て，主にRNA機能を障害すると考えられる．FOLFOXやFOLFIRI療法などは2つの投与法を組み合わせて使用されており，5-FUの作用を最大限に活用していると考えられる．

2)Tegafur/Oteracil/Gimeracil(S-1)

5-FUのプロドラッグであるテガフールに，DPDを阻害するギメラシルを含んだ5-FU系の抗癌剤で，オテラシルカリウムが消化管毒性の増強を軽減することに成功した抗癌剤である．

テガフールは，肝臓にあるチトクロームP-450によって5-FUへと代謝されるが，80～90％が分解されてしまうため，分解を抑えるためにギメラシルが配合されている．また，オテラシルは経口投与されると消化管粘膜に局在し，OPRTを選択的に阻害することにより5-FUからフルオロウリジン一リン酸(FUMP)への生成を選択的に抑制し，下痢や口内炎などの消化管障害を軽減させる．骨髄細胞での活性化は，配合という手法では抑制できなかったため，TS-1の用量制限毒性は好中球減少を主体とした骨髄抑制となる．

3)Capecitabine

副作用を軽減し，また抗腫瘍効果を高めるため，腫瘍組織内で高発現の酵素により5-FUに変換されることを目的として開発された5FUのプロドラッグの5'DFUR(フルツロン)の，さらにプロドラッグであるフルオロシチジン誘導体である．以下に述べる3つの代謝過程を経て5-FUに変換され，はじめて薬理活性を示す．

まず腸管から未変化体のまま吸収され，肝臓で活性の高いカルボキシルエステラーゼ(CE)により5'-deoxy-5-fluorocytidine(5'-DFCR)に変換され，次に肝臓および腫瘍で活性の高いシチジンデアミナーゼ(CD)で5'-DFURに変換される．さらに，腫瘍組織で活性の高いチミジンホスホリラーゼ(TP)で5-FUに変換されて抗腫瘍効果を示す．

本剤に特徴的な副作用として手足症候群(Hand—foot syndrome)があげられる．手足症候群では手掌および足底に湿性落屑，皮膚潰瘍，水疱，疼痛，知覚不全，有痛性紅斑，腫脹等の症状が見られる．

4)Tegafur-Uracil(UFT)

5-FUのプロドラッグであるテガフール(TS-1の項参照)に，5-FUの分解阻害作用を有するウラシルを配合した抗悪性腫瘍剤である．

2. プラチナ化合物

L-OHP(oxaliplatin)

プラチナ原子に1,2-ジアミノシクロヘキサン(DACH)およびシュウ酸基が付加したものである．作用機序は他の白金系抗癌剤と同様，DNA塩基との架橋形成によるDNA合成阻害，蛋白合成阻害と考えられている．白金系抗癌剤は，脱離基が水あるいは環境中の求核分子に置換されることにより，生体内分子との反応性を発現させる．この変換過程は酵素反応を伴わないことから，biotransformation(生体内変換)と定義されている．オキサリプラチンは，モノアクオモノクロロ1,2-ジアミノシクロヘキサン(DACH)白金やジアクオDACH白金等の生体内変換体を形成し，腫瘍細胞内のDNA鎖と共有結合することにより，DNA鎖内および鎖間の両者に白金-DNA架橋を形成する．これらの架橋がDNAの複製および転写を阻害し，細胞増殖抑制作用を発現すると考えられている．

本剤は主に尿中に排泄され，投与後48時間に一掃される．また，本剤投与にあたって大量輸液は不要である．特徴的な副作用としては，手足や口唇周囲部等の感覚異常または知覚不全(末梢神経症状)が，本剤投与直後からほぼ全例にあらわれる．また，咽頭喉頭の絞扼感(咽頭喉頭感覚異常)があらわれることもある．

3. トポイソメラーゼⅠ阻害剤

Irinotecamn hydrochloride(CPT-11)

CPT-11は，中国原産の喜樹(Camptothecaacuminuta)から抽出された植物アルカロイド20(S)-camptothecin(CPT)の半合成誘導体である．CPT-11の抗腫瘍効果は，topoisomeraseⅠ活性を

選択的に阻害することによってもたらされる．topoisomerase は，DNA の複製/転写のときに重要な働きを示す蛋白であり，このうち topoisomerase Ⅰは，DNA の1本鎖に nick を生じさせる．DNA のリン酸基に共有結合し，DNA のホシホジルエステル結合を切断し，DNA 複製がスムースに行われるようにする働きをする．CPT は，このDNA と toroisomerase Ⅰの共有結合部(DNA topoisomerase Ⅰ cleavable complex)に結合し，DNA 複製を停止させることで細胞増殖を抑制する．CPT-11は静脈内投与後 carboxylesterase により活性型 SN-38に変換される．SN-38の約70％は，肝臓にて glucuronidation を受け SN-38 glucuronide となり，SN-38や CPT-11とともに胆汁中に排泄される．副作用としては，骨髄抑制と遅発性下痢が特徴的である．

4．分子標的薬

1)抗 VEGF 抗体(Bevacizumab)

Bevacizumab は，VEGF(VEGF-A)に特異的に結合するヒト化 IgG1モノクローナル抗体で，VEGF がその受容体(VEGF-1および VEGF-2)に結合するのを阻止することで VEGF の生物活性を抑制する．VEGF の生物学的活性は，血管内皮細胞増殖，血管内皮細胞の遊走，未熟な内皮細胞の生存などである．半減期は2～3週間である．血管新生阻害剤のなかで，他に先んじてその有効性が証明された薬剤である．

抗体医薬の効果や副作用は，細胞毒性抗がん剤と異なり個体差の大きいことが知られている．Bevacizumab の毒性は比較的軽微とされるが，時に消化管穿孔・血痰などの出血症状がみられる．とくに消化管穿孔に関しては，種々の固形癌のなかでも大腸癌症例においてリスクが高いとされており，ハイリスク症例には慎重な判断が必要である[1]．

2)抗 EGFR 抗体(Cetuximab)

上皮増殖因子受容体(EGFR)の構造的特性やシグナル伝達，癌における発現調節異常については専門書を参照されたい．Cetuximab はマウスとヒト IgG1のキメラ型モノクローナル抗体(ヒト95％，マウス5％)であり，ヒト EGFR の細胞外ドメインに EGF の5倍の親和性をもって特異的に結合し，内因性の増殖因子である EGF や TGF-α を競合阻害する．EGFR は Cetuximab と結合すると細胞内に取り込まれ(Internalization)ライソゾームにより分解され，細胞内への増殖シグナルが遮断される．その他のメカニズムとして，宿主免疫を介した ADCC(antibody-dependent cellular cytotoxicity)や補体を介した CDC(complement dependent cytotoxicity)などの関与も想定されている．また免疫染色により大腸癌の75～82％に EGFR の過剰発現がみられることが報告されている．

