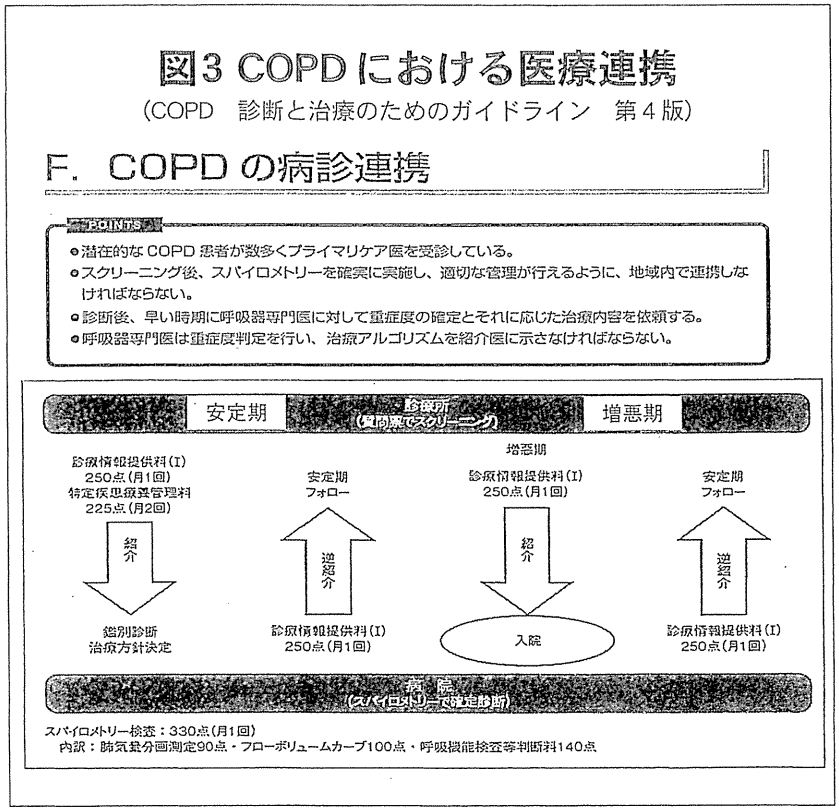
悪抑制や進行抑制，さらには生存率の改善効果まで期待できることが明らかとなり，COPDが積極的に治療介入すべき疾患になってきたことの一因ともなっている。一方，COPDに対する包括的アプローチを考える場合，最も重要な治療は「禁煙」である。禁煙補助薬を活用し，COPD治療として，まず禁煙を進めなければならない。ワクチンの接種（肺炎球菌ワクチン＋インフルエンザワクチン）も忘れてはならないポイントである。

本年度から徳島市における研究事業としてCOPD検診が開始されている。早期COPD患者には自覚症状に乏しい症例もあり，COPDの早期診断には積極的なスパイロメトリーの実施が欠かせない。検診に留まらず，日常的に診療している患者に潜むCOPDを早期に診断するために，ぜひスパイロメトリーを行っていただきたい。早期症例であっても基幹病院でまず一度診療を受けることは，精密肺機能検査（拡散能検査を含む），合併する可能性のある喘息に加え，肺がんおよび肺高血圧のスクリーニングのための胸部CTと心臓超音波検査を受けておく意義がある。徳島県のCOPD診療の向上に向けて，ガイドラインにも示されているような病診連携を進めていきたい（図3）。



綜説

肺線維症と増殖因子シグナル*

西岡　安彦[1]

はじめに

特発性肺線維症(idiopathic pulmonary fibrosis; IPF)は，特発性間質性肺炎(idiopathic interstitial pneumonias; IIPs)に分類される7疾患のなかで最も頻度が高く，5年生存率が30～50%と予後不良の難治性肺線維化疾患である．IPFは，肺胞領域の炎症，すなわち胞隔炎に始まり組織修復に伴って線維化が進行する疾患であると考えられてきたが，近年慢性の肺胞上皮傷害とそれに続く異常修復がIPFの本態であるとする炎症を重視しない仮説が提唱された．

この異常修復において様々な線維化関連因子が報告されているが，そのなかで線維芽細胞の増殖，筋線維芽細胞への分化，細胞外基質(extracellular matrix; ECM)産生などの作用を有する増殖因子が重要な役割を果たしていると考えられている．これらの増殖因子には，transforming growth factor-β (TGF-β)，platelet-derived growth factor(PDGF)，fibroblast growth factor (FGF)，vascular endothelial growth factor (VEGF)，connective tissue growth factor (CTGF)，insulin-like growth factor-Ⅰ(IGF-Ⅰ)などがある．特にTGF-βとPDGFは中心的な役割を担っていると考えられており，TGF-βは線維芽細胞に作用し強力なECM産生刺激作用と筋線維芽細胞への分化促進作用をもつ一方，PDGFは線維芽細胞に対し強い増殖活性および遊走活性をもつことが特徴である．

現在，様々な角度から抗線維化薬の開発研究が進められているが，増殖因子は治療薬開発の最も有望な分子標的の一つと考えられている．本稿では，間質性肺炎/肺線維症における増殖因子の役割について概説する．

TGF-β

1. TGF-β/TGF-β受容体シグナル

TGF-βは，12.5 kDのポリペプチドがS-S結合した25 kDの二量体分子である[1]．線維芽細胞，筋線維芽細胞，マクロファージ，再生Ⅱ型肺胞上皮から産生され，TGF-β1，2，3の3つのアイソフォームが存在する．*in vitro*においてはTGF-βの3つのアイソフォームは同様に線維芽細胞におけるプロコラーゲン産生を促進するが，*in vivo*においては発現の部位やbleomycin(BLM)刺激による発現の仕方がそれぞれ異なっている[2]．

TGF-β受容体(TβR)にはⅠ型(約53 kD)とⅡ型(約70～80 kD)があり，セリン/スレオニンキ

* Growth Factor Signals in Pulmonary Fibrosis

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ナーゼ活性を持つ1回膜貫通型レセプターである[1]．TβR-Ⅰは，activin receptor-like kinases 5 (ALK5)としても知られている．TGF-β二量体は，まずTβR-Ⅱ二量体に結合し，そこにTβR-Ⅰの2分子が結合し，四量体を形成する．TβR-Ⅱが，TβR-Ⅰをリン酸化し，TβR-Ⅰのキナーゼが活性化され，シグナル伝達分子であるSmadがリン酸化される．特異型Smadと呼ばれるSmad2/3がリン酸化されると共有型SmadであるSmad4と結合して，核内に移行し転写因子として作用する．一方，Smad6/7は，Smad2/3のリン酸化抑制活性を持つ抑制型Smadである．

一方，TβR下流のシグナル伝達経路として，最近non-Smad経路が注目されている[3]．これらの経路には，mitogen-activated protein kinase (MAPK)であるERK，JNK，p38の活性化，Rho-like GTPase経路，phosphatidylinositol-3-kinase (PI3K)/Akt/mTOR経路などが知られている．また，focal adhesion kinase (FAK)はTGF-β刺激下でPI3K p85と結合し，c-ablはPI3Kの下流でTGF-βの作用に関与している[4]．

2. 肺線維芽細胞とTGF-β

TGF-βは線維芽細胞のコラーゲンなどの細胞外基質産生を亢進し，筋線維芽細胞への分化を促進する一方，筋線維芽細胞のアポトーシス抑制作用を持つ[5]．またTGF-βは線維芽細胞や筋線維芽細胞に対する遊走能も有している．単球やマクロファージの遊走能も有し，これらの細胞からのフィブロネクチン，interleukin-1(IL-1)，tumor necrosis factor-α(TNF-α)，PDGFなどの産生とともにTGF-β自身の産生を亢進させる．TGF-βは低濃度では線維芽細胞や肺胞マクロファージからのPDGFなどの増殖因子の産生刺激を介して間接的な増殖作用を有している．また，マトリックスメタロプロテアーゼ(MMP)を減少させ組織インヒビター(TIMP)やplasminogen activator inhibitor-Ⅰ(PAI-1)を増加させる作用も有していることから，ECMの産生と分解の抑制作用によっても線維化を促進させる．

TGF-βの肺線維芽細胞に対する作用は，正常肺由来の線維芽細胞とIPF患者由来の肺線維芽細胞では差異が認められる．Huangらの報告では，TGF-βの添加でIPF患者由来の肺線維芽細胞ではコラーゲン産生の増強が認められるが，正常肺由来の線維芽細胞では認められない[6]．Ramosらの報告においても，IPF患者由来の線維芽細胞では正常肺由来に比較してα平滑筋アクチンの陽性率が高く筋線維芽細胞の特徴を有しているだけでなく，増殖速度が遅くTGF-β産生量が多いなどの相違点が報告されている[7]．

