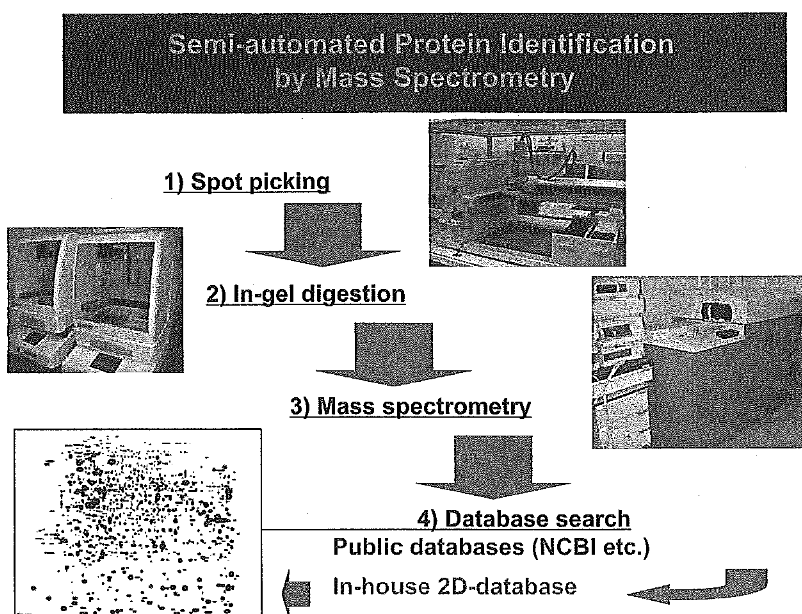

図5　ロボット化したゲル内消化システムとPMF法によるタンパク質同定

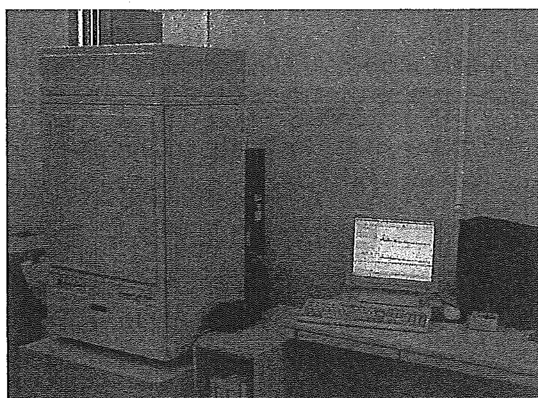

図6　MALDI-TOF型の質量分析機

パク質の物理化学的な性状をすべて均一にして網羅的に解析することから，ショットガン法とも呼ばれている。特に微小なカラムを用い，nl/minレベルの低流速で液体クロマトグラフィーから直接質量分析機に資料を導入することで，高感度が得られる[7]。

従来LC-MS法は定量性に問題があったが，安定同位体による代謝標識[8]やICAT（isotope-coded affinity tag）法による化学標識[9]を行うことで，定量的な解析も可能となった。

Ⅳ．プロテインチップ法による腫瘍マーカー開発

進行がんの治療は現在利用可能な医療技術では困難な場合が多く，高感度な検出方法を用いて微少のがんを発見し，早期に治療を開始することが患者のQuality of lifeや予後の改善に必須であることは論を待たない。そのため多数の被験者からがん罹患者を効率よくスクリーニングできるがん検診技術の開発と整備が国民から強く要望されている。国立がんセンターでは平成16年2月よりがん予防・検診研究センターを開設し，既存のがん検診方法の検証と新しいがん検診技術の開発を行っている。がん予防・検診研究センターでは従来の放射線やエコー，内視鏡などに加えて，PET（positron emission tomography）によるがん検診を行っている。しかし施設と機器に膨大な経費がかかるため，がん検診の目的でPETが全国的に普及するかどうかは現時点では不明である。一方，血液中の腫瘍マーカーは，どこの病院や診療所でも容易に検体が採取できることから，大多数の被験者のスクリーニングに有利である。自己採血などによるがん診断キットも市販されるようになってきたが，その有用性は不明である。

現在腫瘍プロテオミクスプロジェクトはがんの早期診断のための腫瘍マーカー開発に特に力を入れている。腫瘍マーカーといえば，CEA（carcinoembryonic antigen）やCA19-9のように治療効果の判定や，再発の発見など限られた範囲で臨床に有用であるが，がんの早期発見には，前立腺がんにおけるPSA（prostatic specific antigen）[10]以外に有用なマーカーはないというのが今までの常識であった。しかしこの医学常識が最近崩れつつある。米国のCiphergen社のプロテインチップシステム（**図3**）

とCorrelogic社が開発したアルゴリズムを用いた多変量解析を用い，米国NCIとFDAの共同で行われているClinical Proteomics Projectは治癒可能性の高いStage Iの早期症例を含め，100％に近い正診率で卵巣癌が診断できたと報告している[11]。この成果は米国のマスコミに大きく取り上げられ（図7），注目を浴びているが，チップの消耗品が高価であり，またピークの解析ソフトが市販されていなかったため，国内では少数例を用いた散発的な研究しか行われていなかった。われわれはピーク同定の独自のアルゴリズムを開発し，またロボットシステムによる分画法を改良し，わずか20μlほどの血漿（血清）から約1,000の低分子タンパク質とペプチドを検出することができるようになっている。

V．ロボット化したモノクローナル抗体作製システムとタンパク質アレイ

96穴マイクロタイタープレート内でアレイ化した小さな組織切片に対し，免疫染色を迅速に行えるロボットシステムを独自に開発した。がんの浸潤部や患者血清と反応する多数の抗体を分離し，病態の診断やスクリーニングへの応用を検討している。われわれは膵がん細胞株を免疫源として，本システムを用いた大規模なスクリーニングで，膵がん患者血清と高率に反応する新しい8種の新規モノクローナル抗体を選択した。これらの抗体パネルを用いた血漿のタンパク質アレイで膵がん患者の98例中73例（74.4％）が陽性を示したが，健常人においても9.7％が陽性を示した（第62回日本癌学会総会記事)。膵がんは進行して発見された場合著しく予後が不良であり，早期発見の効果的な戦略が重要である。治癒率を向上させるためには，現時点ではある程度の偽陽性があっても検出率を向上させる必要がある。本方法は偽陽性が高く，現状ではそのままではがん検診には使えないが，膵がん患者の高い検出率を示し，今後重要な診断法の1つになる可能性がある。

Ⅵ．その他のプロテオーム解析技術

組織の凍結切片に直接レーザーをあて，レーザーが照射された局所にあるタンパク質の質量をMALDI-TOF MSで測定する方法が行われている。プロテオームの結果で肺癌の組織型や予後を判定できたと報告されている[12]。

図7　プロテインチップに関する米国マスコミの報道

おわりに

バイオテクノロジーの基礎研究の領域では近年急速な技術革新が行われ，cDNAマイクロアレイ法などのトランスクリプトーム技術や本稿で述べたプロテインチップ法，質量分析法，抗体・タンパク質マイクロアレイ法などのプロテオーム技術の臨床応用が期待されている。またバイオインフォマティクスの技術が進歩し，一見特異性のないように思われる数値データからも，診断に有用な情報が引き出せる可能性がでている。われわれはこれらの新技術を単なる基礎研究に終わらせないことが責務であると感じている。邦人がノーベル賞をとったことを機会に，日本独自の画期的なプロテオーム解析によるがん診断法を開発したいと思う。モダンメディア読者であられる全国の病院臨床検査部の皆様からの協力で，この目標を達成できればと願う。

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トピックス

難治がんの早期診断マーカーの探索

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検 査 と 技 術

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TNF-α)である．TNF-α は脂肪細胞でも高発現すること[9]，TNF-α 遺伝子や受容体の欠損マウスを肥満状態にしてもインスリン抵抗性が惹起されないこと，すなわち TNF-α はその受容体を介してインスリン抵抗性を惹起することが明らかにされている[10]．

▩症　例

図に筆者らが経験した，歯周炎を合併した2型糖尿病患者に対する歯周病治療に伴う上記検査値の変動を示す．本患者は50歳代の男性で糖尿病歴は10年である．体格指数(body mass index，BMI)は26.4(kg/m^2)と軽度肥満であり，内因性のインスリン分泌能は保たれているのみでなくむしろ亢進していた．歯科治療開始前3か月間のヘモグロビン A_{1c} は7%前後であり，重度の歯周炎を併発していた．患者の歯周ポケット内細菌を局所抗生剤で徹底的に駆逐するとともに，歯表面の汚れを機械的に除去した．治療開始3か月後，ヘモグロビン A_{1c} は5.8%に低下し，上記3種類の検査項目(歯周病菌に対する血清 IgG 抗体価，高感度 CRP，TNF-α)はいずれも検査値が低下した．内因性のインスリン分泌が著明に低下したことから，ヘモグロビン A_{1c} の低下はインスリン抵抗性の改善を介してもたらされたものと考えられた．

おわりに

一般にインスリン感受性を低下させる軽微な慢性炎症の代表格は，肥満症であると考えられている．実際，肥満患者の内臓脂肪組織は TNF-α に代表されるインスリン抵抗性惹起分子を複数，高発現すること，また肥満患者では高感度 CRP 値が上昇しており体重減少とともに低下することが知られている．しかしながら，わが国においても糖尿病患者数が激増しているとはいえ，実際に欧米人にみられるような著明な肥満症を基盤として発症する糖尿病は，むしろ稀である．歯周炎は糖尿病患者で高頻度に発症・重症化しやすいことから，今後肥満のみでなく口腔内の炎症にも十分配慮する必要がある．