Cetuximab は呼吸困難，低血圧を含む重篤な副作用を引き起こしうるため，投与前に抗ヒスタミン剤を前投薬することが望ましい．

5．その他

LV

本邦では，大腸癌に対して LV の光学異性体の活性体であるl体のみを成分としたアイソボリン(l-LV)が1999年に承認された．還元型葉酸である LV は細胞内で還元され5,10-CH2-FH4に代謝され，上述のように FdUMP，TS と三者複合体を形成し TS の解離を遅延させ，5-FU の抗腫瘍効果を増強させる．

化学療法のレジメン

1．5-FU/LV

5-FU 単独と5-FU/LV のメタ・アナリシスの結果，奏効率は5-FU 単独11％，5-FU/LV 群21％で併用群の奏効率は約2倍であった($P<0.0001$)．さらに生存期間の中央値(mean survival time；MST)においても，5-FU 単独群10.5ヵ月に対して5-FU/LV 群は11.7ヵ月と有意な延長($P<0.004$)が示された[2]．

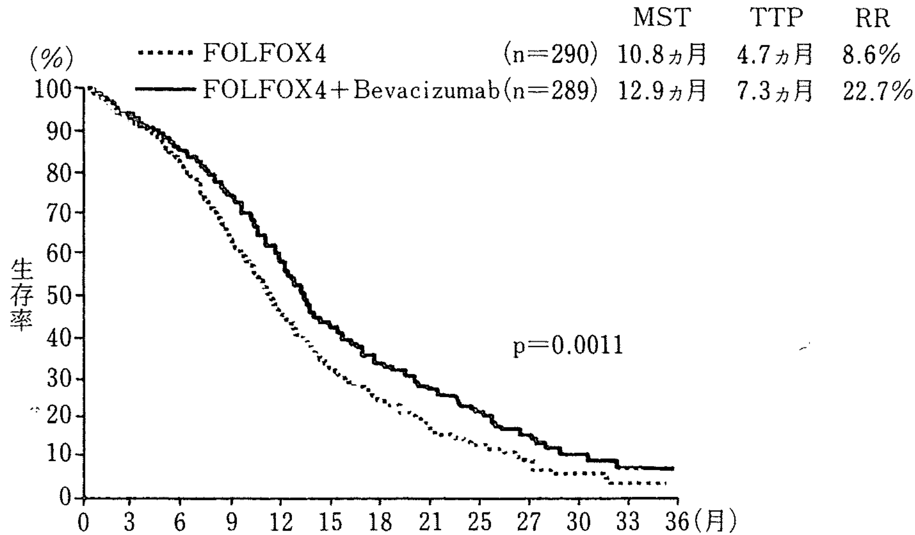

図2 E3200：FOLFOX4 vs FOLFOX4+Bevacizumab

2. IFL

Saltz らは，低用量 LV と5-FUbolus 投与を併用する regimen に CPT-11を組み合わせ，転移性大腸癌に対する first line 治療としての効果を LV/5-FU (Mayo regimen) と CPT-11単独を比較検討した結果，IFL の RR は39%，PFS 中央値が7ヵ月，MST が14.8ヵ月ときわめて高かった[3]．この時の LV/5-FU+CPT-11 を Saltz regimen (IFL) と呼ぶが，投与後60日以内の死亡率が6.7%と高く問題視された．その後 CPT-11の投与量を抑えた Modified IFL が多くの施設で行われ，死亡率も1.3%に減少することも報告された．しかし，補助化学療法としての有用性は確立されていない．

3. FOLFOX regimen

LV と5-FU 持続静注の併用 regimen に L-OHP を投与する regimen であり，de Gramont らによって ASCO1994で報告された．その後改良 regimen が次々と報告されてきている．Goldberg らは，当時米国で主流であった Saltz レジメン (IFL) と FOLFOX および CPT-11+l-OHP の3群比較試験を行った結果，FOLFOX レジメンの奏効率が明らかに高く，副作用も少なかった[4]．

E3200試験では，FOLFOX4療法に Bevacizumab を追加投与された患者は，FOLFOX4のみの患者に比べて，主要評価ポイントである死亡のリスクを22%減少させることが明らかになっている．FOLFOX4と Bevacizumab の併用投与を受けた患者の平均生存期間は13.0ヵ月で，FOLFOX4単独投与患者の10.8ヵ月と比べて長かった(図2)[5]．

また，近年種々の固形癌に対する化学療法において好中球減少と予後との関連に関して報告がなされているが，大腸癌においても一次治療として FOLFOX 療法を受けた転移性大腸癌症例において，好中球減少が良好な予後予測因子となることが示唆されている[6]．

4. FOLFIRI

FOLFOX と並ぶ標準的レジメンである．5-FU/LV (5-FU 持続点滴静注) に CPT-11を併用する phaseⅢが行われ，RR，MST，TTP ともに LV/5-FU より優れた成績が得られた[7]．2日間の5-FU 持続静注+LV に CPT-11を併用する regimen を FOLFIRI と総称としている．

一次治療としての FOLFIRI に対して Cetuximab の上乗せ効果を検討した CRYSTAL 試験では，Cetuximab 群において無再発生存期間の改善がみられ，とくに後述のように KRAS 野生型群において明らかな上乗せ効果がみられた(表2)[8]．

表2 The CRYSTAL trial

	ITT		KRAS野生型		KRAS変異型	
	FOLFIRI (n=599)	Cetuximab +FOLFIRI (n=599)	FOLFIRI (n=176)	Cetuximab +FOLFIRI (n=172)	FOLFIRI (n=87)	Cetuximab +FOLFIRI (n=105)
奏効率(%)	39	47	43	59	40	36
	p=0.0038		p=0.0025		p=0.46	
mPFS*(月)	8.0	8.9	8.7	9.9	8.1	7.6
HR**	0.85 p=0.048		0.68 p=0.017		1.07 p=0.75	

