human DNA topoisomerase II [11]. Amrubicinol is 10–100 times more cytotoxic than amrubicin [9]. The potent therapeutic activity of amrubicin is caused by the selective distribution of its highly active metabolite, amrubicinol, in tumors [9]. In an experimental animal model, amrubicin did not exhibit any chronic cardiotoxicity potential, and no deleterious effects on doxorubicin-induced cardiotoxicity in dogs was observed [14]. In a phase II study of amrubicin using a schedule of 45 mg/m^2 on days 1–3 every 3 weeks, in 33 previously untreated ED-SCLC patients, an overall response rate of 76% and a complete response (CR) rate of 9% were reported [15]. Moreover, median survival time (MST) was 11.7 months in the single-agent phase II study of amrubicin. Amrubicin is one of the most active new agents for SCLC. Thus, we conducted a phase I/II study of amrubicin plus cisplatin for untreated ED-SCLC, because cisplatin is considered as one of the most important drugs in the treatment of SCLC. The aims of this trial were to determine the maximum-tolerated doses (MTD) of combination therapy of amrubicin with cisplatin, to assess the efficacy and safety for ED-SCLC at their recommended doses (RD), and to examine the pharmacokinetics of the drug combination.

Patients and methods

Patient selection

Patients with histologically and/or cytologically documented SCLC were eligible for this study. Each patient was required to meet the following criteria: extensive-stage disease [16]; no prior therapy for primary lesion; measurable lesion; Eastern Cooperative Oncology Group (ECOG) performance status (PS) 0–2; expected survival time >2 months; age 20–74 years; adequate hematological function [white blood cell (WBC) count 4000–12 000/mm^3, neutrophils ≥2000/mm^3, platelets ≥100 000/mm^3, hemoglobin ≥10 g/dl]; adequate hepatic function [total bilirubin within 1.5× the upper limit of normal; aspartate aminotransferase (AST) and alanine aminotransferase (ALT) within 2.5× the upper limit of normal]; adequate renal function (creatinine within the upper limit of normal); partial pressure of arterial oxygen 60 torr; no abnormality requiring treatment on electrocardiogram; left ventricle ejection fraction >60%; written informed consent. Patients with symptomatic brain metastasis, pleural effusion that required drainage, non-steroidal anti-inflammatory drug or glucocorticoid use for >50 days, pericarditis carcinomatous, active infection, varicella, superior vena cava syndrome, syndrome of inappropriate secretion of antidiuretic hormone (SIADH), gastric and/or duodenal ulcer, severe heart disease, severe renal disease, active concomitant malignancy, symptomatic pneumonitis and/or pulmonary fibrosis and pregnant/nursing women were excluded. This study was approved by the Institutional Review Board at each hospital.

Patient evaluation

Pretreatment evaluation consisted of complete blood cell counts, differential, routine chemistry measurements, progastrin-releasing peptide (ProGRP), neuron-specific enolase, electrocardiogram, echocardiography, chest radiograph, chest and abdominal computed tomography (CT) scan, whole-brain magnetic resonance imaging (MRI) or CT scan, and isotope bone scan. Complete blood cell counts, differential and routine chemistry measurements were performed at least once a week during the chemotherapy.

Treatment schedule

At level 1, chemotherapy consisted of cisplatin 60 mg/m^2 on day 1 and amrubicin 40 mg/m^2 on days 1–3. Amrubicin was administered as an intravenous injection over 5 min and cisplatin was administered as a drip infusion over 60–120 min with adequate hydration. At level 2 the dose of amrubicin was increased to 45 mg/m^2 on days 1–3. Level 3 was planned with cisplatin 80 mg/m^2 on day 1 and amrubicin 45 mg/m^2 on days 1–3. The chemotherapy was repeated every 3 weeks for four to six courses. Intrapatient dose escalation was not allowed. Administration of granulocyte colony-stimulating factor (G-CSF) was permitted prophylactically for patients expected to experience grade 3 neutropenia during the first course. Prophylactic administration of G-CSF was only permitted at second or later courses.

The administrations of both cisplatin and amrubicin were postponed if patients met the following criteria: WBC <3000/mm^3; neutrophils <1500/mm^3; platelets <100 000/mm^3; AST and ALT >5× the upper limit of normal; total bilirubin >1.5× the upper limit of normal; creatinine >1.3× the upper limit of normal; ECOG PS 3 or 4; active infection; grade 2 or worse non-hematological toxicity, except for alopecia, anorexia, nausea, vomiting or fatigue.

The administrations of both cisplatin and amrubicin were withdrawn if patients met the following criteria: tumor regression <15% after first course or <30% after second course; WBC <3000/mm^3; neutrophils <1500/mm^3; platelets <100 000/mm^3; no recovery from grade 3 or 4 non-hematological toxicity at 6 weeks after the start of previous chemotherapy; abnormality of electrocardiogram requiring treatment for more than 6 weeks; left ventricle ejection fraction <48%; treatment delay of >4 weeks.

The dose of amrubicin was decreased 5 mg/m^2/day if patients met the following criteria: grade 4 leukopenia or neutropenia for ≥4 days; grade 3 neutropenia with fever; platelets <20 000/mm^3 during the previous course. The dose of cisplatin was decreased to 75% if creatinine increased to >1.5× the upper limit of normal during the previous course.

The dose-limiting toxicity (DLT) was defined as follows: grade 4 leukopenia or neutropenia for ≥4 days; grade 3 febrile neutropenia; platelets <20 000/mm^3; grade 3 or worse non-hematological toxicity except for nausea, vomiting, anorexia, fatigue, hyponatremia and infection. Initially, three patients were treated at each dose level. If DLT was not observed in any of the three patients, dose escalation was carried out. If DLT was observed in one of three patients, an additional three patients were entered at the same dose level. If DLT was observed in three or more of six patients, or two or three of the initial three patients, we considered that dose to be the MTD. If DLT was observed in one or two of six patients, dose escalation was also carried out. Dose escalation was determined based only on the data from the first course of chemotherapy.

Response and toxicity evaluation

Response was evaluated according to Response Evaluation Criteria in Solid Tumors (RECIST) and tumor markers were excluded from the criteria [17]. CR was defined as the complete disappearance of all clinically detectable tumors for at least 4 weeks and no new lesions. Partial response (PR) was defined as at least a 30% decrease in the sum of the longest diameters of target lesion, taking as reference the baseline sum longest diameter, the required non-progression in non-target lesions and no new lesions for at least 4 weeks. Stable disease (SD) included: regression of target lesions insufficient to meet the criteria for PR, a <20% increase in the sum of the longest diameter of target lesion, taking as reference the smallest sum longest diameters recorded since the treatment started, the required non-progression in non-target lesions and no new lesions for at least 6 weeks. Progressive disease (PD) indicated a >20% increase in the sum of the longest diameters of target lesion, taking as reference the smallest sum longest diameter recorded since the treatment started

and/or unequivocal progression of existing non-target lesions and/or appearance of new lesions. The evaluation of objective tumor response for all patients was performed by an external review committee.

Toxicity grading criteria of the National Cancer Institute Common Toxicity Criteria (version 2.0) was used for evaluation of toxicity.

