occurrence, surgical treatment and survival. *Eur J Cardiothorac Surg* 2003;23:818–823.
125. Lee HY, Lee KS, Han J, et al. Mucinous versus nonmucinous solitary pulmonary nodular bronchioloalveolar carcinoma: CT and FDG PET findings and pathologic comparisons. *Lung Cancer* 2009;65:170–175.
126. Miyake H, Matsumoto A, Terada A, et al. Mucin-producing tumor of the lung: CT findings. *J Thorac Imaging* 1995;10:96–98.
127. Casali C, Rossi G, Marchioni A, et al. A single institution-based retrospective study of surgically treated bronchioloalveolar adenocarcinoma of the lung: clinicopathologic analysis, molecular features, and possible pitfalls in routine practice. *J Thorac Oncol* 2010;5:830–836.
128. Akira M, Atagi S, Kawahara M, et al. High-resolution CT findings of diffuse bronchioloalveolar carcinoma in 38 patients. *AJR Am J Roentgenol* 1999;173:1623–1629.
129. Kodama K, Higashiyama M, Yokouchi H, et al. Natural history of pure ground-glass opacity after long-term follow-up of more than 2 years. *Ann Thorac Surg* 2002;73:386–392.
130. Nagao M, Murase K, Yasuhara Y, et al. Measurement of localized ground-glass attenuation on thin-section computed tomography images: correlation with the progression of bronchioloalveolar carcinoma of the lung. *Invest Radiol* 2002;37:692–697.
131. Saito H, Yamada K, Hamanaka N, et al. Initial findings and progression of lung adenocarcinoma on serial computed tomography scans. *J Comput Assist Tomogr* 2009;33:42–48.
132. Yabuuchi H, Murayama S, Murakami J, et al. High-resolution CT characteristics of poorly differentiated adenocarcinoma of the peripheral lung: comparison with well differentiated adenocarcinoma. *Radiat Med* 2000;18:343–347.
133. Im JG, Han MC, Yu EJ, et al. Lobar bronchioloalveolar carcinoma: 'Angiogram sign' on CT scans. *Radiology* 1990;176:749–753.
134. Tateishi U, Muller NL, Johkoh T, et al. Mucin-producing adenocarcinoma of the lung: thin-section computed tomography findings in 48 patients and their effect on prognosis. *J Comput Assist Tomogr* 2005; 29:361–368.
135. Clayton F. The spectrum and significance of bronchioloalveolar carcinomas. *Pathol Annu* 1988;23:361–394.
136. Shah RN, Badve S, Papreddy K, et al. Expression of cytokeratin 20 in mucinous bronchioloalveolar carcinoma. *Hum Pathol* 2002;33:915–920.
137. Lau SK, Desrochers MJ, Luthringer DJ. Expression of thyroid transcription factor-1, cytokeratin 7, and cytokeratin 20 in bronchioloalveolar carcinomas: an immunohistochemical evaluation of 67 cases. *Mod Pathol* 2002;15:538–542.
138. Sarantopoulos GP, Gui D, Shintaku P, et al. Immunohistochemical analysis of lung carcinomas with pure or partial bronchioloalveolar differentiation. *Arch Pathol Lab Med* 2004;128:406–414.
139. Simsir A, Wei XJ, Yee H, et al. Differential expression of cytokeratins 7 and 20 and thyroid transcription factor-1 in bronchioloalveolar carcinoma: an immunohistochemical study in fine-needle aspiration biopsy specimens. *Am J Clin Pathol* 2004;121:350–357.
140. Finberg KE, Sequist LV, Joshi VA, et al. Mucinous differentiation correlates with absence of *EGFR* mutation and presence of *KRAS* mutation in lung adenocarcinomas with bronchioloalveolar features. *J Mol Diagn* 2007;9:320–326.
141. Sakuma Y, Matsukuma S, Yoshihara M, et al. Distinctive evaluation of nonmucinous and mucinous subtypes of bronchioloalveolar carcinomas in *EGFR* and *K-ras* gene-mutation analyses for Japanese lung adenocarcinomas: confirmation of the correlations with histologic subtypes and gene mutations. *Am J Clin Pathol* 2007;128:100–108.
142. Marchetti A, Buttitta F, Pellegrini S, et al. Bronchioloalveolar lung carcinomas: *K-ras* mutations are constant events in the mucinous subtype. *J Pathol* 1996;179:254–259.
143. Maeshima A, Sakamoto M, Hirohashi S. Mixed mucinous-type and non-mucinous-type adenocarcinoma of the lung: immunohistochemical examination and *K-ras* gene mutation. *Virchows Arch* 2002;440:598–603.
144. Tam IY, Chung LP, Suen WS, et al. Distinct epidermal growth factor receptor and *KRAS* mutation patterns in non-small cell lung cancer patients with different tobacco exposure and clinicopathologic features. *Clin Cancer Res* 2006;12:1647–1653.
145. Copin MC, Buisine MP, Leteurtre E, et al. Mucinous bronchioloalveolar carcinomas display a specific pattern of mucin gene expression among primary lung adenocarcinomas. *HumPathol* 2001;32:274–281.
146. Awaya H, Takeshima Y, Yamasaki M, et al. Expression of MUC1, MUC2, MUC5AC, and MUC6 in atypical adenomatous hyperplasia, bronchioloalveolar carcinoma, adenocarcinoma with mixed subtypes, and mucinous bronchioloalveolar carcinoma of the lung. *Am J Clin Pathol* 2004;121:644–653.
147. Sato K, Ueda Y, Shikata H, et al. Bronchioloalveolar carcinoma of mixed mucinous and nonmucinous type: immunohistochemical studies and mutation analysis of the p53 gene. *Pathol Res Pract* 2006;202: 751–756.
148. Tsuta K, Ishii G, Nitadori J, et al. Comparison of the immunophenotypes of signet-ring cell carcinoma, solid adenocarcinoma with mucin production, and mucinous bronchioloalveolar carcinoma of the lung characterized by the presence of cytoplasmic mucin. *J Pathol* 2006; 209:78–87.
149. Sica GL, Yoshizawa AK, Downey RJ, et al. Reassessment of the histologic spectrum of mucinous bronchioloalveolar carcinoma (mBAC). *Mod Pathol* 2008;21:351A.
150. Gaeta M, Blandino A, Scribano E, et al. Mucinous cystadenocarcinoma of the lung: CT-pathologic correlation in three cases. *J Comput Assist Tomogr* 1999;23:641–643.
151. Deshpande CG, Yoshizawa A, Motoi N, et al. Clear cell change in lung adenocarcinoma: a cytologic change rather than a histologic variant. *Mod Pathol* 2009;22(Suppl 1):1595.
152. Cohen PR, Yoshizawa A, Motoi N, et al. Signet ring cell features (SRCF) in lung adenocarcinoma: a cytologic feature or a histologic subtype? *Mod Pathol* 2010;23:404A.
153. Rodig SJ, Mino-Kenudson M, Dacic S, et al. Unique clinicopathologic features characterize ALK-rearranged lung adenocarcinoma in the western population. *Clin Cancer Res* 2009;15:5216–5223.
154. Inamura K, Satoh Y, Okumura S, et al. Pulmonary adenocarcinomas with enteric differentiation: histologic and immunohistochemical characteristics compared with metastatic colorectal cancers and usual pulmonary adenocarcinomas. *Am J Surg Pathol* 2005;29:660–665.
155. Moran CA, Hochholzer L, Fishback N, et al. Mucinous (so-called colloid) carcinomas of lung. *Mod Pathol* 1992;5:634–638.
156. Rossi G, Murer B, Cavazza A, et al. Primary mucinous (so-called colloid) carcinomas of the lung: a clinicopathologic and immunohistochemical study with special reference to CDX-2 homeobox gene and MUC2 expression. *Am J Surg Pathol* 2004;28:442–452.
157. Gao ZH, Urbanski SJ. The spectrum of pulmonary mucinous cystic neoplasia: a clinicopathologic and immunohistochemical study of ten cases and review of literature. *Am J Clin Pathol* 2005;124:62–70.
158. Nakatani Y, Kitamura H, Inayama Y, et al. Pulmonary adenocarcinomas of the fetal lung type: a clinicopathologic study indicating differences in histology, epidemiology, and natural history of low-grade and high-grade forms. *Am J Surg Pathol* 1998;22:399–411.
159. Nakatani Y, Masudo K, Miyagi Y, et al. Aberrant nuclear localization and gene mutation of beta-catenin in low-grade adenocarcinoma of fetal lung type: up-regulation of the Wnt signaling pathway may be a common denominator for the development of tumors that form morules. *Mod Pathol* 2002;15:617–624.
160. Sekine S, Shibata T, Matsuno Y, et al. Beta-catenin mutations in pulmonary blastomas: association with morule formation. *J Pathol* 2003;200:214–221.
161. Li HC, Schmidt L, Greenson JK, et al. Primary pulmonary adenocarcinoma with intestinal differentiation mimicking metastatic colorectal carcinoma: case report and review of literature. *Am J Clin Pathol* 2009;131:129–133.
162. Hatanaka K, Tsuta K, Watanabe K, et al. Primary pulmonary adenocarcinoma with enteric differentiation resembling metastatic colorectal carcinoma: a report of the second case negative for cytokeratin 7. *Pathol Res Pract.* In press.
163. Yousem SA. Pulmonary intestinal-type adenocarcinoma does not show enteric differentiation by immunohistochemical study. *Mod Pathol* 2005;18:816–821.
164. Rossi G, Pelosi G, Graziano P, et al. A reevaluation of the clinical significance of histological subtyping of non–small-cell lung carcinoma: diagnostic algorithms in the era of personalized treatments. *Int J Surg Pathol* 2009;17:206–218.
165. Rossi G, Papotti M, Barbareschi M, et al. Morphology and a limited

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number of immunohistochemical markers may efficiently subtype non-small-cell lung cancer. *J Clin Oncol* 2009;27:e141–e142; author reply e3–e4.

166. Suh J, Rekhtman N, Ladanyi M, et al. Testing of new IASLC/ATS/ERS criteria for diagnosis of lung adenocarcinoma (AD) in small biopsies: minimize immunohistochemistry (IHC) to maximize tissue for molecular studies. *Mod Pathol.* 2011:24 (Supplement 1). In press.
167. Hanna N, Shepherd FA, Fossella FV, et al. Randomized phase III trial of pemetrexed versus docetaxel in patients with non-small-cell lung cancer previously treated with chemotherapy. *J Clin Oncol* 2004;22: 1589–1597.
168. Yatabe Y, Mitsudomi T, Takahashi T. TTF-1 expression in pulmonary adenocarcinomas. *Am J Surg Pathol* 2002;26:767–773.
169. Lau SK, Luthringer DJ, Eisen RN. Thyroid transcription factor-1: a review. *Appl Immunohistochem Mol Morphol* 2002;10:97–102.
170. Camilo R, Capelozzi VL, Siqueira SA, et al. Expression of p63, keratin 5/6, keratin 7, and surfactant-A in non-small cell lung carcinomas. *Hum Pathol* 2006;37:542–546.
171. Wu M, Wang B, Gil J, et al. p63 and TTF-1 immunostaining. A useful marker panel for distinguishing small cell carcinoma of lung from poorly differentiated squamous cell carcinoma of lung. *Am J Clin Pathol* 2003;119:696–702.
172. Chu PG, Weiss LM. Expression of cytokeratin 5/6 in epithelial neoplasms: an immunohistochemical study of 509 cases. *Mod Pathol* 2002;15:6–10.
173. Ordonez NG. Value of thyroid transcription factor-1, E-cadherin, BG8, WT1, and CD44S immunostaining in distinguishing epithelial pleural mesothelioma from pulmonary and nonpulmonary adenocarcinoma. *Am J Surg Pathol* 2000;24:598–606.
174. Kaufmann O, Dietel M. Thyroid transcription factor-1 is the superior immunohistochemical marker for pulmonary adenocarcinomas and large cell carcinomas compared to surfactant proteins A and B. *Histopathology* 2000;36:8–16.
175. Kargi A, Gurel D, Tuna B. The diagnostic value of TTF-1, CK 5/6, and p63 immunostaining in classification of lung carcinomas. *Appl Immunohistochem Mol Morphol* 2007;15:415–420.
176. Khayyata S, Yun S, Pasha T, et al. Value of P63 and CK5/6 in distinguishing squamous cell carcinoma from adenocarcinoma in lung fine-needle aspiration specimens. *Diagn Cytopathol* 2009;37:178–183.
177. Chu P, Wu E, Weiss LM. Cytokeratin 7 and cytokeratin 20 expression in epithelial neoplasms: a survey of 435 cases. *Mod Pathol* 2000;13: 962–972.
178. Rivera MP, Mehta AC. Initial diagnosis of lung cancer: ACCP evidence-based clinical practice guidelines (2nd edition). *Chest* 2007;132: 131S–148S.
179. Rekhtman N, Brandt SM, Sigel CS, et al. Suitability of thoracic cytology for new therapeutic paradigms in non-small cell lung carcinoma: high accuracy of tumor subtyping and feasibility of *EGFR* and *KRAS* molecular testing. *J Thorac Oncol.* In press.
180. Sigel CS, Friedlander MA, Zakowski MF, et al. Subtyping of non-small cell lung carcinoma (NSCLC): comparison of cytology and small biopsy specimens. *Mod Pathol* 2010;23:414A.
181. Zhang X, Zhao Y, Wang M, et al. Detection and comparison of epidermal growth factor receptor mutations in cells and fluid of malignant pleural effusion in non-small cell lung cancer. *Lung Cancer* 2008;60:175–182.
182. Zakowski MF, Hussain S, Pao W, et al. Morphologic features of adenocarcinoma of the lung predictive of response to the epidermal growth factor receptor kinase inhibitors erlotinib and gefitinib. *Arch Pathol Lab Med* 2009;133:470–477.
183. Wu SG, Gow CH, Yu CJ, et al. Frequent *EGFR* mutations in malignant pleural effusion of lung adenocarcinoma. *Eur Respir J* 2008;32:924–930.
184. Au NH, Gown AM, Cheang M, et al. P63 expression in lung carcinoma: a tissue microarray study of 408 cases. *Appl Immunohistochem Mol Morphol* 2004;12:240–247.
185. Ang DC, Ghaffar H, Zakowski MF, et al. Expression of squamous markers in lung adenocarcinoma (AD): clinicopathologic and molecular correlates, and implications for differentiation from squamous cell carcinoma (SqCC). *Mod Pathol* 2010;23:397A.
186. Ionescu DN, Treaba D, Gilks CB, et al. Nonsmall cell lung carcinoma with neuroendocrine differentiation–an entity of no clinical or prognostic significance. *Am J Surg Pathol* 2007;31:26–32.
187. Sterlacci W, Fiegl M, Hilbe W, et al. Clinical relevance of neuroendocrine differentiation in non-small cell lung cancer assessed by immunohistochemistry: a retrospective study on 405 surgically resected cases. *Virchows Arch* 2009;455:125–132.
188. Chung CK, Zaino R, Stryker JA, et al. Carcinoma of the lung: evaluation of histological grade and factors influencing prognosis. *Ann Thorac Surg* 1982;33:599–604.
189. Kobayashi N, Toyooka S, Soh J, et al. Risk factors for recurrence and unfavorable prognosis in patients with stage I non-small cell lung cancer and a tumor diameter of 20 mm or less. *J Thorac Oncol* 2007;2:808–812.
190. Nakazato Y, Minami Y, Kobayashi H, et al. Nuclear grading of primary pulmonary adenocarcinomas—correlation between nuclear size and prognosis. *J Thorac Oncol* 2009;4:S495.
191. Petersen I, Kotb WF, Friedrich KH, et al. Core classification of lung cancer: correlating nuclear size and mitoses with ploidy and clinicopathological parameters. *Lung Cancer* 2009;65:312–318.
192. Aida S, Shimazaki H, Sato K, et al. Prognostic analysis of pulmonary adenocarcinoma subclassification with special consideration of papillary and bronchioloalveolar types. *Histopathology* 2004;45:468–476.
193. Gleason DF. Histologic grading of prostate cancer: a perspective. *Hum Pathol* 1992;23:273–279.
194. Kadota K, Suzuki K, Rusch VW, et al. Nuclear grading system predicts recurrence in stage I lung adenocarcinoma patients. *Mod Pathol.* 2011;24 (Supplement 1). In press.
195. Li AR, Chitale D, Riely GJ, et al. *EGFR* mutations in lung adenocarcinomas: clinical testing experience and relationship to *EGFR* gene copy number and immunohistochemical expression. *J Mol Diagn* 2008;10:242–248.
196. Lim EH, Zhang SL, Li JL, et al. Using whole genome amplification (WGA) of low-volume biopsies to assess the prognostic role of *EGFR*, *KRAS*, *p53*, and *CMET* mutations in advanced-stage non-small cell lung cancer (NSCLC). *J Thorac Oncol* 2009;4:12–21.
197. Savic S, Tapia C, Grilli B, et al. Comprehensive epidermal growth factor receptor gene analysis from cytological specimens of non-small-cell lung cancers. *Br J Cancer* 2008;98:154–160.
198. Miller VA, Riely GJ, Zakowski MF, et al. Molecular characteristics of bronchioloalveolar carcinoma and adenocarcinoma, bronchioloalveolar carcinoma subtype, predict response to erlotinib. *J Clin Oncol* 2008; 26:1472–1478.
199. Kimura H, Fujiwara Y, Sone T, et al. *EGFR* mutation status in tumour-derived DNA from pleural effusion fluid is a practical basis for predicting the response to gefitinib. *Br J Cancer* 2006;95:1390–1395.
200. Borczuk AC, Shah L, Pearson GD, et al. Molecular signatures in biopsy specimens of lung cancer. *Am J Respir Crit Care Med* 2004;170:167–174.
201. Zudaire I, Lozano MD, Vazquez MF, et al. Molecular characterization of small peripheral lung tumors based on the analysis of fine needle aspirates. *Histol Histopathol* 2008;23:33–40.
202. Gordon GJ, Richards WG, Sugarbaker DJ, et al. A prognostic test for adenocarcinoma of the lung from gene expression profiling data. *Cancer Epidemiol Biomarkers Prev* 2003;12:905–910.
203. Solomon SB, Zakowski MF, Pao W, et al. Core needle lung biopsy specimens: adequacy for *EGFR* and *KRAS* mutational analysis. *AJR Am J Roentgenol* 2010;194:266–269.
204. Asano H, Toyooka S, Tokumo M, et al. Detection of *EGFR* gene mutation in lung cancer by mutant-enriched polymerase chain reaction assay. *Clin Cancer Res* 2006;12:43–48.
205. Otani H, Toyooka S, Soh J, et al. Detection of *EGFR* gene mutations using the wash fluid of CT-guided biopsy needle in NSCLC patients. *J Thorac Oncol* 2008;3:472–476.
206. Bepler G, Kusmartseva I, Sharma S, et al. RRM1 modulated in vitro and in vivo efficacy of gemcitabine and platinum in non-small-cell lung cancer. *J Clin Oncol* 2006;24:4731–4737.
207. Olaussen KA, Dunant A, Fouret P, et al. DNA repair by ERCC1 in non-small-cell lung cancer and cisplatin-based adjuvant chemotherapy. *N Engl J Med* 2006;355:983–991.
208. Chang MH, Ahn JS, Lee J, et al. The efficacy of pemetrexed as a third- or fourth-line therapy and the significance of thymidylate synthase