*median progression free survival **hazard ratio

分子標的治療に関する最近の話題

第45回 Annual Meeting of the American Society of Clinical Oncology (ASCO 2009, 5/29-6/2)において，Ⅱ期またはⅢ期の結腸癌に対する術後療法として，mFOLFOX6療法とmFOLFOX6+Bevacizumab併用療法とを比較する第Ⅲ相試験(NSABP C-08試験)の結果が報告され，術後補助療法としてのBevacizumabの有用性は否定された．しかしながら，同様の試験であるAVANT BO17920の結果報告はまだされておらず，この試験の結果も加味して術後補助化学療法におけるBevacizumabの効果を判断すべきだと思われる．

また，未治療の転移性大腸癌症例に対するCapecitabine+L-OHP+Bevacizumab±Cetuximab療法を比較検討した結果，KRAS野生型群においてもCetuximabの上乗せ効果は認められなかった[9]．このように非常に有用であると思われる分子標的治療においても，適応症例と各種薬剤の組み合わせを十分に吟味する必要があると考えられる．

ポストゲノム研究

ポストゲノム時代に突入し，臓器・個体レベルでの解析の重要性が再認識されてきている．その一例としてK-ras変異と治療効果に関する臨床試験等を概説する．

一次治療として，FOLFIRI±cetuximab治療を受けた転移性結腸直腸癌患者のKRAS遺伝子型と有効性に与える影響に関する検討(The CRYSTAL trial)では，1,198例のうち540例の患者でKRASの評価が行われた．KRASの野生型は540例中348例(64.4%)，KRASの変異型は540例中192例(35.6%)で認められた．野生型と変異型では，FOLFIRIにcetuximabを併用するとFOLFIRI単独と比較して，野生型では無増悪生存期間が9.9ヵ月と8.7ヵ月でFOLFIRI+Cetuximabで有意に延長し(p=0.0167)，奏功率も59%と43%でFOLFIRI+Cetuximabで有意(p=0.0025)に上昇した．変異型では，Cetuximabを併用しても上乗せ効果は認められなかった[8]．このようにK-ras変異の有無がcetuximabの効果予測に利用できる可能性が複数の試験にて示唆されていた．この結果を受けASCOは，Clinical opinionとしてCetuximab投与予定症例においてはKRAS変異の有無を検索すべきであり，KRAS変異症例においてはCetuximabを投与すべきではないと結論付けている[10]．

また，ASCO 2009において進行再発大腸癌におけるKRASと新バイオマーカーに関する報告が複数なされ，KRAS野生型でEpiregulin(EREG)やAmphiregulin(AREG)が高発現群ではCetuximabの効果が高いことや，BRAF変異症例ではKRAS野生型であってもCetuximabの効果が減弱することが示唆された．

終わりに

大腸癌は，過去においては化学療法があまり効かないタイプの固形腫瘍と考えられていたが，

5FU/LV, CPT-11, Oxaliplatinの開発利用によって化学療法がひとつの治療オプションとし位置づけされるようになり多剤併用療法時代を迎えた．それからほどなくBevacizumabやCetuximabのような分子標的薬が登場し，治療選択肢が増え治療成績の明らかな向上がみられてきている．大腸がん治療に携わる医師や医療関係者は，続々と発信される新しい治療効果・副作用情報を常に更新し，患者にup to dateな治療を提供せねばならないと言える．

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外科学会会員のための企画

癌幹細胞研究の最前線

消化器癌領域における癌幹細胞研究の現状

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キーワード　消化器癌，癌幹細胞

I. 内容要旨

癌組織を構成する細胞は等しく腫瘍形成能を有するのではなく，腫瘍形成能を有する癌幹細胞と腫瘍形成能を有しない細胞に区別できるという癌幹細胞仮説が提唱され，ここ数年注目されている．癌幹細胞は癌の発生や維持のメカニズム解明に重要な鍵を握るだけでなく，外科的根治術後もしくは抗癌剤など術後補助療法後でも再発・転移をきたす原因として，癌幹細胞の残存が深く関係していると考えられ，癌治療の真の標的細胞として注目されるようになった．最初に白血病で癌幹細胞の存在が報告された後，大腸癌，膵臓癌，肝臓癌など消化器癌でも癌幹細胞もしくはこれに近い性質をもつとされるTI-C（tumor initiating cells）が同定されているが，固形腫瘍でも血液腫瘍と同様の知見が当てはまるのかまだ不明な点が多く精力的な検証が進められている段階である．また，宿主側の癌幹細胞の微小環境（ニッチ）が癌の転移形成に重要であることも指摘されるようになった．消化器癌幹細胞研究の成果を治療に臨床応用するためには，正常組織幹細胞の存在の証明と分化メカニズムの解明など基礎的知見を十分に集積し，癌細胞側因子としての癌幹細胞と宿主側環境因子としての微小環境（ニッチ）の両面の細胞学的・遺伝子学的特徴を俯瞰的に解明することが必要であると考えられる．今後，癌幹細胞仮説の導入による新たな腫瘍学に基づいた消化器癌治療法の確立が期待される．

II. はじめに

癌組織は多様性（heterogeneity）のある細胞の集団である．従来，癌細胞はすべてに自己複製能があり，その結果として多様性のある癌が発生，増殖すると考えられてきた[1)]．これに対して，腫瘍を構成する細胞は等しく腫瘍形成能を有するのではなく，腫瘍形成能を有する癌幹細胞と腫瘍形成能を有しない細胞に区別できるという癌幹細胞仮説が提唱され，ここ数年注目されている．癌幹細胞は自己複製能，多分化能に加え腫瘍形成能をあわせもつ少数の細胞で，正常組織での分化過程と同様に，癌幹細胞を頂点として階層的に分化することにより，細胞形態学的・細胞機能学的に多様性のある癌組織を形成するという考え方である（図1）．

この癌幹細胞仮説は，すでに1960年代に提唱されていたが[2)]，それを実験的に証明することが困難であった．しかし，近年の技術的な進歩として，フローサイトメトリー（FACS：fluorescence activated cell sorting）の解析精度が向上し任意の細胞集団の分離が可能となったこと，組織体性幹細胞が発見されその解明が進んだこと，NOD/SCID（nonobese diabetic/severe combined immunodeficient）マウスの開発で