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難治がんの早期診断マーカーの探索

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はじめに

進行がんの治療は現在利用可能な医療技術をもってしても治癒困難な場合は少なくない．高感度で高特異度を有する検出方法を用いて微小がんを発見し，早期に治療を開始する以外には，予後の改善に大きな期待が持てないのが現状である．そのためには，多数の被検者を効率よく非侵襲的にスクリーニングできる診断マーカーの開発が急務である．近年，バイオテクノロジーの基礎研究領域において急速な技術革新がなされ，蛋白質を全体としてとらえて研究するプロテオミ

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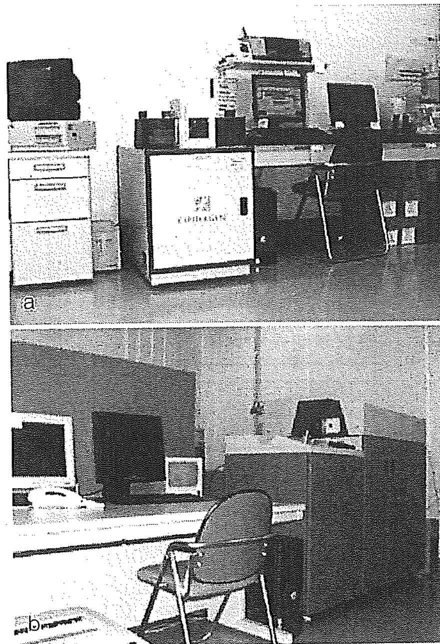

図1　プロテインチップ測定用質量分析装置
a：飛行時間型質量分析装置．b：四重極飛行時間型質量分析装置．

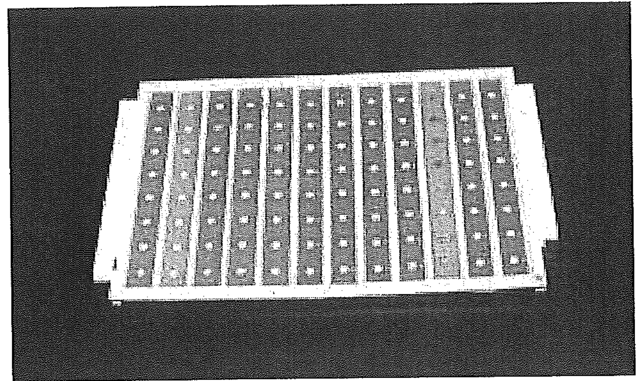

図2　プロテインチップ
直径2mmの金属板の表面に蛋白質に親和性を持つ各種官能基が修飾されている．チップ上でアフィニティークロマトグラフィーが可能でチップに親和性を持つ，蛋白質・ペプチドを選択的に捕捉できる．

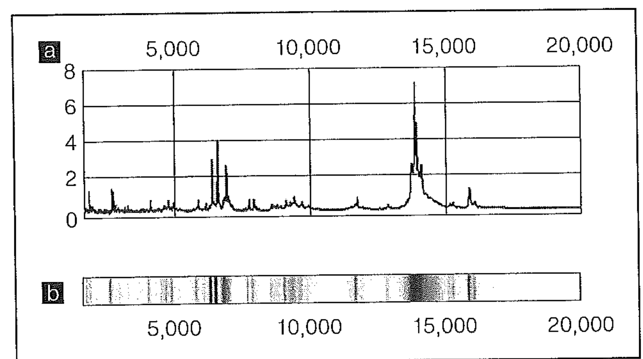

図3　SELDI-TOF-MSで測定されたピークイメージ
a：ヒト血漿サンプルから得られたピークプロファイル．
b：ピークプロファイルをゲルイメージで観察したもの．

クス技術が診断マーカーの探索法として注目を集めるようになってきた．プロテインチップと質量分析装置を組み合わせたSELDI-TOF-MS(surface-enhanced laser desorption/ionization time-of-flight mass spectrometry，表面エンハンス型レーザー脱イオン化質量分析法)で得られた質量データを，機械学習アルゴリズムを用いて解析することにより，一見特異性がないように思われる血漿・血清中に含まれる蛋白質・ペプチドの数値データからも早期がんが高い判別率で診断できるという報告がなされている[1]．

本稿ではSELDI-TOF-MSの原理，MSデータからのマーカー探索法，臨床検査への応用について述べる．

SELDI-TOF-MSの原理

SELDI-TOF-MSは金属板表面に蛋白質を分離・捕捉するための性質を付加してあるプロテインチップと飛行時間型質量分析装置を組み合わせた解析法である(図1)．血液サンプルを解析する場合，化学修飾チップがよく利用される．化学修飾チップには逆相，陽イオン交換，陰イオン交換，IMAC(金属イオン固定化Cu^{2+}，Ni^{2+}，Ga^{3+}など)，順相などのチップがある．血液サンプルをあらかじめ変性，イオン交換などを用いて分画し複雑度を低下させる．この分画液をプロテインチップ上に微量(20 μl程度)添加し，チップ上でアフィニティークロマトグラフィーを行う．チップに捕捉された蛋白質・ペプチドは，特化された質量分析装置を用いて定量的に質量分析される(図2)．

マーカー探索

イオン化された蛋白質・ペプチドの質量情報は図3が示すような波形として定量的に可視化される．これらピークの中からがん特異的な蛋白質を探索する．実際には単一のピークだけでがんとがん以外の疾患を区別することは困難なことが多い．そこで，単一の生物学マーカーから診断するという従来の考えかたではなく，人工知能などに応用される機械学習アルゴリズム(サポートベクターマシーン，ニューラルネットワーク，ファジーニューラルネットワーク)を利用する．年齢，性別，採血時期，採血方法，保存方法などを合致させた疾患群と対象群を学習セットとして用意する．この学習セットを用いてコンピューターにがん患者群と対象群のピークパターンを学習させる．さらに学習セットから得られたがんとそれ以外を区別するパターンに寄与するデータセットを抽出する．データセットが厳密に定義されれば，その後に臨床情報が付

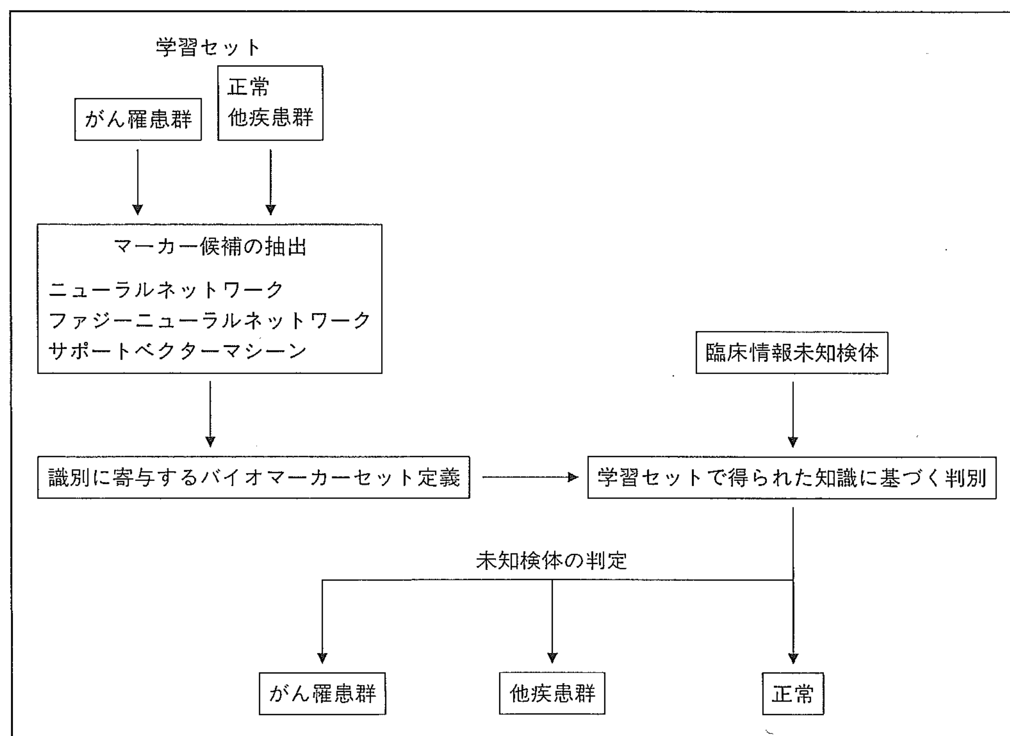

図4　マーカー探索の流れ

帯していないサンプルが持ち込まれたとしても高い判別率でがんか否かを判定することが可能である(図4)[2]．

▩臨床検査への応用

SELDI-TOF-MS によるデータに高い再現性が得られれば，ピークパターンを用いた臨床検査法の開発が期待される．機械学習法で抽出されたピークは，より精密な質量分析法を用いることにより，そのアミノ酸構造を決定することが可能である．決定されたアミノ酸構造から特異抗体を作製すれば，酵素免疫測定法(enzyme-linked immunosorbent assay，ELISA)などによる生化学検査を用いることにより，質量分析装置を有しない一般施設などでもスクリーニング検査が可能になると考えられる．

おわりに

筆者らは現在，上記の方法を用いて臨床的に早期発見が難しいとされる膵臓がんの診断マーカーの開発を行っている．たしかに罹患者と非罹患者を区別することができるピークプロファイルは存在するようである．臨床検査として本法を成功に導くには，ピークプロファイルを厳密に判別するための多数の学習セットサンプルが必要である．さらに症例数を拡大させ，より正確なピークプロファイルを抽出したいと考えている．