一方，TGF-βは線維芽細胞だけでなく肺胞上皮細胞に対する作用も有している．TGF-β1は肺胞上皮細胞の増殖を抑制し，マウス胎生期にTGF-β1を過剰発現させると肺胞の形成不全を引き起こす．さらにTGF-β1は，Ⅱ型肺胞上皮細胞からのサーファクタントプロテインBとCのmRNAの発現を低下させることも知られている[8]．

3. 肺線維症とTGF-β

肺線維症動物モデルにおけるTGF-β1のピークはそれぞれのモデルで異なっており，BLMの経気管投与では7日目頃に，TNF-αの過剰発現モデルや放射線照射では14日目頃にみられ，その後漸減する[9]．IPF患者の肺組織おいては線維芽細胞の増殖が活発なfibroblastic foci周囲にECMの沈着と一致してTGF-βの産生がみられる．

動物モデルでの肺線維化とTGF-βの関係についても多くの報告がある．活性型のTGF-βをアデノウイルスベクターにより肺に導入すると高度の線維化を来し[10]，逆に可溶性TβR[11]やTβR-1キナーゼ阻害剤[12]，シグナル伝達を抑制するSmad7の遺伝子導入[13]，アンタゴニストであるデコリンの強発現により肺線維化が抑制される．

しかしながらTGF-β阻害は，線維化が抑制される一方，炎症が惹起・遷延する問題が指摘されている．

4. EMTとTGF-β

EMTは上皮細胞が間葉系の細胞へ変化する現象である．TGF-β刺激により肺胞上皮細胞はaquaporin-5，cytokeratinsなどの上皮細胞のマーカーを喪失し，α-SMA，vimentin，desmin，コラーゲンⅠなどの間葉系のマーカーを発現し，fibroblast様に形態変化しEMTを引き起こすことが報告されている．Willisらは，ラットⅡ型肺胞上皮細胞をTGF-β存在下の *in vitro* で培養す

ることで，EMTが生じ，fibroblast様の形態と間葉系のマーカー発現がみられることを報告した[14]．同時にIPF肺組織の免疫染色で，αSMAとpro-SP-BあるいはTTF-1を共発現する細胞の存在を示唆するデータを報告している[14]．Kimらは，pro-SPCのプロモーターの下流にlacZ遺伝子を発現させるトランスジェニックマウスを作製し，肺線維症モデルにおいてαSMA発現細胞の40%までがX-gal陽性であることを示し，*in vivo*でEMTが生じていることを報告した[15]．一方，最近の報告ではBLMモデルにおいてEMTは検出されないとする報告もあり[16]，今後さらなる検討が必要である．

PDGF

1. PDGF/PDGF受容体シグナル

PDGFには約15 kDのA，B，C，Dの4種類のポリペプチドがあり，AA，BB，AB，CC，DDの5種類の2量体を形成する[17]．PDGF受容体は170～180 kDのαとβがありα-α，β-β，α-βの3種類の2量体を形成する．PDGF-AAはα-α，BBはα-α，α-β，β-βに，ABとCCはα-α，α-βに，DDはα-β，β-βにそれぞれ結合することが知られている．PDGF受容体からのシグナルは，Ras-MAPKやPI3K，PLC-γを介して伝達される．

2. 肺線維芽細胞とPDGF

PDGFは肺線維芽細胞の最も強力な増殖因子および遊走因子である[18]．PDGFもTGF-βと同様に線維芽細胞，筋線維芽細胞，マクロファージ，再生Ⅱ型肺胞上皮から産生される．肺線維芽細胞には，PDGF受容体のαとβがともに発現している．5つのPDGFアイソフォームはすべて肺線維芽細胞に対して増殖活性，遊走活性を有するが，なかでもPDGF-BBが最も強力な作用を有する．

3. 肺線維症とPDGF

BLM誘発肺線維症モデルの気管支肺胞洗浄液のcell pelletにおいてPDGF-A mRNAの発現量の増加が報告されている[19]．また同モデルにおいて肺組織のPDGF-A，B，C，DのmRNAの発現を検討した結果，8日目をピークにPDGF-Cの高発現が認められ，また同時期にPDGF受容体αのリン酸化亢進を認めた報告もある[20]．一方，マウス肺においてPDGF-B遺伝子を過剰発現させると肺線維化を引き起こすことが報告されている[21]．

IPF患者の肺胞マクロファージでは，PDGFの産生が亢進しており，早期のIPF患者の線維芽細胞やⅡ型肺胞上皮細胞にPDGF αおよびβ受容体が高発現していたとの報告もある[22]．また，リウマチ肺の解析から，肺線維化が進行期にある患者肺の気道被服液中においてPDGFの増加が報告されており，肺線維症の進行に関与していることが示唆されている[23]．

一方，肺線維症動物モデルにおいてPDGF-PDGF受容体阻害による抗線維化効果が報告されている．バナジウム投与によるラットの肺線維症モデルにおいてPDGF受容体阻害薬であるAG1296の投与による肺線維化抑制効果が報告されている[24]．また放射線肺臓炎モデルにおいてもPDGF受容体リン酸化阻害剤を有する複数の低分子化合物(SU9518, SU11657, imatinib)を用いて，これらの薬剤による抗線維化効果が実証されている[25]．われわれもブレオマイシン肺線維症モデルにおいてimatinibの強い抗線維化効果を確認している[26]．

その他の増殖因子

IGF-ⅠはIPF患者の組織およびBLMモデルでの発現増強が認められており，PDGF同様の作用が推測されている[22]．マウスブレオマイシン肺線維症モデルにおいてIGF-Ⅰ受容体抗体(A12)の抗線維化効果が報告されており，治療標的となる可能性がある[27]．

FGFは，その名称どおり線維芽細胞の増殖を促進する分子として発見された増殖因子であるが，現在18のFGFファミリー分子と各アイソフォームを含む7つの主要FGF受容体が同定されており，その生理機能，特に肺線維化病態における役割については不明な点も多い[28]．線維化病態との関連が最も多く報告されている分子はFGF-2であり，線維芽細胞の増殖および遊走活性が示されている[26,29]が，これらはすべて*in vitro*の作用であり臓器線維化における役割は明らかではない．

一方，VEGFは主要な血管新生因子であり急

性肺傷害における血管透過性亢進にも重要な役割が推測されているが，肺線維芽細胞に対する作用を検討した報告は少ない．Farkesらは，TGF-βによって誘導される肺線維芽細胞のコラーゲンI産生をVEGFが相乗的に増強させると報告しており，血管透過性亢進作用に加えて線維化促進に作用しうる可能性がある[30]．実際VEGFの可溶性受容体(sflt-1)投与による抗線維化効果も報告されている[31]．

さらにTGF-βの下流にある増殖因子としてCTGFが注目されている．CTGFはTGF-βによるコラーゲン合成に必須であることが示されている．IPF患者の肺組織やBLMモデルにおいても発現が亢進している[32]．しかし肺組織で過剰発現させた場合に線維化は生じるが一過性であるとの報告[33]があり，肺の線維化における役割に関してはさらなる検討が必要である．

増殖因子シグナルと抗線維化療法

以上のようなデータから増殖因子が肺線維症の有望な治療標的であることが容易に推測される．しかしながら，現在まで明らかに増殖因子を治療標的とする抗線維化療法は確立されていない．われわれをはじめとする前臨床試験データからimatinibがIPF治療薬として期待され，IPFに対する最初の分子標的治療として米国において臨床試験が実施された[34]．しかし，残念ながらその結果はnegative studyであった．この試験は，試験デザイン自体にもいくつか問題点があったのは事実であり，また肺線維症におけるα1-acid glycoproteinなどのimatinib抵抗性因子の増加も関係していた可能性もある[35]．しかし，2番目の分子標的治療薬として第II相臨床試験が実施されたPDGF受容体，FGF受容体，VEGF受容体に対するマルチキナーゼ阻害薬Nintedanib(BIBF1120)は，IPF進行抑制と急性増悪の抑制という有望な結果を示した[36]．本年の米国胸部学会(ATS)で報告される第III相国際共同臨床試験の結果が期待される．