PERSPECTIVES ON CURRENT STATUS AND FUTURE DIRECTIONS FOR CANCER STEM CELLS THEORY IN GASTROINTESTINAL CANCER

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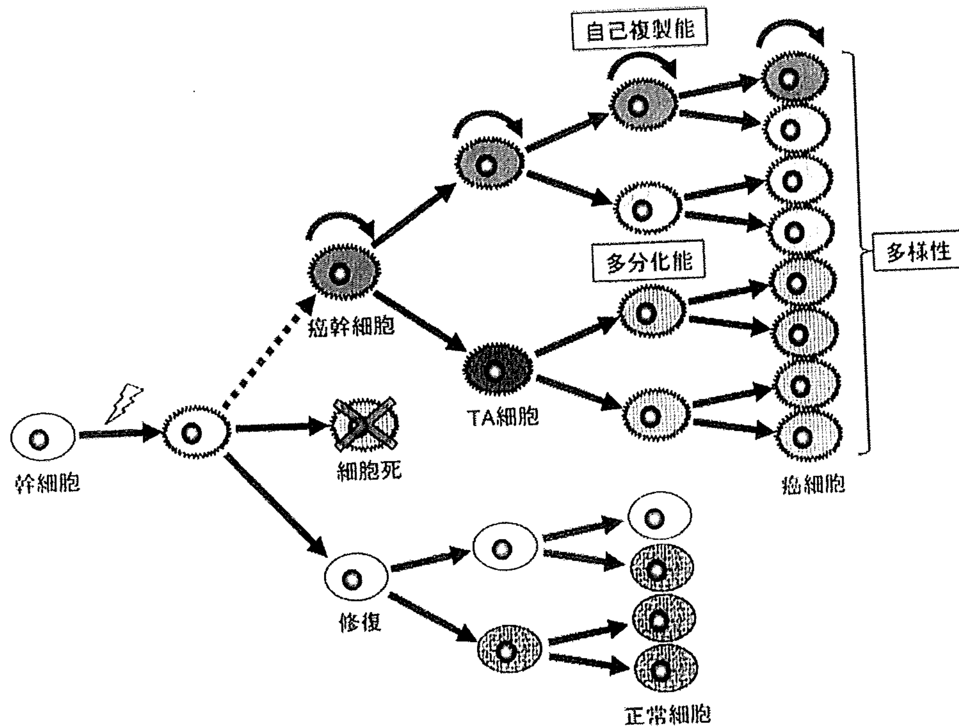

図 1

腫瘍の移植実験が容易にできるようになったことにより，1997年に急性骨髄性白血病（AML）におけるCD34＋/CD38－細胞が癌幹細胞として報告された[3]．それ以来，癌幹細胞は癌の発生や維持のメカニズム解明に重要な鍵を握るだけでなく，癌幹細胞の残存が治療後の癌の再発・転移と深く関係していると考えられ，癌治療の真の標的細胞として注目されるようになった．アメリカでは白血病幹細胞を標的とした治療が開発され臨床応用に向けて準備が進んでいる．一方，固形腫瘍では，2003年に乳癌でCD24－/CD44＋細胞が癌幹細胞として報告され[4]，引き続き脳腫瘍，消化器癌，頭頸部を含む固形腫瘍で報告された（文献5に総説）．特に消化器癌では，われわれが大腸癌・肝臓癌などの癌幹細胞研究を報告し[6)~8)]，引き続いて膵臓癌[9]，肝臓癌[10]でも癌幹細胞もしくはこれに近い性質をもつとされるTI-C（tumor initiating cells）が報告された．このようにこの分野は開発競争が激しい．消化器癌のような固形腫瘍では，癌幹細胞の発生起源が推定困難なこと，癌幹細胞同定のための表面抗原が十分に明らかになっていないこと，異種細胞移植，移植経路など癌幹細胞を同定する過程での技術的な問題点も指摘されており[11]，血液腫瘍と同様の知見が当てはまるのかまだ不明な点が多く精力的な検証が進められている段階である．

本稿では，消化器癌を中心に癌幹細胞研究の現状と今後の展望について，当科での知見を含め概説する．

III. 正常と癌の幹細胞

正常組織幹細胞は各組織に僅かに存在し，一定の幹細胞を保つための自己複製能と多系統の分化細胞への多分化能を有する細胞と定義される．正常な腸管上皮組織では，腸管上皮幹細胞が階層的な非対称性分裂により増殖し，細胞死，損傷組織の修復に関わり，組織としての恒常的な機能維持と，腸管上皮を構成する全分化細胞の由来となると考えられている．癌幹細胞仮説に基づけば，癌の発生，進展は癌幹細胞の発生から始まることになる．現時点で癌幹細胞の起源は明らかになっていないが，癌幹細胞と正常組織幹細胞の性質には自己複製能や多分化能など共通点が多ことより，癌が発生する組織の組織幹細胞もしくは前駆細胞から発生するという考え方が有力視されている．一方，幹細胞を頂点とした分化は一方通行的な事象とされてきたが，分化細胞の先祖帰り（リプログラミング）が実際に起こりうることも指摘されている．2007年，Yamanakaらグループは，マウス線維芽細胞にOct3/4・Sox2・Klf4・c-Mycの4遺伝子を導入し，ES（Embryonic stem cell）細胞様の人工多能性幹細胞（iPS：Induced pluripotent stem cells）細胞の樹立に成功し

表1 固形腫瘍で同定されている癌幹細胞の表面マーカー

乳癌	CD44＋/CD24－/lowESA＋
脳腫瘍	CD133＋
大腸癌	CD133＋
	EpCAMhigh/CD44＋/（CD166＋）
	CD44＋CD133＋
肝細胞癌	CD90＋/CD45－
頭頸部癌	CD44＋
	CD20＋
	ABCB5
膵臓癌	CD44＋/CD24＋/ESA＋
	CD133＋
前立腺癌	CD44＋/a2b1high/CD133＋

（clinical sampleを用いたもののみ表記）

た[12]．このiPS細胞の概念は分化した細胞を人工的に幹細胞に戻すものであり，癌の発生過程においても，遺伝子変異によって分化した癌細胞もしくは正常細胞がゲノム変化を経て幹細胞化する可能性を示している．このような分化異常の過程には，消化器癌のような上皮性悪性腫瘍の場合，癌の先進部細胞のEMT（epitherial-mesenchymal transition：上皮―間葉転換）との関係も注目されている[13]．