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Prognostic Significance of Tissue Factor in Pancreatic Ductal Adenocarcinoma

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Abstract Tissue factor (TF) is a transmembrane glycoprotein that plays roles in the blood coagulation and intracellular signaling pathways, and has also been suggested to modulate the biological behavior of cancer cells. In order to examine the clinicopathologic significance of TF expression in pancreatic ductal adenocarcinoma, TF expression was determined by immunohistochemistry using a newly raised anti-TF monoclonal antibody in 113 patients who had undergone surgical resection of pancreatic ductal adenocarcinoma. According to the incidence of tumor cell immunopositivity, patients were divided into "negative TF" (0%), "weak TF" (<25%), or "high TF" (25% or more) groups, which accounted for 11.6% ($n = 13$), 44.2% ($n = 50$), and 44.2% ($n = 50$) of the total, respectively. Increased TF expression was correlated with the extent of the primary tumor ($P = 0.0043$), lymph node metastasis ($P = 0.0043$), lymphatic distant metastasis ($P = 0.0039$), advanced tumor-node-metastasis stage ($P = 0.0002$), and high tumor grade ($P = 0.0164$). Multivariate analysis using the Cox proportional hazards model showed that high TF expression was an independent negative predictor for survival (hazard ratio, 2.014; $P = 0.0076$). Moreover, patients with TF-negative tumors had a significantly better prognosis even if lymph node metastasis was present ($P < 0.0001$). We also showed that TF knockdown by RNA interference suppressed the invasiveness of a pancreatic adenocarcinoma cell line *in vitro*. These results indicate that TF expression may contribute to the aggressiveness of pancreatic ductal adenocarcinoma by stimulating tumor invasiveness, and that evaluation of the primary tumor for TF expression may identify patients with a poor prognosis.

Tissue factor (TF) is a transmembrane glycoprotein that functions as a cellular receptor for coagulation factor VII (FVII) and modulates it to produce the activated form, FVIIa. The TF/FVIIa complex is regarded as the initiator of the extrinsic blood coagulation cascade, which ultimately leads to the generation of thrombin (1). In normal human tissues, TF is expressed only in extravascular cells, including the vascular adventitia and organ capsules (2). Based on this cellular distribution, under physiologic conditions, TF is thought to act mainly as a hemostatic barrier to prevent blood loss. In addition to its role as a hemostatic initiator, the binding of FVIIa with TF has been suggested to be involved in intracellular signaling mechanisms (3), such as the mitogen-activated protein kinase pathway (4) and the Src family member/PI3K/Rac-dependent signaling pathway (5), at least in some cell types.

TF is also involved in many pathophysiologic conditions, such as inflammation, atherosclerosis, and malignancies. With regard to malignancies, it has been well recognized that patients with malignant diseases are predisposed to hypercoagulation since Trousseau (6) first reported the increased frequency of thrombosis in patients with gastrointestinal cancers, and this hypercoagulable state is associated with TF (7). Immunohistochemical analysis has revealed that TF is expressed in a wide variety of malignancies (8). Metastatic melanoma cells express higher levels of TF than nonmetastatic cells (9), and a metastatic rectal carcinoma subline showed enhanced TF expression in comparison to its parental line (10). Transfection of TF promoted the metastasis of melanoma in a mouse model (11), and enhanced primary tumor growth in a pancreatic adenocarcinoma cell line (12). Therefore, TF not only contributes to the development of a hypercoagulable state in cancer patients but also modulates the biological behavior of cancer cells.

Pancreatic adenocarcinoma is one of the most clinically aggressive malignancies; indeed, the 3-year survival rate after surgical resection of the primary tumor has been reported as only 17% (13). Therefore, identification of molecules that

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might predict a poor prognosis is important in selecting patients who would benefit from radical treatment or molecular targeting therapy. Although a few immunohistochemical studies on TF expression in pancreatic ductal carcinoma have been done (8, 14, 15), no detailed clinicopathologic study using multivariate-type analysis has been carried out to date. In the present immunohistochemical study, we used a newly raised anti-TF antibody named NCC-7C11 to examine TF expression in a large series of surgically resected pancreatic ductal adenocarcinomas, and investigated the correlations between TF expression and various clinicopathologic parameters, including the clinical outcome. Furthermore, we investigated the effect of TF knockdown on the invasiveness of a pancreatic cancer cell line using RNA interference, a new gene-silencing technique.

Materials and Methods

Production of the monoclonal antibody. Female BALB/c (*nu/nu*) mice were immunized with the scirrhous gastric carcinoma cell line HSC-44PE by means of a rejection method, and hybridomas were produced as described previously (16). The hybridomas were then selected on the basis of their immunohistochemical reactivity with various cancerous tissues, and a hybridoma that produced the monoclonal antibody (mAb) NCC-7C11 (IgG_1, k), which reacted with the invasive front of pancreatic ductal adenocarcinoma, was obtained.

Cell lines and reagents. All pancreatic cancer cell lines (BxPC-3, SU 86.86., AsPC-1, Capan-1, Capan-2, PK-59, HPAC, MPanc-96, CFPAC-1, PANC-1, and MIAPaCa-2) were obtained from the American Type Culture Collection (Rockville, MD). The scirrhous gastric carcinoma cell line HSC-44PE was established by Yanagihara (17). The cells were maintained in RPMI 1640 (BxPC-3, SU86.86., AsPC-1, Capan-1, PK-59, HPAC, CFPAC-1 and HSC-44PE) or DMEM (Capan-2, MPanc-96, PANC-1, and MIAPaCa-2), supplemented with either 20% (Capan-1) or 10% (others) heat-inactivated fetal bovine serum (Sigma Chemical Co., St. Louis, MO), 100 units/mL penicillin and 100 μg/mL streptomycin (Invitrogen Corp., Carlsbad, CA) at 37°C in a humidified atmosphere containing 5% carbon dioxide. Another murine anti-human TF mAb (TFE), recombinant human TF apoprotein, and normal murine IgG_1k were purchased from Enzyme Research Laboratories, Inc. (South Bend, IN), Angiopharm (O'Fallon, MO), and Becton Dickinson and Company (Franklin Lakes, NJ), respectively.

Immunoprecipitation. The BxPC-3 pancreatic carcinoma cell line was used for immunoprecipitation. The cells were washed with ice-cold Ca^{2+}/Mg^{2+}-free PBS and treated with radioimmunoprecipitation assay buffer containing a proteinase inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany) on ice for 30 minutes. After centrifugation (15,000 rpm for 30 minutes), the supernatant was collected and precleared with protein G sepharose (50% slurry) at 4°C overnight. To conjugate the primary antibodies, 1 μg primary antibody and 25 μL protein G sepharose beads suspended in RIPA buffer were incubated with mixing at 4°C overnight. After centrifugation, ~500 μg of total cellular protein from the precleared supernatant and the antibody-sepharose conjugate were incubated with mixing at 4°C for 3 hours. The immunoprecipitates were collected by centrifugation at 2,500 rpm for 5 minutes at 4°C. After washing four times with RIPA buffer, the supernatant was carefully removed and the pellets were resuspended in 40 μL of 2× electrophoresis sample buffer.

Protein identification by mass spectrometry. The protein immunoprecipitated by mAb NCC-7C11 from the BxPC-3 lysate was subjected to SDS-PAGE. The protein was visualized using a negative gel stain kit (Wako Pure Chemical Industries, Ltd., Japan) and its band was excised from the gel. In-gel digestion was carried out with trypsin (Promega, Madison, WI), as described in the literature (18). Mass spectrometric analyses of the trypsin digests were done using Voyager (Applied Biosystems, Framingham, MA), and peptide mass mapping was carried out with reference to the MASCOT database.

Western blot analysis. Samples were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). After blocking, the filters were incubated with the primary antibodies, then with peroxidase-conjugated secondary antibodies (Amersham Biosciences Corp., Piscataway, NJ). The peroxidase-labeled bands were visualized using an electrochemiluminescence kit (Amersham Biosciences). As a loading control, the same membrane was reprobed with an anti--actin mAb (Sigma-Aldrich), as described in the literature (19).