おわりに

肺線維症の細胞分子病態に関与する増殖因子シグナルについて概説した．数多くの研究結果からTGF-β，PDGFをはじめとする増殖因子の線維化病態における重要な役割には疑いの余地はない．しかし一方，ヒト肺線維症に対してこれらの増殖因子が治療標的となるか否かはより複雑な要素を含んだ重要な課題である．実際に，肺線維症の治療薬開発が困難を極めている原因の一つは，その治療標的が同定されていないことにある．Nintedanibの第III相臨床試験結果により，IPFの治療標的としてPDGF，FGF，VEGFの重要性が臨床的に証明されれば，今後この分野の研究がさらに活性化され，細胞分子病態の解明が進む可能性がある．肺線維症と増殖因子シグナルに関する基礎研究および臨床研究の両面から注目される臨床試験である．

追記：本稿執筆後の本年5月のATSおよびN Engl J Med誌において，IPFに対するNintedanibの第III相臨床試験結果が発表され，有意な進行抑制(努力性肺活量の低下抑制)効果が示された[37]．

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ひとくちメモ

新規治療薬の開発状況と将来展望

西岡安彦

はじめに

間質性肺炎・肺線維症に対する治療薬開発は，本特集でも中心的に取り上げられている特発性肺線維症（IPF）を対象に進められている．その理由は，IPFが特発性間質性肺炎のなかで最も頻度が高く，かつ難治性であることによる．2000年代初頭に，それまで経験した副腎皮質ステロイドの有効性の低さや細胞分子病態解析データなどから，IPFに対する治療薬開発のコンセプトが「抗炎症」から「抗線維化」へと転換された．その後10年以上にわたる数多くの臨床試験を経て，複数の抗線維化薬が臨床現場で使用される時代を迎えようとしている．現在開発中の代表的薬剤について紹介する．

1. ニンテダニブ（BIBF1120）

IPFに対する治療薬開発の難点は，細胞という視点からも分子という視点からも，明らかな治療標的が特定されていないことにある．このような現状のなか，古くから線維芽細胞の増殖や機能を活性化する増殖因子と呼ばれる分子群が治療標的となることが想定されてきた．

ニンテダニブは，血小板由来増殖因子（platelet-derived growth factor；PDGF）受容体，線維芽細胞増殖因子（fibroblast growth factor；FGF）受容体，血管内皮細胞増殖因子（vascular endothelial growth factor；VEGF）受容体の3種類の受容体のチロシンリン酸化を阻害するマルチキナーゼ阻害薬である．PDGFおよびFGFには肺線維芽細胞の増殖刺激作用があり，またVEGFは血管新生ならびに血管透過性亢進作用をもつ．

2011年にIPFを対象とした国際多施設共同第II相臨床試験（TOMORROW試験）結果が報告され，有意なFVC低下抑制効果および急性増悪抑制効果が確認された[1]．さらに本年5月には，第III相臨床試験であるINPULSIS-1，2試験の結果が発表され，両試験における確実なFVC低下抑制効果が認められた[2]．現在，ピルフェニドンに次ぐIPF治療薬として承認申請が開始されている．一方，ニンテダニブの臨床試験結果は3種類の増殖因子（PDGF，FGF，VEGF）の受容体阻害がIPFに対する有効な治療戦略であることを示しており，今後の薬剤開発という観点からも重要な知見と言える．

2. レシチン化スーパーオキシドジスムターゼ（PC-SOD）

IPFの病態に活性酸素による上皮傷害が関与していることが報告されており，活性酸素を除去する物質（ラジカルスカベンジャー）の治療薬への応用が期待されてきた．PC-SODは，活性酸素除去作用をもつSODをレシチン化することで，活性を維持したまま血中安定性と組織親和性を高めた製剤である．当初，静注用製剤として開発され，IPF患者を対象とした第II相臨床試験では有望な結果が得られた．その後，実臨床への応用を目的に吸入製剤が開発され，現在，日韓両国で第II相臨床試験（無作為多施設二重盲検試験）が行われている．

3. その他

米国で実施された臨床試験では抗酸化作用をもつN-アセチルシステイン経口薬の有用性は示されなかったが[3]，本邦では吸入薬の有効性が期待されている．そのほか，抗IL-13抗体，抗CTGF（connective tissue growth factor）抗体，抗CCL（CC chemokine ligand）2抗体などの抗体製剤の第II相臨床試験も実施されており，その結果が注目されている．

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Developing novel therapeutic agents and future perspectives. Yasuhiko Nishioka: Department of Respiratory Medicine and Rheumatology, Institute of Health Biosciences, The University of Tokushima Graduate School. 徳島大学大学院ヘルスバイオサイエンス研究部教授（呼吸器・膠原病内科学）

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Original Article

Tumor-size-based morphological features of metastatic lymph node tumors from primary lung adenocarcinoma

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Most primary lung adenocarcinomas show histological diversity, however, histological diversity in the metastatic lymph node tumors (LNT) is not well defined. The aim of this study was to explore the histological characteristics of the metastatic LNT based on their sizes. We analyzed 163 primary tumors and 509 metastatic LNTs. When the primary tumor showed papillary-predominant subtype, the most frequent histological subtype in the metastatic LNT that were ≤2 mm in diameter was solid subtype (49%), followed by papillary subtype (35%); on the other hand, in the metastatic LNT measuring >2 mm in size, the frequency of tumors showing papillary-predominant subtype increased significantly to 52% ($P = 0.04$). When the primary tumor showed acinar-predominant subtype, the most predominant subtype in the ≤2 mm metastatic LN tumors was acinar subtype (55%), followed by solid subtype (40%), with the frequency of acinar subtype increasing significantly to 76% in the metastatic LNT that were >2 mm in diameter ($P = 0.04$). These results indicate that solid subtype is the characteristic histological subtype in the early phase of the LN metastatic process, and that as the metastatic LNT grow larger, they develop morphological features resembling those in the primary tumor.

Key words: diversity, lung adenocarcinoma, lymph node metastasis, micrometastasis

Adenocarcinoma is the most common histologic type of primary lung cancer and is a major focus of research to improve the patients' survival. In the new adenocarcinoma classification, International Multidisciplinary Lung Adenocarcinoma Classification, published in 2011 by the International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society (IASLC/ATS/ERS), invasive adenocarcinoma is divided into five predominant subtypes: the lepidic-, acinar-, papillary-, micropapillary- and solid-predominant subtypes.[1] Most adenocarcinomas are histologically heterogeneous, consisting of two or more histological subtypes. Matching the new adenocarcinoma classification, many studies have reported the prognosis and characteristics of the gene mutations according to this histological typing.[2–6] However, most previous studies focused on the primary tumors, and few studies have focused on the metastatic lesions.

Metastasis is considered as a complex and multistep process that ultimately results in the formation of a secondary mature tumor.[7–9] At the metastatic site, neoplastic cancer cells first go through an avascular growth phase to reach a size not much more than a few millimeters in diameter. During this phase, a small number of tumor cells survive and interact with the surrounding stromal cells, to then develop into macroscopically detectable metastatic lesions. During this metastatic tumor development, effective and dynamic molecular changes, including the epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET) have been reported.[10,11]We previously reported that as intrapulmonary metastatic lesions grow larger, the constituent cancer cells exhibit diverse growth patterns, which results in histological diversity in the secondary tumors, just as in the case of primary tumors.[12] However, the morphological changes in early and small metastatic lymph node tumors have not yet been clarified, and it is not yet known whether larger metastatic lymph node tumors exhibit as much histological diversity as the primary tumors. In the present report, we attempt to elucidate the morphological characteristics of the metastatic lymph nodes with special reference to their size and histological diversity. In the TNM Classification of Malignant Tumors, 7th Edition, published in 2009, for breast cancer, if the size of the cancer spread is larger than 0.2 mm and/or more than 200 cells, but not larger than 2 mm, it is

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defined as *micrometastasis*.[13] Based on this model, we evaluated the morphological features of metastatic lymph node tumors in relation to their sizes: > 2 mm vs. ≤ 2 mm.

MATERIALS AND METHODS

Patient selection

Lymph node metastasis from primary lung adenocarcinoma was detected in 184 consecutive patients who underwent surgical resection without preoperative therapy at the National Cancer Center Hospital East, Chiba, Japan, between January 2008 and December 2012. After excluding 21 cases for which only incomplete clinical information was available, the remaining 163 cases with 509 metastatic lymph nodes were histologically evaluated. The data collection and analyses were performed with the approval of the institutional review board.

Pathological studies

All surgical specimens were fixed with 10% formalin and embedded in paraffin. The tumors were cut at approximately 5-mm intervals, and serial 4-μm sections were stained with hematoxylin-eosin. The median number of tissue blocks per resected specimen was 25 (range: 10–90). The materials were subsequently reviewed by two pathologists (E.Y and G.I.) to confirm the presence of lymph node metastasis and to assess the histopathological features of both the primary tumors and the metastatic lymph nodes.