大腸癌では正常大腸粘膜上皮から腺腫を経て癌に至るadenoma carcinoma sequence[14]が多段階発癌のモデルとしてよく知られている．大腸の正常幹細胞は陰窩の基底膜直上に存在し，分化した細胞は消化管内皮下に向かって移動し約2～5日でアポトーシスを起こして消化管内に脱落する．このような短時間に段階的な遺伝子異常の蓄積が起こる確率は低いと考えられる．よって，腸管上皮に長い期間存在する正常幹細胞もしくは前駆細胞に遺伝子変化が蓄積され，通常は厳密にコントロールされている細胞周期がこの遺伝子変化によって破綻することで癌化するという考え方には矛盾がないと思われる．実際に造血系幹細胞における研究では加齢に伴い癌化につながる遺伝子変異が正常幹細胞に蓄積されるという報告があり[15]，消化器癌でも幹細胞の存在は癌の発生に深く関与していると考えられている．

IV. 消化器癌における癌幹細胞研究の動向

臨床検体を用いた最も一般的な癌幹細胞の同定法は，各種表面マーカーを用いた方法である．フローサイトメトリーを用いて細胞膜上に存在する表面蛋白マーカーを指標として，検体癌組織細胞を分画し，NOD/SCIDマウスに移植して腫瘍形成能を解析する．CD133（prominin-1）は造血系および神経系の幹細胞/前駆細胞だけでなく，白血病，脳腫瘍，肝臓癌，前立腺癌などさまざまな癌腫における幹細胞マーカーとして知られている（表1）．この他，side population（SP）分離法，浮遊細胞塊（sphere）を用いる方法が癌幹細胞の同定法として用いられている．SP分離法は組織幹細胞濃縮方法のひとつで，Hoechst33342という色素を細胞内に取り込ませ，Verapamilなど ABCトランスポーター阻害剤を用いて薬剤の排出能力をフローサイトメトリーで解析する方法である．SP細胞は抗癌剤抵抗性をもち腫瘍形成能が高く，癌幹細胞様の特徴をもっていることが明らかになっている．浮遊細胞塊は細胞を特殊条件下で培養することで得られる球形の細胞集団であり，幹細胞様の性質をもつ細胞を濃縮することが報告されている．

大腸癌では，ヒト大腸癌切除組織よりフローサイトメトリーを用いて分離したCD133＋細胞が，EGFおよびFGF2を添加した無血清培養条件下で浮遊細胞塊（sphere）を形成し，NOD/SCIDマウスにおいて造腫瘍能が高いことが報告され，CD133＋細胞が大腸癌の前駆細胞を発生させている可能性が示唆された[6)～8]．一方，DalerbaらはEpCAM（high)/CD44＋細胞の腫瘍形成能が高いことを示し，CD133－の腫瘍でもEpCAM（high)/CD44＋細胞は腫瘍形成能を示し，CD133よりEpCAMやCD44の方が大腸癌幹細胞の分離マーカーとして重要である可能性を示した[16]．また，Shmelkovらは，大腸癌の肝転移症例からCD133＋細胞とCD133－細胞を分離し，それぞれNOD/SCIDマウスへ繰り返し移植した結果，どちらの細胞からも腫瘍を形成し，むしろCD133－細胞の方がCD133＋細胞よりも大きな腫瘍を形成し悪性度が高かったと報告している[17]．われわれは，臨床大腸癌手術検体を用いて，CD133とCD44を用いたmulti-colorによるフローサイトメトリー解析を行い，NOD/SCIDマウスへの皮下投与による腫瘍形成能を検討した．その結果，CD133＋およびCD44＋細胞では腫瘍形成を認めたが，CD133－およびCD44－細胞では造腫瘍能を認めず，またCD133とCD44の発現による4群展開では，CD133＋/CD44＋細胞においてのみ腫瘍形成を認めたことより，CD133＋/CD44＋が大腸癌幹細胞の絞り込みに有用であると報告した[18]．

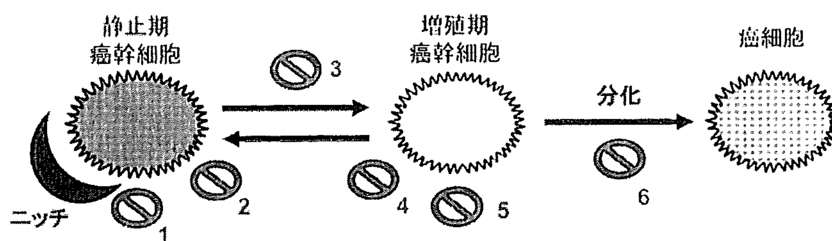

図 2

膵癌では，Liらがヒト膵癌組織をNOD/SCIDマウスの皮下に移植し単離された癌細胞からフローサイトメトリーを用いて分離したCD44+/CD24+/ESA+細胞が限定的に高い腫瘍増殖能を示し，膵癌の発生に関与する重要な増殖シグナルのHedgehogのリガンドであるsonic hedgehog（SHH）の発現が亢進していたことより，膵癌幹細胞である可能性が高いと報告している[9]．Hermannらはヒト膵癌組織の癌浸潤先進部から分離したCD133+/CXCR4+細胞だけが肝転移巣を形成すること，CXCR4中和抗体が肝転移を抑制することを示し，膵癌の転移にSDF-1/CXCR4経路が深く関与することを報告した[19]．

肝細胞癌では，Chibaらがフローサイトメトリーを用いて肝癌細胞株Huh7，PLC/PRF/5細胞中にわずかしか存在しないSP細胞をNOD/SCIDマウスに移植することによって，限局した腫瘍形成能と多分化能を有する癌幹細胞の分離が可能であることを示した[20]．またMaらは，ヒト肝細胞癌細胞にCD133+細胞には高頻度にprogenitor cellsが存在し，β-カテニン，Oct-3/4，Bmi1，SMO，Notchなどのstemness geneの発現が亢進していることを報告した[21]．Yamashitaらはフローサイトメトリーを用いてヒト肝細胞癌を2種のsubtype（EpCAM+/AFP+肝細胞癌細胞とEpCAM−/AFP−肝細胞癌細胞）に分離し，遺伝子発現プロファイルとpathway分析し，EpCAM+/AFP+に肝細胞癌幹細胞が含まれる可能性を示した[10]．