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expression in patients with advanced non-small cell lung cancer. *Lung Cancer* 2010;69:323–329.
209. Monica V, Scagliotti GV, Ceppi P, et al. Differential thymidylate synthase expression in different variants of large-cell carcinoma of the lung. *Clin Cancer Res* 2009;15:7547–7552.
210. Kang CH, Jang BG, Kim DW, et al. The prognostic significance of ERCC1, BRCA1, XRCC1, and betaIII-tubulin expression in patients with non-small cell lung cancer treated by platinum- and taxane-based neoadjuvant chemotherapy and surgical resection. *Lung Cancer* 2010; 68:478–483.
211. Rosell R, Perez-Roca L, Sanchez JJ, et al. Customized treatment in non-small-cell lung cancer based on *EGFR* mutations and *BRCA1* mRNA expression. *PLoS One* 2009;4:e5133.
212. Savci-Heijink CD, Kosari F, Aubry MC, et al. The role of desmoglein-3 in the diagnosis of squamous cell carcinoma of the lung. *Am J Pathol* 2009;174:1629–1637.
213. Monica V, Ceppi P, Righi L, et al. Desmocollin-3: a new marker of squamous differentiation in undifferentiated large-cell carcinoma of the lung. *Mod Pathol* 2009;22:709–717.
214. Bishop JA, Sharma R, Illei PB. Napsin A and thyroid transcription factor-1 expression in carcinomas of the lung, breast, pancreas, colon, kidney, thyroid, and malignant mesothelioma. *Hum Pathol* 2010;41: 20–25.
215. Paez JG, Janne PA, Lee JC, et al. *EGFR* mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004; 304:1497–1500.
216. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004;350:2129–2139.
217. Pao W, Miller V, Zakowski M, et al. *EGF* receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci USA* 2004;101:13306–13311.
218. Azzoli CG, Baker S Jr, Temin S, et al. American Society of Clinical Oncology Clinical Practice Guideline update on chemotherapy for stage IV non-small-cell lung cancer. *J Clin Oncol* 2009;27:6251–6266.
219. Pao W, Kris MG, Iafrate AJ, et al. Integration of molecular profiling into the lung cancer clinic. *Clin Cancer Res* 2009;15:5317–5322.
220. Shaw AT, Yeap BY, Mino-Kenudson M, et al. Clinical features and outcome of patients with non-small-cell lung cancer who harbor *EML4-ALK*. *J Clin Oncol* 2009;27:4247–4253.
221. Detterbeck FC, Jantz MA, Wallace M, et al. Invasive mediastinal staging of lung cancer: ACCP evidence-based clinical practice guidelines (2nd edition). *Chest* 2007;132:202S–220S.
222. Schrump DS, Giaccone G, Kelsey CR, et al. Non-small cell lung cancer. In VT DeVita, TS Lawrence, SA Rosenberg (Eds.). Cancer, Principles and Practice of Oncology, 7th Ed. Philadelphia: Wolters Kluwer; Lippincott, Williams & Wilkins, 2008. Pp. 887–895.
223. Sculier JP, Chansky K, Crowley JJ, et al. The impact of additional prognostic factors on survival and their relationship with the anatomical extent of disease expressed by the 6th Edition of the TNM Classification of Malignant Tumors and the proposals for the 7th Edition. *J Thorac Oncol* 2008;3:457–466.
224. Chansky K, Sculier JP, Crowley JJ, et al. The International Association for the Study of Lung Cancer Staging Project: prognostic factors and pathologic TNM stage in surgically managed non-small cell lung cancer. *J Thorac Oncol* 2009;4:792–801.
225. Janjigian YY, McDonnell K, Kris MG, et al. Pack-years of cigarette smoking as a prognostic factor in patients with stage IIIB/IV Nonsmall cell lung cancer. *Cancer* 2010;116:670–675.
226. Miller VA, Kris MG, Shah N, et al. Bronchioloalveolar pathologic subtype and smoking history predict sensitivity to gefitinib in advanced non-small-cell lung cancer. *J Clin Oncol* 2004;22:1103–1109.
227. West HL, Franklin WA, McCoy J, et al. Gefitinib therapy in advanced bronchioloalveolar carcinoma: Southwest Oncology Group Study S0126. *J Clin Oncol* 2006;24:1807–1813.
228. Sequist LV, Bell DW, Lynch TJ, et al. Molecular predictors of response to epidermal growth factor receptor antagonists in non-small-cell lung cancer. *J Clin Oncol* 2007;25:587–595.
229. Sutani A, Nagai Y, Udagawa K, et al. Gefitinib for non-small-cell lung cancer patients with epidermal growth factor receptor gene mutations screened by peptide nucleic acid-locked nucleic acid PCR clamp. *Br J Cancer* 2006;95:1483–1489.
230. Inoue A, Suzuki T, Fukuhara T, et al. Prospective phase II study of gefitinib for chemotherapy-naive patients with advanced non-small-cell lung cancer with epidermal growth factor receptor gene mutations. *J Clin Oncol* 2006;24:3340–3346.
231. Inoue A, Kobayashi K, Usui K, et al. First-line gefitinib for patients with advanced non-small-cell lung cancer harboring epidermal growth factor receptor mutations without indication for chemotherapy. *J Clin Oncol* 2009;27:1394–1400.
232. Tamura K, Okamoto I, Kashii T, et al. Multicentre prospective phase II trial of gefitinib for advanced non-small cell lung cancer with epidermal growth factor receptor mutations: results of the West Japan Thoracic Oncology Group trial (WJTOG0403). *Br J Cancer* 2008;98: 907–914.
233. Yoshida K, Yatabe Y, Park JY, et al. Prospective validation for prediction of gefitinib sensitivity by *epidermal growth factor receptor* gene mutation in patients with non-small cell lung cancer. *J Thorac Oncol* 2007;2:22–28.
234. Douillard JY, Shepherd FA, Hirsh V, et al. Molecular predictors of outcome with gefitinib and docetaxel in previously treated non-small-cell lung cancer: data from the randomized phase III INTEREST trial. *J Clin Oncol* 2010;28:744–752.
235. Hirsch FR, Varella-Garcia M, Bunn PA Jr, et al. Molecular predictors of outcome with gefitinib in a phase III placebo-controlled study in advanced non-small-cell lung cancer. *J Clin Oncol* 2006;24:5034–5042.
236. Zhu CQ, da Cunha Santos G, Ding K, et al. Role of *KRAS* and *EGFR* as biomarkers of response to erlotinib in National Cancer Institute of Canada Clinical Trials Group Study BR. 21. *J Clin Oncol* 2008;26: 4268–4275.
237. Sholl LM, Xiao Y, Joshi V, et al. *EGFR* mutation is a better predictor of response to tyrosine kinase inhibitors in non-small cell lung carcinoma than FISH, CISH, and immunohistochemistry. *Am J Clin Pathol* 2010;133:922–934.
238. Kawahara A, Yamamoto C, Nakashima K, et al. Molecular diagnosis of activating EGFR mutations in non-small cell lung cancer using mutation specific antibodies for immunohistochemical analysis. *Clin Cancer Res* 2010;16:3163–3170.
239. Brevet M, Arcila M, Ladanyi M. Assessment of *EGFR* mutation status in lung adenocarcinoma by immunohistochemistry using antibodies specific to the two major forms of mutant EGFR. *J Mol Diagn* 2010;12:169–176.
240. Yu J, Kane S, Wu J, et al. Mutation-specific antibodies for the detection of *EGFR* mutations in non-small-cell lung cancer. *Clin Cancer Res* 2009;15:3023–3028.
241. Fukuoka M, Wu Y, Thongprasert S, et al. Biomarker analyses from a phase III, randomized, open-label, first-line study of gefitinib (G) versus carboblatin/paclitaxel (C/P) in clinically selected patients (pts) with advanced non-small-cell lung cancer (NSCLC) in Asia (IPASS). *J Clin Oncol* 2009;27:521S.
242. Kubota K, Niho S, Enatsu S, et al. Efficacy differences of pemetrexed by histology in pretreated patients with stage IIIB/IV non-small cell lung cancer: review of results from an open-label randomized phase II study. *J Thorac Oncol* 2009;4:1530–1536.
243. Zinner RG, Novello S, Peng G, et al. Comparison of patient outcomes according to histology among pemetrexed-treated patients with stage IIIB/IV non-small-cell lung cancer in two phase II trials. *Clin Lung Cancer* 2010;11:126–131.
244. Gronberg BH, Bremnes RM, Flotten O, et al. Phase III study by the Norwegian lung cancer study group: pemetrexed plus carboplatin compared with gemcitabine plus carboplatin as first-line chemotherapy in advanced non-small-cell lung cancer. *J Clin Oncol* 2009;27:3217–3224.
245. Sandler A, Gray R, Perry MC, et al. Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. *N Engl J Med* 2006;355:2542–2550.
246. Soda M, Choi YL, Enomoto M, et al. Identification of the transforming *EML4-ALK* fusion gene in non-small-cell lung cancer. *Nature* 2007; 448:561–566.
247. Wong DW, Leung EL, So KK, et al. The *EML4-ALK* fusion gene is

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involved in various histologic types of lung cancers from nonsmokers with wild-type *EGFR* and *KRAS*. *Cancer* 2009;115:1723–1733.
248. Kwak EL, Bang YJ, Camidge DR, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med* 2010;363:1693–1703.
249. Choi YL, Soda M, Yamashita Y, et al. *EML4-ALK* mutations in lung cancer that confer resistance to ALK inhibitors. *N Engl J Med* 2010; 363:1734–1739.
250. Mao C, Qiu LX, Liao RY, et al. *KRAS* mutations and resistance to EGFR-TKIs treatment in patients with non-small cell lung cancer: a meta-analysis of 22 studies. *Lung Cancer* 2010;69:272–278.
251. Linardou H, Dahabreh IJ, Kanaloupiti D, et al. Assessment of somatic *k-RAS* mutations as a mechanism associated with resistance to EGFR-targeted agents: a systematic review and meta-analysis of studies in advanced non-small-cell lung cancer and metastatic colorectal cancer. *Lancet Oncol* 2008;9:962–972.
252. Goldstraw P. IASLC Staging Manual in Thoracic Oncology. Orange Park, FL: International Association for the Study of Lung Cancer, Editorial Rx Press, 2009.
253. Goldstraw P, Crowley J, Chansky K, et al. The IASLC Lung Cancer Staging Project: proposals for the revision of the TNM stage groupings in the forthcoming (seventh) edition of the TNM Classification of malignant tumours. *J Thorac Oncol* 2007;2:706–714.
254. Pignon JP, Tribodet H, Scagliotti GV, et al. Lung adjuvant cisplatin evaluation: a pooled analysis by the LACE Collaborative Group. *J Clin Oncol* 2008;26:3552–3559.
255. Pao W, Wang TY, Riely GJ, et al. *KRAS* mutations and primary resistance of lung adenocarcinomas to gefitinib or erlotinib. *PLoS Med* 2005;2:e17.
256. Kim CF, Jackson EL, Woolfenden AE, et al. Identification of bronchioalveolar stem cells in normal lung and lung cancer. *Cell* 2005;121: 823–835.
257. Weir BA, Woo MS, Getz G, et al. Characterizing the cancer genome in lung adenocarcinoma. *Nature* 2007;450:893–898.
258. Tanaka H, Yanagisawa K, Shinjo K, et al. Lineage-specific dependency of lung adenocarcinomas on the lung development regulator TTF-1. *Cancer Res* 2007;67:6007–6011.
259. Colby TV, Leslie KO, Yousem SA. Lungs. In SE Mills (Ed.). Histology for Pathologists. 3rd ed. Philadelphia: Lippincott Williams & Wilkins, 2007, pp 473–504.
260. Wang BY, Gil J, Kaufman D, et al. P63 in pulmonary epithelium, pulmonary squamous neoplasms, and other pulmonary tumors. *Hum Pathol* 2002;33:921–926.
261. Takeuchi T, Tomida S, Yatabe Y, et al. Expression profile-defined classification of lung adenocarcinoma shows close relationship with underlying major genetic changes and clinicopathologic behaviors. *J Clin Oncol* 2006;24:1679–1688.
262. Colby TV, Wistuba II, Gazdar A. Precursors to pulmonary neoplasia. *Adv Anat Pathol* 1998;5:205–215.
263. Westra WH. Early glandular neoplasia of the lung. *Respir Res* 2000; 1:163–169.
264. Tang X, Varella-Garcia M, Xavier AC, et al. Epidermal growth factor receptor abnormalities in the pathogenesis and progression of lung adenocarcinomas. *Cancer Prev Res (Phila Pa)* 2008;1:192–200.
265. Tang X, Shigematsu H, Bekele BN, et al. EGFR tyrosine kinase domain mutations are detected in histologically normal respiratory epithelium in lung cancer patients. *Cancer Res* 2005;65:7568–7572.
266. Soh J, Toyooka S, Ichihara S, et al. Sequential molecular changes during multistage pathogenesis of small peripheral adenocarcinomas of the lung. *J Thorac Oncol* 2008;3:340–347.
267. Yatabe Y, Takahashi T, Mitsudomi T. *Epidermal growth factor receptor* gene amplification is acquired in association with tumor progression of *EGFR*-mutated lung cancer. *Cancer Res* 2008;68:2106–2111.
268. Tang ZQ, Han LY, Lin HH, et al. Derivation of stable microarray cancer-differentiating signatures using consensus scoring of multiple random sampling and gene-ranking consistency evaluation. *Cancer Res* 2007;67:9996–10003.
269. Koga T, Hashimoto S, Sugio K, et al. Clinicopathological and molecular evidence indicating the independence of bronchioloalveolar components from other subtypes of human peripheral lung adenocarcinoma. *Clin Cancer Res* 2001;7:1730–1738.
270. Marchetti A, Pellegrini S, Bertacca G, et al. *FHIT* and *p53* gene abnormalities in bronchioloalveolar carcinomas. Correlations with clinicopathological data and *K-ras* mutations. *J Pathol* 1998;184:240–246.
271. Yoshida Y, Kokubu A, Suzuki K, et al. Molecular markers and changes of computed tomography appearance in lung adenocarcinoma with ground-glass opacity. *Jpn J Clin Oncol* 2007;37:907–912.
272. Terasaki H, Niki T, Matsuno Y, et al. Lung adenocarcinoma with mixed bronchioloalveolar and invasive components: clinicopathological features, subclassification by extent of invasive foci, and immunohistochemical characterization. *Am J Surg Pathol* 2003;27:937–951.
273. Huang CL, Taki T, Adachi M, et al. Mutations of *p53* and *K-ras* genes as prognostic factors for non-small cell lung cancer. *Int J Oncol* 1998;12:553–563.
274. Shigematsu H, Lin L, Takahashi T, et al. Clinical and biological features associated with *epidermal growth factor receptor* gene mutations in lung cancers. *J Natl Cancer Inst* 2005;97:339–346.
275. Marchetti A, Martella C, Felicioni L, et al. *EGFR* mutations in non-small-cell lung cancer: analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment. *J Clin Oncol* 2005;23:857–865.
276. Ohtsuka T, Watanabe K, Kaji M, et al. A clinicopathological study of resected pulmonary nodules with focal pure ground-glass opacity. *Eur J Cardiothorac Surg* 2006;30:160–163.
277. Sonobe M, Manabe T, Wada H, et al. Mutations in the *epidermal growth factor receptor* gene are linked to smoking-independent, lung adenocarcinoma. *Br J Cancer* 2005;93:355–363.
278. Bryant CM, Albertus DL, Kim S, et al. Clinically relevant characterization of lung adenocarcinoma subtypes based on cellular pathways: an international validation study. *PLoS One* 2010;5:e11712.
279. Yousem SA, Nikiforova M, Nikiforov Y. The histopathology of *BRAF-V600E*-mutated lung adenocarcinoma. *Am J Surg Pathol* 2008;32: 1317–1321.
280. Conde E, Angulo B, Tang M, et al. Molecular context of the *EGFR* mutations: evidence for the activation of *mTOR/S6K* signaling. *Clin Cancer Res* 2006;12:710–717.
281. Ohtsuka K, Ohnishi H, Furuyashiki G, et al. Clinico-pathological and biological significance of tyrosine kinase domain gene mutations and overexpression of epidermal growth factor receptor for lung adenocarcinoma. *J Thorac Oncol* 2006;1:787–795.
282. Sonobe M, Manabe T, Wada H, et al. Lung adenocarcinoma harboring mutations in the ERBB2 kinase domain. *J Mol Diagn* 2006;8:351–356.
283. Ninomiya H, Hiramatsu M, Inamura K, et al. Correlation between morphology and *EGFR* mutations in lung adenocarcinomas Significance of the micropapillary pattern and the hobnail cell type. *Lung Cancer* 2009;63:235–240.
284. Hirsch FR, Varella-Garcia M, McCoy J, et al. Increased epidermal growth factor receptor gene copy number detected by fluorescence in situ hybridization associates with increased sensitivity to gefitinib in patients with bronchioloalveolar carcinoma subtypes: a southwest oncology group study. *J Clin Oncol* 2005;23:6838–6845.
285. Tanaka R, Horikoshi H, Nakazato Y, et al. Magnetic resonance imaging in peripheral lung adenocarcinoma: correlation with histopathologic features. *J Thorac Imaging* 2009;24:4–9.
286. Stenhouse G, Fyfe N, King G, et al. Thyroid transcription factor 1 in pulmonary adenocarcinoma. *J Clin Pathol* 2004;57:383–387.
287. Kim YT, Kim TY, Lee DS, et al. Molecular changes of *epidermal growth factor receptor* (*EGFR*) and *KRAS* and their impact on the clinical outcomes in surgically resected adenocarcinoma of the lung. *Lung Cancer* 2008;59:111–118.
288. Ang DC, Zakowski MF, Ladanyi M, et al. Characteristic morphology and immunoprofile of lung adenocarcinoma with *KRAS* mutations: propensity for solid growth pattern and correlation with TTF-1 expression. *Mod Pathol* 2010;23(Suppl):396A.
289. Saad RS, Cho P, Silverman JF, et al. Usefulness of Cdx2 in separating mucinous bronchioloalveolar adenocarcinoma of the lung from metastatic mucinous colorectal adenocarcinoma. *Am J Clin Pathol* 2004; 122:421–427.
290. Shrestha B, Ebihara Y, Osakabe Y, et al. Immunohistochemical, ultrastructural and molecular study of well differentiated adenocarcinomas of the lung predominantly composed of goblet cells. *Lung Cancer* 1998;22:103–117.
291. Yatabe Y, Koga T, Mitsudomi T, et al. CK20 expression, CDX2