Histological subtyping of the primary tumors

Histological subtyping of the primary tumors was based on the IASLC/ATS/ERS International Multidisciplinary Lung Adenocarcinoma Classification published in 2011. The lepidic subtype is defined as growth of neoplastic cells along preexisting alveolar structures. The papillary subtype is defined as growth of glandular cells along central fibrovascular cores. If a tumor shows lepidic growth, but the alveolar spaces are filled with papillary structures, the tumor is classified as the papillary subtype. The acinar subtype is defined by the formation of round to oval-shaped glandular structures with a central luminal space surrounded by tumor cells. A cribriform arrangement is regarded as representing the acinar subtype of adenocarcinoma. The micropapillary subtype is defined as tumor cells growing in papillary tufts lacking fibrovascular cores, which may appear detached and/or connected to the alveolar walls. Ring-like glandular structures floating within the alveolar spaces are also regarded as representing micropapillary components. The solid-predominant subtype is characterized by the appearance of polygonal tumor cells arranged in sheets, lacking the features of any of the other recognizable histologic subtypes of adenocarcinoma.

Comprehensive histologic subtyping was performed through a process in which the percent area occupied by each histopathological subtype present in a tumor was estimated in 10% increments, followed by identification and classification of that tumor according to the histologic subtype. The predominant subtype was defined as the subtype accounting for the largest percent area in a tumor. In this cohort, none of the primary tumors were identified as showing the micropapillary-predominant subtype. The typical appearances of the histological subtypes of the primary tumors are shown in Fig. 1(a,h,o).

Histological subtyping of the metastatic lymph node tumors

Histological subtyping of the metastatic lymph node tumors was performed according to the same classification that was used for the primary tumor, and the predominant subtype was also identified. In the case of small metastatic tumors, isolated and small clusters of tumor cells which lacked clear differentiation into the papillary or acinar pattern were divided into the solid-predominant subtype. Also, as the micropapillary-predominant subtype, characterized by tumor cells growing in papillary tufts lacking a fibrovascular core, frequently coexisted with the papillary component, it was included in the papillary-predominant subtype.

The sizes of the metastatic lymph node tumors were evaluated based on the maximum diameter of the metastatic lesion in the lymph nodes. According to the TNM classification of Malignant Tumors, 7th Edition, published in 2009, we evaluated the morphological features of the metastatic lymph node tumors in relation to their sizes, > 2 mm vs. ≤ 2 mm. The typical appearances of the histological subtypes of the metastatic lymph node tumors are shown in Fig. 1(b–g,i–n,p–s).

Histological diversity according to the sizes of the metastatic tumors

The number of histological subtypes in the metastatic lymph node tumors of the size two groups (≤2 mm vs. >2 mm) was compared statistically according to the number of subtypes in the primary tumor. As the micropapillary subtype was observed in only a very small proportion of cases, and frequently coexisted with the papillary component, it was included in the papillary subtype.

Clinical information

All available clinical information was obtained from the clinical records and reports of the referring physicians. The records were reviewed for the patient age, sex, smoking history, pathological T and N classification, stage, and number of

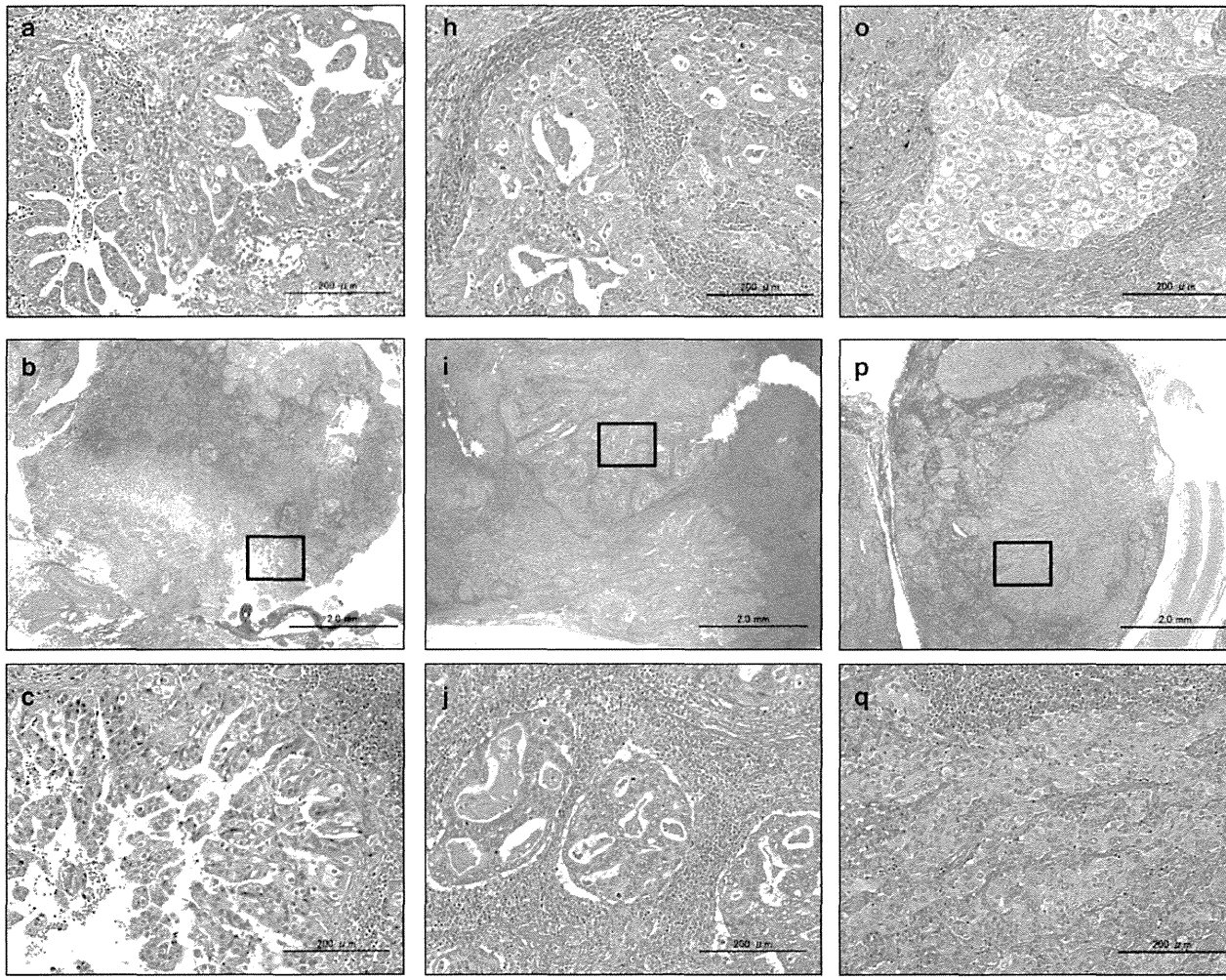

Figure 1 Histopathological features of the primary tumors and metastatic lymph node tumors(H & E). (**a**) Primary tumor showing the papillary-predominant subtype. (**b**) Metastatic lymph node tumor that was >2 mm in diameter. (**c**) Higher magnification view of the tumor shown in **b**. The tumor was mainly composed of the papillary component. (**d,f**) Metastatic lymph node tumors that werre ≤2 mm in diameter. The tumor was mainly composed of the (**d**) solid or the (**f**) papillary component. (**e,g**) Higher magnification views of the tumors shown in **d** and **f**, respectively. (**h**) Primary tumor showing the acinar-predominant subtype. (**i**) Metastatic lymph node tumor that was >2 mm in diameter. (**j**) Higher magnification view of the tumor shown in (**i**). The tumor was mainly composed of the acinar component. (**k**) and (**m**) Metastatic lymph node tumors that were ≤2 mm in diameter. The tumor was mainly composed of the (**k**) solid or the (**m**) acinar component. (**l**) and (**n**) Higher magnification views of the tumors shown in **k** and **m**, respectively. (**o**) Primary tumor showing the solid-predominant subtype. (**p**) and (**r**) Metastatic lymph node tumors that were (**p**) > 2 mm and (**r**) ≤ 2 mm in diameter. (**q**) and (**s**) Higher magnification views of the tumors shown in **p** and **r**, respectively. The tumor was mainly composed of the solid component, irrespective of the tumor size.

metastatic lymph nodes. Pathological staging was based on the TNM classification of the International Union Against Cancer (UICC).[13]

Statistical analysis

Differences in the patient characteristics between the two groups were compared by the Pearson's chi-square test. The unpaired t-test was performed to calculate the statistical significance of the differences. All *P* values are two-sided, and the significance level was set at <0.05. The analyses were performed with the SPSS 11.0 statistical software program (Dr. SPSS II for Windows, standard version 11.0, SPSS Inc., Chicago, IL, USA).