V. 癌幹細胞を標的とした治療法の開発

外科的根治術後の再発や術後長期経過後の再発には，臨床的に同定困難な微量の遊離癌細胞が遺残していることが示唆される．癌細胞が他の臓器に転移するためには，その細胞が原発巣から遊離するだけではなく，到達した部位で新しく癌を形成する能力が必要となることから，転移においても癌幹細胞が関与することが示唆されている．また抗癌剤など術後補助療法を附加したにも関わらず再発転移を来し，再発した場合多くは完治しえないことも知られている．抗癌剤や放射線に対する耐性獲得は，癌治療における最大の問題点の一つであり，治療耐性に関わる一群の細胞集団を含む癌組織の多様性がその原因と考えられている．癌幹細胞には強力な抗癌剤排出・中和能力があること，また癌幹細胞は通常，細胞分裂を停止したG0期にあり，正常組織幹細胞と同様に特別な微小環境（ニッチ）内で休眠状態として存在するため，従来使用されている多くの細胞周期依存性の抗癌剤や，増殖が活発な細胞に効果的な放射線に耐性を示すと考えられている（図2，3）．また，循環血液中に出現する骨髄由来細胞が幹細胞ニッチを形成し，癌の転移形成に重要な役割を担う可能性も指摘されはじめた[22]．このように，癌克服のためには癌細胞側因子として癌幹細胞と宿主側環境因子としてニッチの両面の俯瞰的研究が必要であり，癌幹細胞を選択的に除去する方法，癌幹細胞を分化誘導する方法，ニッチを制御する方法などの開発が進められている．

白血病幹細胞研究は，造血幹細胞システムを正常モデルとして対比させることにより進められ，治療法が開発されている．CMLでは，チロシンキナーゼ阻害剤であるImanitibが臨床応用され，画期的治療薬として治療成績の向上に貢献している．またAML，ALLでは，静止期の造血幹細胞を高率に細胞周期に入らせるmTOR阻害剤のRapamysinの有効性が確認されつつある．AMLではニッチで働く接着分子のCD44に対する抗体療法や，CMLでは幹細胞維持に重要なPMLを亜ヒ素により阻害する新規治療法が考按されている．しかし，いずれも服用を中止した後に多くの症例で再発し，完全に白血病幹細胞を根絶できていないと考えられ，より直接幹細胞を標的とした治療法の開発が必要とされる．

固形腫瘍では乳癌で，CD44+/CD24−細胞を取り

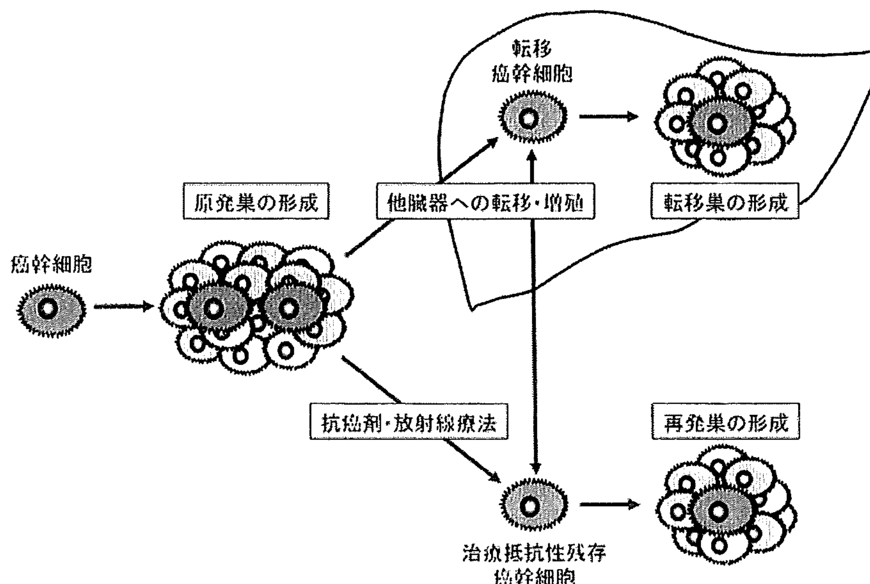

図 3

除く治療法が重要であり，チロシンキナーゼ阻害剤でAkt経路，MAPキナーゼ経路を阻害できるLapatinibと化学療法の併用により高い治療効果が得られることが報告された[23]．また，正常幹細胞と同様に癌幹細胞では活性酸素（ROS：reactive oxygen species）濃度が低く遺伝子損傷が発生しにくいことが放射線治療抵抗性の一因となっていること，ROSスカベンジャーの抑制により抵抗性を克服できる可能性が示された[24]．大腸癌細胞株ではOxaliplatinの持続曝露により耐性化にEMTが関与する可能性が報告されている[25]．消化器領域での組織正常幹細胞と癌幹細胞の同定，それぞれの類似点と相違点，癌の遺伝子変化がどの段階でおこるのか理解がすすめば，癌幹細胞を標的とした新たな消化器癌の治療法の開発につながると考えられる．

VI. おわりに

近年，消化器癌領域においても癌幹細胞研究が急速に進み始め，正常組織幹細胞の存在の証明，癌幹細胞の発生起源の推定，分化メカニズムの解明など基礎的知見が徐々に集積されつつある．また，癌幹細胞および宿主側の微小環境（ニッチ）両者間におけるシグナル伝達あるいは細胞間反応，EMT，miR-124やmiR-137など一部のmicroRNAなども含め，癌幹細胞仮説を中心とした消化器癌の再発・転移メカニズム，抗癌剤や放射線抵抗性のメカニズムの解明も進められている．さらには，癌遺伝子のc-Mycやレトロウィルスを用いずにiPS細胞の樹立が可能であることが示され，癌研究と再生医療の接点としても新たな局面を迎えている．今後，癌幹細胞仮説の導入による新たな腫瘍学に基づいた消化器癌治療法の確立が期待される．

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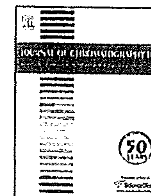

Review

A new strategy for protein biomarker discovery utilizing 2-nitrobenzenesulfenyl (NBS) reagent and its applications to clinical samples☆

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ABSTRACT

For the purpose of biomarker discovery, we originally developed a novel method for quantitative proteome analysis utilizing both tryptophan-targeted stable isotope tagging and mass spectrometry. The method has now been refined by replacing detergents and an enrichment column and further utilizing a novel matrix that is specifically suitable for tagged peptides. A total analytical system has been constructed by combining this method with HPLC, an automatic spotter, MALDI-TOF MS and analytical software. Clinical tissue samples such as colorectal carcinoma and renal cell carcinoma were analyzed using this system, and the results demonstrated that it is useful for discovering novel biomarker candidates. Here, we review a series of these studies and also discuss future directions for development of this technology.