Statistical analysis

This study was designed to reject response rates of 70% (P0) at a significance level of 0.05 (one-tailed) with a statistical power of 80% to assess the activity of the regimen as a 85% response rate (P1) at the recommended dose. The upper limit of rejection was 29 responses (CR + PR) among 37 evaluable patients. Overall survival was defined as the interval between the first administration of the drugs in this study and death or the last follow-up visit. Median overall survival was estimated using the Kaplan–Meier method [18].

Table 1. Characteristics of treated patients

	Phase I	Phase II	Total
Number of patients	7	37	44
Gender			
Male	5	31	36
Female	2	6	8
Age (years)			
Median	65	64	64.5
Range	54–73	50–74	50–74
ECOG PS			
0	0	5	5
1	7	32	39
2	0	0	0
Stage			
IIIB	0	2	2
IV	7	35	42
Prior therapy			
Yes	0	1	1
No	7	36	43
Serum ALP			
Normal	7	29	36
Elevated	0	7	7
Serum LDH			
Normal	3	14	17
Elevated	4	23	27
Na			
Normal	6	35	41
Decreased	1	2	3
Number of metastases			
0	0	2	2
1	4	27	31
2	3	6	9
3	0	1	1
4 or more	0	1	1

In one patient, serum ALP level could not be measured.
ECOG PS, Eastern Cooperative Oncology Group performance status; LDH, lactate dehydrogenase; ALP, alkaline phosphatase.

Pharmacokinetic analysis

Pharmocokinetic analysis was performed in patients entering the phase I section of this study. One milliliter of the blood was taken from the patients before administration of amrubicin, and at 0 min, 15 min, 1, 2, 3, 4, 8 and 24 h after administration on days 1 and 3 in the first course of chemotherapy. Concentrations of amrubicin and its active metabolite, amrubicinol, in plasma and red blood cells were measured as reported elsewhere [9].

Results

Patient characteristics

Between April 2001 and December 2002, 45 patients with ED-SCLC were enrolled and 44 were treated in this study (Table 1). One patient did not receive the protocol treatment because atrial fibrillation was observed just before administration on day 1 of the first course. All treated patients were assessed for response, survival and toxicity. The median age of the treated patients was 64.5 years (range 50–74). There were 36 males and eight females. Five patients had an ECOG PS 0 and 39 patients had PS 1. Only one patient received surgery for brain metastasis as a prior therapy.

MTD and DLT in the phase I study

Four patients were enrolled at dose level 1 (amrubicin 40 mg/m^2 on days 1–3 and cisplatin 60 mg/m^2 on day 1) and three patients at level 2 (amrubicin 45 mg/m^2 on days 1–3 and cisplatin 60 mg/m^2 on day 1). Toxicities in the phase I study are listed in Table 2. No DLT were observed during the first course of level 1. At level 2, grade 4 neutropenia for ≥4 days and febrile neutropenia occurred in one patient, and febrile neutropenia and grade 3 constipation occurred in another patient. Consequently, the MTD and RD were determined to be level 2 and level 1, respectively.

Pharmacokinetics of amrubicin and its active metabolite, amrubicinol

Pharmacokinetic parameters of amrubicin in plasma were almost identical on days 1 and 3 at the two dose levels (Table 3). No clear dose relationship in the area under the concentration–time curve (AUC) of amrubicin in the plasma was observed. The AUC of amrubicinol in red blood cells tended to increase on day 3 at both doses (Table 4). No clear dose relationship in the AUC of amrubicinol in red blood cells was observed. Combination with cisplatin did not alter the pharmacokinetics of amrubicin and amrubicinol (data not shown).

Treatment received in patients treated at the RD

Forty-one patients were treated at the RD: amrubicin 40 mg/m^2 on days 1–3 and cisplatin 60 mg/m^2 on day 1. Of 41 patients, 32 (78%) patients received more than three

Table 2. Toxicities during the first course in the phase I study

	Level 1 ($n=4$)					Level 2 ($n=3$)				
Amrubicin	40 mg/m^2 days 1–3					45 mg/m^2 days 1–3				
Cisplatin	60 mg/m^2 day 1					60 mg/m^2 day 1				
	Grade (NCI CTC)					Grade (NCI CTC)				
	0	1	2	3	4	0	1	2	3	4
Leukopenia	0	1	1	2	0	0	0	1	1	1
Neutropenia	0	0	0	2	2	0	0	0	0	3
Febrile neutropenia	4	–	–	0	0	1	–	–	2	0
Hemoglobin	1	1	2	0	0	2	1	0	0	0
Thrombocytopenia	1	2	0	1	0	0	2	0	1	0
Stomatitis	3	0	1	0	0	3	0	0	0	0
Nausea	1	1	2	0	–	1	1	0	1	–
Constipation	3	0	1	0	0	1	0	1	1	0
Hyponatremia	2	1	0	0	1	1	2	0	0	0
Hypocalcemia	3	0	1	0	0	3	0	0	0	0

Dose limiting toxicity at level 2: febrile neutropenia, two patients; grade 4 neutropenia ≥4 days, one patient; grade 3 constipation, one patient.
NCI CTC, National Cancer Institute Common Toxicity Criteria.

Table 3. Pharmacokinetics of amrubicin in plasma

Dose	n	Day	$T_{1/2\alpha}$ (h)	$T_{1/2\beta}$ (h)	V_d (l)	CL (l/h)	AUC_{0-24h} (ng h/ml)
40 mg/m^2	4	1	0.11 ± 0.04	2.29 ± 0.31	46.6 ± 11.0	13.6 ± 1.8	2995 ± 434
	4	3	0.08 ± 0.01	2.89 ± 0.34	50.0 ± 10.6	11.6 ± 1.9	3511 ± 514
45 mg/m^2	3	1	0.13 ± 0.05	2.39 ± 0.34	56.3 ± 10.6	14.9 ± 1.8	3052 ± 402
	3	3	0.09 ± 0.03	2.27 ± 0.18	51.9 ± 3.7	14.2 ± 2.3	3217 ± 479

$T_{1/2\alpha}$, half-life at distribution phase; $T_{1/2\beta}$, half-life at elimination phase; V_d, volume of distribution; CL, clearance; AUC, area under the concentration–time curve.

courses of chemotherapy, and 10 (31%) of these 32 patients needed dose reduction of amrubicin at the fourth course (Table 5). Of 41 patients, 22 (54%) patients completed four courses of chemotherapy without dose modification. The main cause of dose reduction was myelosuppression, especially leukopenia and neutropenia.

Objective tumor response and overall survival

The objective tumor responses are given in Table 6. Four CRs and 32 PRs occurred, for an objective response rate of 87.8% [95% confidence interval (CI) 73.8% to 95.9%] in 41 patients treated at the RD. The objective response rate for all 44 patients was 88.6% (95% CI 75.4% to 96.2%). The overall survival times of the 41 patients treated at the RD are shown in Figure 1. The MST of the 41 patients was 13.6 months (95% CI 11.1–16.6), with a median follow-up time for eight censored patients of 16.4 months (95% CI 14.2–18.8). The 1- and 2-year survival rates were 56.1% and 17.6%, respectively. The MST of all 44 patients was 13.8 months (95% CI 11.1–16.6). The 1- and 2-year survival rates of all 44 patients were 56.8% and 21.4%, respectively.