RESULTS

Clinicopathological characteristics of the patients with lymph node metastasis

The clinicopathological characteristics of the 163 adenocarcinoma patients diagnosed as having lymph node metastasis

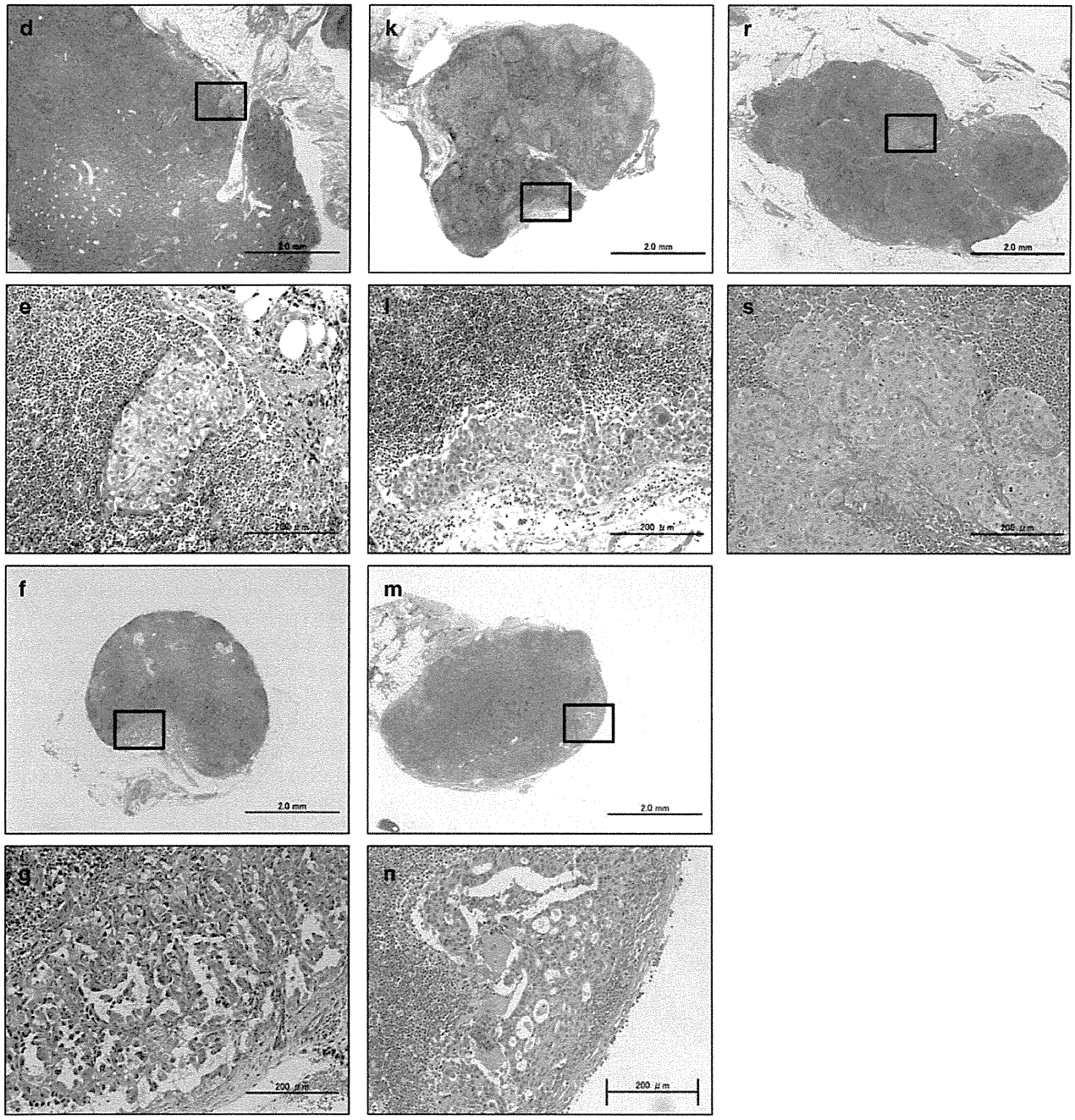

Figure 1 Continued

are summarized in Table 1. In all, 54 patients (33.1%) and 109 patients (66.9%) were pathologically diagnosed as belonging to the N1 and N2 categories, respectively.

The histologic subtypes of the primary tumors were distributed as follows: lepidic-predominant subtype, 6 tumors (3.7%); papillary-predominant subtype, 59 tumors (36.2%); acinar-predominant subtype, 35 tumors (21.5%); solid-predominant subtype, 63 tumors (38.6%).

Pathological characteristics of the metastatic lymph node tumors

Clinicopathological information pertaining to the 509 metastatic lymph node tumors is summarized in Table 2. In all, 286 (56.2%) and 223 (43.8%) of the metastatic lymph node tumors were pathologically diagnosed as belonging to the N1 region and N2 region, respectively. The histologic subtypes

Table 1 Characteristics of the patients with lymph node metastasis

Factors	n = 163 (%)
Age (years)	
<65	71 (43.6)
≥65	92 (56.4)
Sex	
Male	100 (61.3)
Female	63 (38.7)
Smoking history	
Never smoker	59 (36.2)
Current/Previous smoker	104 (63.8)
Pathological T classification	
pT1	40 (24.5)
pT2	81 (49.7)
pT3	33 (20.3)
pT4	9 (5.5)
Pathological N classification	
pN1	54 (33.1)
pN2	109 (66.9)
Pathological Stage (UICC7)	
Stage IIA	34 (20.8)
Stage IIB	6 (3.7)
Stage IIIA	111 (68.1)
Stage IIIB	7 (4.3)
Stage IV	5 (3.1)
Predominant subtype in primary tumor	
Lepidic	6 (3.7)
Papillary	59 (36.2)
Acinar	35 (21.5)
Solid	63 (38.6)
Number of metastatic lymph nodes	
1	47 (28.8)
2–5	82 (50.3)
≥6	34 (20.9)

Table 2 Characteristics of metastatic lymph node tumors

Factors	n = 509 (%)
Region	
Hilar, lobar and segmental (N1)	286 (56.2)
Mediastinal (N2)	223 (43.8)
Predominant subtype	
Papillary	107 (21.0)
Acinar	119 (23.4)
Solid	283 (55.6)
Metastatic lesion size	
≤2 mm	122 (24.0)
>2 mm, ≤5 mm	165 (32.4)
>5 mm	222 (43.6)

of the metastatic lymph node tumors were distributed as follows: papillary-predominant subtype, 107 lymph nodes (21.0%); acinar-predominant subtype, 119 lymph nodes (23.4%); solid-predominant subtype, 283 lymph nodes (55.6%).

Size distribution of the metastatic lymph node tumors

The median diameter of the lesions was 5 mm. The numbers of metastatic lymph node tumors belonging to the three size categories of ≤2 mm, >2 mm but ≤5 mm (median size) and >5 mm were 122 (24.0%), 165 (32.4%) and 222 (43.6%)(Table 2).

The number of histologic subtypes in the metastatic lymph node tumors divided according to the size

We compared the number of component histological subtypes in the primary tumors and metastatic lymph node tumors by dichotomizing the metastases according to the tumor diameter into ≤2 mm or >2 mm. The number of subtypes in the metastatic lymph node tumors in the group in which the primary tumor was composed of one subtype was 1.00 ± 0, indicating that all metastatic tumors arising from primary lesions composed of a single histologic subtype also showed a single histologic subtype (Fig. 2a). Fig. 2(b–d) shows the comparisons between the two metastatic tumor groups (divided according to the tumor size) in each case of the primary tumor being composed of two, three, or four subtypes, respectively. No significant differences between the two size groups were observed in the metastatic tumors arising from primary tumors composed of two histologic subtypes. On the other hand, significant differences between the two size groups were observed in the metastatic tumors arising from primary tumors composed of three or four subtypes (Fig. 2c,d; $P < 0.01$ and $P < 0.01$). The mean numbers ± standard error (SE) of subtypes in the metastatic lymph node tumors that were ≤2 mm and >2 mm in diameter were 1.18 ± 0.06 and 1.60 ± 0.05, respectively, and 1.32 ± 0.11 and 1.75 ± 0.06, respectively.