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expression, K-ras mutation, and goblet cell morphology in a subset of lung adenocarcinomas. *J Pathol* 2004;203:645–652.
292. Sasaki H, Kawano O, Endo K, et al. Uncommon V599E *BRAF* mutations in Japanese patients with lung cancer. *J Surg Res* 2006;133: 203–206.
293. Naoki K, Chen TH, Richards WG, et al. Missense mutations of the *BRAF* gene in human lung adenocarcinoma. *Cancer Res* 2002;62: 7001–7003.
294. Tang Z, Du R, Jiang S, et al. Dual *MET-EGFR* combinatorial inhibition against T790M-EGFR-mediated erlotinib-resistant lung cancer. *Br J Cancer* 2008;99:911–922.
295. Cappuzzo F, Janne PA, Skokan M, et al. *MET* increased gene copy number and primary resistance to gefitinib therapy in non-small-cell lung cancer patients. *Ann Oncol* 2009;20:298–304.
296. Engelman JA, Zejnullahu K, Mitsudomi T, et al. *MET* amplification leads to gefitinib resistance in lung cancer by activating *ERBB3* signaling. *Science* 2007;316:1039–1043.
297. Pao W, Miller VA, Politi KA, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med* 2005;2:e73.
298. Sequist LV, Martins RG, Spigel D, et al. First-line gefitinib in patients with advanced non-small-cell lung cancer harboring somatic *EGFR* mutations. *J Clin Oncol* 2008;26:2442–2449.
299. Nguyen KS, Kobayashi S, Costa DB. Acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non-small-cell lung cancers dependent on the epidermal growth factor receptor pathway. *Clin Lung Cancer* 2009;10:281–289.
300. Takahashi T, Sonobe M, Kobayashi M, et al. Clinicopathologic features of non-small-cell lung cancer with *EML4-ALK* fusion gene. *Ann Surg Oncol* 2010;17:889–897.
301. Inamura K, Takeuchi K, Togashi Y, et al. *EML4-ALK* lung cancers are characterized by rare other mutations, a TTF-1 cell lineage, an acinar histology, and young onset. *Mod Pathol* 2009;22:508–515.
302. Takeuchi K, Choi YL, Togashi Y, et al. *KIF5B-ALK*, a novel fusion oncokinase identified by an immunohistochemistry-based diagnostic system for *ALK*-positive lung cancer. *Clin Cancer Res* 2009;15:3143–3149.
303. Yoshida A, Tsuta K, Watanabe SI, et al. Frequent *ALK* rearrangement and TTF-1/p63 co-expression in lung adenocarcinoma with signet-ring cell component. *Lung Cancer.* In press.
304. Jokoji R, Yamasaki T, Minami S, et al. Combination of morphological feature analysis and immunohistochemistry is useful for screening of EML4-ALK-positive lung adenocarcinoma. *J Clin Pathol* 2010;63: 1066–1070.
305. Inamura K, Takeuchi K, Togashi Y, et al. *EML4-ALK* fusion is linked to histological characteristics in a subset of lung cancers. *J Thorac Oncol* 2008;3:13–17.
306. Mino-Kenudson M, Chirieac LR, Law K, et al. A novel, highly sensitive antibody allows for the routine detection of *ALK*-rearranged lung adenocarcinomas by standard immunohistochemistry. *Clin Cancer Res* 2010;16:1561–1571.
307. Sakairi Y, Nakajima T, Yasufuku K, et al. *EML4-ALK* fusion gene assessment using metastatic lymph node samples obtained by endobronchial ultrasound-guided transbronchial needle aspiration. *Clin Cancer Res* 2010;16:4938–4945.
308. Boland JM, Erdogan S, Vasmatzis G, et al. Anaplastic lymphoma kinase immunoreactivity correlates with *ALK* gene rearrangement and transcriptional up-regulation in non-small cell lung carcinomas. *Hum Pathol* 2009;40:1152–1158.
309. Beer DG, Kardia SL, Huang CC, et al. Gene-expression profiles predict survival of patients with lung adenocarcinoma. *Nat Med* 2002;8:816–824.
310. Borczuk AC, Kim HK, Yegen HA, et al. Lung adenocarcinoma global profiling identifies type II transforming growth factor-(beta) receptor as a repressor of invasiveness. *Am J Respir Crit Care Med* 2005;172:729–737.
311. Shibata T, Hanada S, Kokubu A, et al. Gene expression profiling of *epidermal growth factor receptor/KRAS* pathway activation in lung adenocarcinoma. *Cancer Sci* 2007;98:985–991.
312. Berrar D, Sturgeon B, Bradbury I, et al. Survival trees for analyzing clinical outcome in lung adenocarcinomas based on gene expression profiles: identification of neogenin and diacylglycerol kinase alpha expression as critical factors. *J Comput Biol* 2005;12:534–544.
313. Bianchi F, Nuciforo P, Vecchi M, et al. Survival prediction of stage I lung adenocarcinomas by expression of 10 genes. *J Clin Invest* 2007; 117:3436–3444.
314. Endoh H, Tomida S, Yatabe Y, et al. Prognostic model of pulmonary adenocarcinoma by expression profiling of eight genes as determined by quantitative real-time reverse transcriptase polymerase chain reaction. *J Clin Oncol* 2004;22:811–819.
315. Guo L, Ma Y, Ward R, et al. Constructing molecular classifiers for the accurate prognosis of lung adenocarcinoma. *Clin Cancer Res* 2006;12: 3344–3354.
316. Hayes DN, Monti S, Parmigiani G, et al. Gene expression profiling reveals reproducible human lung adenocarcinoma subtypes in multiple independent patient cohorts. *J Clin Oncol* 2006;24:5079–5090.
317. Inamura K, Shimoji T, Ninomiya H, et al. A metastatic signature in entire lung adenocarcinomas irrespective of morphological heterogeneity. *Hum Pathol* 2007;38:702–709.
318. Larsen JE, Pavey SJ, Passmore LH, et al. Gene expression signature predicts recurrence in lung adenocarcinoma. *Clin Cancer Res* 2007;13: 2946–2954.
319. Liu H, Kho AT, Kohane IS, et al. Predicting survival within the lung cancer histopathological hierarchy using a multi-scale genomic model of development. *PLoS Med* 2006;3:e232.
320. Sun Z, Wigle DA, Yang P. Non-overlapping and non-cell-type-specific gene expression signatures predict lung cancer survival. *J Clin Oncol* 2008;26:877–883.
321. Xi L, Lyons-Weiler J, Coello MC, et al. Prediction of lymph node metastasis by analysis of gene expression profiles in primary lung adenocarcinomas. *Clin Cancer Res* 2005;11:4128–4135.
322. Potti A, Mukherjee S, Petersen R, et al. A genomic strategy to refine prognosis in early-stage non-small-cell lung cancer. *N Engl J Med* 2006;355:570–580.
323. Chen HY, Yu SL, Chen CH, et al. A five-gene signature and clinical outcome in non-small-cell lung cancer. *N Engl J Med* 2007;356:11–20.
324. Chitale D, Gong Y, Taylor BS, et al. An integrated genomic analysis of lung cancer reveals loss of DUSP4 in EGFR-mutant tumors. *Oncogene* 2009;28:2773–2783.
325. Tonon G, Brennan C, Protopopov A, et al. Common and contrasting genomic profiles among the major human lung cancer subtypes. *Cold Spring Harb Symp Quant Biol* 2005;70:11–24.
326. Aviel-Ronen S, Coe BP, Lau SK, et al. Genomic markers for malignant progression in pulmonary adenocarcinoma with bronchioloalveolar features. *Proc Natl Acad Sci USA* 2008;105:10155–10160.
327. Chang JW, Liu HP, Hsieh MH, et al. Increased epidermal growth factor receptor (*EGFR*) gene copy number is strongly associated with EGFR mutations and adenocarcinoma in non-small cell lung cancers: a chromogenic in situ hybridization study of 182 patients. *Lung Cancer* 2008;61:328–339.
328. Cappuzzo F, Hirsch FR, Rossi E, et al. Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. *J Natl Cancer Inst* 2005;97:643–655.
329. Dacic S, Ionescu DN, Finkelstein S, et al. Patterns of allelic loss of synchronous adenocarcinomas of the lung. *Am J Surg Pathol* 2005;29: 897–902.
330. Wang X, Wang M, MacLennan GT, et al. Evidence for common clonal origin of multifocal lung cancers. *J Natl Cancer Inst* 2009;101:560–570.
331. Girard N, Ostrovnaya I, Lau C, et al. Genomic and mutational profiling to assess clonal relationships between multiple non-small cell lung cancers. *Clin Cancer Res* 2009;15:5184–5190.
332. van Rens MT, Eijken EJ, Elbers JR, et al. p53 mutation analysis for definite diagnosis of multiple primary lung carcinoma. *Cancer* 2002; 94:188–196.
333. Matsuzoe D, Hideshima T, Ohshima K, et al. Discrimination of double primary lung cancer from intrapulmonary metastasis by *p53* gene mutation. *Br J Cancer* 1999;79:1549–1552.
334. Wang X, Christiani DC, Mark EJ, et al. Carcinogen exposure, *p53* alteration, and *K-ras* mutation in synchronous multiple primary lung carcinoma. *Cancer* 1999;85:1734–1739.
335. Lau DH, Yang B, Hu R, et al. Clonal origin of multiple lung cancers: *K-ras* and *p53* mutations determined by nonradioisotopic single-strand conformation polymorphism analysis. *Diagn Mol Pathol* 1997;6:179–184.
336. Girard N, Deshpande C, Azzoli CG, et al. Use of *epidermal growth factor*

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receptor/Kirsten rat sarcoma 2 viral oncogene homolog mutation testing to define clonal relationships among multiple lung adenocarcinomas: comparison with clinical guidelines. *Chest* 2010;137:46–52.
337. Nonami Y, Ohtuki Y, Sasaguri S. Study of the diagnostic difference between the clinical diagnostic criteria and results of immunohistochemical staining of multiple primary lung cancers. *J Cardiovasc Surg (Torino)* 2003;44:661–665.
338. Vansteenkiste JF, De Belie B, Deneffe GJ, et al. Practical approach to patients presenting with multiple synchronous suspect lung lesions: a reflection on the current TNM classification based on 54 cases with complete follow-up. *Lung Cancer* 2001;34:169–175.
339. Yoshino I, Nakanishi R, Osaki T, et al. Postoperative prognosis in patients with non-small cell lung cancer with synchronous ipsilateral intrapulmonary metastasis. *Ann Thorac Surg* 1997;64:809–813.
340. Chung JH, Choe G, Jheon S, et al. *Epidermal growth factor receptor* mutation and pathologic-radiologic correlation between multiple lung nodules with ground-glass opacity differentiates multicentric origin from intrapulmonary spread. *J Thorac Oncol* 2009;4:1490–1495.
341. Balak MN, Gong Y, Riely GJ, et al. Novel D761Y and common secondary *T790M* mutations in *epidermal growth factor receptor*-mutant lung adenocarcinomas with acquired resistance to kinase inhibitors. *Clin Cancer Res* 2006;12:6494–6501.
342. Jackman DM, Holmes AJ, Lindeman N, et al. Response and resistance in a non-small-cell lung cancer patient with an epidermal growth factor receptor mutation and leptomeningeal metastases treated with high-dose gefitinib. *J Clin Oncol* 2006;24:4517–4520.
343. Schmid K, Oehl N, Wrba F, et al. *EGFR/KRAS/BRAF* mutations in primary lung adenocarcinomas and corresponding locoregional lymph node metastases. *Clin Cancer Res* 2009;15:4554–4560.
344. Monaco SE, Nikiforova MN, Cieply K, et al. A comparison of *EGFR* and *KRAS* status in primary lung carcinoma and matched metastases. *Hum Pathol* 2010;41:94–102.
345. Meert AP, Martin B, Delmotte P, et al. The role of *EGF-R* expression on patient survival in lung cancer: a systematic review with meta-analysis. *Eur Respir J* 2002;20:975–981.
346. Berghmans T, Paesmans M, Mascaux C, et al. Thyroid transcription factor 1—a new prognostic factor in lung cancer: a meta-analysis. *Ann Oncol* 2006;17:1673–1676.
347. Mascaux C, Iannino N, Martin B, et al. The role of *RAS* oncogene in survival of patients with lung cancer: a systematic review of the literature with meta-analysis. *Br J Cancer* 2005;92:131–139.
348. Nakamura H, Kawasaki N, Taguchi M, et al. Association of HER-2 overexpression with prognosis in nonsmall cell lung carcinoma: a metaanalysis. *Cancer* 2005;103:1865–1873.
349. Mitsudomi T, Hamajima N, Ogawa M, et al. Prognostic significance of p53 alterations in patients with non-small cell lung cancer: a meta-analysis. *Clin Cancer Res* 2000;6:4055–4063.
350. Steels E, Paesmans M, Berghmans T, et al. Role of p53 as a prognostic factor for survival in lung cancer: a systematic review of the literature with a meta-analysis. *Eur Respir J* 2001;18:705–719.
351. Martin B, Paesmans M, Mascaux C, et al. Ki-67 expression and patients survival in lung cancer: systematic review of the literature with meta-analysis. *Br J Cancer* 2004;91:2018–2025.
352. Martin B, Paesmans M, Berghmans T, et al. Role of Bcl-2 as a prognostic factor for survival in lung cancer: a systematic review of the literature with meta-analysis. *Br J Cancer* 2003;89:55–64.
353. Mascaux C, Martin B, Paesmans M, et al. Has Cox-2 a prognostic role in non-small-cell lung cancer? A systematic review of the literature with meta-analysis of the survival results. *Br J Cancer* 2006;95:139–145.
354. Marks JL, Broderick S, Zhou Q, et al. Prognostic and therapeutic implications of *EGFR* and *KRAS* mutations in resected lung adenocarcinoma. *J Thorac Oncol* 2008;3:111–116.
355. Kosaka T, Yatabe Y, Onozato R, et al. Prognostic implication of *EGFR, KRAS,* and *TP53* gene mutations in a large cohort of Japanese patients with surgically treated lung adenocarcinoma. *J Thorac Oncol* 2009;4:22–29.
356. Yanaihara N, Caplen N, Bowman E, et al. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer Cell* 2006;9:189–198.
357. Raponi M, Dossey L, Jatkoe T, et al. MicroRNA classifiers for predicting prognosis of squamous cell lung cancer. *Cancer Res* 2009;69:5776–5783.
358. Hansell DM, Bankier AA, MacMahon H, et al. Fleischner Society: glossary of terms for thoracic imaging. *Radiology* 2008;246:697–722.
359. Godoy MC, Naidich DP. Subsolid pulmonary nodules and the spectrum of peripheral adenocarcinomas of the lung: recommended interim guidelines for assessment and management. *Radiology* 2009;253:606–622.
360. Lee HY, Goo JM, Lee HJ, et al. Usefulness of concurrent reading using thin-section and thick-section CT images in subcentimetre solitary pulmonary nodules. *Clin Radiol* 2009;64:127–132.
361. Takashima S, Sone S, Li F, et al. Small solitary pulmonary nodules (< or =1 cm) detected at population-based CT screening for lung cancer: reliable high-resolution CT features of benign lesions. *AJR Am J Roentgenol* 2003;180:955–964.
362. Ishikawa H, Koizumi N, Morita T, et al. Ultrasmall pulmonary opacities on multidetector-row high-resolution computed tomography: a prospective radiologic-pathologic examination. *J Comput Assist Tomogr* 2005;29:621–625.
363. Kishi K, Homma S, Kurosaki A, et al. Small lung tumors with the size of 1cm or less in diameter: clinical, radiological, and histopathological characteristics. *Lung Cancer* 2004;44:43–51.
364. Kim HY, Shim YM, Lee KS, et al. Persistent pulmonary nodular ground-glass opacity at thin-section CT: histopathologic comparisons. *Radiology* 2007;245:267–275.
365. Kim TJ, Goo JM, Lee KW, et al. Clinical, pathological and thin-section CT features of persistent multiple ground-glass opacity nodules: comparison with solitary ground-glass opacity nodule. *Lung Cancer* 2009;64:171–178.
366. Ikeda K, Awai K, Mori T, et al. Differential diagnosis of ground-glass opacity nodules: CT number analysis by three-dimensional computerized quantification. *Chest* 2007;132:984–990.
367. Choi JA, Kim JH, Hong KT, et al. CT bronchus sign in malignant solitary pulmonary lesions: value in the prediction of cell type. *Eur Radiol* 2000;10:1304–1309.
368. Takashima S, Maruyama Y, Hasegawa M, et al. CT findings and progression of small peripheral lung neoplasms having a replacement growth pattern. *AJR Am J Roentgenol* 2003;180:817–826.
369. Gould MK, Fletcher J, Iannettoni MD, et al. Evaluation of patients with pulmonary nodules: when is it lung cancer?: ACCP evidence-based clinical practice guidelines (2nd edition). *Chest* 2007;132:108S–130S.
370. Nakazono T, Sakao Y, Yamaguchi K, et al. Subtypes of peripheral adenocarcinoma of the lung: differentiation by thin-section CT. *Eur Radiol* 2005;15:1563–1568.
371. Zwirewich CV, Vedal S, Miller RR, et al. Solitary pulmonary nodule: high-resolution CT and radiologic-pathologic correlation. *Radiology* 1991;179:469–476.
372. Yang ZG, Sone S, Takashima S, et al. High-resolution CT analysis of small peripheral lung adenocarcinomas revealed on screening helical CT. *AJR Am J Roentgenol* 2001;176:1399–1407.
373. Tateishi U, Uno H, Yonemori K, et al. Prediction of lung adenocarcinoma without vessel invasion: a CT scan volumetric analysis. *Chest* 2005;128:3276–3283.
374. Kojima Y, Saito H, Sakuma Y, et al. Correlations of thin-section computed tomographic, histopathological, and clinical findings of adenocarcinoma with a bubblelike appearance. *J Comput Assist Tomogr* 2010;34:413–417.
375. Yoshino I, Nakanishi R, Kodate M, et al. Pleural retraction and intra-tumoral air-bronchogram as prognostic factors for stage I pulmonary adenocarcinoma following complete resection. *Int Surg* 2000;85:105–112.
376. Kondo T, Yamada K, Noda K, et al. Radiologic-prognostic correlation in patients with small pulmonary adenocarcinomas. *Lung Cancer* 2002;36:49–57.
377. Sakao Y, Nakazono T, Sakuragi T, et al. Predictive factors for survival in surgically resected clinical IA peripheral adenocarcinoma of the lung. *Ann Thorac Surg* 2004;77:1157–1161.
378. Kuriyama K, Seto M, Kasugai T, et al. Ground-glass opacity on thin-section CT: value in differentiating subtypes of adenocarcinoma of the lung. *AJR Am J Roentgenol* 1999;173:465–469.
379. Castro CY, Coffey DM, Medeiros LJ, et al. Prognostic significance of

