Prognostic Stratification of Stage IIIA pN2 Non-small Cell Lung Cancer by Hierarchical Clustering Analysis of Tissue Microarray Immunostaining Data

An Alpe Adria Thoracic Oncology Multidisciplinary Group Study (ATOM 014)

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Introduction: Stage IIIA non-small cell lung cancer (NSCLC) with ipsilateral mediastinal lymph node metastases (N2) is a heterogeneous disease with differing prognoses. In this study, we retrospectively investigated the prognostic value of the expression of 10 molecular markers in 87 patients with stage IIIA pN2 NSCLC treated with radical surgery.

Methods: Primary tumor tissue microarrays (TMAs) were constructed and sections used for immunohistochemical analysis of epidermal growth factor receptor, ErbB-2, c-kit, cyclooxygenase-2, survivin, bcl-2, cyclin D1, cyclin B1, metalloproteinase (MMP)-2, and MMP-9. Univariate and multivariate analyses and unsupervised hierarchical clustering analysis of clinical pathologic and immunostaining data were performed.

Results: Bcl-2 ($p < 0.0001$) and cyclin D1 ($p = 0.015$) were more highly expressed in squamous cell carcinoma (SCC), whereas MMP-2 ($p = 0.009$), MMP-9 ($p = 0.005$), and survivin ($p = 0.032$) had increased expression in other histologic subtypes. In univariate analysis, SCC histology and cyclin D1 expressions were favorable prognostic factors ($p = 0.015$ and $p < 0.0001$, respectively); by contrast, MMP-9 expression was associated with worse prognosis ($p = 0.042$). In multivariate analysis, cyclin D1 was the only positive prognostic factor ($p < 0.0001$). Unsupervised hierarchical clustering analysis of TMA immunostaining data identified five distinct clusters. They formed two subsets of patients with better (clusters 1 and 2) and worse (clusters 3, 4, and 5) prognoses, and median survival of 51 and 10 months, respectively ($p < 0.0001$). The better prognosis subset mainly comprised patients with SCC (80%).

Conclusions: Hierarchical clustering of TMA immunostaining data using a limited set of markers identifies patients with stage IIIA pN2 NSCLC at high risk of recurrence, who may benefit from more aggressive treatment.

Key Words: Stage IIIA NSCLC, Prognostic markers, Tissue microarrays, Unsupervised hierarchical clustering analysis.

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Patients with stage IIIA non-small cell lung cancer (NSCLC) involving ipsilateral mediastinal nodes (pN2) represent a heterogeneous population with differing clinical presentations and prognoses, ranging from those with incidental N2 disease found after surgery by pathologic examination of the resected tissue, to patients with bulky or fixed multistation, pathologically confirmed N2 disease that is clearly unresectable. Treatment guidelines for stage IIIA pN2 NSCLC are evolving,[1] and the management of these patients remains challenging. Although the superiority of multimodality treatment is well established, the optimal combination and sequence of chemotherapy, surgery, and radiation therapy are still controversial. Platinum-based combination chemoradiotherapy is the recommended primary treatment for patients with N2 disease detected at staging, although surgery combined with either adjuvant or neoadjuvant chemotherapy is acceptable in selected patients.[1,2] Nevertheless, overall survival for patients with stage IIIA pN2 NSCLC is still unsatisfactory, with a 5-year survival rate of just 22%.[3]

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Subclassification of this heterogeneous population, and the identification of distinct prognostic subgroups, may allow the optimization of clinical trial design, with the potential to improve treatment outcomes. As a first step in this direction, a revision of nodal (N) descriptors in NSCLC was recently proposed by the International Association for the Study of Lung Cancer. This establishes the following three prognostic groups: single-zone N1 (N1a); either multiple zone N1 (N1b) or single-zone N2 (N2a); and multiple zone N2 lymph node disease.[3] Preliminary validation of this new classification has been reported,[4] but larger prospective studies are required. Molecular markers, including those involved in the regulation of cell proliferation, differentiation, apoptosis, and in invasion, angiogenesis, and metastasis, have the potential to further refine this process. The assessment of the expression pattern of such molecules in each individual tumor sample by molecular biology or traditional immunohistochemistry has been relatively expensive and time consuming, but this is changing. Already these limitations can be overcome by the use of immunohistochemistry on tissue microarrays (TMAs), a useful tool for the rapid and efficient analysis of multiple markers on large numbers of paraffin-embedded tissues.[5,6] Using TMA technology, samples from hundreds of different tumors can be analyzed at the same time to assess the expression profile of potentially relevant prognostic markers and/or therapeutic targets.