Comparison between the predominant histologic subtypes in the primary tumors and metastatic lymph node tumors

We compared the predominant histologic subtypes of the primary tumors and 509 metastatic lymph node tumors (Table 3). The predominant subtypes of the metastatic lymph node tumors in the cases in which the primary tumors showed the lepidic-predominant subtype were the papillary subtype (50.0%) and acinar (50.0%) subtype. The predominant subtype of the metastatic tumors in the cases in which the primary tumors showed the papillary-predominant subtype was the original papillary subtype (47.4%), followed by the solid (39.7%) and acinar (12.9%) subtypes. The predominant subtype of the metastatic tumors in the cases in which the primary tumors showed the acinar-predominant subtype was the original acinar subtype (72.0%), followed by the solid (23.4%) and papillary (4.6%) subtypes. The predominant subtype of the metastatic lymph node tumors in the cases in which the primary tumors showed the solid-predominant

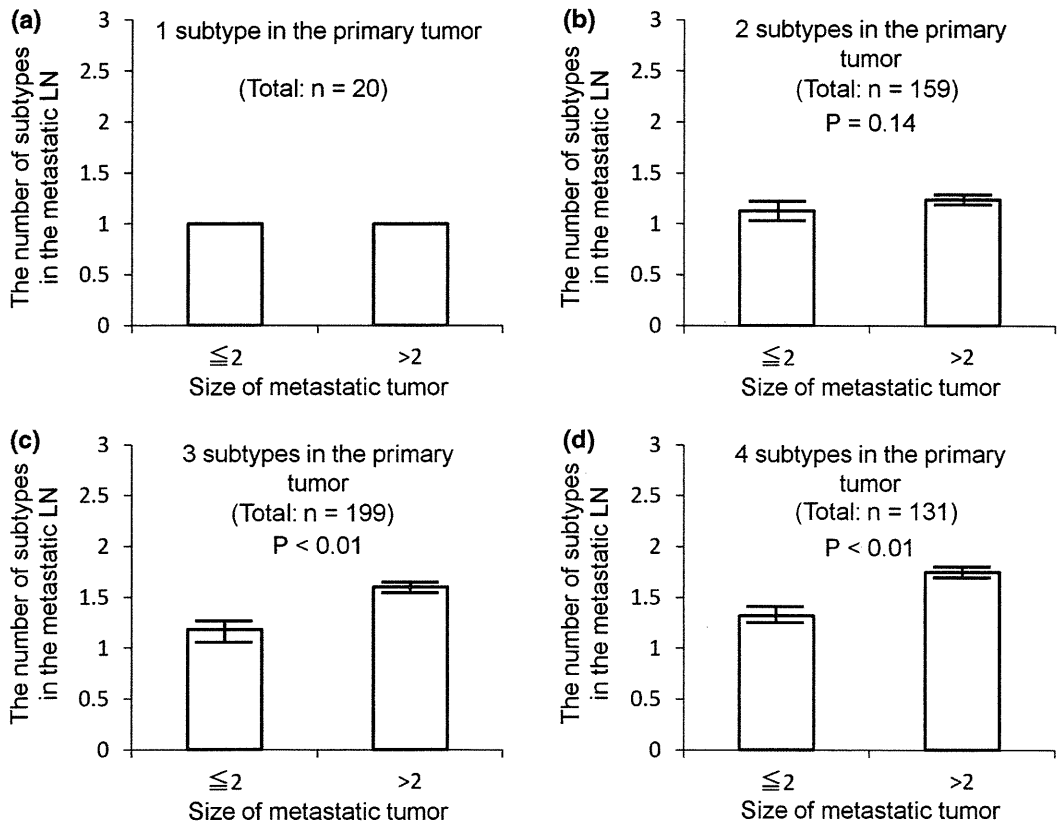

Figure 2 The number of component histologic subtypes in the metastatic lymph node tumors. (**a**) Comparison of the histologic subtypes in the group in which the primary tumor was composed of a single subtype. The mean number of histologic subtypes in both metastatic tumor size groups was 1.00. (**b**) Comparison in the group in which the primary tumor was composed of 2 subtypes (≤2 mm: 1.1, > 2 mm: 1.2: $P = 0.14$). (**c**) Comparison of the histologic subtypes in the group in which the primary tumor was composed 3 subtypes (≤2 mm: 1.2, > 2 mm: 1.6: $P < 0.01$). (**d**) Comparison of the histologic subtypes in the group in which the primary tumor was composed of 4 subtypes (≤2 mm: 1.3, > 2 mm: 1.8: $P < 0.01$).

Table 3 Comparison between the predominant histrological subtypes in the primary tumors and the metastatic lymph node tumors

Predominant subtype		
Primary tumor	Metastatic LN tumor	(%)
Lepidic	Papillary	4 (50.0)
	Acinar	4 (50.0)
	Solid	0 (0.0)
Papillary	Papillary	92 (47.4)
	Acinar	25 (12.9)
	Solid	77 (39.7)
Acinar	Papillary	5 (4.6)
	Acinar	77 (72.0)
	Solid	25 (23.4)
Solid	Papillary	6 (3.0)
	Acinar	13 (6.5)
	Solid	181 (90.5)

subtype was the original solid subtype (90.5%), followed by the acinar (6.5%) and papillary (3.0%) subtypes. The solid-predominant subtype was identified at a high frequency in the metastatic lymph node tumors, even in cases in which the primary tumor showed other predominant subtypes.

Predominant histologic subtypes according to the sizes of the metastatic lymph node tumors

Next, we compared the predominant histologic subtype of the primary tumors and matched metastatic lymph node tumors in relation to the sizes of the metastatic lymph node tumors (Fig. 3).

1 Primary tumor showing the lepidic-predominant subtype; one case of the papillary-predominant and one of the acinar-predominant subtype were identified in metastatic

Table 4 The correlation between the metastatic lymph node predominant subtype and metastatic tumor size (≤2 mm and >2 mm) in a) papillary and b) acinar predominant primary tumor

a) in papillary predominant primary tumor			
	Predominant subtype		
Metastatic lymph node tumors	Papillary (%)	Non-Papillary (%)	*P*
Size			
≤2 mm	18 (35)	33 (65)	0.04
>2 mm	74 (52)	69 (48)	
b) in acinar predominant primary tumor			
	Predominant subtype		
Metastatic lymph node tumors	Acinar (%)	Non-Acinar (%)	*P*
Size			
≤2 mm	12 (55)	10 (45)	0.04
>2 mm	65 (76)	20 (24)	

lymph node tumors that were more than 5 mm in size (data not shown).

2 Primary tumor showing the papillary-predominant subtype; the most frequent subtype in the metastatic tumors that were ≤2 mm in size was the solid-predominant subtype (25/51, 49.0%), followed by the papillary subtype 35.3% (18/51). However, when the metastatic tumors grew to more than 5 mm in size, the most frequent subtype was the papillary subtype in 54.3% (44/81), followed by the solid subtype in 39.5% (32/81) (Fig. 3a).

3 Primary tumor showing the acinar-predominant subtype; the acinar- and solid-predominant subtypes were identified in 54.5% (12/22) and 36.4% (8/22) of metastatic lymph node tumors that were ≤ 2 mm in diameter, respectively. On the other hand, the acinar and solid subtypes were seen in 83.8% (31/37) and 10.8% (4/37), respectively, of metastatic lymph node that were more than 5 mm in diameter (Fig. 3b).

4 Primary tumors showing the solid-predominant subtype; the solid-predominant subtype was identified in 98.0% (48/49) of metastatic lymph node tumors that were ≤2 mm in diameter. The solid subtype was seen in 86.3% (88/102) of metastatic tumors that were more than 5 mm in diameter (Fig. 3c).

Comparison of the predominant histologic subtypes between metastatic lymph node tumors that were 2 cm or under and over 2 mm in diameter

Table 4 shows the correlation between the predominant histological subtype and the metastatic tumor size (≤2 mm vs. >2 mm) in cases in which the primary tumor showed the papillary- or acinar- predominant subtype.

When the predominant histologic subtype of the primary tumor was the papillary, 35% of metastatic tumors that were ≤2 mm in diameter showed the papillary-predominant subtype; however, the frequency of tumors showing the papillary-predominant subtype increased to 52% in the metastatic tumors that were >2 mm in diameter ($P = 0.04$).