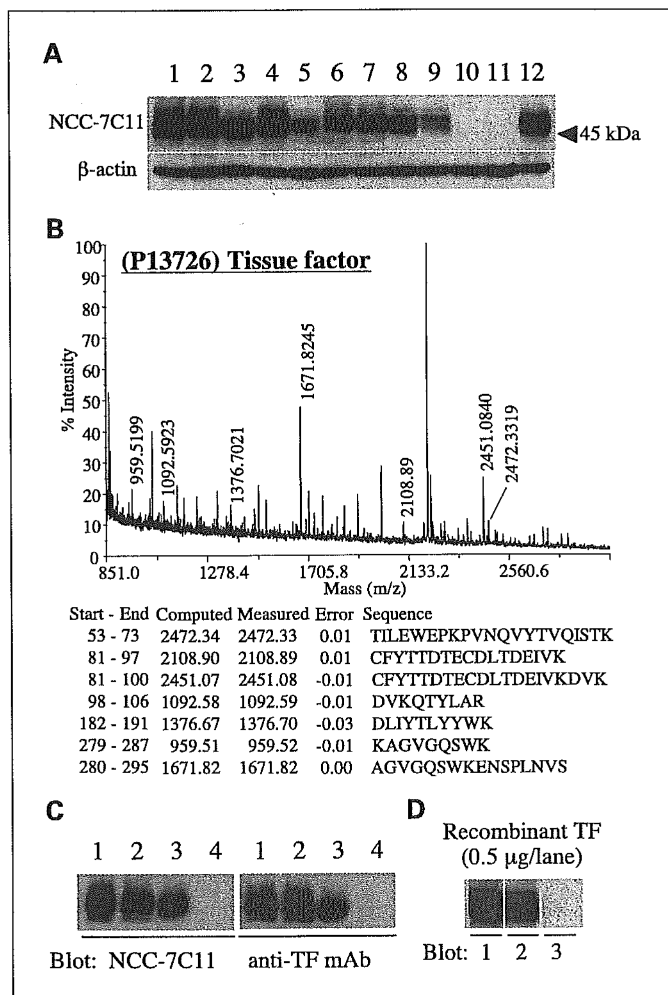

Fig. 1. Identification of the antigen recognized by mAb NCC-7C11. *A,* Western blot analysis of the NCC-7C11 antigen in various pancreatic cancer cell lines. *Lane 1,* BxPC-3; *lane 2,* SU 86.86.; *lane 3,* AsPC-1; *lane 4,* Capan-1; *lane 5,* Capan-2; *lane 6,* PK-59; *lane 7,* HPAC; *lane 8,* MPanc-96; *lane 9,* CFPAC-1; *lane 10,* PANC-1; *lane 11,* MIAPaCa-2; *lane 12,* HSC-44 (scirrhous gastric carcinoma cell line; Immunogen). Forty micrograms of whole cell lysate were applied to each lane and separated by SDS-PAGE under reducing condition. Position of the 45 kDa molecular size marker (*right*); *B,* identification of the protein immunoprecipitated by NCC-7C11 using mass spectrometry. After trypsin digestion, the ion peak spectra matched the seven peptide sequences of TF (P13726); *C,* reciprocal coimmunoprecipitations from BxPC-3 cells. Immunoprecipitates with NCC-7C11 (*lane 1*), a commercially available anti-TF mAb (TFE; *lane 2*) and mouse immunoglobulin (*lane 4;* negative control) were subjected to SDS-PAGE under nonreducing conditions. Whole cell lysates served as a positive control (*lane 3*); *D,* reactivity of antibodies with recombinant human TF apoprotein (0.5 μg/lane) by Western blot analysis under reducing condition. *Lane 1,* NCC-7C11; *lane 2,* the anti-TF mAb TFE; *lane 3,* mouse immunoglobulin.

Patients and tissue specimens. Formalin-fixed, paraffin-embedded tumor specimens were obtained from a series of 113 consecutive patients with pancreatic ductal adenocarcinoma who had undergone surgical resection at the National Cancer Center Hospital in Tokyo, Japan between 1990 and 1999. Patients with pancreatic tumors of a special type, such as mucinous cystadenocarcinoma, intraductal papillary-mucinous adenocarcinoma, or adenosquamous carcinoma, were excluded. Three patients who died in the immediate postoperative period were also excluded. The patients consisted of 72 men (63.7%) and 41 women (36.3%), who ranged in age from 45 to 82 years, with a mean age of 63.1 years. The median duration of follow-up was 16 months (range 2.9-72 months). The surgical procedures were total pancreatectomy in 6 patients, distal pancreatectomy in 35 patients, pylorus-preserving pancreaticoduodenectomy in 20 patients, and pancreaticoduodenectomy in 52 patients. Intraoperative radiation was done in 77 patients and postoperative chemotherapy was given to 44 patients. The resected specimens were staged according to the International Union against Cancer tumor-node-metastasis (TNM) classification (20). Histologic grading of the tumors was done according to the WHO classification system (21). Other pathologic variables (lymphatic invasion, vascular invasion, perineural invasion, and growth pattern) were based on the Japan Pancreas Society's classification system for pancreatic carcinoma (22).

Immunohistochemistry. The avidin-biotin-peroxidase complex method was used for immunostaining, as described in the literature (23). Briefly, formalin-fixed, paraffin-embedded sections (4 μm thick) containing the maximum diameter of the tumor were deparaffinized using a graded ethanol and xylene series, treated with 0.3% hydrogen peroxide in methanol and immersed in 10 mmol/L citrate buffer (pH 6.0). After autoclaving, the sections were incubated with normal swine serum for 10 minutes to block nonspecific antibody reactions, exposed to the primary antibody (final concentration, 1 μg/mL) overnight at 4°C, then incubated sequentially with biotinylated goat anti-mouse IgG and avidin-biotinylated-peroxidase complex as supplied in the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). The color reaction was developed over 5 minutes using diaminobenzidine tetrahydrochloride and 0.02% hydrogen peroxide, and nuclear counterstaining with hematoxylin was done. The positive control included in every assay was a section composed of formalin-fixed, paraffin-embedded cell pellets of the human pancreatic carcinoma cell line BxPC-3, which was confirmed to express the NCC-7C11 antigen by Western blot analysis. Negative control staining, which was done using the same class of mouse immunoglobulin as the primary antibody, yielded negative results in every specimen.

RNA interference, immunocytochemistry, and invasion assays. The sequences used to design the small interfering RNAs (siRNA) were selected according to a previously described strategy (24-26). The siRNA sequences chosen to target TF (Genbank accession number NM 001993) were positions 489 to 509 ($siRNA_{TF}489$) and 653 to 673 ($siRNA_{TF}653$), numbered from the start codon, and the siRNAs were purchased from Dharmacon, Inc. (Lafayette, CO). Control experiments were done using two unrelated siRNAs. $siRNA_{Luc}$ was Cy3 labeled siRNA directed against Luciferase mRNA (Dharmacon) and $siRNA_{NC}$ (mock) was Nonspecific Control Duplex X (Dharmacon). The sequence of the latter (5′-NNATTCTATCACTAGCGTGAC-3′) was confirmed to have no homology with any known mRNA by a BLAST search; however, it had the same GC content as $siRNA_{TF}$.

At first, we examined transfection efficiencies among the TF-positive cell lines BxPC-3, SU 86.86., and AsPC-1 by using Cy3-labeled siRNA against luciferase. In >60% of BxPC-3 cells, Cy3 was observed by fluorescence microscopy, and therefore the BxPC-3 cell line was selected. This Cy3-labeled siRNA against luciferase was used as a negative control in each experiment, so we confirmed the transfection efficiency every time we did the siRNA knockdown and invasion assay. Reduction of TF expression on the surface of cells was confirmed by immunocytochemistry using anti-TF antibody NCC-7C11, biotinylated goat anti-mouse IgG, and avidin-FITC (Vector Laboratories) under fluorescence microscopy.

RNA interference and invasion assays were done as described in the literature (27). BxPC-3 cells were exposed to 40 nmol/L siRNA, in the presence of Lipofectamine 2000 (Invitrogen), for 6 hours. The transfected cells were subjected to either immunoblot assays or invasion assays 24 hours after the removal of the transfection reagent.

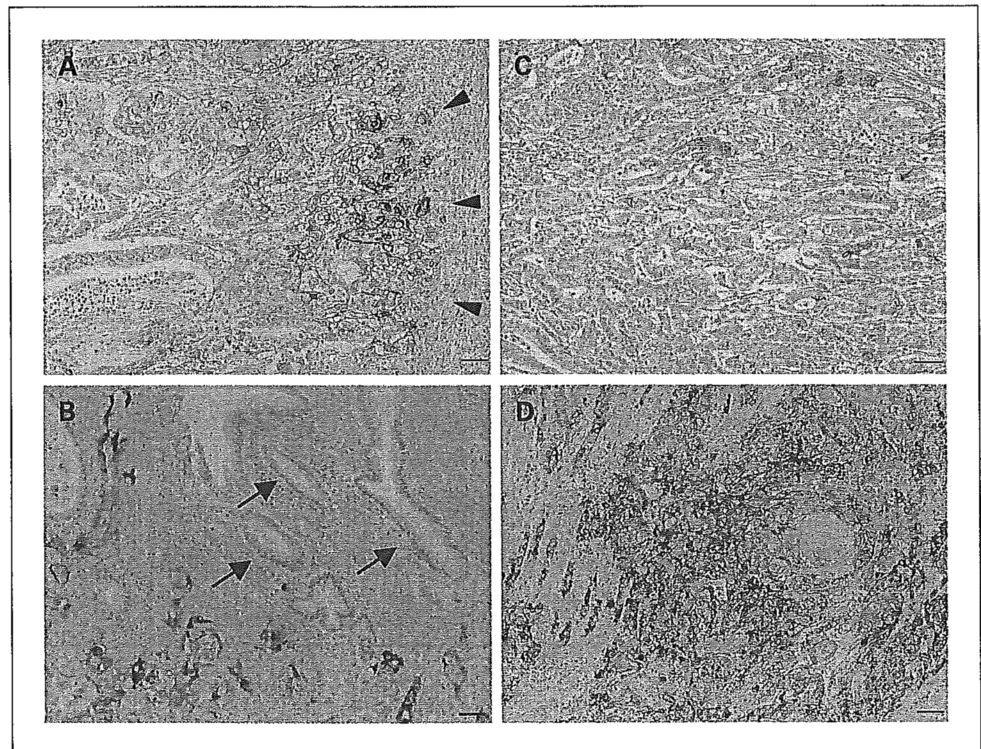

Fig. 2. Immunohistochemical staining pattern of the anti-TF mAb NCC-7C11. *A,* NCC-7C11 reacted preferentially with the invasive tumor front, as shown in this moderately differentiated pancreatic ductal adenocarcinoma (*arrowheads*); *B,* pancreatic ductal adenocarcinoma cells were stained by NCC-7C11, whereas the adjacent normal pancreatic ducts showed no immunoreactivity (*arrows*). Representative staining patterns (*C* and *D*); *C,* this moderately differentiated adenocarcinoma was classified as showing low TF expression; *D,* this poorly differentiated adenocarcinoma was markedly stained by NCC-7C11 and was classified as showing high TF expression. *Bar,* 100 μm.