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1. Introduction

With the advent of whole genome sequencing of human [1–3] and other species [4–6], both transcriptome and proteome analyses have been increasingly performed to discover genes and proteins related to various biological phenomena or diseases [7–9]. Transcriptome analyses, along with the development of microarray systems, have led to the discovery of candidates responsible for diseases and have highlighted features of special cells [10,11]. However, it is often difficult to select effective biomarker proteins from the results of these experiments, because mRNA expression levels do not necessarily correlate with cellular protein abundance [12,13]. In addition, many proteins receive post-translational modifications [14] and/or processing that cannot be predicted only from genome or transcriptome information. Proteome analyses can directly identify a set of proteins whose abundance is altered, and thus this method is well suited to biomarker discovery, although the procedure is somewhat complex and sometimes time-consuming. For many years, two-dimensional gel electrophoresis (2-DE) has been applied to proteome analysis, and biomarkers have been discovered using this technology [15]. However, this approach has some experimental and operational limitations [16]. For example, higher molecular weight proteins, basic proteins, and membrane proteins are difficult to separate effectively. In addition, it is laborious to deal with many samples and it is sometimes difficult to obtain reproducible results. In the last decade, mass spectrometry (MS)-based proteome analysis has become a mainstream method as instrumental and methodological aspects have progressed [17–19], and novel methodologies utilizing stable isotope labeling and MS detection have been developed to perform quantitative proteome analysis

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Table 1
Comparison among isotopic-labeling methods for quantitative proteome analysis.

Methods	Labeling	Target	Protein coverage	Peptide coverage	Simplification of analysis	Applicability to samples	Advantage to use MALDI-MS	Isolation of labeled peptides	Data used for quantitation
SILAC	*In vivo*	n.a.[a]	+++[a]	+++[a]	+[a]	+	++	n.a.	MS
cICAT	*In vitro*	Cys residue	++(+)	+	++(+)	++(+)	++	+++	MS
iTRAQ	*In vitro*	Amino group	+++	+++	++	+++	++	n.a.	MS/MS
NBS	*In vitro*	Trp residue	++(+)	+	+++	++(+)	+++	++	MS

Marks indicate as follows: +++; excellent > ++(+) > ++; good > +(+) > +; poor, n.a.; not applicable. All the methods can be combined with other method and thus each evaluation can be changed.
[a] Indicate that the evaluation can be changed depending on which amino acids are isotopically labeled and which protease is used. Shown here is a typical example of using Lys and Arg for labeled amino acid and trypsin for digestion.

[20–23]. This approach has an advantage in that it can be combined with liquid chromatography and automated. Thus it is expected to have great potential for more powerful analyses of complex samples. In our efforts to discover novel biomarkers related to diseases such as cancers, we have developed a novel quantitative proteome method employing this approach [24]. We have now refined our original method [25,26] and constructed an analytical system [27]. Here, we describe the method development, establishment of the analytical system and its applications to clinical samples.

2. NBS method development

A number of methods using stable isotope labeling for quantitative proteome analysis have been developed, and three commonly used methods, as well as ours, are summarized in Table 1. These methods can be roughly classified into two categories: *in vivo* and *in vitro* labeling [21]. *In vivo* labeling techniques utilize stable isotope-labeled nutrients, *e.g.* amino acids for SILAC (stable isotope labeling with amino acids in cell culture [28]), that are metabolically incorporated into cellular proteins. The labeled nutrients are relatively inexpensive and easily used, but these techniques are limited to samples such as cultured cells. On the other hand, *in vitro* labeling techniques utilize stable isotope-labeled reagents that are bound to proteins via a chemical reaction. Therefore, they are applicable to almost all protein samples, including human tissues, and thus are matched to our purpose. In these methods, proteins are generally digested with an enzyme (endopeptidase) that cleaves peptide bonds next to specific residue(s), and then a number of peptides are generated whose lengths are desirable for MS analysis. Since cell or tissue samples are expected to contain thousands of proteins, the digests after enzymatic cleavage include tens of thousands or even more peptides. Therefore, it seems difficult to analyze all of them, although some methods, such as iTRAQ, label and target all peptides present in a given mixture [29]. Theoretically, this type of method can cover all the peptide fragments resulting from protein digests, and thus can be applied even to peptidome analysis. However, it seems advantageous to adopt a strategy where only a specific residue is labeled and the resultant labeled peptides are somehow isolated. Using this type of strategy, only part of the digest is tagged but the labeled peptides are representative of their parent proteins, allowing quantification of protein levels. We have developed a novel *in vitro* labeling method that utilizes tryptophan as a target residue [24]. Because tryptophan is the least abundant amino acid in proteins [30], isolation of tryptophan-labeled peptides reduces the number of analytes and the complexity of the entire analysis. Most proteins (>90%) in *Homo sapiens* contain at least one tryptophan residue [30], so this method is suitable for global proteome analysis. However, there is a limitation of this method: it is not suitable to peptidomic application (and sometimes to other applications), because the coverage of tryptophan labeling becomes less and less as the sequences of targets become shorter and shorter.

Several arylsulfenyl halides are known for their selective reactivity towards the indole ring of tryptophan under acidic conditions [31–33]. Some of these chemicals were tested for their reactivity, and it was found that 2-*n*itro*b*enezene*s*ulfenyl chloride (NBSCl) effectively labeled tryptophan residues [24] (Fig. 1). NBSCl also reacts, to some extent, with sulfhydryl groups of cysteine residues. However, the resulting labeled cysteine residues, in which sulfhydryl and NBS groups are linked through disulfide bonds, are all converted to carbamidomethyl cysteins after subsequent reduction and alkylation steps [24] (Fig. 2). Thus, "tryptophan specific" labeling was finally achieved using NBSCl. We prepared a set of "heavy" and "light" NBSCls (referred to as "NBS reagent"; this reagent is commercially available from Shimadzu Corporation as "^{13}CNBS Stable Isotope Labeling Kit-N") that incorporated six ^{13}C and six ^{12}C in their benzene rings, respectively (Fig. 1). The two protein samples were then processed according to the procedure shown in Fig. 2: sample 1 was labeled with a heavy NBS reagent and sample 2 was labeled with a light NBS reagent, leading to a mass difference of 6 Da between sample 1 and sample 2 for all of the tryptophan-containing peptides. The labeled peptides were enriched by taking advantage of the relatively stronger affinity of NBS-labeled tryptophan-containing peptides for Sephadex media (LH-20) [34]. Relative quantitation of the proteins in the two samples was calculated from the intensities of paired peaks having a 6 Da mass difference in the MS spectra; proteins were then identified by a database search using queries based on data from the MS/MS spectra (Fig. 3).