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percentage of bronchioloalveolar pattern in adenocarcinomas of the lung. *Ann Diagn Pathol* 2001;5:274–284.
380. Hashizume T, Yamada K, Okamoto N, et al. Prognostic significance of thin-section CT scan findings in small-sized lung adenocarcinoma. *Chest* 2008;133:441–447.
381. Dong B, Sato M, Sagawa M, et al. Computed tomographic image comparison between mediastinal and lung windows provides possible prognostic information in patients with small peripheral lung adenocarcinoma. *J Thorac Cardiovasc Surg* 2002;124:1014–1020.
382. Matsuguma H, Yokoi K, Anraku M, et al. Proportion of ground-glass opacity on high-resolution computed tomography in clinical T1 N0 M0 adenocarcinoma of the lung: a predictor of lymph node metastasis. *J Thorac Cardiovasc Surg* 2002;124:278–284.
383. Ohde Y, Nagai K, Yoshida J, et al. The proportion of consolidation to ground-glass opacity on high resolution CT is a good predictor for distinguishing the population of non-invasive peripheral adenocarcinoma. *Lung Cancer* 2003;42:303–310.
384. Okada M, Nishio W, Sakamoto T, et al. Correlation between computed tomographic findings, bronchioloalveolar carcinoma component, and biologic behavior of small-sized lung adenocarcinomas. *J Thorac Cardiovasc Surg* 2004;127:857–861.
385. Sakao Y, Nakazono T, Tomimitsu S, et al. Lung adenocarcinoma can be subtyped according to tumor dimension by computed tomography mediastinal-window setting. Additional size criteria for clinical T1 adenocarcinoma. *Eur J Cardiothorac Surg* 2004;26:1211–1215.
386. Seki N, Sawada S, Nakata M, et al. Lung cancer with localized ground-glass attenuation represents early-stage adenocarcinoma in nonsmokers. *J Thorac Oncol* 2008;3:483–490.
387. Takashima S, Maruyama Y, Hasegawa M, et al. High-resolution CT features: prognostic significance in peripheral lung adenocarcinoma with bronchioloalveolar carcinoma components. *Respiration* 2003;70:36–42.
388. Nishio R, Akata S, Saito K, et al. The ratio of the maximum high attenuation area dimension to the maximum tumor dimension may be an index of the presence of lymph node metastasis in lung adenocarcinomas 3 cm or smaller on high-resolution computed tomography. *J Thorac Oncol* 2007;2:29–33.
389. Shim HS, Park IK, Lee CY, et al. Prognostic significance of visceral pleural invasion in the forthcoming (seventh) edition of TNM classification for lung cancer. *Lung Cancer* 2009;65:161–165.
390. Ikehara M, Saito H, Kondo T, et al. Comparison of thin-section CT and pathological findings in small solid-density type pulmonary adenocarcinoma: prognostic factors from CT findings. *Eur J Radiol.* In press.
391. Gaeta M, Vinci S, Minutoli F, et al. CT and MRI findings of mucin-containing tumors and pseudotumors of the thorax: pictorial review. *Eur Radiol* 2002;12:181–189.
392. Nakata M, Sawada S, Saeki H, et al. Prospective study of thoracoscopic limited resection for ground-glass opacity selected by computed tomography. *Ann Thorac Surg* 2003;75:1601–1605; discussion 5–6.
393. Takashima S, Maruyama Y, Hasegawa M, et al. Prognostic significance of high-resolution CT findings in small peripheral adenocarcinoma of the lung: a retrospective study on 64 patients. *Lung Cancer* 2002;36:289–295.
394. Hiramatsu M, Inagaki T, Matsui Y, et al. Pulmonary ground-glass opacity (GGO) lesions-large size and a history of lung cancer are risk factors for growth. *J Thorac Oncol* 2008;3:1245–1250.
395. Austin JHM, Mujoomdar A, Powell CA, et al. Carcinoma of the lung and metastatic disease of the central nervous system. *Am J Respir Crit Care Med* 2008;178:1090.
396. Mujoomdar A, Austin JHM, Malhotra R, et al. Clinical predictors of metastatic disease to the brain from non-small cell lung carcinoma: primary tumor size, cell type, and lymph node metastases. *Radiology* 2007;242:882–888.
397. MacMahon H, Austin JHM, Gamsu G, et al. Guidelines for management of small pulmonary nodules detected on CT scans: a statement from the Fleischner Society. *Radiology* 2005;237:395–400.
398. Eisenberg RL, Bankier AA, Boiselle PM. Compliance with Fleischner Society guidelines for management of small lung nodules: a survey of 834 radiologists. *Radiology* 2010;255:218–224.
399. MacMahon H. Compliance with Fleischner Society guidelines for management of lung nodules: lessons and opportunities. *Radiology* 2010;255:14–15.
400. Zhao B, James LP, Moskowitz CS, et al. Evaluating variability in tumor measurements from same-day repeat CT scans of patients with non-small cell lung cancer. *Radiology* 2009;252:263–272.
401. Ravenel JG, Leue WM, Nietert PJ, et al. Pulmonary nodule volume: effects of reconstruction parameters on automated measurements—a phantom study. *Radiology* 2008;247:400–408.
402. Jennings SG, Winer-Muram HT, Tarver RD, et al. Lung tumor growth: assessment with CT–comparison of diameter and cross-sectional area with volume measurements. *Radiology* 2004;231:866–871.
403. Winer-Muram HT, Jennings SG, Meyer CA, et al. Effect of varying CT section width on volumetric measurement of lung tumors and application of compensatory equations. *Radiology* 2003;229:184–194.
404. Yankelevitz DF, Reeves AP, Kostis WJ, et al. Small pulmonary nodules: volumetrically determined growth rates based on CT evaluation. *Radiology* 2000;217:251–256.
405. de Hoop B, Gietema H, van de Vorst S, et al. Pulmonary ground-glass nodules: increase in mass as an early indicator of growth. *Radiology* 2010;255:199–206.
406. Nakata M, Sawada S, Yamashita M, et al. Surgical treatments for multiple primary adenocarcinoma of the lung. *Ann Thorac Surg* 2004; 78:1194–1199.
407. Zwirewich CV, Miller RR, Muller NL. Multicentric adenocarcinoma of the lung: CT-pathologic correlation. *Radiology* 1990;176:185–190.
408. Park CM, Goo JM, Kim TJ, et al. Pulmonary nodular ground-glass opacities in patients with extrapulmonary cancers: what is their clinical significance and how can we determine whether they are malignant or benign lesions? *Chest* 2008;133:1402–1409.
409. Okada M, Tauchi S, Iwanaga K, et al. Associations among bronchioloalveolar carcinoma components, positron emission tomographic and computed tomographic findings, and malignant behavior in small lung adenocarcinomas. *J Thorac Cardiovasc Surg* 2007;133:1448–1454.
410. Higashi K, Ueda Y, Seki H, et al. Fluorine-18-FDG PET imaging is negative in bronchioloalveolar lung carcinoma. *J Nucl Med* 1998;39: 1016–1020.
411. Higashi K, Ueda Y, Yagishita M, et al. FDG PET measurement of the proliferative potential of non-small cell lung cancer. *J Nucl Med* 2000;41:85–92.
412. Higashi K, Ueda Y, Ayabe K, et al. FDG PET in the evaluation of the aggressiveness of pulmonary adenocarcinoma: correlation with histopathological features. *Nucl Med Commun* 2000;21:707–714.
413. Ohtsuka T, Nomori H, Watanabe K, et al. Prognostic significance of [(18)F]fluorodeoxyglucose uptake on positron emission tomography in patients with pathologic stage I lung adenocarcinoma. *Cancer* 2006; 107:2468–2473.
414. Raz DJ, Odisho AY, Franc BL, et al. Tumor fluoro-2-deoxy-D-glucose avidity on positron emission tomographic scan predicts mortality in patients with early-stage pure and mixed bronchioloalveolar carcinoma. *J Thorac Cardiovasc Surg* 2006;132:1189–1195.
415. Sagawa M, Higashi K, Sugita M, et al. Fluorodeoxyglucose uptake correlates with the growth pattern of small peripheral pulmonary adenocarcinoma. *Surg Today* 2006;36:230–234.
416. Pastorino U, Landoni C, Marchiano A, et al. Fluorodeoxyglucose uptake measured by positron emission tomography and standardized uptake value predicts long-term survival of CT screening detected lung cancer in heavy smokers. *J Thorac Oncol* 2009;4:1352–1356.
417. Nakayama H, Okumura S, Daisaki H, et al. Value of integrated positron emission tomography revised using a phantom study to evaluate malignancy grade of lung adenocarcinoma: a multicenter study. *Cancer* 2010;116:3170–3177.
418. Um SW, Kim H, Koh WJ, et al. Prognostic value of 18F-FDG uptake on positron emission tomography in patients with pathologic stage I non-small cell lung cancer. *J Thorac Oncol* 2009;4:1331–1336.
419. Berghmans T, Dusart M, Paesmans M, et al. Primary tumor standardized uptake value (SUVmax) measured on fluorodeoxyglucose positron emission tomography (FDG-PET) is of prognostic value for survival in non-small cell lung cancer (NSCLC): a systematic review and meta-analysis (MA) by the European Lung Cancer Working Party for the IASLC Lung Cancer Staging Project. *J Thorac Oncol* 2008;3:6–12.
420. Birchard KR, Hoang JK, Herndon JE Jr, et al. Early changes in tumor size in patients treated for advanced stage nonsmall cell lung cancer do not correlate with survival. *Cancer* 2009;115:581–586.
421. Sohn HJ, Yang YJ, Ryu JS, et al. [18F]Fluorothymidine positron emission tomography before and 7 days after gefitinib treatment pre-