In this study, we used TMA to evaluate the expression and prognostic significance of a panel of 10 molecular markers in patients with stage IIIA pN2 NSCLC treated surgically with curative intent who did not receive adjuvant chemotherapy or biologic therapies. The panel of markers included cell cycle regulators (cyclin D1 and cyclin B1), growth factor receptors (c-erbB-1 and c-erbB-2, c-kit), antiapoptotic factors (bcl-2 and survivin), an enzyme involved in the arachidonic acid cascade with angiogenic properties (cyclooxygenase-2 [COX-2]), and proteins involved in the degradation of the extracellular matrix metalloproteinases (MMPs)-2 and -9. These 10 markers were chosen among the ones that were targets for biologic agents under evaluation in clinical trials at the time this study was designed, and for which antibodies suitable for immunohistochemistry were available.[7–12]

PATIENTS AND METHODS

Patients and Clinical Samples

A search of the prospective S. Maria della Misericordia General Hospital Thoracic Surgery database identified 196 consecutive patients with stage IIIA pN2 NSCLC who underwent radical surgery at that Center between 1985 and 1997. Eighty seven of these patients satisfied the following additional selection criteria: (i) at least 4 weeks survival after surgery; (ii) no preoperative radiotherapy or chemotherapy; (iii) microscopically negative resection margins (R0); and (iv) availability of archival formalin-fixed paraffin-embedded tumor tissue suitable for TMA preparation. Regional lymph node staging was performed by systematic mediastinal lymph node sampling; nodal stations were classified according to Naruke's map.[13] Routine preoperative staging included clinical examination, chest radiography, and computed tomography scan of the chest and upper abdomen; positron emission tomography was not available at the time. Patients did not receive adjuvant chemotherapy. Demographic, clinical, and pathologic characteristics are listed in Table 1.

Formalin-fixed, paraffin-embedded primary tumor tissue samples were retrieved from the archives of the local Histopathology Department. To confirm the diagnosis of NSCLC, histologic slides from all patients were independently reviewed by two pathologists (V.C. and C.A.B.). Tumor size and nodal status were obtained from the original pathology reports. The tumors were staged according to the International Union Against Cancer's tumor node metastasis classification.[14] Histologic subtype and grade were classified according to the World Health Organization guidelines.[15] Survival data were available for all patients from hospital records or local registries. Ethics approval was obtained according to local practice.

TMA Construction

TMA were constructed as described by Kononen et al.[5] Briefly, 4-μm sections stained with hematoxylin and eosin were prepared from each formalin-fixed paraffin-embedded block to select the most representative tumor areas to be

TABLE 1. Clinical and Pathological Characteristics (n = 87)

Parameter	n (%)
Age, yr	
Median	62
Range	35–74
Gender	
Male	71 (82)
Female	16 (18)
Histology	
Adenocarcinoma, BAC	33 (38)
Squamous cell carcinoma	50 (58)
Large cell carcinoma	4 (5)
pT	
T1	25 (29)
T2	48 (55)
T3	14 (16)
Number of N2 lymph node stations involved	
1	45 (52)
2	23 (26)
3	16 (18)
4	2 (2)
5	1 (1)
Surgical Intervention	
Lobectomy	39 (45)
Pneumonectomy	48 (55)
Postoperative thoracic radiotherapy	
Yes	44 (51)
No	23 (26)
Unknown	20 (23)
Survival, mo	
Median	13.2
Range	1.1–215

BAC, bronchioloalveolar carcinoma.