Table 1. Association between TF expression and clinicopathologic variables

Variables	TF expression			P value (Low vs. High)
	Low TF (<25%, n = 63)			
	Negative TF (0%, n = 13)	Weak TF (0%<, n = 50)	High TF (≥ 25%, n = 50)	
Age (y)				
<65	8	26	24	
≥65	5	24	26	0.5284
Gender				
Male	7	31	34	
Female	6	19	16	0.3989
Extent of the primary tumor spread				
pT_1	3	3	1	
pT_2	3	9	5	
pT_3	4	20	15	
pT_4	3	18	29	0.0043*
Lymph node metastasis				
pN_0	2	7	5	
pN_{1a}	10	20	11	
pN_{1b}	1	23	34	0.0043*
Distant metastasis				
pM_0	9	42	28	
pM_1	4	8	22	0.0041*
M_1 (LYM)†	2	7	19	0.0039*
M_1 (HEP)†	1	1	1	0.9999
M_1 (PER)†	1	0	2	0.5508
Stage				
I	1	4	1	
II	0	2	2	
III	5	23	7	
IVA	3	13	18	
IVB	4	8	22	0.0002*
Histopathologic tumor grade				
G_1	7	24	16	
G_2	5	23	23	
G_3	1	3	11	0.0164*
Lymphatic invasion‡				
Negative	4	5	8	
Positive	9	45	42	0.8001
Vascular invasion‡				
Negative	8	14	12	
Positive	5	36	38	0.2087
Perineural invasion‡				
Negative	2	6	4	
Positive	11	44	46	0.5443
Growth pattern‡				
Expansive + intermediate	9	30	21	
Infiltrative	4	20	29	0.0392*
Surgical margin				
Negative	10	34	30	
Positive	3	16	20	0.2744

*Significant.
†LYM, lymphatic metastasis; HEP, hepatic metastasis; PER, peritoneal metastasis.
‡Classified according to the classification of Pancreatic Carcinoma of Japan Pancreas Society.

The relative density of the chemiluminescence signal was determined using Image Gauge Software (Fuji Photo Film Co., Ltd., Japan) and standardized by using the relative density of the β-actin signal. For the invasion assays, Biocoat Matrigel Invasion Chambers (Becton Dickinson Labware) were utilized according to the manufacturer's instructions. We used Accutase (Innovative Cell Technologies, Inc., San Diego, CA) to harvest cells for use in the invasion assay, and the harvested cells were washed with ice-cold PBS containing 0.1% bovine serum albumin before seeding. Transfected cells (4×10^5) in 500 μL RPMI 1640 containing 0.1% bovine serum albumin were seeded into each insert chamber. Then, 750 μL RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum was added to each lower chamber, and the plates were incubated at 37°C in a 5% CO_2/95% air incubator for 18 hours. After incubation, the noninvading cells were carefully removed from the top of each insert chamber with a cotton swab. The invading cells were then fixed and stained using a Diff-Quik kit (Sysmex Corp., Japan), and the total number of invading cells was counted under a microscope. Each run was done in triplicate, and the experiment was repeated independently thrice.

Statistical analysis. Correlations between TF immunoreactivity and patients' clinicopathologic variables were analyzed using the Mann-Whitney *U* test for the extent of the primary tumor spread (pT), lymph node metastasis, histologic tumor grade, and pTNM stage, and either the χ^2 test or Fisher's exact test for the remaining variables. The Kaplan-Meier method was used to generate survival curves, and differences in survival were analyzed using the log-rank test, based on the TF expression status. Univariate and multivariate analyses were done using the Cox proportional hazards model. Matrigel invasion assays and densitometric analyses were compared using the Mann-Whitney *U* test. Probability values <0.05 were considered statistically significant. All analyses were done using statistical analysis software (Statview, version 5.0; SAS Institute, Inc., Cary, NC).

Results

Monoclonal antibody characterization. Western blotting under reducing condition showed that about half of the pancreatic cancer cell lines expressed moderate to high levels of the NCC-7C11 antigen (Fig. 1A). A peptide mass fingerprint of tryptic digests of the antigen immunoprecipitated from the BxPC-3 cell lysates was obtained by mass spectrometry and a search of the MASCOT database identified this antigen as TF (Fig. 1B). To confirm the identity of TF, we did reciprocal coimmunoprecipitation assays using a commercially available anti-TF mAb TFE under nonreducing conditions (Fig. 1C). We also showed the reactivity of NCC-7C11 and TFE mAbs to recombinant TF apoprotein by immunoblotting (Fig. 1D). Together, these data confirmed that NCC-7C11 was an anti-TF mAb. We examined the TF expression pattern of the cell lines by Western blotting with a commercially available polyclonal antibody against TF (clonal, American Diagnostic, Inc, Greenwich, CT), and thus confirmed the results of our Western blot analysis (data not shown).

Immunohistochemical analysis of tissue factor expression in pancreatic ductal adenocarcinoma. The immunostaining pattern of NCC-7C11 is shown in Fig. 2. TF expression occurred preferentially at the invasive front of the tumor (Fig. 2A), whereas no TF was expressed in adjacent normal ductal cells (Fig. 2B), as previously described in the literature (14). According to the proportion of TF-positive cancer cells, TF expression was classified as "low TF" (0-25% of cells showing immunopositivity, Fig. 2C) or "high TF" (25% or more of cells showing immunopositivity, Fig. 2D). Low TF included patients with completely TF-negative tumors ("negative TF", 0% of cells showing immunopositivity), and those with weakly TF-positive tumors ("weak TF", >0% and <25% of cells showing immunopositivity). The cutoff point for weak/high TF was set at the median value for the entire sample without the TF-negative sample. When comparing the high TF group with the low TF group, increased TF expression was positively correlated with the extent of primary tumor spread, lymph node metastasis, the presence of lymphatic distant metastasis, high tumor grade, advanced TNM stage, and an infiltrative growth pattern (Table 1).

Prognostic significance of tissue factor expression. The survival curves of the patients, grouped according to the level of TF staining in their tumors, are shown in Fig. 3A. The high TF expression group had a significantly poorer prognosis than the

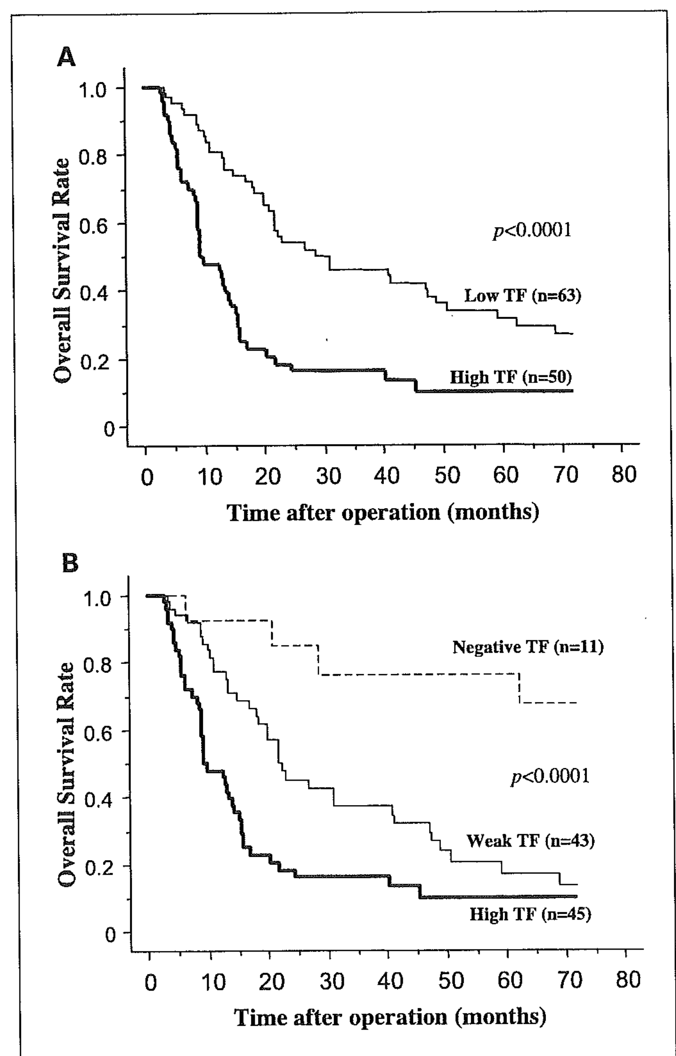

Fig. 3. Kaplan-Meier survival curves for patients who had undergone surgical resection of pancreatic ductal adenocarcinoma, stratified according to the level of expression of TF in their tumors. *A*, overall survival of patients with pancreatic ductal adenocarcinoma (low TF, 0-25%; high TF, 25% or more of the cells showing immunopositivity; log-rank test, $P < 0.0001$); *B*, overall survival of patients who had tumors with lymph node metastasis (negative TF, 0%; weak TF, >0% and <25%; high TF, 25% or more of the cells showing immunopositivity; log-rank test, $P < 0.0001$).

low TF expression group (log-rank test, $P < 0.0001$). Upon univariate analysis with the Cox proportional hazards model, the extent of the primary tumor ($P = 0.0497$), lymph node metastasis ($P = 0.0102$), distant metastasis ($P = 0.0027$), histologic tumor grade ($P = 0.0070$), growth pattern ($P = 0.0173$), and TF immunopositivity ($P < 0.0001$) were all positively correlated with a poor prognosis. Multivariate analyses indicated that TF expression was an independent predictor of an unfavorable prognosis ($P = 0.0076$; risk ratio, 2.014; 95% confidence interval, 1.205-3.366), as were the presence of lymph node metastasis ($P = 0.0103$) and histologic tumor grade ($P = 0.0154$; Table 2). The survival of the patients with lymph node metastasis was further analyzed, grouped according to three TF staining levels, i.e., negative TF, weak TF, and high TF (Fig. 3B). The survival of the TF-negative group was markedly better and increased TF expression was significantly correlated with a poor prognosis (log-rank test, $P < 0.0001$).