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dicts response in patients with advanced adenocarcinoma of the lung. *Clin Cancer Res* 2008;14:7423–7429.
422. Cloran FJ, Banks KP, Song WS, et al. Limitations of dual time point PET in the assessment of lung nodules with low FDG avidity. *Lung Cancer* 2010;68:66–71.
423. Ohno Y, Hatabu H, Takenaka D, et al. Dynamic MR imaging: value of differentiating subtypes of peripheral small adenocarcinoma of the lung. *Eur J Radiol* 2004;52:144–150.
424. van Klaveren RJ, Oudkerk M, Prokop M, et al. Management of lung nodules detected by volume CT scanning. *N Engl J Med* 2009;361: 2221–2229.
425. Oda S, Awai K, Murao K, et al. Computer-aided volumetry of pulmonary nodules exhibiting ground-glass opacity at MDCT. *AJR Am J Roentgenol* 2010;194:398–406.
426. Henschke CI, McCauley DI, Yankelevitz DF, et al. Early Lung Cancer Action Project: overall design and findings from baseline screening. *Lancet* 1999;354:99–105.
427. Henschke CI, Naidich DP, Yankelevitz DF, et al. Early lung cancer action project: initial findings on repeat screenings. *Cancer* 2001;92: 153–159.
428. Lindell RM, Hartman TE, Swensen SJ, et al. Five-year lung cancer screening experience: CT appearance, growth rate, location, and histologic features of 61 lung cancers. *Radiology* 2007;242:555–562.
429. Hasegawa M, Sone S, Takashima S, et al. Growth rate of small lung cancers detected on mass CT screening. *Br J Radiol* 2000;73:1252–1259.
430. Henschke CI, Yankelevitz DF, Libby DM, et al. Survival of patients with stage I lung cancer detected on CT screening. *N Engl J Med* 2006;355:1763–1771.
431. Kakinuma R, Ohmatsu H, Kaneko M, et al. Progression of focal pure ground-glass opacity detected by low-dose helical computed tomography screening for lung cancer. *J Comput Assist Tomogr* 2004;28:17–23.
432. Sone S, Nakayama T, Honda T, et al. Long-term follow-up study of a population-based 1996–1998 mass screening programme for lung cancer using mobile low-dose spiral computed tomography. *Lung Cancer* 2007;58:329–341.
433. Pelosi G, Sonzogni A, Veronesi G, et al. Pathologic and molecular features of screening low-dose computed tomography (LDCT)-detected lung cancer: a baseline and 2-year repeat study. *Lung Cancer* 2008;62:202–214.
434. Wang JC, Sone S, Feng L, et al. Rapidly growing small peripheral lung cancers detected by screening CT: correlation between radiological appearance and pathological features. *Br J Radiol* 2000;73:930–937.
435. Infante M, Cavuto S, Lutman FR, et al. A randomized study of lung cancer screening with spiral computed tomography: three-year results from the DANTE trial. *Am J Respir Crit Care Med* 2009;180:445–453.
436. Bepler G. Are we coming full circle for lung cancer screening a second time? *Am J Respir Crit Care Med* 2009;180:384–385.
437. McMahon PM, Kong CY, Johnson BE, et al. Estimating long-term effectiveness of lung cancer screening in the Mayo CT screening study. *Radiology* 2008;248:278–287.
438. McMahon PM, Kong CY, Weinstein MC, et al. Adopting helical CT screening for lung cancer: potential health consequences during a 15-year period. *Cancer* 2008;113:3440–3449.
439. Gatsonis CA. The National Lung Screening Trial: overview and study design. *Radiology*. 2011;258:243–253.
440. Park EA, Lee HJ, Kim YT, et al. *EGFR* gene copy number in adenocarcinoma of the lung by FISH analysis: investigation of significantly related factors on CT, FDG-PET, and histopathology. *Lung Cancer* 2009;64:179–186.
441. Yano M, Sasaki H, Kobayashi Y, et al. *Epidermal growth factor receptor* gene mutation and computed tomographic findings in peripheral pulmonary adenocarcinoma. *J Thorac Oncol* 2006;1:413–416.
442. Chantranuwat C, Sriuranpong V, Huapai N, et al. Histopathologic characteristics of pulmonary adenocarcinomas with and without *EGFR* mutation. *J Med Assoc Thai* 2005;88(Suppl 4):S322–S329.
443. Huang CT, Yen RF, Cheng MF, et al. Correlation of F-18 fluorodeoxyglucose-positron emission tomography maximal standardized uptake value and *EGFR* mutations in advanced lung adenocarcinoma. *Med Oncol* 2010;27:9–15.
444. Watanabe K, Nomori H, Ohtsuka T, et al. [F-18]Fluorodeoxyglucose positron emission tomography can predict pathological tumor stage and proliferative activity determined by Ki-67 in clinical stage IA lung adenocarcinomas. *Jpn J Clin Oncol* 2006;36:403–409.
445. Vesselle H, Salskov A, Turcotte E, et al. Relationship between non-small cell lung cancer FDG uptake at PET, tumor histology, and Ki-67 proliferation index. *J Thorac Oncol* 2008;3:971–978.
446. Schuchert MJ, Pettiford BL, Keeley S, et al. Anatomic segmentectomy in the treatment of stage I non-small cell lung cancer. *Ann Thorac Surg* 2007;84:926–932.
447. Shapiro M, Weiser TS, Wisnivesky JP, et al. Thoracoscopic segmentectomy compares favorably with thoracoscopic lobectomy for patients with small stage I lung cancer. *J Thorac Cardiovasc Surg* 2009;137: 1388–1393.
448. Yan TD, Black D, Bannon PG, et al. Systematic review and meta-analysis of randomized and nonrandomized trials on safety and efficacy of video-assisted thoracic surgery lobectomy for early-stage non-small-cell lung cancer. *J Clin Oncol* 2009;27:2553–2562.
449. Watanabe T, Okada A, Imakiire T, et al. Intentional limited resection for small peripheral lung cancer based on intraoperative pathologic exploration. *Jpn J Thorac Cardiovasc Surg* 2005;53:29–35.
450. Higashiyama M, Kodama K, Takami K, et al. Intraoperative lavage cytologic analysis of surgical margins in patients undergoing limited surgery for lung cancer. *J Thorac Cardiovasc Surg* 2003;125:101–107.
451. Utsumi T, Sawabata N, Inoue M, et al. Optimal sampling methods for margin cytology examination following lung excision. *Interact Cardiovasc Thorac Surg* 2010;10:434–436.
452. Asamura H, Suzuki K, Watanabe S, et al. A clinicopathological study of resected subcentimeter lung cancers: a favorable prognosis for ground glass opacity lesions. *Ann Thorac Surg* 2003;76:1016–1022.
453. Ginsberg RJ, Rubinstein LV. Randomized trial of lobectomy versus limited resection for T1 N0 non-small cell lung cancer. Lung Cancer Study Group. *Ann Thorac Surg* 1995;60:615–622.
454. Miller DL, Rowland CM, Deschamps C, et al. Surgical treatment of non-small cell lung cancer 1 cm or less in diameter. *Ann Thorac Surg* 2002;73:1545–1550; discussion 50–51.
455. Rami-Porta R, Wittekind C, Goldstraw P. Complete resection in lung cancer surgery: proposed definition. *Lung Cancer* 2005;49:25–33.
456. Ishiguro F, Matsuo K, Fukui T, et al. Effect of selective lymph node dissection based on patterns of lobe-specific lymph node metastases on patient outcome in patients with resectable non-small cell lung cancer: a large-scale retrospective cohort study applying a propensity score. *J Thorac Cardiovasc Surg* 2010;139:1001–1006.
457. Darling GE, Allen MS, Landreneau RJ, et al. Randomized trial of mediastinal lymph node sampling versus complete lymphadenectomy during pulmonary resection in the patient with N0 or N1 (less than hilar) non-small cell carcinoma: results of the ACOSOG Z0030 Trial. *J Thorac Cardiovasc Surg*. In press.
458. Nomori H, Iwatani K, Kobayashi H, et al. Omission of mediastinal lymph node dissection in lung cancer: its techniques and diagnostic procedures. *Ann Thorac Cardiovasc Surg* 2006;12:83–88.
459. Finley DJ, Yoshizawa A, Travis W, et al. Predictors of outcomes after surgical treatment of synchronous primary lung cancers. *J Thorac Oncol* 2010;5:197–205.
460. Hayes DF, Allred C, Anderson BO, et al. Breast. In: SB Edge, DR Byrd, CC Compton, et al. (Eds.). AJCC Cancer Staging Manual, 7th Ed. New York: Springer, 2009. Pp. 347–376.

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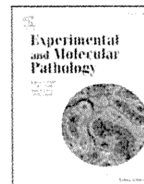

MT1-MMP plays an important role in an invasive activity of malignant pleural mesothelioma cell ☆

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ABSTRACT

Malignant pleural mesothelioma (MPM) has a poor prognosis and is a treatment resistant tumor, which is increasing in frequency throughout the world. The poor prognosis is due to the aggressive local invasiveness rather than distant metastasis. In this study, we established a cell line of malignant mesothelioma from a clinical specimen and assessed the relationship between the expression of MT1-MMP and the invasion ability of that line, as well as the cultured cells of several other lines, using the simple method that we created previously. We established a cell line from a clinical specimen from a patient with malignant mesothelioma. We assessed the invasive activities of MPM cells in an easy-to-prepare double-layered collagen gel hemisphere (DL-CGH) system that enabled us to visualize cell movements during invasion. To assess the role of MT1-MMP in the invasive activity of MPM cells, we knocked down its expression by RNA interference (RNAi). The invasion assay with DL-CGH revealed that a high expression of MT1-MMP in MPM cells was associated with aggressive invasive activity. The RNAi of MT1-MMP indicated that the expression of MT1-MMP might have a crucial role in the invasiveness of MPM cells. The MT1-MMP expression in MPM cells is related to their capacity for locally aggressive spreading into the pleura and the surrounding tissues, and MT1-MMP should be a suitable molecular target for the suppression of the invasiveness of MPM.

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Introduction

Malignant pleural mesothelioma (MPM) has a poor prognosis and is a treatment resistant tumor, which is increasing in frequency throughout the world (Robinson et al., 2005). MPM is not likely to metastasize distantly to other organs; its malignancy is due to its locally aggressive spreading into the pleura and surrounding tissues (Zhong et al., 2006; Pistolesi and Rusthoven, 2004).

It is said that the microenvironment (both cellular and extracellular elements) of the local host tissue plays an important role in the process of tumor cell invasion and that interaction between the ECM and tumor cells is essential for the degradation of ECM by the tumor cells (Liotta and Kohn, 2001). Matrix metalloproteinases (MMPs) are proteins that play an important role in this process (Curran and Murray, 2000).

The MMP family consists of more than 25 structurally related, zinc-dependent endopeptidases that are capable of degrading the basement membrane and the ECM (Konstantinopoulos et al., 2008). Among the members of this family, MMP-2 and MMP-14 play important roles in the MPM, and some epithelial malignant tumors show the overexpression of MMP-14 (Atkinson et al., 2007; Edwards et al., 2003). MMP-14, which is known as a membrane-type matrix metalloproteinase (MT-MMP), is mainly concentrated at the surface of the cells (Sato et al., 1994; Takino et al., 2007), so it is possible that MT1-MMP directly contributes to the degradation of the ECM. Because of these characteristics we focused on MMP-14 (MT1-MMP) as one of the potentially important factors that help MPM spread directly into other organs. Moreover, various methods for *in vitro* 3-D studies of cell invasion using a collagen gel have been described (Albini et al., 1987; Nyström et al., 2005; Duong et al., 2005; Takata et al., 2007), and we believe that these methods are very useful for the assessment of the invasion ability of MPM.

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In this study, we established a cell line of malignant mesothelioma from a clinical specimen. We then assessed the relationship between the expression of MT1-MMP and the invasion ability of this established cell line and other cell lines using the simple method that we created previously (Takata et al., 2007).

Materials and methods

Cell lines

The A549 (bronchiolo-alveolar carcinoma of lung) cell line was obtained from the Health Science Research Resources Bank (Osaka, Japan); the WI-38 cell line was obtained from the RIKEN Bioresource Center (Tsukuba, Japan). NCI-H28 (pleural effusion), NCI-H2452 (epithelial mesothelioma) and MSTO-211H (biphasic mesothelioma) were obtained from the American Type Culture Collection (Manassas, USA). Cells were maintained in RPMI-1640 medium supplemented with penicillin (100 U/mL), streptomycin (100 U/mL), and 10% bovine calf serum.

Establishment of a cell line of malignant mesothelioma

A clinical specimen from a patient with malignant mesothelioma was minced finely using scalpel or razor blade and digested in a cell dispersion enzyme solution (EZ; Nitta Gelatin Inc., Osaka, Japan) for 2 h. The dispersed cancer cells were treated with ethylene-glycol-tetra-acetic acid (EGTA)-trypsin and filtered through a 200-μm nylon mesh. The cells were then incubated in a collagen-gel-coated flask (CG-flask; Nitta Gelatin Inc., Osaka, Japan) containing a preculture medium with 10% fetal bovine serum (FBS; PCM-1; Nitta Gelatin Inc., Osaka, Japan) at 37 °C in 5% CO_2 overnight. We collected the viable cancer cells that adhered to the collagen gel and performed repeated subculturing until fibroblasts and other normal cells had disappeared.

Immunohistochemistry

Immunohistochemistry was performed to detect the MT1-MMP expression in paraffin sections, and tissue microarray samples were analyzed immunohistochemically. The MT1-MMP primary antibody (MAB3328, Chemicon International a Serologicals Company) was diluted 1:100 in a blocking solution before use. This diluted primary antibody was added to the tissue sections and incubated overnight at 4 °C. Antigen–antibody complexes were detected by the avidin–biotin peroxidase method (Vectastain Elite ABC Kit, Vector Laboratories, Inc., Burlingame, CA) and diaminobenzidine tetrahydrochloride reagents (DAKO EnVision™/HRP, Dako, Japan). Sections were counterstained with hematoxylin.

Western blotting

Cultured cells washed with PBS^- were lysed with 100-μl Laemmli sample buffer, and 10 μl of these samples were analyzed by SDS-PAGE. Then, the separated bands were transferred to nitrocellulose membranes (Amersham Biosciences Corp.). After washing the membranes with PBS-T, they were blocked for 30 minutes (5% skim milk, diluted by PBS-T). Following 2 rinses with PBS-T, membranes were incubated (1 hour, room temperature) with the primary antibody for MT1-MMP (MAB3328, Chemicon International a Serologicals Company), which was diluted 1:500 with 5% BSA/PBS-T. After washing with PBS-T, membranes were incubated (30 minutes, room temperature) with the secondary peroxidase-labeled sheep anti-mouse Ig whole antibody (Amersham Biosciences Corp.), which was diluted 1:5000 with PBS-T. Membranes were then washed with PBS-T and visualized using the luminoimage analyzer LAS-3000(Fuji film Inc., Tokyo, Japan) treated with a detection kit (Amersham Biosciences Corp.).

As a control assay, we performed Western blotting using the same membranes. The primary antibody was directed against β-actin (#AB6276, Abcam, Cambridge, UK), and the secondary antibody was peroxidase-labeled sheep anti-mouse Ig whole antibody (Amersham Biosciences Corp.).

Preparation of double-layered collagen gel hemispheres

Acid-soluble collagen I (Nitta Gelatin Inc., Osaka, Japan), tenfold concentrated Ham's F-12 medium, and reconstruction buffer (2.2-g $NaHCO_3$ + 4.77-g HEPES in 100 ml of 0.05-N NaOH) were mixed at a volume ratio of 8:1:1 and then seeded with cultured cells at a density of 3.0×10^6 cells/ml. Five microliters of the mixture, containing 3.0×10^4 cells, were dropped onto a plastic dish. Once the mixture had gelled, a second 30-μl drop of collagen was placed exactly on the top of the first gel drop, encapsulating it completely. The gel hemisphere was then submerged in medium and cultured. Cells were then stained with neutral red, and the gel was allowed to dry. The invasive activity of the cells was evaluated by measuring the expansion of red stain into the outer collagen layer.

RNA interference (RNAi) in WI38 and established mesothelioma cells

RNAi was performed with commercially available siRNAs (HP-validated siRNA for MT1-MMP; Qiagen GmbH, Hilden, Germany) and a non-silencing control siRNA (target sequence; AAT TCT CCG AAC GTG TCA CGT, Qiagen GmbH) according to the manufacturer's instructions. Briefly, 24 μl of transfection reagent (Hiperfect; Qiagen GmbH) was suspended in 200 μl of serum-free culture medium containing 6 μg siRNA. After a 10-minute incubation at room temperature, the mixture was added to WI38 and established mesothelioma cell culture (60-mm-round dish with 4-ml culture medium containing 10% fetal bovine serum and antibiotics mentioned above) grown to 60% confluence; the final concentration of the siRNA was 100 nM. After 24 hours (at 37 °C, 5% CO_2), these cells were suspended in phosphate buffered saline (PBS) and the cell density was calculated to prepare for the encapsulation of the cells in DL-CGH.

Time-lapse motion picture

A Moticam 2000 digital microscopy system (Shimadzu Rika Corp., Tokyo, Japan) was used to create motion pictures of cell invasion. The camera head was set at the position of the eyepiece on an inverted microscope (CKX31; Olympus Corp., Tokyo, Japan), and the entire microscope was then installed in a 37 °C, 5% CO_2 incubator without humidity (to prevent dew formation in the instruments). A DL-CGH prepared in the well of an ordinary 6-well plastic culture plate was submerged in proper medium; the residual 5 wells were filled with water to maintain humidity inside the plate. Cells were observed microscopically using a 10× objective lens, and the camera was operated from a personal computer running the Moticam 2000 software to capture and display images of living cells. Recording initiated 24 hours after DL-CGH culture continued for 96 hours. Images were captured automatically every 20 minutes, with 288 consecutive images stored as 800 × 600 pixel JPEG files. Using the Windows Movie Maker software (Microsoft Corp., Redmond, WA), we created a 30-second movie (saved as a WMV file) that displayed 288 consecutive images for 0.125 seconds each.

Result

Establishment of a cell line of malignant pleural mesothelioma from clinical specimen

We established an MPM cell line from a clinical specimen. To prove that these cells indeed were MPM, we sent samples of them to the

department of pathology in our hospital and requested an immunohistochemical analysis with calretinin, D2-40, CAM5.2, and AE1/AE3, which are useful markers of MPM (Mimura et al., 2007). While D2-40 was not identified, calretinin, AE1/AE3 and CAM5.2 were stained (Fig. 1). Thus, these cells were proved to be MPM immunochemically, and we had obtained a primary culture of MPM cells.

Expression of MT1-MMP in a clinical sample of MPM

To establish whether MT1-MMP was expressed in the MPM specimen we performed immunohistochemistry on the clinical samples of the MPM patient, following the technique described in Materials and methods (Fig. 2). We show the difference between normal cell structures and tumor cells in Fig. 2a. MT1-MMP was strongly expressed in the tumor cells, especially at the edge of the cells (Fig. 2b). In contrast, MT1-MMP was not expressed in the normal vascular endothelial cells.

Relationship between the MT1-MMP expression and the ability to invade, using cancer cells and normal fibroblasts

Western blotting was performed to determine if MT1-MMP was expressed in the cell lines of lung adenocarcinoma, fibroblasts and MPM (among them, the MPM cell line established in our laboratory). We detected a strong expression of MT1-MMP in WI38 and the established MPM cell line. But the expression of MT1-MMP was very weak in A549, the cell line of lung adenocarcinoma and NCI-H28, one of the acquired MPM cell lines. In the other MPM cell lines (NCI-H2452 and MSTO-211H), the expression of MT1-MMP was moderate (Fig. 3).

Then, we performed invasion assays with these cell lines using DL-CGH. A549 and NCI-H28, which showed a weak expression of MT1-MMP, showed only a minimal tendency to invade into the outer layer of collagen gel, whereas the other 4 cell lines, in which a strong expression of MP1-MMP was observed, showed a high tendency of invasion (Fig. 4). These invasive cells spread by extending their podocyte into the outer layer (time lapse).

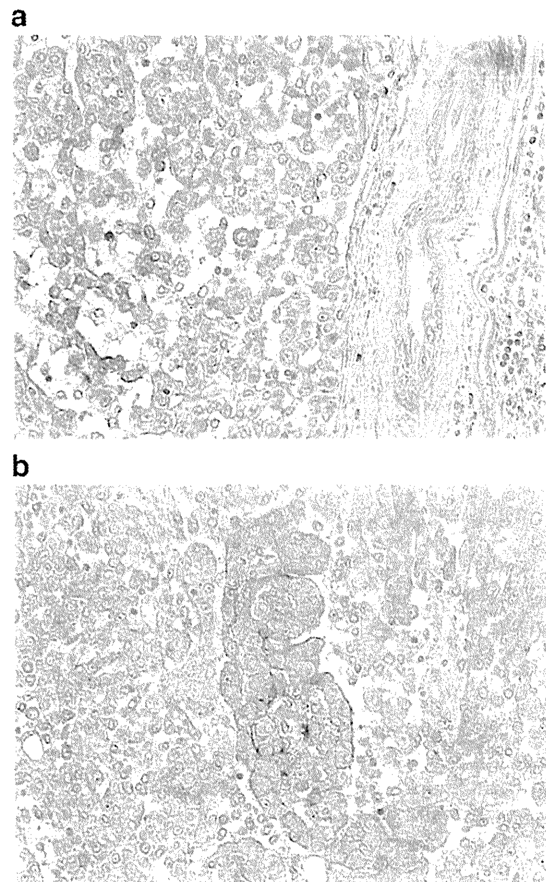

Fig. 2. Result of immunohistochemistry for MT1-MMP using surgical specimens of malignant pleural mesothelioma. Tumor cells expressed MT1-MMP strongly, but the normal vascular endothelial cells did not. (b) Especially, MT1-MMP was more expressed at the edge of the tumor cells than the inner area.