In addition, when the predominant subtype of the primary tumor was the acinar subtype, 55% of metastatic tumors that were ≤2 mm in size showed the acinar-predominant subtype, with the frequency of this histologic subtype increasing to 76% in metastatic tumors that were >2 mm in diameter ($P = 0.04$).

DISCUSSION

In the present report, we found that the predominant histologic subtype of metastatic lymph node tumors that were 2 mm or less in size often differed from that of the primary tumor. Actually, even when the primary tumor showed the acinar- or papillary-predominant subtype, metastatic lymph node tumors that were small in size often showed a solid-predominant subtype. However, as the metastatic lymph node tumors increased in size, the original subtype began to appear. These findings suggest that the solid morphology could be the initiating feature of metastasis in the early phase of the lymph node metastatic process. Then, as the metastatic tumor cells engraft and grow in the lymph nodes, the tumor cell populations regain the original morphological features and diversity. This sequence implies that the histological and biological characteristics of tumor cells in the engraftment process are largely different from those of tumor cells in the development process of metastatic tumor formation.[14–16] This is the first study to evaluate the correlation between the sizes of the metastatic tumors and the histologic subtype and tumor cell diversity in cases of adenocarcinoma of the lung.

In a previous report about the histological characteristics of metastatic lymph node tumors, Sica *et al.* reported that the

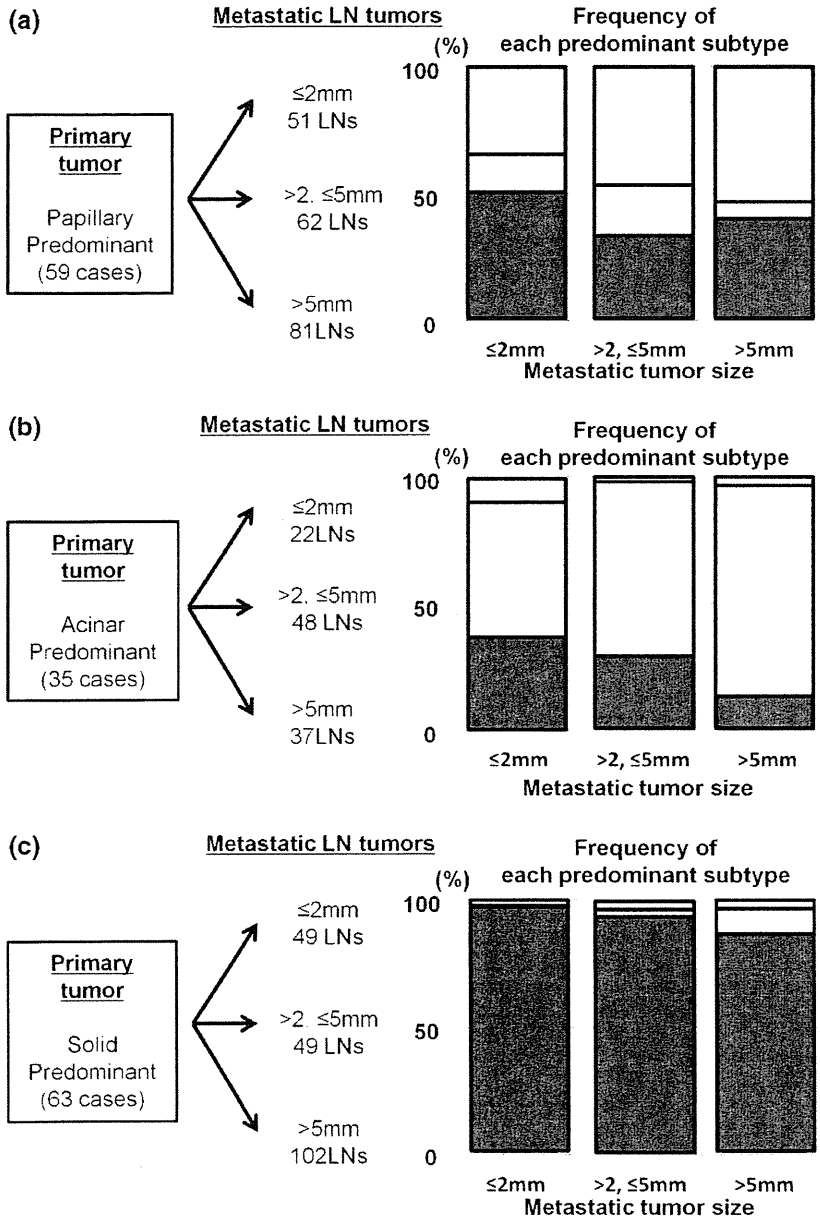

Figure 3 Comparison of the predominant histologic subtypes according to the sizes of the metastatic lymph node tumors. (**a**) Cases in which the primary tumor showed the papillary-predominant subtype. There were 51, 62 and 81 metastatic lymph node tumors that were ≤2 mm, > 2 mm but ≤5 mm and >5 mm in diameter respectively. Of the 51 metastatic tumors that were <2 mm in diameter, 25 (49%) showed the solid-predominant subtype and 18 (35%) showed the papillary-predominant subtype. Of the 81 metastatic tumors that were >5 mm in diameter, 32 (39%) showed the solid-predominant subtype and 44 (54%) showed the papillary-predominant subtype. (**b**) Cases in which the primary tumor showed the acinar-predominant subtype. There were 22, 48 and 37 metastatic lymph node tumors that were ≤2 mm, > 2 mm but ≤5 mm and >5 mm in diameter, respectively. Of the 22 metastatic tumors that were <2 mm in diameter, 12 (55%) showed the acinar-predominant subtype and 8 (36%) showed the solid-predominant subtype. Furthermore of the 37 metastatic tumors that were >5 mm in diameter, 31 (84%) showed the acinar-predominant subtype and 4 (11%) showed the solid-predominant subtype. (**c**) Cases in which the primary tumor showed the solid-predominant subtype. There were 49, 49 and 102 metastatic lymph node tumors that were ≤2 mm, > 2 mm but ≤5 mm and >5 mm in diameter, respectively. Of the 49 metastatic tumors that were <2 mm in diameter, 48 (90%) showed the solid-predominant subtype, and of the 102 metastatic tumors that were >5 mm in diameter, 87 (86%) showed the solid-predominant subtype. ▭, Papillary; □, Acinar; ▬, Solid.

predominant pattern of any primary tumor is more likely to be seen at the metastatic site, and even when the micropapillary and solid patterns are present only at a small percentage in the primary tumor, these patterns are often seen in the metastatic lymph node tumors.[17] While these findings were partly consistent with our current results, there was no reference to the correlation of the metastatic tumor size with the predominant histologic subtype in that study. Taking these observations and our current results into consideration, it may be speculated that the solid morphology may be an important feature reflecting metastasis-initiating cancer cells during the process of development of lymph node metastasis. Takuwa *et al.* reported that solid subtype in lung adenocarcinomas exhibits the invasive immunophenotype, including increased laminin-5 expression, which may, in part, be consistent with our hypothesis.[3]

In this study, the solid predominant subtype in the metastatic lymph node tumor is morphologically defined according to the primary tumor criterion. However, considering cancer stem/initiating cell theory, it is possible to think that biological features of the cancer cells showing 'solid' morphology in metastatic lymph node tumor are different from those of

primary lung tumor showing solid morphology. Further investigation will be needed whether these two morphologically similar tumors display different biological features.

Aokage, one of our colleagues, examined a large number of small intrapulmonary metastases in detail by applying the histological classification of primary adenocarcinoma of the lung to the metastatic tumors.[12] In this report, most intrapulmonary metastatic tumors arising from primary adenocarcinoma exhibited a lepidic-predominant histologic subtype in the early phase and the morphological diversity of the original tumor began to be reproduced as the tumor grew in size. This phenomenon was similar to our finding of recapitulation of the original morphological features as the metastatic tumors grew, but differed in that the major hisotologic subtype in the early phase of pulmonary metastatic tumors was the lepidic-predominant subtype, whereas in our study, it was the solid subtype. This difference suggests the possibility of the histological environment of the metastatic organs decisively affecting the tumor morphological features in the early phase of the metastatic process.

Recent studies have indicated the importance of transient EMT-MET switches in the metastatic process.[18–22] Chaffer *et al.* showed, using a bladder cell line, spontaneous shift from mesenchymal to epithelial characteristics and its association with increased metastatic ability in advanced malignancies.[23] Tsai *et al.* also reported, using a spontaneous squamous cell carcinoma in Twist 1 transgenic mouse model, that activation of Twist1 is sufficient to promote EMT of cancer cells and to disseminate the cells into the circulation, and also that turning off Twist1 is essential to allow reversal of EMT for dissemination of tumors to distant sites.[24] We also previously investigated the immunophenotypes of cancer cells in small metastatic lesions of the lung, and concluded that a dynamic phenotypic change that includes both EMT and MET occurs in the early phase of metastatic tumor formation.[25] Therefore, the solid histologic subtype is the important phenotype corresponding to the transitional phase of EMT-MET in the early stage of the metastatic process.