Table 4. Pharmacokinetics of amrubicinol in red blood cells

Dose	n	Day	$T_{1/2}$ (h)	AUC_{0-24h} (ng·h/ml)
40 mg/m^2	4	1	21.0 ± 3.1	1412 ± 314
	4	3	20.7 ± 4.8	2159 ± 622
45 mg/m^2	3	1	19.6 ± 6.1	1098 ± 277
	3	3	18.1 ± 5.7	2027 ± 332

$T_{1/2}$, elimination half-life; AUC, area under the concentration–time curve.

Table 5. Treatment received in patients treated at the recommended dose

Cycle	n	Amrubicin (mg/m^2)			Cisplatin (mg/m^2)	
		40	35	30	60	45
1	41	41			41	
2	36	30	6		36	
3	33	26	5	2	33	
4	32	22	8	2	32	
5	18	9	5	4	18	
6	13	6	3	4	12	1

Table 6. Response rates

	n	CR	PR	SD	PD	NE	Response rate (%) (95% CI)
All	44	4	35	3	0	2	88.6 (75.4–96.2)
Treated at RD	41	4	32	3	0	2	87.8 (73.8–95.9)

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluated; 95% CI, 95% confidence interval; RD, recommended dose.

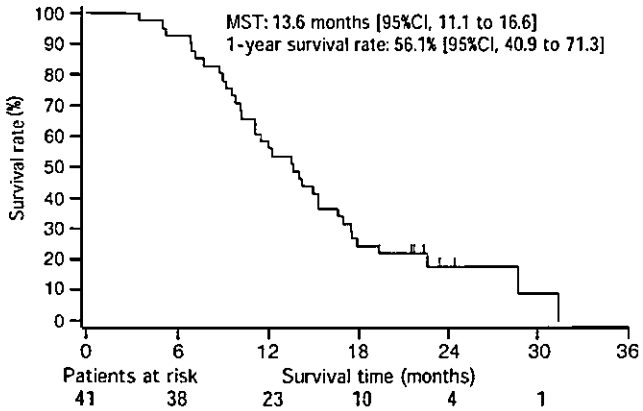

Figure 1. Overall survival of patients with extensive-stage small-cell lung cancer who were treated with amrubicin and cisplatin at the recommended dose. MST, median survival time; 95% CI, 95% confidence interval.

Toxicity in patients treated at the RD

The worst grades of hematological and non-hematological toxicities experienced by each patient are listed in Table 7. Hematological toxicity, especially leukopenia and neutropenia, was common and relatively severe. Grade 3 or worse leukopenia and neutropenia occurred in 65.9% and 95.1% of patients, respectively. Febrile neutropenia was observed in two patients at level 2. Grade 3 or worse anemia and thrombocytopenia occurred in 53.7% and 24.4% of patients, respectively. Four patients received platelet transfusions. Common non-hematological toxicities were gastrointestinal toxicity, such as anorexia, nausea, vomiting, constipation, diarrhea and stomatitis. Gastric ulcers developed in three patients. Hepatic and renal toxicity were not common in this study. Grade 3 or worse hyponatremia and hypokalemia occurred in 22% and 9.8% of patients, respectively. One patient developed myocardial infarction; however, cardiac toxicity was not common. No treatment-related deaths were observed.

Discussion

Doxorubicin and epirubicin are classified as active agents for SCLC, for which single-agent activity is a >20% response rate [19]. Doxorubicin has been used as a constituent of combination therapy for SCLC in the CAV (cyclophospamide, doxorubicin and vincristine) and CAP (cyclophosphamide, doxorubicin and cisplatin) regimens. Epirubicin has shown

Table 7. Toxicity in patients treated at the recommended dose ($n=41$)

	Grade (NCI CTC)					Grade 3/4 (%)
	0	1	2	3	4	
Leukopenia	1	0	13	20	7	65.9
Neutropenia	0	1	1	7	32	95.1
Febrile neutropenia	41	–	–	0	0	0.0
Hemoglobin	1	8	10	17	5	53.7
Thrombocytopenia	9	14	8	10	0	24.4
Stomatitis	22	13	5	1	0	2.4
Anorexia	1	14	13	13	0	31.7
Nausea	3	15	14	9	0	22.0
Vomiting	20	8	11	2	0	4.9
Constipation	24	1	13	3	0	7.3
Diarrhea	26	12	1	2	0	4.9
Gastric ulcer	38	0	1	2	0	4.9
Bilirubin	24	12	4	1	0	2.4
Hyponatremia	18	14	–	7	2	22.0
Hypokalemia	31	6	–	4	0	9.8
Hyperkalemia	33	3	4	1	0	2.4
Hypocalcemia	31	5	4	0	1	2.4

NCI CTC, National Cancer Institute Common Toxicity Criteria.

50% and 48% response rates in two clinical studies in 41 and 80 previously untreated patients, respectively, with ED-SCLC [20, 21]. However, currently, combination modalities containing doxorubicin or epirubicin are not being used in the therapy of SCLC, in preference to combination therapy with cisplatin and etoposide. Since amrubicin has shown excellent single-agent activity [15], it can be expected to be superior to other anthracyclines in the treatment of SCLC. Additionally, the present results of combination therapy with cisplatin support the view that amrubicin may be a promising agent that overcomes the therapeutic plateau of SCLC.

Amrubicin is one of the most promising new agents for the treatment of SCLC. In a previous phase II study of amrubicin 45 mg/m^2 on days 1–3 every 3 weeks as a monotherapy for chemonaive ED-SCLC, a 76% overall response rate and 11.7 month MST were observed [15]. The overall response rate and MST were comparable to those achieved with standard combination chemotherapy, such as etoposide plus cisplatin [5, 6]. Moreover, only a few patients treated in the phase II study received salvage chemotherapy consisting of cisplatin and etoposide [15]. The major toxicity of amrubicin as a monotherapy was hematological toxicity: grade 4 leukopenia and neutropenia were seen in 12.1% and 39.4% of patients, respectively, and thrombocytopenia and anemia of grade 3 or worse in 21.2%. Hepatic, renal and cardiac toxicities with amrubicin were not common. Cisplatin is a key drug for the treatment of SCLC and its hematological toxicity, such as leukopenia and neutropenia, is not severe. Thus, we conducted a phase I–II study of amrubicin and cisplatin treatment for chemonaive ED-SCLC to determine the MTD of this combination therapy, to

assess the efficacy and safety of the drugs delivered at their RD in chemonaive ED-SCLC, and to examine pharmacokinetics.

The topoisomerase I inhibitor, irinotecan, is also very effective for SCLC [6]. Combinations of topoisomerase I and topoisomerase II inhibitors, such as irinotecan plus etoposide, have been reported as active combination chemotherapy for SCLC [22]. Thus, combination of irinotecan and amrubicin is another candidate for new combination chemotherapy for SCLC. A phase I study of irinotecan and amrubicin for chemonaive non-SCLC was performed in National Cancer Center Hospital (unpublished data). However, the MTD was less than irinotecan 60 mg/m^2 on days 1 and 8 and amrubicin 35 mg/m^2 on days 2–4, due to relatively severe myelotoxicity. We considered that amrubicin <35 mg/m^2 on days 2–4 with irinotecan 60 mg/m^2 on days 1 and 8 was insufficient to treat SCLC.