The effects of small interfering RNAs targeted against tissue factor on tumor invasion. TF overexpression proved to be linked with the aggressiveness of pancreatic cancer in our immunohistochemical analysis. In order to determine whether down-regulation of endogenous TF would suppress the invasive behavior of pancreatic cancer, we synthesized siRNAs that, when transfected into cells, target TF mRNA for degradation, thus reducing the expression of TF protein. High transfection efficiency of siRNAs into BxPC-3 cells has been achieved with Lipofectamine 2000 (Fig. 4A, *top*) and reduction of TF expression by $siRNA_{TF}653$ against TF, compared with control $siRNA_{NC}$, has been ascertained under fluorescence microscopy by immunocytochemistry (Fig. 4A, *middle* and *bottom*). Densitometric analyses (Fig. 4B) and invasion assays (Fig. 4C) showed that transfection with either $siRNA_{TF}489$ or $siRNA_{TF}653$ significantly reduced TF expression by, and the invasiveness of, BxPC-3 cells compared with mock-transfected cells ($siRNA_{NC}$), whereas transfection with a siRNA targeted to an unrelated mRNA ($siRNA_{Luc}$) had no effect on TF expression or invasiveness.

Discussion

In the present study, we showed the clinicopathologic significance of TF expression in pancreatic ductal adenocarcinoma in an immunohistochemical analysis using a newly raised

Table 2. Prognostic factors in Cox's proportional hazards model

Variables	Univariate			Multivariate		
	Hazard ratio	95% Confidence interval	P	Hazard ratio	95% Confidence interval	P
Age (y)						
≥65/<65	1.202	0.782-1.846	0.4014			
Gender						
Female/male	0.909	0.582-1.420	0.6762			
Extent of the primary tumor spread						
pT_4/pT_1-pT_3	1.545	1.001-2.385	0.0497*	1.280	0.793-2.066	0.3125
Lymph node metastasis						
pN_{1a}, pN_{1b}/pN_0	2.770	1.274-6.023	0.0102*	2.953	1.292-6.752	0.0103*
Distant metastasis						
pM_1/pM_0	2.301	1.279-3.223	0.0027*	1.501	0.912-2.471	0.1101
Histologic tumor grade						
G_2, G_3/G_1	1.845	1.182-2.879	0.0070*	1.882	1.128-3.318	0.0154*
Lymphatic invasion						
Positive/negative	1.429	0.757-2.700	0.2708			
Vascular invasion						
Positive/negative	1.412	0.877-2.273	0.1554			
Tumor diameter (cm)						
≥3.5/<3.5	1.366	0.884-2.111	0.1604			
Growth pattern						
Infiltrative/expansive, intermediate	1.638	1.096-2.584	0.0173*	1.211	0.742-1.976	0.4446
Surgical margin						
Positive/negative	1.168	0.747-1.824	0.4959			
Chemoradiotherapy						
Not received/received	0.957	0.562-1.630	0.8708			
TF expression						
High TF/low TF	2.723	1.748-4.243	<0.0001*	2.014	1.205-3.366	0.0076*

*Significant.

anti-TF antibody. Our findings indicate that TF has prognostic significance in patients with resectable tumors. Moreover, we confirmed that TF contributed to the invasiveness of a pancreatic cancer cell line by inhibiting TF expression using the RNA interference technique *in vitro*.

It is well recognized that cancer cells at the invasive front express invasion-related molecules such as matrix metalloproteinases (28) and the laminin γ2 chain (29, 30). We confirmed that TF is another of these invasion-related molecules, since TF immunopositivity was clearly observed at the invasive fronts of the pancreatic ductal adenocarcinomas. Our immunohistochemical study also showed that TF expression in the primary tumors was correlated significantly with many aggressiveness-related factors, including the extent of primary tumor spread, lymph node metastasis, lymphatic distant metastasis, TNM stage, tumor grade, and growth pattern. Among previous immunohistochemical studies of TF expression in pancreatic ductal adenocarcinoma, only that reported by Kakkar et al. (14) showed correlations between TF expression and clinicopathologic characteristics, showing that TF expression is correlated with histologic tumor grade and possibly with lymph node metastasis. In agreement with their results, the present study clarified that TF expression was indeed correlated with tumor grade and the extent of lymph node metastasis. Although there was a tendency for TF to be frequently expressed in G_3 cells, it was also expressed in some well or moderately differentiated tumors. Moreover, it is very disconcerting that the least differentiated cell lines examined, such as MIAPaCa-2 and Panc-1, proved TF-negative. However, in agreement with the present study, MIAPaCa-2 and Panc-1 have actually been reported to express hardly any TF mRNA (31). Therefore, we speculate that TF is not merely an indicator of grade. It is unclear what value this spectrum of cell lines adds to the current proposal and whether they are incapable of expressing TF. Further analysis will be needed to reconcile this discrepancy between *in vitro* and *in situ* conditions. On the other hand, TF expression in lymph node metastases is of great interest since our immunohistochemical analysis seemed to indicate that TF was involved in lymph node metastasis. Therefore, we have additionally examined 10 lymph node metastases to determine whether TF expression is enriched in comparison with the expression in the primary tumor. We found that TF expression in lymph node metastases reflected that in the primary tumor, although it was not necessarily enriched (data not shown). Immunohistochemical studies on other cancers have also revealed correlations between TF expression and clinicopathologic characteristics. In colorectal carcinoma, TF expression was positively correlated with lymph node metastasis, liver metastasis, and Dukes' stage (32). In non-small cell lung cancers, TF expression was also associated with hematogenous or lymphogenous metastasis (33). These observations are consistent with our findings, in that TF