The cancer stem cell theory may explain the phenomenon of metastatic tumor cells reproducing the morphological diversity of the original tumor as the metastatic tumors grew in size.[26–30] According to the cancer stem cell concept, a single cell or a small number of cancer cells have the potential to reconstitute a primary tumor under favorable conditions and diversity of the tumor cell populations develops during tumor progression.

Successful formation of macroscopic metastatic tumors requires angiogenesis and several extrinsic factors in the microenvironment. Therefore, tumor microenvironment of micrometastases and macrometastasis would be obviously different. To examine the differences of cancer cell phenotype and the stromal reaction between in micrometastases and macrometastases gives us very important information to know the dynamism of tumor metastatic process. For further investigation, we are planning to examine the immunophenotypic differences between lymph node micrometastasis and macrometastasis.

In conclusion, we found a high percentage of cases showing the solid phenotype in the early phase of the lymph node metastatic process, and that the metastatic tumor cells tended to regain the original morphological features and diversity as the tumors grew in size. As a prognostic factor, the small lymph node metastasis is recently noted and prospective cohort surveys have been performed.[31–33] Clarification of the biological features of small lymph node metastases is important for the development of new strategies for early cancer detection and for the development of effective cancer treatment approaches.

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ORIGINAL ARTICLE - CANCER RESEARCH

Ezrin-expressing lung adenocarcinoma cells and podoplanin-positive fibroblasts form a malignant microenvironment

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Abstract
Purpose Cancer cells and cancer-associated fibroblasts (CAFs) together create the tumor microenvironment, which affects malignant behavior. Lung adenocarcinomas with CAFs expressing podoplanin (PDPN) are clinically aggressive, but the molecular mechanism underlying this phenomenon has not been established. So we identified the characteristic immunophenotype of lung adenocarcinoma cells coexisting with PDPN-expressing CAFs (PDPN-CAFs) and examined how it relates to an aggressive clinicopathological outcome.
Methods We analyzed the clinicopathological characteristics of 119 adenocarcinomas with a uniform size (2–3 cm). The expression levels of ten invasiveness-related proteins which related to cell adhesion and invasiveness, such as Ezrin, were examined in cancer cells from PDPN-CAFs (+) cases and from PDPN-CAFs (−) cases ($n = 20$ each). To examine the functional importance of the identified protein on the invasion phenotype, we performed wound healing and a Matrigel invasion assay using shRNA-knockdown lung adenocarcinoma cells (PC-9).
Results The PDPN-CAFs (+) cases had significantly higher rates of node metastasis ($p < 0.01$) and vascular invasion ($p < 0.01$). The cancer cells from the PDPN-CAFs (+) cases also had a significantly higher staining score for Ezrin ($p < 0.01$) than those from the PDPN-CAFs (−) cases. The migration and invasion activities of the shEzrin-induced PC-9 cells were significantly lower than those of the control cells.
Conclusions Our results indicated that within a tumor microenvironment composed of PDPN-CAFs, increased Ezrin expression in cancer cells might play a key role in the invasiveness of lung adenocarcinoma.

Keywords Cancer microenvironment · Ezrin · Podoplanin · CAFs · Lung cancer

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Introduction

Primary adenocarcinoma of the lung is known to have a poor prognosis, even if surgery is successful, and approximately 15 % of patients develop recurrences (Asamura et al. 2008; Devesa et al. 2005). The 5-year disease-free survival rate for patients with stage I disease is about 75 % (Yoshizawa et al. 2011). Thus, improved knowledge of the molecular mechanisms underlying the development and progression of lung adenocarcinoma is essential to the development and establishment of effective therapeutic modalities.

Cancer tissue is composed of not only cancer cells, but also stromal cells, such as fibroblasts, endothelial cells,

and inflammatory cells; together, these cells create the tumor microenvironment. Previous studies have demonstrated that the biological behavior of cancers is influenced by the tumor microenvironment (Ishii et al. 2005; Ito et al. 2007). Extensive clinical evidence and experimental models have shown that certain types of cancer-associated fibroblasts (CAFs) may have a tumor-promoting phenotype (Gottschling et al. 2013; Ito et al. 2007; Korc 2007; Okusa et al. 1999; Xing et al. 2010).

We previously reported that CAFs expressing podoplanin (PDPN), a mucin-like transmembrane glycoprotein that is known as a lymphatic endothelial marker, was correlated with a poor prognosis in patients with all-stage lung adenocarcinoma,(Kawase et al. 2008) and a high rate of recurrence in patients with stage I lung adenocarcinoma (Ito et al. 2012a). Moreover, the co-transplantation of A549 (a human lung adenocarcinoma cell line) and PDPN-positive fibroblasts significantly increased the efficiency of tumor implantation, and the short hairpin RNA (shRNA) knockdown of PDPN in fibroblasts decreased the augmenting effect on A549 tumor formation in a mouse xenograft model (Hoshino et al. 2011). These results suggested that both cancer cells and CAFs expressing PDPN might create a more malignant microenvironment for tumor tissue. The biological characteristics of PDPN-CAFs are gradually being elucidated (Ito et al. 2012b), but the characteristics of cancer cells in a microenvironment containing PDPN-CAFs have never been analyzed.

Accordingly, we attempted to identify the immunohistochemical characteristics of lung adenocarcinoma cells coexisting with PDPN-expressing CAFs (PDPN-CAFs) and to examine how these immunohistochemical characteristics relate to aggressive outcomes.

Materials and methods

Patient selection

A total of 695 consecutive patients underwent surgical resection for primary lung cancer between January 2011 and June 2013 at the National Cancer Center Hospital East, Chiba, Japan. We reviewed the patients' clinicopathological information in their medical records and selected 119 cases of invasive lung adenocarcinoma with a tumor size of 2–3 cm in diameter. Patients who received preoperative treatment and cases with multiple primary lung cancer were excluded. All the surgical specimens were collected and analyzed after receiving the approval of the Institutional Review Board of the National Cancer Center Hospital East.

Histological studies

The surgical specimens were fixed in 10 % formalin and were serially sectioned at 5-mm intervals; all the sections were then embedded in paraffin. The sections were stained using the hematoxylin and eosin (HE) method, the Alcian blue-periodic acid-Schiff (AB-PAS) method for the detection of cytoplasmic mucin production, or the Victoria-blue van Gieson (VVG) method for the detection of elastic fibers. All the histological materials included in this series were reviewed by two pathologists (S.S. and G.I.). The pathological stage was determined based on the TNM classification of the International Union Against Cancer (UICC), seventh edition. Histological typing of the primary tumors was performed based on the World Health Organization classification of cell types, third edition. Invasive lung adenocarcinomas were classified into the following subtypes: lepidic predominant, papillary predominant, acinar predominant, micropapillary predominant, and solid predominant. The predominant component was defined as the histological component that comprised the largest percentage among the components.

Antibodies and immunohistochemical staining

The antibodies used in this study are summarized in Supplementary Table 1. The slides were deparaffinized in xylene and dehydrated in a graded ethanol series, and endogenous peroxidase was blocked with 3 % hydrogen peroxide in absolute methyl alcohol. After epitope retrieval, the slides were washed with phosphate-buffered saline and were incubated overnight at 4 °C using mouse or rabbit antihuman antibodies at their final dilution in the blocking buffer. The slides were washed again and incubated with EnVision (Dako, Glostrup, Denmark) for 1 h at room temperature. The reaction products were stained with diaminobenzidine; lastly, the slides were counterstained with Meyer hematoxylin.

Calculation of immunohistochemical scores

All the stained tissue sections were semiquantitatively scored and evaluated independently under a light microscope by two pathologists (S.S. and G.I.); when the evaluation results differed, the final report was determined based on a consensus reached between the two pathologists who evaluated the slides together under a conference microscope. We graded the podoplanin expression by the CAFs as follows: grade 0, podoplanin-positive CAF area/stromal area $\times$ 100 = <10 %; grade 1, podoplanin-positive CAF area/stromal area $\times$ 100 = 10–50 %; and grade 2, podoplanin-positive CAF area/stromal area $\times$ 100 = >50 %.