In this study, we determined the RD to be amrubicin 40 mg/m^2 on days 1–3 and cisplatin 60 mg/m^2 on day 1 every 3 weeks, and 41 patients were treated at the RD. Main toxicities of this combination chemotherapy were myelosuppression, especially leukopenia and neutropenia, and gastrointestinal toxicities including anorexia, nausea, vomiting, constipation, diarrhea, stomatitis and gastric ulcer. Of 41 patients, 32 (78%) patients received four or more courses of chemotherapy, and 22 (54%) patients completed four courses of chemotherapy without dose modification. One patient developed myocardial infarction; however, other cardiac toxicity, including decrease in left ventricle ejection fraction, was not observed in up to six courses of chemotherapy. The total dose of amrubicin was 720 mg/m^2. Grade 3 or 4 hyponatremia occurred in nine (22%) patients; however, most of the patients were asymptomatic. No unexpected toxicities and no treatment-related deaths were observed in this study. Toxicities observed in this study were manageable.

Four CRs and 32 PRs occurred, for an objective response rate of 87.8% (95% CI 73.8% to 95.9%) in 41 patients treated at the RD. In most patients, ProGRP levels changed in parallel with tumor responses. The MST of the 41 patients was 13.6 months, and the 1-year survival rate was 56.1%. These results were better than recently reported results for irinotecan and cisplatin in chemonaive ED-SCLC: an objective response rate of 84% and MST of 12.8 months [6]. The combination of amrubicin and cisplatin has demonstrated an impressive response rate and MST in patients with previously untreated ED-SCLC. A possible reason for the better results is overselection of patients, because we used unusual exclusion criteria such as non-steroidal anti-inflammatory drug or adrenal cortical steroid use for >50 days, and gastric and/or duodenal ulcer. However, in a phase II study, this kind of bias is not uncommon.

Combination chemotherapy with etoposide plus cisplatin or etoposide plus cisplatin, alternating with cyclophosphamide, doxorubicin and vincristine, had been considered as standard chemotherapy for SCLC in North America and Japan. A Japanese phase III trial (JCOG 9511) demonstrated that treatment with four cycles of irinotecan plus cisplatin every 4 weeks yielded a highly significant improvement in survival in ED-SCLC patients over standard etoposide plus cisplatin, with less myelosuppression [6]. Based on the results of the JCOG 9511 trial, irinotecan plus cisplatin is considered to be the reference chemotherapy arm for ED-SCLC in future trials in Japan [23]. The JCOG are preparing a phase III clinical trial of amrubicin and cisplatin for previously untreated ED-SCLC to compare combination therapy of irinotecan with cisplatin.

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Topographical distribution of allelic loss in individual lung adenocarcinomas with lymph node metastases

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Adenocarcinomas of the lung are characterized by morphological heterogeneity, and since carcinogenesis has been suggested to be a multistep process involving sequential accumulation of multiple genetic alterations, the morphological heterogeneity may represent a cross-sectional view of genetic alterations within individual tumors. Therefore, to elucidate whether, and which, genetic alterations accumulated in relation to morphological cancer progression, we examined 56 microdissected sites for topographical distribution of loss of heterozygosity (LOH) in 12 adenocarcinomas of the lung with bronchioloalveolar (BA) and invasive components in their primary tumors and metastases to lymph nodes. The morphological changes from noninvasive BA lesions to invasive and metastatic components were characterized by a significant rise in the prevalence of allelic losses ($P<0.05$). Individually, eight cases (67%) showed accumulation of genetic alterations from BA lesions to metastases. LOHs in multiple foci in one case were compared to determine whether they were shared at all tumor sites as an early event or localized in metastases as an additional event. LOHs at 5q and 17p may be crucial steps in the early phase of development to metastasis, while 18q loss may be an additional step. These findings suggested that the cancer cells in some pulmonary adenocarcinomas evolved from the BA lesions to the invasive and metastatic lesions.
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Carcinoma of the lung is one of the most common human cancers and is the predominant cause of cancer-related death throughout the world. Of the four major histological subtypes of lung cancer, the incidence of adenocarcinoma has been gradually increasing, but the morphological and genetic progression schemes of adenocarcinoma of the lung have not been elucidated as clearly as those of colon cancer,[1] which is the human cancer that has been most intensively investigated in this regard. Elucidation of the genetic sequence responsible for the progression of adenocarcinoma of the lung from *in situ* to invasive and metastatic carcinoma is an important goal in understanding the biological basis of this malignancy.

One of the most characteristic features of adenocarcinoma of the lung is its high degree of morphological heterogeneity. We have often observed morphologically noninvasive bronchioloalveolar carcinoma (BAC) components replacing pre-existing alveolar epithelium (the replacing-growth-type) at the periphery of invasive adenocarcinomas. According to the WHO classification,[2] they were diagnosed as 'adenocarcinoma mixed bronchioloalveolar and other subtypes (acinar, solid, papillary).' This morphological heterogeneity may represent a cross-sectional view of clonal evolution within individual tumors. That is, some noninvasive BACs may evolve into invasive adenocarcinoma as they acquire invasiveness during carcinogenesis, as observed in the adenoma–carcinoma sequence of colon carcinogenesis.[1,3–5] Adenocarcinomas mixed bronchioloalveolar (BA) and other subtypes are supposed to contain bronchioloalveolar (BA) components of BACs and

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invasive components evolving from the BAC in the individual tumor. They contain noninvasive components and invasive components that we usually observe in each tumor as part of the adenoma–carcinoma sequence. In addition, it has never been determined whether adenocarcinomas containing a morphologically noninvasive BA component (adenocarcinoma mixed BA and other subtypes) evolve from BAC. We recently examined 66 cases of replacing-growth-type small adenocarcinoma of the lung less than 2 cm in size to investigate allelic losses at eight loci on the eight chromosomes carrying the most important cancer-associated genes by the laser capture microdissection method, in which cancer cells are selectively collected.[6] The 66 cases were divided into three groups according to the Noguchi's classification of small adenocarcinomas of the lung:[7] 12 localized bronchioloalveolar carcinomas (LBACs), 28 LBACs with alveolar collapse, and 26 LBACs with active fibroblastic proliferation. We confirmed that the prevalence of loss of heterozygosity (LOH) increased as adenocarcinoma of the lung underwent histological progression from LBAC (16.7%) to small but advanced LBAC with fibroblastic proliferation (96.2%). Deletions of four of the markers, 3p, 17p, 18q, and 22q, increased significantly during the carcinogenic steps from noninvasive to invasive carcinoma, and these molecular genetic data were consistent with the morphological progression of the adenocarcinoma.

It has been suggested that certain types of genetic alterations may be involved in the early phase of tumorigenesis, whereas others may play a role in late events during tumor progression. However, such inferences have essentially been based on statistical arguments after analysis of a set of different tumors[6,8,9] and whether they are indeed applicable to the progression scheme in individual tumors has not been specifically addressed in regard to adenocarcinoma of the lung. An alternative approach therefore seemed to be necessary, for those relying on the statistical correlation between the frequency of each genetic alteration and histological and/or disease progression, but such an approach has rarely been taken in studies of the genetic changes in adenocarcinoma of the lung.