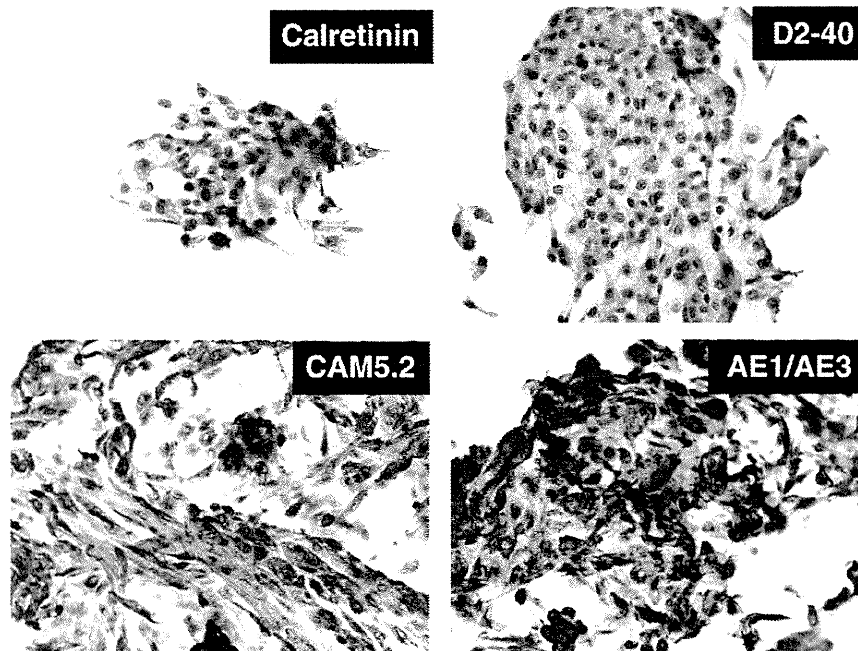

Fig. 1. Cell line established from a sample of malignant pleural mesothelioma. Calretinin, D2-40, CAM5.2, and AE1/AE3 were examined as useful markers of MPM. While D2-40 was not stained, calretinin, AE1/AE3 and CAM5.2 were stained.

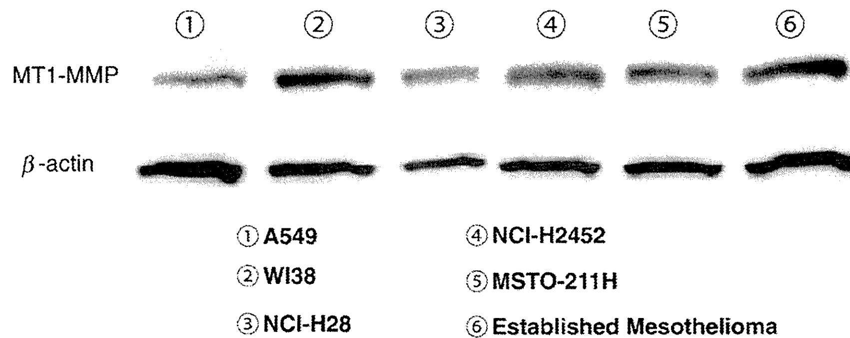

Fig. 3. Western blotting for MT1-MMP and β-actin. We detected a strong expression of MT1-MMP in WI38 and the established MPM cell line. The expression of MT1-MMP was very weak in A549, the cell line of lung adenocarcinoma, and NCI-H28, one of the acquired MPM cell lines. In the other MPM cell lines (NCI-H2452 and MSTO-211H), the expression of MT1-MMP was moderate.

Inhibition of MT1-MMP in the MPM cells and fibroblasts

We performed Western blotting to check if we could inhibit MT1-MMP in WI38 and the established MPM cell line, both of which showed wide spreading in the DL-CGH. The blotting showed about a 50% reduction in the expression of MT1-MMP protein relative to cells transfected with control siRNAs (Fig. 5).

In order to determine their invasive potential cells, transfected with inhibitory RNAs were embedded within the inner layer of DL-CGH and incubated for several days, after which we observed how the cells stained with neutral red. Cells of the established MPM cell line transfected with MT1-MMP RNAi showed only a slight invasion into the outer layer relative to the normal or control RNAi-transfected cells (Fig. 6). We also obtained similar results using WI38 cells (data not shown).

Discussion

In cell culture to establish a primary culture from a clinical specimen is one of the most difficult techniques, so many attempts result in failure. In this study, we succeeded in establishing the MPM cell line with the technique described in Materials and methods.

This is the first study to analyze the invasive activity of cell lines established from clinically resected specimens, with the aim of eventual clinical application. DL-CGH made it possible to visualize the invasive activity of the cells precisely, and the procedure would be useful for deciding a therapeutic strategy and predicting the clinical outcome. Also, the combination of DL-CGH and RNAi treatment of MT1-MMP revealed that the protein was a good candidate for a molecular target that would control the invasive activity of the cancer cells.

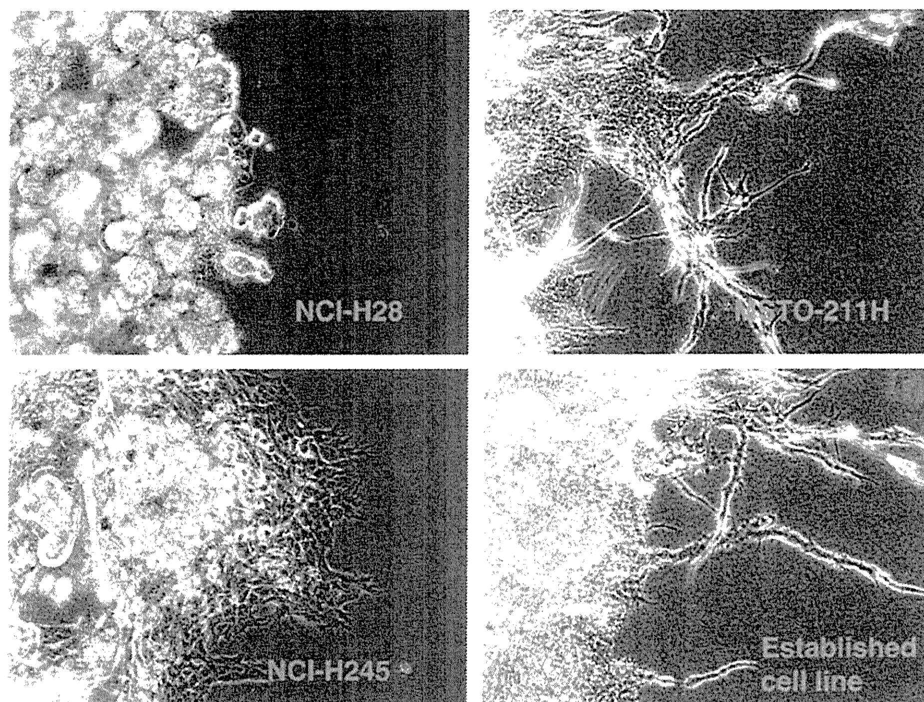

Fig. 4. Invasive activity of malignant mesothelioma cell lines assessed by DL-CGH. NCI-H28, which showed a weak expression of MT1-MMP, showed no tendency to invade the outer layer of collagen gel, whereas the other 3 cell lines showed a high tendency to invade the outer layer.

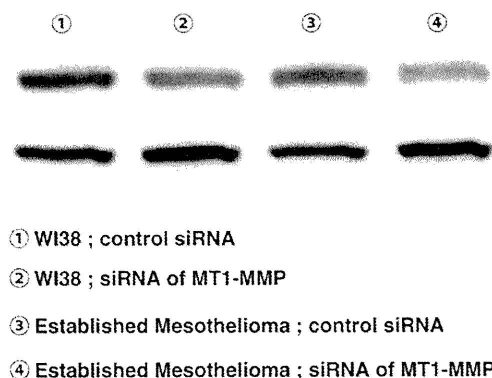

Fig. 5. Western blotting after transfection with siRNA for MT1-MMP. The blotting showed about a 50% reduction in the expression of the MT1-MMP protein relative to cells transfected with control siRNAs.

Invasion occurs within a tumor-host microenvironment, where stroma and tumor cells exchange enzymes and cytokines that modify the local extracellular matrix, stimulate migration, and promote proliferation and survival (Liotta and Kohn, 2001). It has been reported that the presence of fibroblasts is essential in cancer invasion (Olumi et al., 1999; Che et al., 2006; Gaggioli et al., 2007). The fibroblast itself is a benign mesenchymal cell that has no malignancy. Nevertheless, if fibroblasts interact with cancer cells, they play an important role in the tumor cell malignancy. In lung cancer, patients with small-sized bronchiolo-alveolar carcinoma (BAC) of the lung, in which cancer cells spread on the internal surface of alveoli but do not infiltrate interstitially, have a better prognosis than patients with BAC containing actively proliferating fibroblasts; in the latter case, cancer cells invade frequently into micro-vessels (Noguchi et al., 1995). In our study, WI38 cells (a fibroblast cell line) showed the overexpression of MT1-MMP, which indicates that fibroblasts are essential for degenerating the ECM and making tracks and scaffolding for the cancer cells. Not only fibroblasts but also some mesenchymal cells show the overexpression of MT1-MMP. Previous reports have stated that malignant mesothelioma cells produced a broad spectrum of MMPs, which might play an important role in cell invasion (Liu et al., 2001), and that the overexpression of MT1-MMP was observed in malignant mesothelioma (Sivertsen et al., 2006). In the *in vitro* experiments in this study, we observed that the level of MT1-MMP expression in established MPM cells was elevated and that these cells showed active invasion in the assay with DL-CGH.

Cancer-cell migration is typically regulated by integrins, matrix-degrading enzymes, cell–cell adhesion molecules and cell–cell communication (Friedl and Wolf, 2003). Although some tumor cells show sustained protease-independent migration resulting from a flexible amoeba-like shape change (Wolf et al., 2003), it is said that MT1-MMP is the key enzyme in the proteolytic macropatterning of collagen-rich ECM to generate space for the cell masses (Wolf et al., 2007) and that matrix degradation requires MMPs targeted to invadopodia (Sakurai-Yageta et al., 2008). In this study, we were able to establish that the invasive cells spread into the outer layer of the collagen gel by extending their podocyte (dendritic migration). Wolf et al. reported that HT1080 fibrosarcoma showed a spindle-shaped elongation of the cell body for invasion into 3-D collagen matrices (Wolf et al., 2003). We observed a similar phenomenon using the MPM cells and fibroblast. Thus, it is possible that the dendritic migration of mesenchymal cells (such as MPM) results from the overexpression of MT1-MMP.

In conclusion, the overexpression of MT1-MMP in MPM cells is associated with spreading into the surrounding matrix. Furthermore, MT1-MMP expressed in fibroblasts is involved in making a scaffold for the invasion of malignant tumor cells. Thus, we suggest that the degree of MT1-MMP expression is associated with the capacity for locally aggressive spreading into the pleura and the surrounding tissues and that MT1-MMP will be a molecular target for suppressing the invasion of MPM.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.yexmp.2010.10.008.

References

Albini, A., Iwamoto, Y., Kleinman, H.K., Martin, G.R., Aaronson, S.A., Kozlowski, J.M., McEwan, R.N., 1987. A rapid in vitro assay for quantitating the invasive potential of tumor cells. Cancer Res. 47, 3239–3245.

Atkinson, J.M., Pennington, C.J., Martin, S.W., Anikin, V.A., Mearns, A.J., Loadman, P.M., Edwards, D.R., Gill, J.H., 2007. Membrane type matrix metalloproteinases (MMPs) show differential expression in non-small cell lung cancer (NSCLC) compared to normal lung: Correlation of MMP-14 mRNA expression and proteolytic activity. Eur. J. Cancer 43, 1764–1771.

Che, Z.M., Jung, T.H., Choi, J.H., Yoon, D.J., Jeong, H.J., Lee, E.J., Kim, J., 2006. Collagen-based co-culture for invasive study on cancer cells-fibroblasts interaction. Biochem. Biophys. Res. Commun. 346, 268–275.

Curran, S., Murray, G.I., 2000. Matrix metalloproteinases: Molecular aspects of their roles in tumour invasion and metastasis. Eur. J. Cancer 36, 1621–1630.

Duong, H.S., Le, A.D., Zhang, Q., Messadi, D.V., 2005. A novel 3-dimensional culture system as an in vitro model for studying oral cancer cell invasion. Int. J. Exp. Pathol. 86, 365–374.

Edwards, J.G., McLaren, J., Jones, J.L., Waller, D.A., O'Byrne, K.J., 2003. Matrix metalloproteinases 2 and 9 (gelatinases A and B) expression in malignant mesothelioma and benign pleura. Br. J. Cancer 88, 1553–1559.

Friedl, P., Wolf, K., 2003. Tumour-cell invasion and migration: Diversity and escape mechanisms. Nat. Rev. Cancer 3, 362–374.

Gaggioli, C., Hooper, S., Hidalgo-Carcedo, C., Grosse, R., Marshall, J.F., Harrington, K., Sahai, E., 2007. Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells. Nat. Cell Biol. 9, 1392–1400.

Konstantinopoulos, P.A., Karamouzis, M.V., Papatsoris, A.G., Papavassiliou, A.G., 2008. Matrix metalloproteinase inhibitors as anticancer agents. Int. J. Biochem. Cell Biol. 40, 1156–1168.

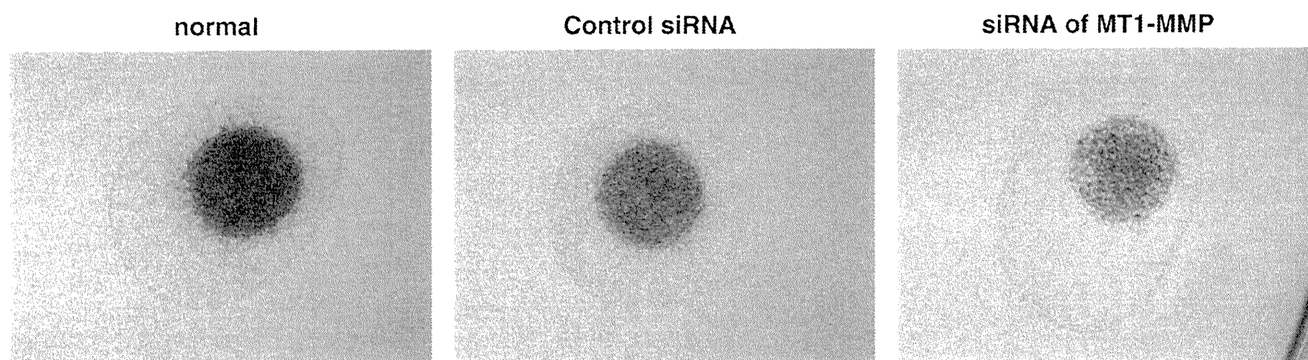

Fig. 6. Result of DL-CGH using MPM cell lines stained with neutral red. In the established MPM cell line, cells transfected with MT1-MMP RNAi showed only a slight invasion into the outer layer relative to that of the normal cells and control-treated cells.

Liotta, L.A., Kohn, E.C., 2001. The microenvironment of the tumour–host interface. Nature 411, 375–379.

Liu, Z., Ivanoff, A., Klominek, J., 2001. Expression and activity of matrix metalloproteases in human malignant mesothelioma cell lines. Int. J. Cancer 91, 638–643.

Mimura, T., Ito, A., Sakuma, T., Ohbayashi, C., Yoshimura, M., Tsubota, N., Okita, Y., Okada, M., 2007. Novel marker D2-40, combined with calretinin, CEA, and TTF-1: An optimal set of immunodiagnostic markers for pleural mesothelioma. Cancer 109, 933–938.

Noguchi, M., Morikawa, A., Kawasaki, M., Matsuno, Y., Yamada, T., Hirohashi, S., Kondo, H., Shimosato, Y., 1995. Small adenocarcinoma of the lung. Histologic characteristics and prognosis. Cancer 75, 2844–2852.

Nyström, M.L., Thomas, G.J., Stone, M., Mackenzie, I.C., Hart, I.R., Marshall, J.F., 2005. Development of a quantitative method to analyse tumour cell invasion in organotypic culture. J. Pathol. 205, 468–475.

Olumi, A.F., Grossfeld, G.D., Hayward, S.W., Carroll, P.R., Tlsty, T.D., Cunha, G.R., 1999. Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. Cancer Res. 59, 5002–5011.

Pistolesi, M., Rusthoven, J., 2004. Malignant pleural mesothelioma: Update, current management, and newer therapeutic strategies. Chest 126, 1318–1329.

Robinson, B.W., Musk, A.W., Lake, R.A., 2005. Malignant mesothelioma. Lancet 366, 397–408.

Sakurai-Yageta, M., Recchi, C., Le Dez, G., Sibarita, J.B., Daviet, L., Camonis, J., D'Souza-Schorey, C., Chavrier, P., 2008. The interaction of IQGAP1 with the exocyst complex is required for tumor cell invasion downstream of Cdc42 and RhoA. J. Cell Biol. 181, 985–998.

Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E., Seiki, M., 1994. A matrix metalloproteinase expressed on the surface of invasive tumour cells. Nature 370, 61–65.

Sivertsen, S., Hadar, R., Elloul, S., Vintman, L., Bedrossian, C., Reich, R., Davidson, B., 2006. Expression of snail, slug and Sip1 in malignant mesothelioma effusions is associated with matrix metalloproteinase, but not with cadherin expression. Lung Cancer 54, 309–317.