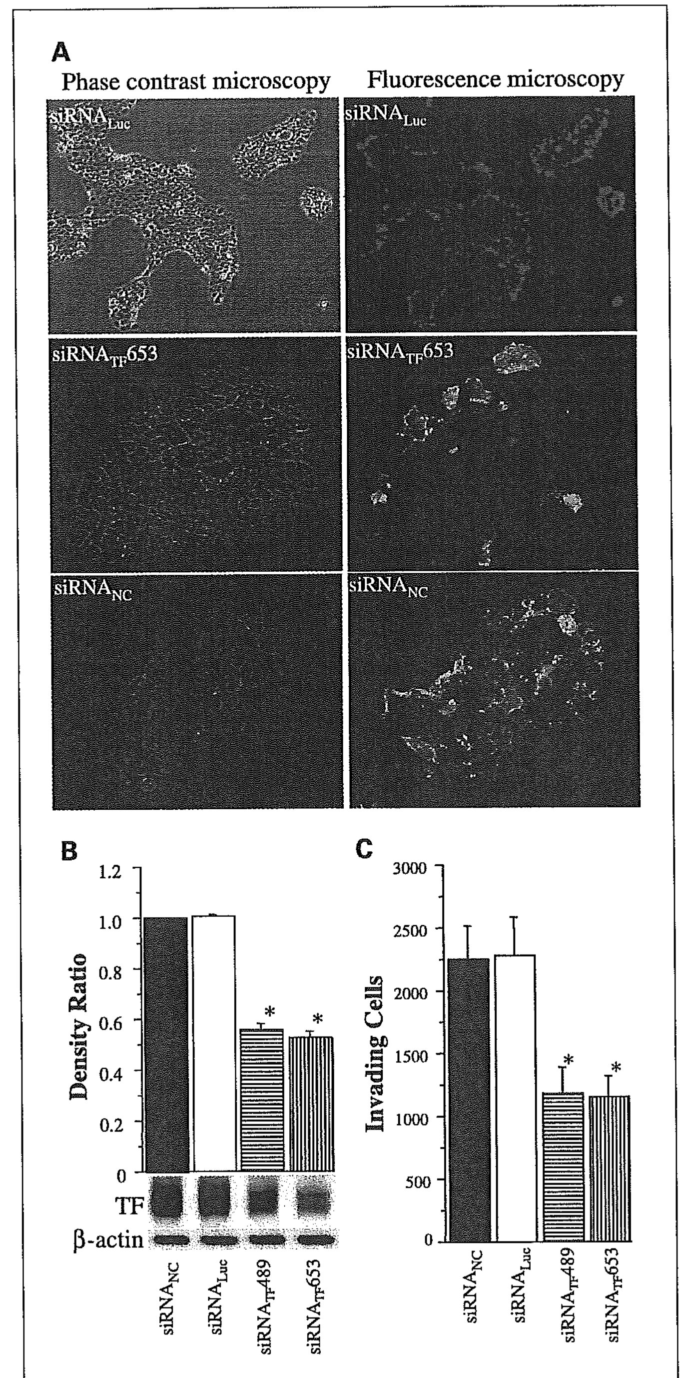

Fig. 4. Effect of TF knockdown by RNA interference on the invasiveness of human pancreatic cancer cells. BxPC-3 cells were transiently transfected with short interfering RNAs and subjected to either Western blot analysis or Matrigel invasion assays. siRNA$_{TF}$489 and siRNA$_{TF}$653 are directed against TF. Control experiments were done with a Cy3-labeled siRNA directed against an unrelated mRNA (Luciferase; siRNA$_{Luc}$) and an irrelevant siRNA (siRNA$_{NC}$; used as a mock-transfectant). Transfection efficiency was confirmed by using Cy3-labeled siRNA$_{Luc}$ in each assay, and representative pictures obtained by phase-contrast microscopy and fluorescence microscopy revealed a high efficiency of transfection of siRNA into BxPC-3 cells (*A, top*). Immunocytochemistry under fluorescence microscopy shows that many cells lack TF expression on their surface as a result of knockdown by siRNA$_{TF}$653 against TF (*A, middle*), whereas control siRNA$_{NC}$ has no effect on TF surface expression (*A, bottom*). Reduction of TF protein expression by siRNA against TF was determined by Western blot analysis and densitometric analysis. The relative density of the chemiluminescence signal was measured and standardized using the relative density of the β-actin signal. Transfection with either siRNA$_{TF}$489 or siRNA$_{TF}$653 significantly reduced TF compared with mock-transfected cells (siRNA$_{NC}$), whereas transfection with a siRNA targeted to an unrelated mRNA (siRNA$_{Luc}$) had no effect on TF expression (*B*). For the invasion assays, the transfectants were seeded onto Matrigel-coated invasion chambers and incubated for 18 hours, then the total number of cells on the underside of each filter was determined. Invading cells were significantly suppressed by siRNA against TF, as reflected in the observed reduction of protein expression (*C*). Columns, means; bars, SE ($n = 9$); *, $P < 0.01$ compared with both control groups.

expression was significantly correlated with lymphatic distant metastasis and TNM stage. In our series, TF expression did not correlate with either hepatic or peritoneal metastasis, but only with lymphatic distant metastasis, suggesting a potential specificity of this protein's role in invasion. However, it is rare for pancreatic tumors with distant metastasis, except lymphatic distant metastasis, to become operable. Therefore, it is difficult to conclude that there is no correlation between TF expression and distant metastasis besides lymphatic distant metastasis. The present study also revealed that high TF expression was associated with the extent of the primary tumor and an infiltrative growth pattern, suggesting that TF overexpression has a proinvasive effect.

The clinical significance of high-level TF expression was further substantiated by its correlation with a shorter overall survival time. Univariate analysis showed that TNM status, tumor grade, tumor size, growth pattern, and TF expression were all significantly correlated with patient survival. Moreover, multivariate analysis also showed that TF expression was an independent prognostic factor. Therefore, TF had significant predictive value for overall survival, suggesting that its expression could be a useful predictor of poor prognosis. Although the hazard ratio of lymph node status was higher than that of TF expression in multivariate analysis, lymph node status and TF expression were proven to be statistically significant and independent prognostic factors. Therefore, we believe that both factors are almost equally important in predicting prognosis in patients with pancreatic cancer. Indeed, among patients with lymph node metastasis, those with TF-negative tumors had a markedly better prognosis, and increased TF was also significantly correlated with a poorer prognosis. Thus, our findings suggest that TF contributes to the aggressiveness of pancreatic ductal adenocarcinoma. To our knowledge, this is the first study to have shown the clinicopathologic significance of TF expression in pancreatic ductal adenocarcinoma using multivariate-type analysis.

The present study revealed that knockdown of endogenous TF could suppress the invasiveness of a pancreatic adenocarcinoma cell line *in vitro*, suggesting that TF plays an important role in tumor invasion. The potential role of coregulation of TF and effector proteases such as matrix metalloproteinases has been reported previously for other cell types (34, 35). In a small cell lung cancer cell line, the transition of a small cell lung cancer from a suspension to adherent and aggressive growth was accompanied by expression of TF as well as matrix metalloproteinases-2 and -9 (35). Other mechanisms by which TF promotes tumor invasion have been suggested previously. Taniguchi et al. (31) showed that binding of FVIIa to TF induced overexpression of the urokinase plasminogen activator receptor gene, which is involved in proteolytic extracellular matrix degradation, resulting in increased migration of pancreatic cancer cells, whereas blockade of TF activity with neutralizing monoclonal antibodies inhibited FVIIa-dependent tumor invasion. Ott et al. (36) showed that the role of TF in cell migration and adhesion is mediated by an interaction with actin-binding protein. TF has also been shown to mediate intracellular signaling leading to the development of lamellipodia and filopodia (5). In our invasion assay, however, the number of invading control cells observed was higher than the levels reported previously (37). One reason for the high invasion may have been that the seeding density we used was more than 10 times higher than that reported previously. Another reason might be that we used Accutase to harvest the cells from culture, although Accutase has also been reportedly utilized for the invasion assay in a study of another cell type (38). Since Accutase is reported to maintain most cell surface antigens and some antibodies including anti-TF antibody and anti-urokinase plasminogen activator receptor antibody work well with Accutase according to the manufacturer (data not shown), cells treated with Accutase might retain their invasive ability. On the other hand, Accutase is a mixture of invasion-relevant proteases that are directly capable of degrading the reconstituted basement membrane used as a barrier in the invasion assay. So, although the cells were washed before being seeded, we cannot rule out the possibility that this assay might not represent an examination of the capability of BxPC-3 cells to invade *de novo*, but rather their ability to use extrinsic enzymes to effect invasion. Although the present study could not prove the mechanism by which TF promotes tumor invasion, our finding of a distinct association between TF and tumor invasiveness may have therapeutic as well as prognostic implications. Since retinoic acid (39), resveratol (40), vitamin D_3 (41), and pentoxifylline (42) have all been reported to down-regulate TF, the effects of these agents on TF expression in pancreatic cancer cells are worth evaluating. Recently, the relationship between TF expression and angiogenesis in various types of malignancies has also been emphasized (43-45); this may occur through regulation of the vascular endothelial growth factor (46). Therefore, down-regulation of TF expression might lead to the suppression of not only tumor invasiveness but also angiogenesis. However, although TF seems to be an attractive target for potential treatments of pancreatic ductal adenocarcinoma, we must always be concerned about the possible side effects of TF targeting therapy, including an increased bleeding tendency.

Finally, Kakkar et al. showed that the level of TF was higher in the plasma of cancer patients, including those with pancreatic cancer, than in healthy controls (47). Furthermore, the plasma concentration of TF was shown to reflect tumor TF, which was correlated with the prognosis of patients with breast cancer (48). Hence, measurement of the plasma TF concentration might be of predictive value for prognosis or selecting candidates for TF-targeting therapy, even in patients with inoperable pancreatic ductal carcinoma.

In conclusion, our present findings indicate that there is a significant association between TF expression and tumor aggressiveness in pancreatic ductal adenocarcinoma and suggest that TF expression is a useful prognostic marker in postoperative patients. In addition, TF expression may contribute to the aggressiveness of pancreatic ductal adenocarcinoma by stimulating tumor invasiveness.

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THE LUNG perspectives

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基礎医学とのダイアローグ

SELDI-TOF-MS法

Surface-enhanced laser desorption / ionization time-of-flight mass spectrometry

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Key words

SELDI-TOF-MS法，病態診断マーカー，ペプチドプロファイル，教師付き機械学習アルゴリズム

Summary

臨床診断マーカー開発の分野でプロテオミクスは注目を集めている。中でもSELDI-TOF-MS法はハイスループットプラットホームとして有用性が期待されている。当研究所では，本システムを稼働させ，がん臨床に有用な診断マーカーの探索を行っている。これまでに血清・血漿中に含まれるペプチドプロファイルを利用して，膵臓がん，腎細胞がんの早期診断マーカーや食道がん術前化学放射線療法奏効性予測マーカーの開発を手がけてきた。本稿では，その原理，実際の解析方法，教師付き機械学習アルゴリズムを用いたマーカー抽出方法，臨床応用の可能性，今後の展望について述べたいと思う。

はじめに

近年，臨床診断マーカーの開発分野では，個々のタンパク質を検討する研究法ではなく，その全体像を包括的に捉えて研究を進めていくプロテオミクスが注目を集めている。特に，SELDI-TOF-MS (surface-enhanced laser desorption/ionization time-of-flight mass spectrometry) 法は，ごく少量の検体からそこに含まれる低分子量タンパク質・ペプチド情報を網羅的に取得できるため，クリニカルプロテオミクス分野で，有用性が期待されている。

難治がんの治癒率を向上させるためには，非侵襲的な方法で採取が可能な検体から検査が行えて，高い信頼性を有する診断マーカーの開発が望まれる。担がん患者血液中に含まれるタンパク質の動態は臨床病態に敏感に反映すると考えられており，この情報を網羅的に解析することは診断マーカーの開発にとって魅力的な戦略になりうる。このような時代背景の中，米国国立がん研究所 (National Cancer Institute ; NCI) と米国食品医薬品局 (Food and Drug Administration ; FDA) の研究グループは，SELDI-TOF-MS法を用いて卵巣がん患者と良性卵巣疾患患者ならびに健常女性の血清中に含まれる低分子量タンパク質とペプチドプロファイルを網羅的に解析し，膨大なデータの中から早期卵巣がんを正確に診断する腫瘍マーカーを抽出した[1]。本研究は診断マーカーを探索する多くの研究者に衝撃を与え，米国などでは