The pathologic staging of malignant tumors, including of non-small cell lung carcinoma (NSCLC), is based on the concept that primary tumor growth and local invasion precede lymphatic dissemination, which is regarded as a later event in lung cancer progression. From the standpoint of molecular genetics as well, the metastatic event is considered to be a relatively late event that follows multiple sequential and selective steps of clonal evolution. Metastases are thought to be established through selected clonal tumor cells that carry all genetic alterations involved in the genesis and progression of carcinoma.[10,11] However, no studies have carefully compared the accumulation of genetic changes and histopathological progression from a noninvasive lesion to an invasive or metastatic lesion in an individual pulmonary adenocarcinoma mixed BA and other subtypes.

In the present study, we examined the topographical distribution of LOH events occurring in the process of neoplastic progression within individual tumors and assessed whether previous inferences are indeed applicable to the progression scheme within individual tumors. To do so, we examined multiple pathologically well-defined specimens from individual tumors with lymph node metastases in order to investigate whether, and which genetic alterations are accumulated in relation to histopathological progression in individual tumors.

Materials and methods

Patients and Tissue Samples

We selected 12 cases of adenocarcinoma of the lung in which the primary tumor measured 3 cm or less in greatest dimension and contained BA components and invasive components (adenocarcinoma mixed BA and other subtypes) and there were metastases in the resected lymph nodes. All tumor samples were obtained from surgical resections at the Department of Thoracic Oncology of the National Cancer Center Hospital East (Chiba, Japan) between 1998 and 2000. All patients underwent lobectomies and lymph node dissections curative intent with no preoperative adjuvant therapy.

A 3-μm-thick section from each block was stained with hematoxylin–eosin (HE) and used as a guide to localize regions of interest for microdissection. We examined all identifiable components that appeared histotopographically different and contained sufficient cells (more than 100 cells). To investigate the clear relationship between pathology and genetic alteration, we collected the lesions whose subtypes we could define clearly and which consisted of the histologically uniform cells. In the microdissection analysis, two or three 10-μm-thick sections from each specimen were deparaffinized with xylene and stained with hematoxylin alone. The stained sections were dried, and multiple lesions in each tumor were microdissected separately with a Pixcell Laser Capture Microdissection System (Arcturus Engineering Inc., Mountainview, CA, USA).[12] Finally, 100–200 tumor cells and the same number of normal cells were microdissected from each specimen, and their genomic DNAs were extracted as described previously. Separated tumor areas (3–6 areas per case) that were histotopographically dissimilar were selected microscopically on HE-stained sections. We identified a total of 56 foci, which included samples from the 12 BA component sites, 30 invasive regions of primary tumors (17 acinar, eight solid, five papillary), and 14 metastases (13 lymph node metastases, one intrapulmonary metastasis). Normal control DNAs were extracted from the corresponding lymph nodes without metastasis.

Multiplex Polymerase Chain Reaction–Loss of Heterozygosity Analysis

To evaluate LOH, we used 24 polymorphic microsatellite markers located at the following sites in the 10 genes reported to play a major role in human carcinogenesis:[13–22] *FHIT* (3p) (D3S1300, D3S1312, and D3S1313), *APC* (5q) (D5S346 and D5S82), *p16* (9p) (D9S171 and D9S162), *TSC-1* (9q) (D9S149, D9S150 and DBH), *Int-2* (11q) (INT-2), *Rb* (13q) (D13S270, D13S273, and D13S176), *TSC-2* (16p) (D16S291 and D16S292), *p53* (17p) (TP53 and D17S520), *Smad 4* (18q) (D18S46, D18S363, and D18S474), and *Band M* (22q) (D22S1140, D22S1170, and D22S1161). The use of more than one microsatellite marker ensured a higher yield of information for each genomic locus. Polymerase chain reaction (PCR) reactions for the fluorescent-labeled markers were carried out in a volume of 20 μl that included 2 or 3 μl of 10 × PCR buffer and 5–25 pmol of each primer, 1 μl of template DNA, 200 μM of each deoxynucleotide triphosphate (dNTP), and 1.0 or 1.5 U of *Taq* DNA polymerase. To detect the amplified fragments, the samples were run on a Model 377 Genetic Analysis System (Applied Biosystems, Foster City, CA, USA) using Gene Scan 377 software (Applied Biosystems, Foster City, CA, USA). Markers that identified two distinguishable alleles of different sizes but similar intensity in normal DNA were termed 'informative' (heterozygous). Markers that yielded a single major peak in normal DNA were termed 'noninformative' (homozygous). LOH was defined as loss of one allele that corresponded to an allele present in informative cases. A result was scored as LOH if there was a greater than 50% reduction in allele ratio in the tumor relative to the normal control DNA.

DNA Analysis

We used two approaches to data analysis:[9] (1) to correlate morphologic changes with allelic losses, we calculated the mean Fractional Regional Loss (FRL) index, defined as: FRL index = total number of chromosomal regions with LOH/total number of informative regions, and (2) to determine whether the deletions in the individual chromosomal regions were progressive in individual foci, we determined the frequencies of loss of individual markers on the basis of a Fractional Allelic Loss (FAL) index defined as: FAL index = total number of markers with LOH/total number of informative markers. Fisher's exact test was used for the statistical analysis in these approaches, and probability values of $P<0.05$ were regarded as statistically significant.

Results

Accumulation of Genetic Alterations in Individual Tumors

Microsatellite markers on 10 chromosome arms were used to perform a PCR-based multifocal LOH assay on a total 56 tumor sites microdissected from 12 adenocarcinomas mixed BA and other subtypes of the lung. The results for each locus are summarized in Table 1. At least one allelic loss was detected in all 56 sites examined. To gain a greater understanding of the relationship between the morphologic and genetic anatomy of each specimen, two representative cases are shown in Figures 1 and 2, respectively. Normal components are designated 'N,' bronchioloalveolar components 'BA,' acinar components 'A,' solid components 'S,' papillary components 'P,' intrapulmonary metastases 'PM,' and lymph node metastases 'LNM.' Multiple foci representing the same histology are distinguished by a numerical designation. We used the changes (including the presence of allelic loss and the specific allele lost) to determine whether the foci in individual cases were clonally related. A diagram shows the least complex pathway of tumor progression compatible with the LOH data in each case. The diagrams were drawn based on the assumption that, whenever compatible with the data, an observed LOH event represents a single occurrence in the evolution of the tumor, so that tumor components with LOH at the same locus are postulated to share a common precursor.

In case 2 (Figure 1), genetic alterations accumulated in the evolution of the tumor from the BA component to the invasive and metastatic tumor components. Case 3 (Figure 2), on the other hand, is characterized by alterations in the primary tumor that were not detectable in the metastasis (TP53 (smaller allele) in A1, D22S1140 in BA). Although genetic alterations accumulated in the evolution of the tumor to metastasis, two subclonal pathways were demonstrated in the primary tumor.

As shown in these representative cases, genetic alterations accumulated during the evolution of the tumors. However, as demonstrated by case 3, tumor evolution along a single clonal pathway from BA to invasive and metastatic components was not detected in every case. Four of the BA components (4/12: 33.3%) and five of the invasive components (5/30: 16.7%) appeared to have evolved through independent subclonal pathways. In regard to the relationship among histological subtypes in the invasive lesions, in seven cases there were multiple morphological subtypes in the primary tumors, even though two of them displayed an identical LOH pattern. The remaining five cases did not show any distinct relationship between morphology and LOH status.