Takata, M., Maniwa, Y., Doi, T., Tanaka, Y., Okada, K., Nishio, W., Ohbayashi, C., Yoshimura, M., Hayashi, Y., Okita, Y., 2007. Double-layered collagen gel hemisphere for cell invasion assay: successful visualization and quantification of cell invasion activity. Cell Commun. Adhes. 14, 157–167.

Takino, T., Saeki, H., Miyamori, H., Kudo, T., Sato, H., 2007. Inhibition of membrane-type 1 matrix metalloproteinase at cell-matrix adhesions. Cancer Res. 67, 11621–11629.

Wolf, K., Mazo, I., Leung, H., Engelke, K., von Andrian, U.H., Deryugina, E.I., Strongin, A.Y., Bröcker, E.B., Friedl, P., 2003. Compensation mechanism in tumor cell migration: Mesenchymal-amoeboid transition after blocking of pericellular proteolysis. J. Cell Biol. 160, 267–277.

Wolf, K., Wu, Y.I., Liu, Y., Geiger, J., Tam, E., Overall, C., Stack, M.S., Friedl, P., 2007. Multi-step pericellular proteolysis controls the transition from individual to collective cancer cell invasion. Nat. Cell Biol. 9, 893–904.

Zhong, J., Gencay, M.M., Bubendorf, L., Burgess, J.K., Parson, H., Robinson, B.W., Tamm, M., Black, J.L., Roth, M., 2006. ERK1/2 and p38 MAP kinase control MMP-2, MT1-MMP, and TIMP action and affect cell migration: A comparison between mesothelioma and mesothelial cells. J. Cell Physiol. 207, 540–552.

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Oncogenic phosphatase Wip1 is a novel prognostic marker for lung adenocarcinoma patient survival

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DNA damage response pathways are important for maintaining genomic stability. The oncogenic phosphatase Wip1 plays a crucial role in DNA damage response by inhibiting several cell cycle proteins, including p53. Although *Wip1* gene amplification has been reported in various primary tumors, including lung cancer, its biological significance for survival of primary lung tumor patients remains unclear. We investigated the expression of Wip1 in cancer epithelial cells immunohistochemically in 84 consecutive resected cases of lung adenocarcinoma. Increased Wip1 expression was observed in 54 (64.3%) of the 84 cases. Wip1 expression was found to be correlated significantly with two clinicopathological factors: γ-H2AX expression, and invasion to the pulmonary vein. A univariate analysis and log–rank test indicated a significant association between Wip1 expression and lower overall survival rate ($P = 0.019$ and $P = 0.0099$, respectively). A multivariate analysis also indicated a statistically significant association between increased Wip1 expression and lower overall survival rate (hazard ratio, 4.3; $P = 0.026$). The Ki67 index level was higher in the Wip1-positive group than in the negative group ($P < 0.04$, Mann–Whitney *U*-test). Moreover, in a subgroup analysis of only stage I patients, increased Wip1 expression was also significantly associated with a lower overall survival rate ($P = 0.023$, log–rank test). These results indicate that the increased expression of Wip1 in cancer epithelial cells has significant value for tumor progression and the clinical prognosis of patients with primary lung adenocarcinoma. (*Cancer Sci* 2011; 102: 1101–1106)

Cellular DNA is constantly exposed to various environmental and endogenous mutagenic insults. To maintain genomic integrity and prevent cancers caused by these potentially mutagenic events, a sophisticated array of damage sensors, signaling molecules, and repair functions have evolved. Among the key sensors of DNA damage are the phosphoinositide-3-kinase-related kinase family, which includes ATM (ataxia-telangiectasia mutated), ATR (ataxia-telangiectasia and Rad3-related), and DNA-PK_{cs} (DNA-dependent protein kinase catalytic subunit).[1,2] A direct role for the ATM/ATR-initiated damage response pathways in cancer prevention has been recently determined.[3,4] Human pre-neoplastic lesions from a variety of different human cancers were shown to express various markers reflecting responses to DNA damage response, including activated and phosphorylated ATM, Chk2, p53, and H2AX.[3,4] In particular, phosphorylated H2AX (called γ-H2AX) plays a crucial role in recruiting DNA damage response factors to damage sites for accurate DNA repair and is considered a specific and sensitive molecular marker of DNA damage and repair.[5–7] Interestingly, late-stage tumors often show loss of these DNA damage response markers, suggesting that a decrease in the activity of DNA damage response pathways may contribute to cancer progression.[3,4]

Wild-type p53-induced phosphatase 1 (Wip1), also called PPM1D, is a member of the magnesium-dependent serine/threonine protein phosphatase (PPM) family.[8] These proteins, whose defining member is PP2Cα, are present in both prokaryotes and eukaryotes.[9] The human *Wip1* gene was first identified as a transcript induced by ultraviolet and ionizing radiation in a p53-dependent manner.[10] To date, Wip1 has been shown to dephosphorylate at least six proteins, ATM, Chk1, Chk2, p53, p38, and Mdm2.[11] A number of studies have shown that the Wip1 phosphatase is a key integrator of a response that attenuates signaling through the ATM and ATR pathways and negatively regulates the stress-responsive p38 MAPK pathway.[11] Furthermore, several reports recently showed that Wip1 directly dephosphorylated γ-H2AX, which might result in attenuating the DNA damage response.[12,13] Thus, Wip1 is considered to be an inhibitor or homeostatic regulator of the DNA damage response that facilitates the return of cells to a normal pre-stress state following DNA damage repair.

In addition, Wip1 is regarded as an oncogenic phosphatase because of the above noted functions. Indeed, amplified levels of *Wip1* have been found in cancer cell lines of the lung, breast, pancreas, bladder, liver, and meninges, and neuroblastomas.[14,15] Moreover, a number of human primary tumors (e.g., breast adenocarcinoma, ovarian clear cell adenocarcinoma, neuroblastoma, and pancreatic adenocarcinoma) contain amplified *Wip1* gene and high levels of Wip1 protein, which appear to correlate with poor prognosis for cancer patients.[16–19] However, it is still unknown whether Wip1 overexpression affects the survival of primary lung carcinoma patients. In this study, we analyzed the expression of Wip1 by immunohistochemistry in surgically resected human primary pulmonary adenocarcinoma tissue from 84 patients. We also investigated whether Wip1 expression in tumor tissues influenced the outcome of these patients.

Materials and Methods

Collection of samples and patient data. Eighty-four patients (46 males, 38 females) examined and treated at Kobe University Hospital (Kobe City, Japan) between 2001 and 2003 for lung adenocarcinoma were evaluated for this study. The study was approved by the Regional Ethics Committee for Clinical Research of Kobe University and conducted according to the principles in the Declaration of Helsinki. All patients gave dated and written informed consent. Primary tumors and adjacent non-neoplastic lung tissue were obtained at the time of surgery. Peripheral portions of resected lung carcinomas were sectioned, evaluated by a pathologist, and used for immunohistochemistry (IHC).

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All patients were consecutively enrolled in this study. Detailed clinical and demographic information, prognostic factors, and disease progression were collected retrospectively.

Immunohistochemistry. Formalin-fixed paraffin-embedded specimens were sectioned in 5 μm-thick slices and sections were deparaffinized with xylene and rehydrated with ethanol. Antigen retrieval was carried out by placing specimens in Dako REAL Target Retrieval Solution (Dako, Glostrup, Denmark) at 98°C for 20 min. Rabbit anti-human Wip1 polyclonal antibodies (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-human phospho-histone H2AX (S139) polyclonal antibodies (5 μg/mL; R&D Systems, Minneapolis, MN, USA) were used as the primary antibodies for detection of Wip1 and γ-H2AX, respectively. The Dako EnVision/HRP Universal (DAB) kit (Dako) was used for endogenous peroxidase blocking, treatment with a secondary antibody against anti-rabbit and anti-mouse immunoglobulin antibody, and the visualization of HRP. Hematoxylin staining was used as the counterstain. Photographs of immunohistochemical stained sections were taken by a camera mounted on a Keyence BZ-8000 digital microscope (Keyence, Osaka, Japan).

Detection of *EGFR* gene mutation. Genomic DNA of tumor cells was successfully extracted from 19 paraffin-embedded tissue specimens.[20] *EGFR* gene (exons 18–21) of the DNA samples was investigated by the peptide nucleic acid-locked nucleic acid PCR clamp method.[21]

Classification of immunohistochemically stained patterns. Immunochemically stained sections were classified by light microscopy. For the assessment of the protein expression of Wip1, samples were classified as Wip1-positive if the ratio of stained cells in total epithelial cancer cells of a tumor tissue was more than 10%; if samples contained <10% stained cells, they were classified as Wip1-negative. Ten percent was used as the cut-off value because of the statistical advantage in this study. For evaluation of γ-H2AX expression, the cut-off value (the ratio of stained cells in total epithelial cancer cells) was set at 3% to obtain high sensitivity for detecting DNA damage. Sample classification was done independently by two pathologists (C.O. and Y.H.) in a blind manner. Ki67 (MIB-1) index (Ki67 expression ratio) and tumor protein p53 (TP53) expression were determined by the Division of Diagnostic Pathology, Kobe University.

Statistical analysis. All statistical analyses were carried out using Stata software version 10.1 (Stata, College Station, TX, USA). Baseline characteristics were reported as percentages for categorical variables and means for ±SD for continuous variables. Fisher's exact or Student's *t*-test were used to examine the association between Wip1 expression and various clinicopathological parameters. For survival analyses, we used the Kaplan–Meier method, and statistical significance between survival curves was assessed by the log–rank test. Overall survival (OS) and relapse-free interval (RFI) were determined from the date of surgery to the time of death or relapse, respectively. The Cox proportional hazards model was used to examine the association between the OS and the RFI and potential prognostic factors. Data were censored at the time of last visit. Significant variables from the univariate analysis were entered into the Cox hazard model analysis. Probability values <0.05 were considered statistically significant in all analyses.

Results

Wip1 expression in epithelial cancer cells of human lung adenocarcinoma. The expression of Wip1 was examined in 84 lung adenocarcinomas and the adjacent normal lung tissues by IHC using anti-human Wip1 polyclonal antibodies. In normal lung tissues, the expression of Wip1 was not detected (Fig. 1A). In some tumor tissues, Wip1 expression was observed in cancer cells (Fig. 1B–D). The frequency of Wip1-stained samples was 64.3% of all samples examined (54/84).

Relationship between Wip1 expression and clinicopathological characteristics of patients. For assessment purposes, we regarded specimens as Wip1 positive if 10% or more cancer cells within a tumor were strongly stained; all other specimens were regarded as negative. Based on this, 54 specimens were classified as Wip1 positive (64.3%) and 30 specimens as Wip1 negative (35.7%).

The relationships between Wip1-positive cases and various clinicopathological characteristics at the time of surgery are shown in Table 1. Expression of γ-H2AX was observed in 38 of 84 specimens (45.2%). Increased expression of Wip1 was significantly associated with γ-H2AX expression ($P < 0.001$) and cancer invasion to the pulmonary vein ($P = 0.019$). Wip1 expression was not significantly related to age ($P = 0.59$),

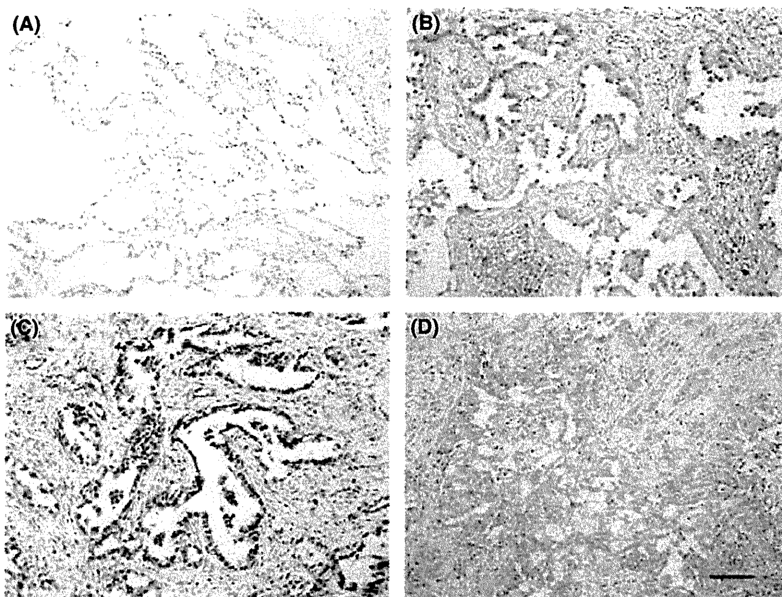

Fig. 1. Immunohistochemical analysis of expression of oncogenic phosphatase Wip1 in epithelial cancer cells of human primary lung adenocarcinoma. (A) Wip1-negative normal lung tissue. (B) Wip1-negative tumor tissue. Cancer cells were not stained. (C,D) Wip1-positive tumor tissues. Cancer cells were diffusely stained. Scale line = 100 μm (magnification, ×200).

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Table 1. Association between increased expression of oncogenic phosphatase Wip1 and clinicopathologic characteristics in 84 patients with lung adenocarcinoma

Variable	Total	Wip1		P-value
		Negative	Positive	
No. patients (%)	84	30 (35.7)	54 (64.3)	NA
Age in years, mean ± SD (range)	67.3 ± 9.1 (42–81)	68.0 ± 8.5 (49–80)	66.9 ± 9.5 (42–81)	0.59*
Gender				
Male/female	46/38	19/11	27/27	0.26
T factor				
T1/T2/T3/T4	45/31/3/4†	20/10/0/0	25/21/3/4	0.17
N factor				
N0/N1/N2/N3	59/8/15/1†	24/3/3/0	35/5/12/1	0.49
M factor				
M0/M1	82/1†	30/0	52/1	1.0
Stage				
I/II/III, IV	56/10/17†	24/3/3	32/7/14	0.14
P factor				
0/1/2/3	56/14/10/4	23/4/3/0	33/10/7/4	0.43
PA invasion				
Negative/positive	67/15‡	26/3	41/12	0.24
PV invasion				
Negative/positive	47/35‡	22/7	25/28	0.019
LY invasion				
Negative/positive	50/32‡	22/7	28/25	0.058
TP53 expression				
Negative/positive	45/39	16/14	29/25	1.0
γ-H2AX expression				
Negative/positive	46/38	25/5	21/33	<0.001

*P-value by Student's t-test. Fisher's exact test was used for statistical analysis. †One sample missing. ‡Two samples missing. LY, lymphatic duct; NA, not applicable; PA, pulmonary artery; PV, pulmonary vein.

gender (P = 0.26), TNM stage (P = 0.14), T factor (P = 0.17), P factor (P = 0.43) according to the criteria of the International Staging System for Lung Cancer, lymph node metastasis (0.49), distant metastasis (P = 1.0), cancer invasion to the pulmonary artery (P = 0.24) or the lymphatic ducts (P = 0.058), or TP53 expression (P = 1.0). *EGFR* mutation was detected in 6 of 19 samples (31.6%). Wip1 expression was not significantly related to *EGFR* mutation (three samples with *EGFR* mutation in nine Wip1-negative samples and 3 in 10 Wip1-positive samples; 33.3% and 30.0%, respectively; P = 1.0).

Increased expression of Wip1 related to poor patient prognosis and proliferation of cancer cells. Using the data collected from 84 study patients, we evaluated their prognosis and its relationship to the expression of Wip1. We examined the OS of Wip1-negative and Wip1-positive groups and found a statistically significant difference between the two groups using the log–rank test (P = 0.0099). As shown, survival of Wip1-negative patients was greater than that observed for Wip1-positive patients (Fig. 2). Moreover, using the Mann–Whitney U-test, the Ki67 index level was higher in the Wip1-positive group than in the negative group (Fig. 3). The median Ki67 index was 6% and 10% in Wip1-negative and Wip1-positive tumors, respectively. A univariate analysis indicated that among clinicopathological factors, tumor classification, lymph node metastasis, and increased Wip1 expression correlated with outcome (Table 2). Further assessment using the Cox multivariate analysis indicated that gender (male), lymph node metastasis, and increased Wip1 expression were statistically significant predictors for OS (Table 2).

We also analyzed the RFI rate for increased Wip1 expression. In our study, the RFI rate in patients positive for increased Wip1 expression was notably lower than that in the negative group (P = 0.013, log–rank test; data not shown). Univariate analysis of RFI also indicated that increased Wip1 expression correlated with outcome (P = 0.018, hazard ratio; 2.9, 95% CI; 1.2–7.2).

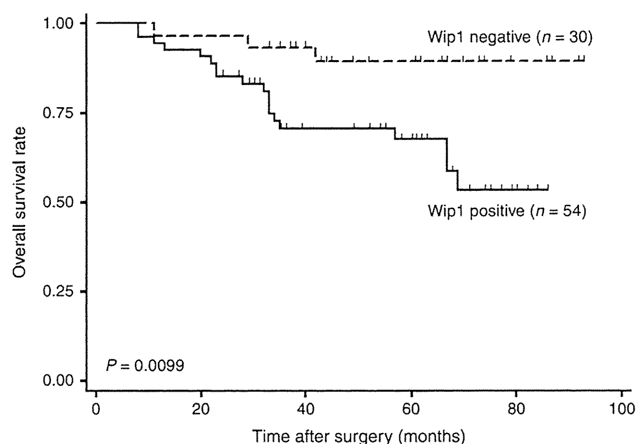

Fig. 2. Kaplan–Meier plot of the overall survival rate in 84 patients with lung adenocarcinoma, and its relationship to expression of oncogenic phosphatase Wip1. P-value determined using the log–rank test.