本法を用いて大規模なマーカー探索プロジェクトが展開中である。当研究所化学療法部・腫瘍プロテオミクスプロジェクトにおいてもSELDI-TOF-MS法が導入され，血液サンプルを使って連日がん病態診断マーカーの探索が行われている。本稿においては，SELDI-TOF-MS法の原理，当研究所で行われているマーカー探索方法，今後の展望について述べたいと思う。

I SELDI-TOF-MS法の原理

SELDI-TOF-MS法とは，ProteinChip®と呼ばれる金属板と飛行時間計測型質量分析装置を組み合わせて行われる分析法のことをいう。ProteinChip®とは，ごく少量の検体から金属表面上でタンパク質の精製を行うために開発されたツールである（図１A）。２mm大の金属円盤上に官能基が修飾されており，少量の体液（血漿，血清，尿など），組織抽出液，細胞破砕液などの検体から，付加された官能基の性質に従ってタンパク質・ペプチドを特異的に捕捉することができる。捕捉されたタンパク質・ペプチドは特化された質量分析装置によって分析される。質量分析装置を用いることにより20 μL程度の検体からも高い分解能で再現性のよい結果を得ることができる。検体ごとの情報は，縦軸がイオン化強度，横軸が質量電荷比としての２次元情報として描出される（図１B）。ここに現れる１本１本のピークが検体中に含まれるペプチドのイオン化強度と質量情報である。さらに数種類の官能基をもつProteinChip®を使用することで，化学的性質の異なったペプチドプロファイルを得ることができる。

II 国立がんセンターで行われている診断マーカー探索法

当研究所化学療法部・腫瘍プロテオミクスプロジェクトでは，血液検体に対して本システムが稼働中である。以下に当研究所で行っている解析方法を記す。

変性剤を用いて変性させた血清・血漿サンプルに，陽イオン交換チップ，銅修飾チップ，逆相チップを使ってアフィニティークロマトグラフィーを行っている。計測には高分解能を有し質量精度の高い４重極搭載飛行時間型質量分析装置を使用し，再現性の高いデータ取得に努めている。得られた結果をすべて大容量のサーバーコンピューターに保存し，データベースを構築していく。ここまでの操作はバイオロボットなどを用いることによりす

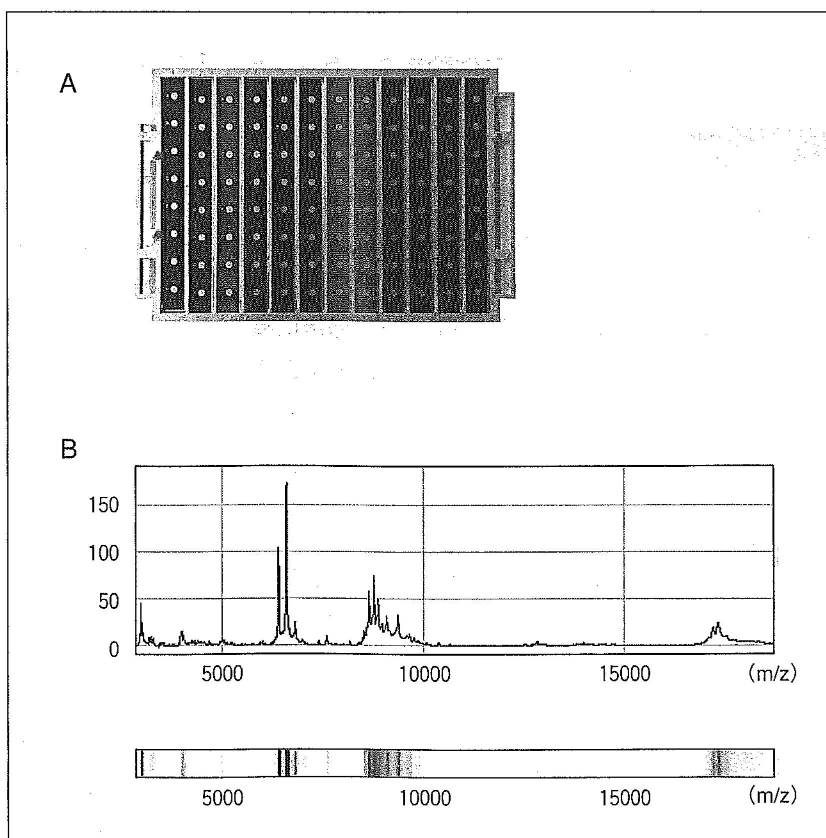

図１．SELDI-TOF-MS法
(A)ProteinChip®：直径約２mmの金属表面に官能基が修飾されていて，チップ表面でアフィニティークロマトグラフィーが可能。
(B)ペプチドプロファイルイメージ：縦軸がイオン化強度，横軸が質量電荷比として表される。下はそのゲルイメージ。

べて自動化され，当研究所では，１日最大192検体の解析が可能である。

このデータベースを利用して，がん罹患者と非罹患者を最も正確に判別するモデルを構築する。モデル構築に使われる検体群を学習セットと呼ぶ。学習セットには，罹患者と非罹患者間に疾患以外にバイアスがかからないよう性差，年齢差などを厳密に一致させた集団を用意する。学習セットで得られたペプチドプロファイルデータベースの中から機械学習法と呼ばれるパターン認識教師付き機械学習アルゴリズムを用いて，判別率の高いマーカー候補を抽出する。高い判別率を有するシングルマーカー候補が抽出できれば最もよいが，複数のマーカーを組み合わせて判別するマルチマーカー候補を抽出することも可能である。

われわれは判別アルゴリズムとしてニューラルネットワーク，サポートベクターマシンなどの数理モデルを使用している。この段階で抽出されたマーカー候補はあくまで候補であって，最終的なマーカーモデルではない。抽出されたマーカー候補は，別に用意した検証セットを使用して判別率の精度が検証される。この作業を数回繰り返し，さらに多施設から採取された検体での全国規模での検証を行うことで，判別マーカーモデルの信頼性を確立できるものと考えている。

Ⅲ 臨床応用

本方法で抽出されたペプチドマーカーを高度に精製し，そのアミノ酸配列を決定することは可能である。この情報をもとに特異抗体を作製し，酵素抗体法などの免疫生化学検査を確立していく戦略も考えられる。しかしながらマーカーとして抽出されてくるペプチドは，腫瘍本体が特異的に産出するペプチドや糖鎖などの従来型の腫瘍マーカーではなく，がん間質相互作用などにより活性化された消化酵素などによって本来生体内に豊富に存在するタンパク質が特異的に消化され，その消化産物を検出している可能性が指摘され始めた。すなわち腫瘍マーカー検査として応用するためには，ペプチドの定量的発現情報だけではなく，そのペプチドの分子量情報が重要であることが理解され始めた。質量分析装置間の分解能，イオン化の再現性ならびに質量情報の安定性を十分整備し，取得データの標準化ができれば，従来型の免疫生化学検査ではなく，質量分析装置から得られる情報そのものと特化された数理アルゴリズムを用いた検査手法の確立も可能であると考えられる。現在われわれは，上記目標のもと新たな技術開発を進めている。

Ⅳ 今後の展開

われわれは，難治がん克服を目標に，臨床に応用できるマーカー開発を進めている。難治性の高い悪性腫瘍の１つに膵臓がんが挙げられる。検診に使用できる膵臓がん腫瘍マーカー開発を目標に，現在までに合計200名を越えるがん罹患者ならびに健常者血漿ペプチドのプロファイル取得を終了した。また，４本のペプチド情報を使うことにより，感度・特異度とも90％を超える正診率をもつマーカーセットの抽出に成功した。このマーカーセットは，従来の膵臓がん腫瘍マーカーであるCA19-9の発現に相補的で，CA19-9の発現と組み合わせると早期膵臓がん患者を含めて100％検出することが可能であった[2]。今後は臨床応用を考えて全国規模での検証実験を検討中である。また膵臓がんだけでなく，他の悪性腫瘍でも検討を進めている。腎細胞がん血清をプロファイルし，腎細胞がんを高率に判別できるペプチドの組み合わせを明らかにしてきた[3]。さらに，多種類の悪性腫瘍でマーカー探索を行いたいと考えている。

また，臨床マーカーとして化学療法奏効性予測マーカーは大きな意義をもつ。なぜならば，施術前にその奏効性を予測できるのであれば，無意味な化学療法で不快な副作用を与えることなく適切な治療の選択が可能になるからである。われわれは，東京医科大学外科学第３講座と共同研究で食道がん術前化学放射線療法奏効性予測マーカーの開発を試みている。現在まで42名の術前療法施行前患者血清のペプチドプロファイルの取得を行ってきたが，奏効性を高特異度で予測できるマーカーの抽出に成功した。今後はさらに検体数を増やし，その信頼性を高めていく予定である[4]。

おわりに

以上述べてきたように，SELDI-TOF-MS法はマーカー探索分野ではパワフルなハイスループットプロテオミクスプラットフォームの１つである。しかしながら，血液中に含まれる微量タンパク質の測定に必ずしも十分な感度が得られているわけではなく，