Allelic Loss in Histopathological Progression (Figure 3)

To elucidate whether accumulation of genetic alterations is related to histopathological progression, we compared mean FRL indices among BA-type lesions, invasive lesions, and metastases. The mean FRL indices for invasive lesions of the primary

Table 1 Genetic analysis of samples from different portions of individual tumors

Case	*Region*	*Histological subtype*	*LOH*									
			3p	*5q*	*9p*	*9q*	*11q*	*13q*	*16p*	*17p*	*18q*	*22q*
1	BA	BA	+	NI	+	+	NI	+	−	+	−	−
	Invasive 1	A1	+	NI	+	+	NI	+	−	+	−	−
	Invasive 2	A2	+	NI	+	+	NI	+	−	+	−	+
	Metastasis 1	PM(A)	+	NI	+	+	NI	+	+	+	−	+
	Metastasis 2	LNM(A)	+	NI	+	+	NI	+	−	+	+	+
2	BA	BA	−	−	−	−	NI	−	NI	−	−	+
	Invasive 1	S1	−	−	−	−	NI	−	NI	+	−	+
	Invasive 2	A1	−	−	−	−	NI	+	NI	+	+	+
	Invasive 3	S2, A2	+	−	−	−	NI	+	NI	+	+	+
	Metastasis	LNM(A)	+	−	−	−	NI	+	NI	+	+	+
3	BA	BA	−	+	NI	+	−	NI	+	−	+	+
	Invasive 1	A1	−	+	NI	−	−	NI	+	−/+	−	−
	Invasive 2	A2	−	+	NI	+	−	NI	+	+/−	+	−
	Invasive 3	A3	−	+	NI	+	+	NI	+	+/−	+	−
	Metastasis	LNM(A)	−	+	NI	+	+	NI	+	+/−	+	−
4	BA	BA	+	+	−/+	−	NI	+	+	+	+	+
	Invasive 1	A1, S	+	+	+/−	−	NI	+	+	+	+	+
	Invasive 2	A2, A3	+	+	+/−	+	NI	+	+	+	+	+
	Metastasis	LNM(A)	+	+	+/−	−	NI	+	+	+	+	+
5	BA	BA	+	+	NI	−/+	−	+	NI	+	+	+
	Invasive 1	A, P	+	+	NI	−/+	−	+	NI	+	+	+
	Invasive 2	S	+	+	NI	+/−	−	+	NI	+	+	+
	Metastasis	LNM(P)	+	+	NI	−/+	−	+	NI	+	+	+
6	BA	BA	+	+	NI	−	−	+	NI	NI	+	−
	Invasive	P	−	+	NI	−	−	+	NI	NI	+	−
	Metastasis	LNM(P)	−	+	NI	−	−	+	NI	NI	+	−
7	BA	BA	+	NI	NI	+	−	+	−	+	−	−
	Invasive	A, S	+	NI	NI	+	−	+	−	+	+	−
	Metastasis	LNM(A, S)	+	NI	NI	+	−	+	−	+	+	−
8	BA	BA	+	+	−	+	−	−	−	+	NI	−
	Invasive	S1, S2	+	+	+	+	+	+	+	+	NI	−
	Metastasis	LNM(S)	+	+	+	+	+	+	+	+	NI	−
9	BA	BA	−	NI	NI	+	−	−	−	+	+	NI
	Invasive	A, P	−	NI	NI	+	−	−	−	+	+	NI
	Metastasis	LNM(A)	−	NI	NI	+	−	−	−	+	+	NI
10	BA	BA	+	+	+	+	−	NI	−	−	+	−
	Invasive	A1, A2	+	+	+	+	+	NI	−	−	+	−
	Metastasis	LNM(A)	+	+	+	+	+	NI	−	−	+	−
11	BA	BA	−	+	NI	−	−	+	−	+	−	−
	Invasive	A, S, P	−	+	NI	+	−	+	−	+	−	−
	Metastasis	LNM(A)	−	+	NI	+	−	+	−	+	−	−
12	BA	BA	+	−	+	+	NI	−	−	+	+	−
	Invasive	A, P	+	−	+	+	NI	−	−	+	+	−
	Metastasis	LNM(A)	+	−	+	+	NI	−	−	+	+	−

BA = bronchioloalveolar component; A = acinar component; S = solid component; P = papillary component; PM = intrapulmonary metastasis; LNM = lymph node metastasis; NI = non-informative; + = LOH; − = no LOH; −/+ or +/− = deletion of smaller or larger allele. (These designations are used only for the case in which heterogenous LOH detected in restricted tumor sites.)
All sites collected were classified into three regions: BA, invasive, and metastasis. 'BA' means only BA lesions. 'Invasive' means invasive lesions in the primary tumor (acinar, solid, and papillary). 'Metastasis' means intrapulmonary metastasis and lymph node metastasis. The sites in each region that had the same LOH pattern were summarized in the same group and numbered. For example, in case 2 the tumor contained S1, A1, S2, and A2 as invasive lesions. They were investigated separately and demonstrated three LOH patterns: invasive 1, 2, and 3. S2 and A2 showed the same LOH patterns and they were classified as invasive 3.

tumor and metastasis (0.66 and 0.69) were significantly higher than that for BA-type lesion (0.55); ($P<0.05$), however, the difference between the mean FRL index of the invasive components and metastatic components was not significant. The histological change from BA to invasive and to metastatic components was characterized by a significant rise in the mean FRL indices. The 12 cases could be divided into three groups according to the patterns of allelic losses. (1) Alleles that were retained in the BA lesion were deleted in either metastatic lesions or invasive and metastatic lesions. Eight cases (67%) showed accumulation of genetic alterations from BA lesions to metastases. (2) All foci had the same allelic losses. Two cases (17%) showed the same LOH patterns in all foci examined. (3) The markers used in this study could not be used to analyze the LOH patterns. Two cases, cases 5 and 6, had allelic loss in only one region in only one component (invasive and BA lesion), and the other components showed the same LOH patterns.