Increased expression of Wip1 also related to poor patient prognosis in stage I lung adenocarcinoma. In the stage I cases, 32 (57.1%) and 24 (42.9%) patients were classified as Wip1 positive and Wip1 negative, respectively (Table 1). A survival analysis that included only stage I patients revealed that the overall survival curve for the Wip1-positive group was lower than the Wip1-negative group. The log–rank test showed that the difference was statistically significant (P = 0.023) (Fig. 4).

Discussion

In the present study, we carried out IHC staining of human primary adenocarcinoma tissue specimens to detect the protein

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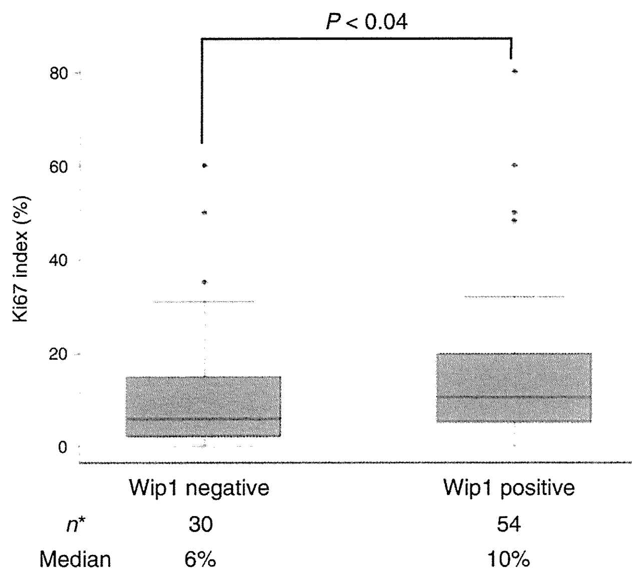

Fig. 3. Ki67 index (%) in lung adenocarcinoma samples and its relationship to the expression of oncogenic phosphatase Wip1. *P*-value determined using the Mann–Whitney *U*-test. **n*, number of lung tumors.

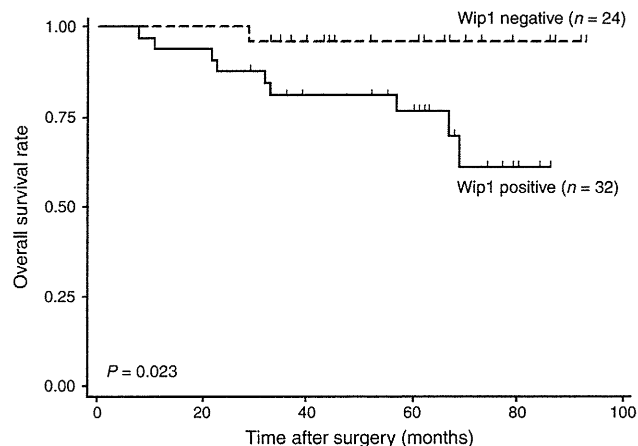

Fig. 4. Kaplan–Meier plot of the overall survival rate in 84 patients with lung adenocarcinoma and its relationship to expression of oncogenic phosphatase Wip1 in stage I patients. *P*-value determined using the log-rank test.

Table 2. Univariate and multivariate analysis of the association between the overall survival of 84 patients with lung adenocarcinoma and prognostic factors, by Cox proportional hazard models

Variable	Hazard ratio	95% Confidence interval	*P*-value
Univariate			
Age	1.0	1.3–14.6	0.93
Gender (male *versus* female)	0.51	0.21–1.3	0.14
T factor (T1<)	2.9	1.2–7.1	0.021
LN (negative *versus* positive)	3.8	1.6–8.9	0.002
PV invasion (negative *versus* positive)	2.2	0.92–5.1	0.077
Wip1 (negative *versus* positive)	4.3	1.3–14.6	0.019
Multivariate			
Age	1.0	0.97–1.1	0.42
Gender (male *versus* female)	0.31	0.11–0.84	0.031
T factor (T1<)	2.2	0.81–5.9	0.12
LN (negative *versus* positive)	3.4	1.3–9.2	0.015
PV (negative *versus* positive)	0.63	0.21–1.9	0.42
Wip1 (negative *versus* positive)	4.3	1.2–15.6	0.026

LN, lymph node metastasis; PV, invasion to pulmonary vein.

expression of oncogenic phosphatase Wip1 and observed the increased expression of Wip1 in tumor tissues, but not in normal lung tissues. The increased Wip1 expression was associated significantly with lower overall survival rate of lung adenocarcinoma patients. To our knowledge, this is the first study to detect protein expression of Wip1 in lung adenocarcinoma and to report that Wip1 expression might be a useful prognostic marker for lung adenocarcinoma patient survival.

Using IHC staining, increased Wip1 protein expression was observed in 64.3% (54/84) of lung adenocarcinoma specimens but was not detected in adjacent non-neoplastic lung tissues (Fig. 1). In order to define the effects of increased Wip1 expression on the prognosis of patients with lung cancer, a prognostic analysis was carried out on follow-up data. The results of the survival analysis showed that the OS rate in patients positive for increased Wip1 expression was notably lower than that of the Wip1-negative group (Fig. 2). These findings indicate that increased Wip1 expression negatively affects the clinical course and that increased Wip1 expression is correlated with malignant behavior of tumors. Our Cox multivariate analysis indicated that increased Wip1 expression, gender (male), and lymph node metastasis were significant prognostic predictors. It has been reported that once lung adenocarcinoma was resected completely, women survived longer than male patients.[22] Furthermore, a prognostic analysis that included only stage I cases revealed that the OS rate of the Wip1-positive group was significantly lower than that of the Wip1-negative group. These findings suggest that increased Wip1 expression may be used as a reference index for molecular staging of patients with a high risk of death who are likely to benefit from intensive adjuvant therapy.

A number of recent reports indicate that Wip1 overexpression in mouse embryonic fibloblasts and transgenic mice promotes cell transformation and accelerated cancer progression.[14,23,24] Furthermore, *Wip1*-disrupted mice are resistant to mammary cancer, and even when tumors form in such mice, their tumor cells have a lower proliferation potential.[15] It has been suggested that the effects of Wip1 overexpression might be due to its dephosphorylation of p38, p53, and regulators of p53 (ATM, Chk1, Chk2).[11] Although the direct downstream effector of Wip1 leading to tumor progression is still unclear, we consider it more likely that increased Wip1 expression contributes to cell proliferation. For this reason, we examined the relationship between increased Wip1 expression and cell proliferation. As an indicator of cell proliferation, we used the Ki67 (MIB-1) expression index (determined by pathologists in the Division of Diagnostic Pathology, Kobe University). Using the Mann–Whitney *U*-test, the Ki67 index level was higher in the Wip1-positive group than in the negative group (Fig. 3). Moreover, the size of tumors (mm^3) was slightly greater in the Wip1-positive group than in the negative group ($P = 0.062$, Mann–Whitney *U*-test, median; 12.0 *vs* 8.4 mm^3, data not shown). In the stage I patients, the Ki67 index levels tended to be higher in the Wip1-positive group than in the negative group ($P = 0.084$, Mann–Whitney *U*-test, data not shown). In our study, increased expression of Wip1 was significantly associated with cancer invasion to the pulmonary vein ($P = 0.019$) and tended to be related to cancer invasion to the pulmonary lymphatic vessel ($P = 0.058$; Table 2). These

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results suggest that increased Wip1 expression may enhance cancer cell proliferation and tumor progression, resulted in cancer invasion to the tumor vessels.

Multiple studies showed that continuous formation of DNA double-strand breaks might contribute to increased genomic instability, leading to tumorigenesis, because of breach of a barrier (such as DNA damage response including *p53* activation).[3,4,25,26] In this study, IHC staining of γ-H2AX protein was carried out to detect presence of DNA damage in the tumor tissues and γ-H2AX expression was observed in 38 of 84 samples (45.2%; Table 1). Interestingly, our result showed that increased expression of Wip1 was significantly associated with γ-H2AX expression ($P < 0.001$). In the presence of DNA damage (indicated by γ-H2AX expression), Wip1 expression might be activated in the process of DNA damage response.[11] It is still unknown whether increased Wip1 expression results from genomic instability or not, and further studies will be required to substantiate these notions.

Alterations of the p53 tumor suppressor gene are the most common genetic changes found in human malignancies, including lung cancer.[27] Although a number of clinical prognostic studies of p53 mutations in lung cancer have been reported, using either IHC or molecular analysis, their effects on survival are unclear. Most studies suggest that the prognosis of patients with mutations in p53 are poorer than those devoid of such alterations,[28] however, others have reported an opposite relationship.[29,30] In our study, overexpression of mutated p53 was observed in 39 of 84 (46.4%) lung adenocarcinoma specimens (Table 1). However, the presence of mutated p53 did not significantly affect the overall survival rate ($P = 0.85$, data not shown). It was previously reported that only one of eight primary breast tumors with elevated levels of Wip1 showed p53 mutations and that Wip1 overexpression correlated with a poor prognosis despite the absence of p53 mutations in the same tumor.[14] In our studies (using IHC staining) we did not observe any association between increased Wip1 expression and p53 mutations in lung adenocarcinoma (Table 1). Recently, it has been reported that activating mutations of *EGFR* are present in a subset of pulmonary adenocarcinomas and also prognostic for survival benefit.[31,32] In this study, *EGFR* mutation was detected in 6 of 19 lung adenocarcinomas (31.6%) and increased Wip1 expression was not significantly related to *EGFR* mutation (three samples with *EGFR* mutation of nine Wip1-negative samples and 3 of 10 Wip1-positive samples; 33.3% and 30.0%, respectively; $P = 1.0$). These results suggest that Wip1 expression itself was not directly related to development of EGFR mutation.

It has been recently reported that p38α MAPK is essential for both proliferation and differentiation of lung stem and progenitor cells, and that the downregulation of p38α might result in human lung tumorigenesis.[33] According to these results, p38 MAPK that is dephosphorylated by Wip1 can negatively regulate the action of EGFR in the proliferation and self-renewal of lung stem and progenitor cells. Interestingly, p38 protein expression was approximately three times lower in human lung tumor samples than that found in normal lung tissues. Thus, p38 dephosphorylation, resulting in upregulation of EGFR, might explain why Wip1 enhances the progression and malignancy of lung adenocarcinoma. Although the downstream factor(s) in the Wip1 pathway that can explain the relationship between increased Wip1 expression and poor prognosis of lung adenocarcinoma patients is presently unknown, dephosphorylation of p38, p53, and γ-H2AX by Wip1 may contribute importantly to tumorigenesis and tumor progression. Thus, Wip1 might be a new lung cancer therapy target.

In conclusion, our results suggest that increased Wip1 expression in cancer cells in primary lung adenocarcinoma plays an important role in the progression of lung adenocarcinoma and acts as a negative factor for the prognosis of patients. These results suggest that increased expression of Wip1 can be used as a reference index of molecular staging to select patients at high risk of death as well as relapsed patients who may benefit from intensive adjuvant therapy.

Disclosure Statement

None of the authors have any interests which may be perceived as posing a conflict or bias.

References

1 Abraham RT. Cell cycle checkpoint signaling through the ATM and ATR kinases. *Genes Dev* 2001; **15**: 2177–96.

2 Shiloh Y. ATM and related protein kinase: safeguarding genome integrity. *Nat Rev Cancer* 2003; **3**: 155–68.

3 Gorgoulis VG, Vassiliou LV, Karakaidos P *et al.* Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. *Nature* 2005; **434**: 907–13.

4 Bartkova J, Horejsí Z, Koed K *et al.* DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature* 2005; **434**: 864–70.

5 Bonner WM, Redon CE, Dickey JS *et al.* γH2AX and cancer. *Nat Rev Cancer* 2008; **8**: 957–67.

6 Mah LJ, El-Osta A, Karagiannis TC. γH2AX: a sensitive molecular marker of DNA damage and repair. *Leukemia* 2010; **24**: 679–86.

7 Yu T, MacPhail SH, Banáth JP *et al.* Endogenous expression of phosphorylated histone H2AX in tumors in relation to DNA double-strand breaks and genomic instability. *DNA Repair (Amst)* 2006; **5**: 935–46.

8 Moorhead GB, Trinkle-Mulcahy L, Ulke-Lemée A. Emerging roles of nuclear protein phosphatases. *Nat Rev Mol Cell Biol* 2007; **8**: 234–44.

9 Jackson MD, Denu JM. Molecular reactions of protein phosphatases – insights from structure and chemistry. *Chem Rev* 2001; **101**: 2313–40.

10 Fiscella M, Zhang H, Fan S *et al.* Wip1, a novel human protein phosphatase that is induced in response to ionizing radiation in a p53-dependent manner. *Proc Natl Acad Sci U S A* 1997; **94**: 6048–53.

11 Lu X, Nguyen TA, Moon SH *et al.* The type 2C phosphatase Wip1: an oncogenic regulator of tumor suppressor and DNA damage response pathways. *Cancer Metastasis Rev* 2008; **27**: 123–35.

12 Cha H, Lowe JM, Li H *et al.* Wip1 directly dephosphorylates γ-H2AX and attenuates the DNA damage response. *Cancer Res* 2010; **70**: 4112–22.

13 Moon SH, Lin L, Zhang X *et al.* Wild-type p53-induced phosphatase 1 dephosphorylates histone variant γ-H2AX and suppresses DNA double strand break repair. *J Biol Chem* 2010; **285**: 12935–47.

14 Bulavin DV, Demidov ON, Saito S *et al.* Amplification of PPM1D in human tumors abrogates p53 tumor-suppressor activity. *Nat Genet* 2002; **31**: 210–5.

15 Bulavin DV, Phillips C, Nannenga B *et al.* Inactivation of the Wip1 phosphatase inhibits mammary tumorigenesis through p38 MAPK-mediated activation of the p16(Ink4a)-p19(Arf) pathway. *Nat Genet* 2004; **36**: 343–50.

16 Rauta J, Alarmo EL, Kauraniemi P *et al.* The serine-threonine protein phosphatase PPM1D is frequently activated through amplification in aggressive primary breast tumours. *Breast Cancer Res Treat* 2006; **95**: 257–63.

17 Hirasawa A, Saito-Ohara F, Inoue J *et al.* Association of 17q21-q24 gain in ovarian clear cell adenocarcinomas with poor prognosis and identification of PPM1D and APPBP2 as likely amplification targets. *Clin Cancer Res* 2003; **9**: 1995–2004.

18 Saito-Ohara F, Imoto I, Inoue J *et al.* PPM1D is a potential target for 17q gain in neuroblastoma. *Cancer Res* 2003; **63**: 1876–83.

19 Loukopoulos P, Shibata T, Katoh H *et al.* Genome-wide array-based comparative genomic hybridization analysis of pancreatic adenocarcinoma: identification of genetic indicators that predict patient outcome. *Cancer Sci* 2007; **98**: 392–400.

20 Coombs NJ, Gough AC, Primrose JN. Optimisation of DNA and RNA extraction from archival formalin-fixed tissue. *Nucleic Acids Res* 1999; **27**: e12.

21 Nagai Y, Miyazawa H, Huqun *et al.* Genetic heterogeneity of the epidermal growth factor receptor in non-small cell lung cancer cell lines revealed by a rapid and sensitive detection system, the peptide nucleic acid-locked nucleic acid PCR clamp. *Cancer Res* 2005; **65**: 7276–82.

22 Minami H, Yoshimura M, Miyamoto Y *et al.* Lung cancer in women: sex-associated differences in survival of patients undergoing resection for lung cancer. *Chest* 2000; **118**: 1603–9.

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23 Nannenga B, Lu X, Dumble M *et al.* Augmented cancer resistance and DNA damage response phenotypes in PPM1D null mice. *Mol Carcinog* 2006; **45**: 594–604.
24 Demidov ON, Kek C, Shreeram S *et al.* The role of the MKK6/p38 MAPK pathway in Wip1-dependent regulation of ErbB2-driven mammary gland tumorigenesis. *Oncogene* 2007; **26**: 2502–6.
25 Halazonetis TD, Gorgoulis VG, Bartek J. An oncogene-induced DNA damage model for cancer development. *Science* 2008; **319**: 1352–5.
26 Beckman RA, Loeb LA. Efficiency of carcinogenesis with and without a mutator mutation. *Proc Natl Acad Sci U S A* 2006; **103**: 14140–5.
27 Bennett WP, Hussain SP, Vahakangas KH *et al.* Molecular epidemiology of human cancer risk: gene-environment interactions and p53 mutation spectrum in human lung cancer. *J Pathol* 1999; **187**: 8–18.
28 Campling BG, El-Deiry WS. Clinical implication of p53 mutation in lung cancer. *Mol Biotechnol* 2003; **24**: 141–56.
29 Passlick B, Izbicki JR, Riethmüller G *et al.* p53 in non-small-cell lung cancer. *J Natl Cancer Inst* 1994; **86**: 801–3.
30 Lee JS, Yoon A, Kalapurakal SK *et al.* Expression of p53 oncoprotein in non-small-cell lung cancer: a favorable prognostic factor. *J Clin Oncol* 1995; **13**: 1893–903.
31 Mitsudomi T, Kosaka T, Endoh H *et al.* Mutations of the epidermal growth factor receptor gene predict prolonged survival after gefitinib treatment in patients with non-small-cell lung cancer with postoperative recurrence. *J Clin Oncol* 2005; **23**: 2513–20.
32 Kosaka T, Yatabe Y, Onozato R *et al.* Prognostic implication of EGFR, KRAS, and TP53 gene mutations in a large cohort of Japanese patients with surgically treated lung adenocarcinoma. *J Thorac Oncol* 2009; **4**: 22–9.
33 Ventura JJ, Tenbaum S, Perdiguero E *et al.* p38alpha MAP kinase is essential in lung stem and progenitor cell proliferation and differentiation. *Nat Genet* 2007; **39**: 750–8.

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