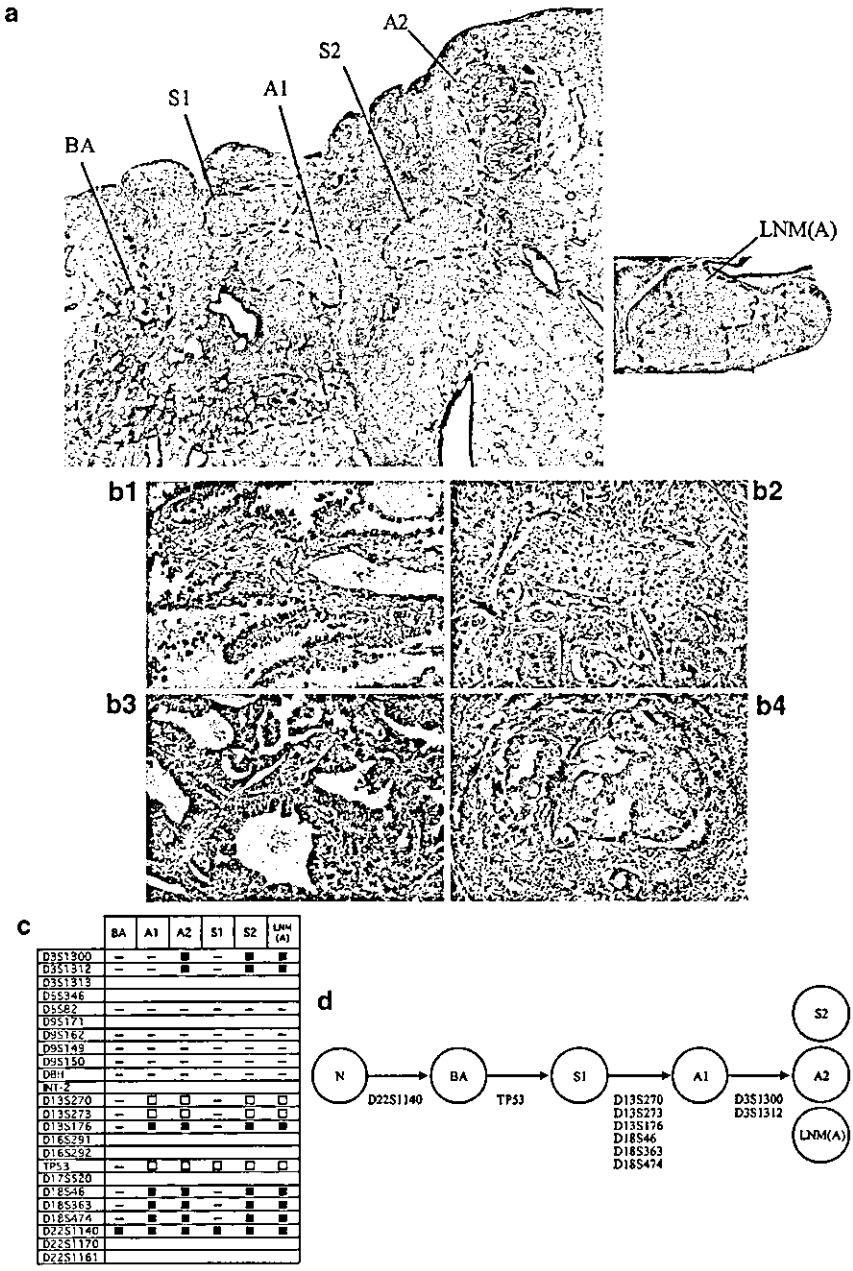

Figure 1 Analysis of LOH in an adenocarcinoma mixed BA, acinar, and solid (case 2). (a), Histology: low-power view showing regions chosen for laser capture microdissection (hematoxylin and eosin staining). BA: bronchioloalveolar component; S1 and S2: solid component dissected from different regions of the specimen; A1 and A2: acinar component dissected from different regions of the specimen; LNM (A): lymph node metastasis (acinar component). Primary tumor, which included a focus of BA-type carcinoma (BA) and two invasive acinar carcinomas (A1 and A2) and two invasive solid carcinomas (S1 and S2), and a metastatic tumor (lymph node metastasis; LNM) were analyzed. The metastatic tumor was acinar carcinoma. (b) Microscopic appearance of the four tumor areas (HE staining; original magnification, ×200). b1: BA component; b2: solid component; b3: acinar component; b4: acinar component in the lymph node metastasis. (c) LOH data obtained from microdissected tumor components. □: LOH of top band, ■: LOH of bottom band, -: no LOH, blank space: noninformative (d) Clonal relationship between microdissected tumor components suggested by LOH data. Open circles indicate a putative intermediate or precursor cell. LOH is indicated on the diagrams by microsatellite markers that showed allelic loss. BA, which contained one allelic loss (marker D22S1140), may be the precursor lesion of other invasive lesions as well as metastatic lesion. In the same manner, S1, which contained one allelic loss (marker TP53) in addition to the loss (marker D22S1140), may be the precursor lesion of other invasive and metastatic lesions. A1, which contained six additional allelic losses, may be the precursor lesion of the remaining lesions. Three lesions (S2, A2, and LNM (A)) had lost alleles at all 10 markers.

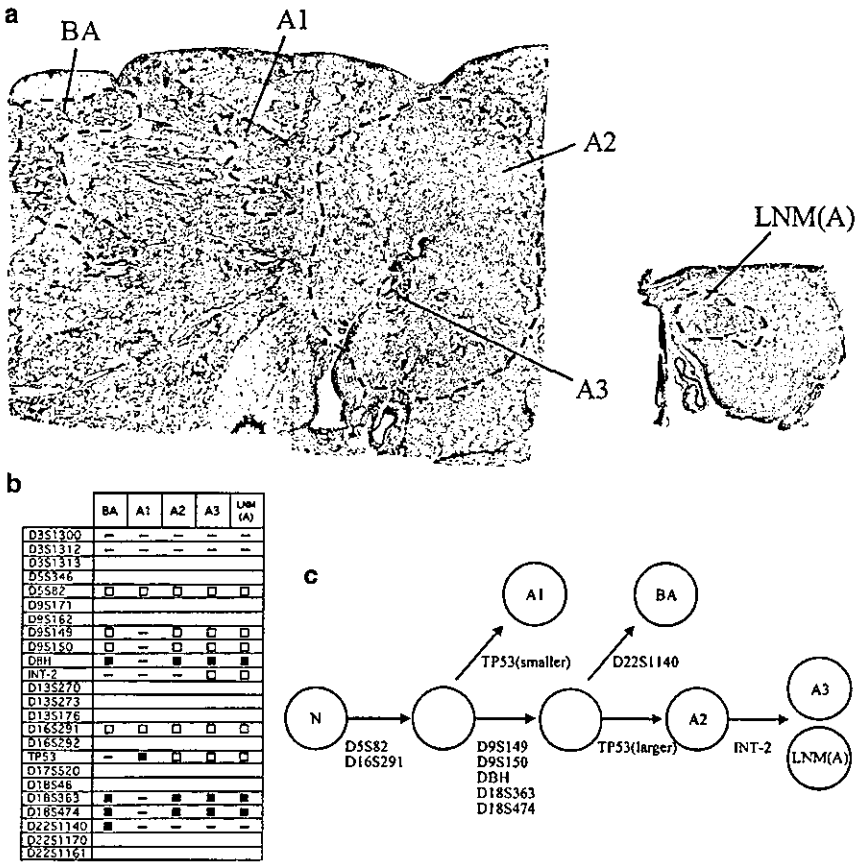

Figure 2 Analysis of LOH in an adenocarcinoma mixed BA and acinar (case 3). (a) Histology: BA: BA component; A1, A2, and A3: acinar component dissected from different regions of the specimen; LNM (A): lymph node metastasis (acinar component). Tumor components included BA-type carcinoma (BA) and three foci of invasive acinar components (A1–3) in the primary tumor and another acinar focus in the lymph node metastasis (LNM (A)). (b) LOH data obtained from microdissected tumor components. □: LOH of top band, ■: LOH of bottom band, -: no LOH, blank space: noninformative. (c) Clonal relationship between microdissected tumor components suggested by LOH data. Designation of LOH results is as in Figure 1. All components contained two allelic losses (marker D5S82 and D16S291). A1 showed LOH at the smaller allele of TP53 that was not detected in other components. In the same manner, only the BA component contained one allelic loss (marker D22S1140). These results suggest that the A1 and BA components must have evolved along subclonal pathways and that they shared common precursors, indicated by the open circle in the diagram. A2 showed LOH at the larger allele of TP53 in addition to the common precursor, and may be the precursor lesion of A3 and LNM (A).

Prevalence and Intratumor Topography of LOH Events

LOH was observed in 75.0% (126/168) of the informative markers on the 10 chromosome arms, including 3p (77.3%), 5q (80.0%), 9p (88.9%), 9q (85.2%), 11q (37.5%), 13q (83.3%), 16p (28.6%), 17p (93.8%), 18q (91.7%), and 22q (46.7%). In order to investigate the sequence of molecular genetic changes involved in the development of metastasizing adenocarcinoma mixed BA and other subtypes of the lung, we analyzed the topographical distribution of allelic losses in the tumors. Multiple foci from the same case were compared to determine whether the LOH events were shared at all tumor sites as a relatively early event derived from a progenitor cell, or localized in either metastatic lesions or invasive and metastatic lesions as an additional event directing toward invasion and metastasis. Losses on 5q were detected uniformly in the various regions of cases that tested positive for LOH (two cases were uniformly negative and three were noninformative), while homogeneous distribution of LOH was observed on 3p (70.6%), 9p (25.0%), 9q (43.5%), 11q (0.0%), 13q (66.7%), 16p (50.0%), 17p (80.0%), 18q (59.1%), and 22q (71.4%; and Figure 4). Losses on 11q were not detected uniformly at all tumor sites tested, but localized in either metastatic lesions or invasive and metastatic lesions, while heterogeneous distribution of LOH was observed on 3p (11.8%), 9p (37.5%), 9q (39.1%), 13q (33.3%), 16p (50.0%), 17p (13.3%), 18q (40.9%), and 22q (14.3%; and Figure 4). As shown in Figure 4, 5q loss was observed frequently (80.0%) and most uniformly (100%) in multiple sites, and was therefore interpreted as an obligatory early event in the progression of metastasizing pulmonary adenocarcinoma mixed BA and other subtypes. As 17p loss was most frequent (93.3%) and relatively uniform (80.0%), it was also interpreted as an early event. The loss of 18q was

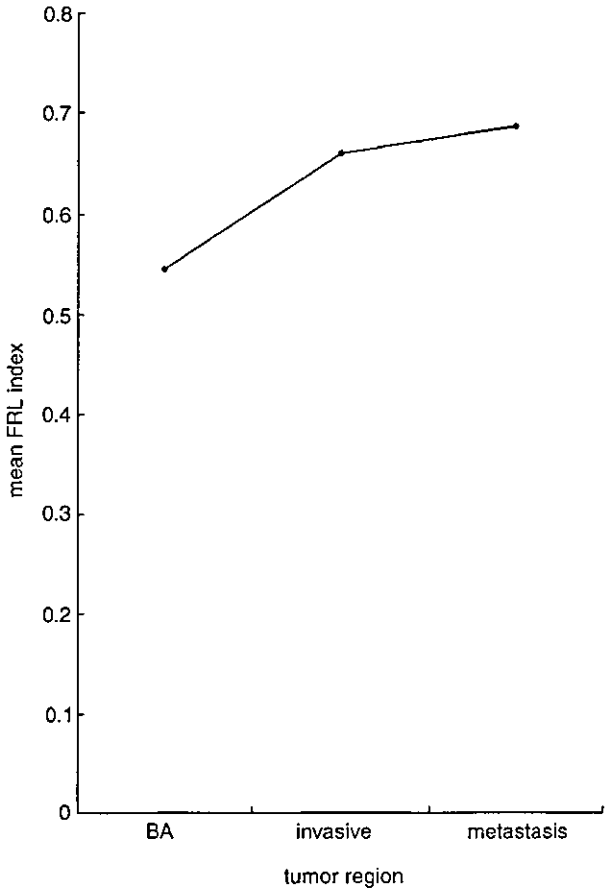

Figure 3 Mean fractional regional loss indices for the BA, invasive, and metastatic components in 10 chromosomal regions.

relatively frequent (91.7%) and heterogeneous (40.9%), and therefore indicative of late alterations required for malignant progression.

Discussion

Invasive and/or metastatic components contained more additional allelic losses than BA components. It has already been postulated that tumor cells evolved from BA to invasive and metastatic components because of the difference in histology between the lepidic pattern along the alveolar walls in BA components and invasiveness in other components.[7] Almost all metastases contained the same or more allelic losses when compared with invasive lesions in each individual tumor, although no significant difference was found in the mean FRL indices of the invasive and metastatic lesions. This suggests that tumor cells that showed noninvasive BA-type morphology evolved into invasive lesions and then to metastases, acquiring the invasive and metastatic phenotype through the process of clonal evolution occurring during multistep tumor progression. Eight cases (67%) actually showed accumulation of genetic alterations during morphological progression, but the tumor components examined thus far in the remaining four cases have shown genetic homogeneity or no obvious accumulation of genetic alterations despite great morphological divergence. This might be explained by the genetic alterations being present on loci other than those examined in this study. Although the additional allelic losses found in the metastases can be explained by the accumulation of genetic aberrations during the

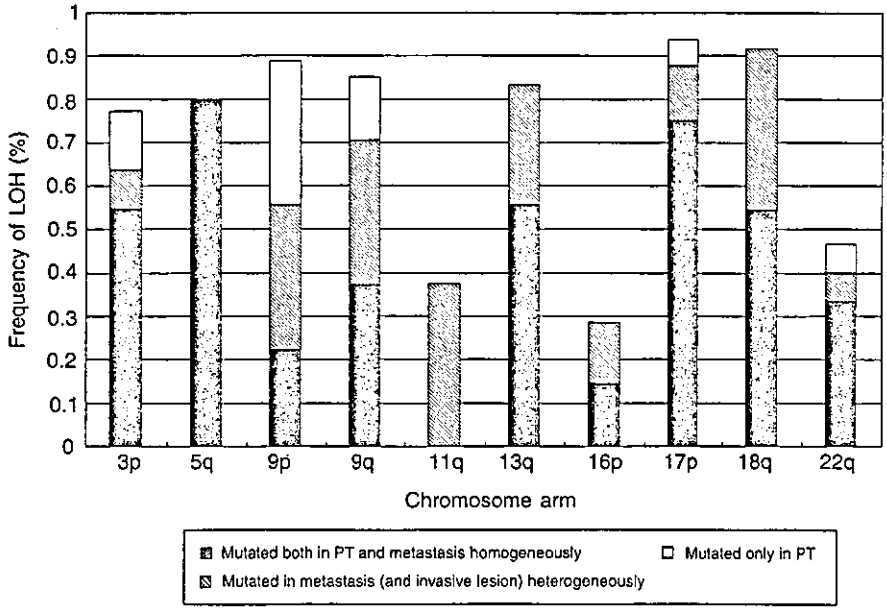

Figure 4 Prevalence and intratumor heterogeneity of allelic loss in primary tumors and lymph node metastases. PT, primary tumor.