Several feasibility studies were performed, demonstrating that this method is well suited to quantitative proteome analyses [24]. Basic properties of the analyses were evaluated, such as accurate quantitation, simple enrichment of labeled peptides, availability of both MALDI-TOF and ESI-MS analysis, compatibility with MS/MS analysis without any undesirable fragmentation, and co-elution of

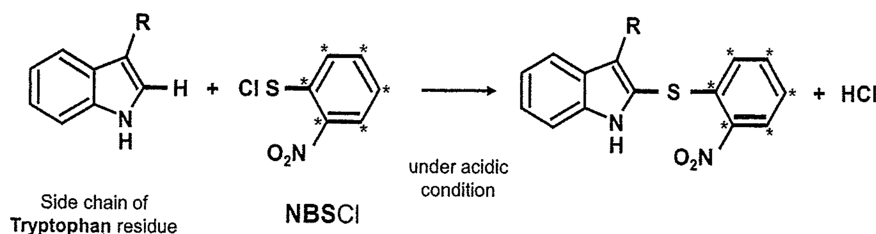

Fig. 1. Structural and reaction formula of the NBS reagent. Asterisk (*) indicates ^{12}C for the light reagent and ^{13}C for the heavy reagent.

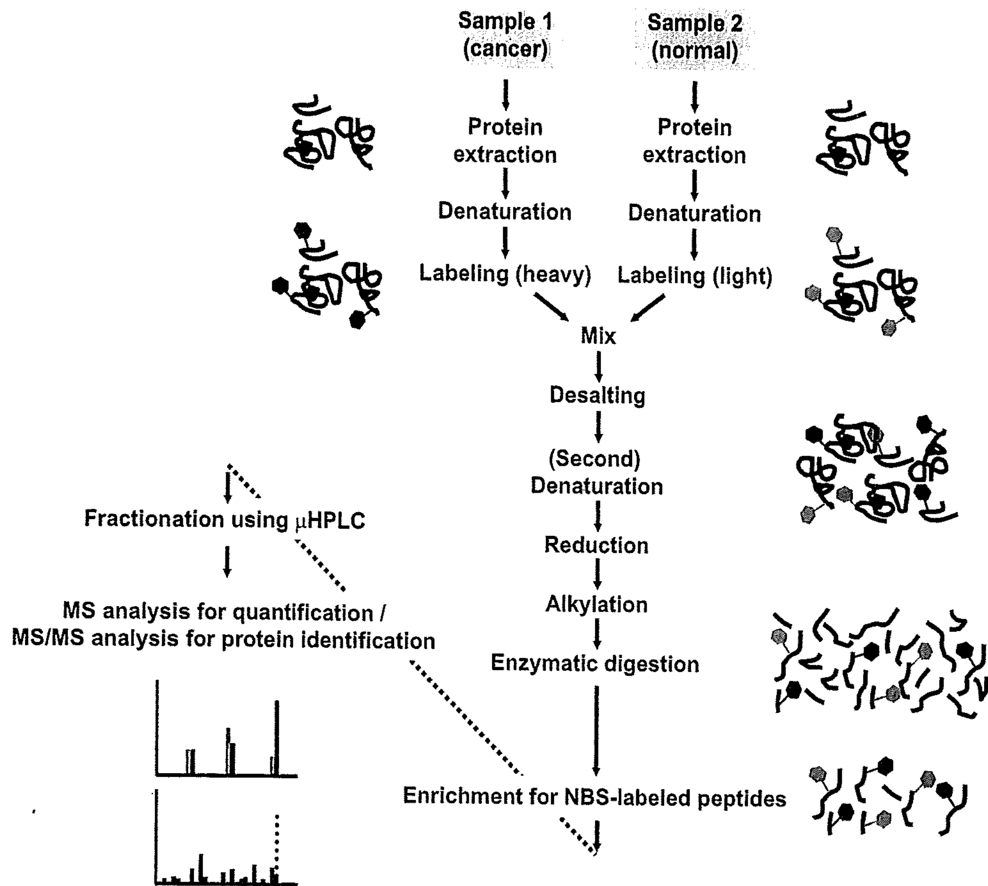

Fig. 2. The NBS method procedure is illustrated. Both proteins and peptides are indicated by black lines. Heavy and light NBS reagents are drawn as blue and red hexagons connected to the peptides with a bar, respectively.

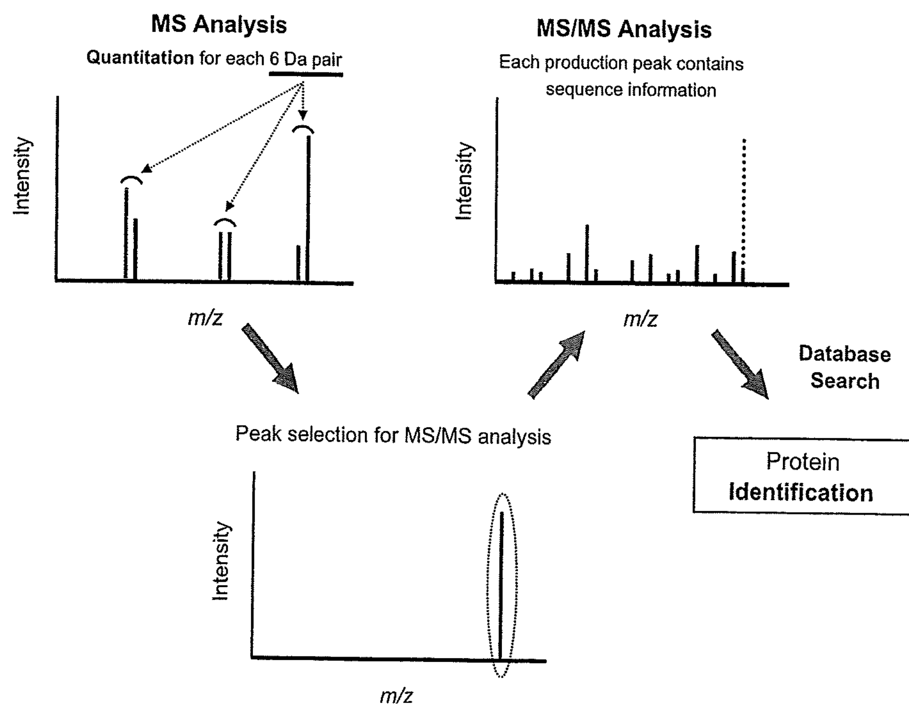

Fig. 3. MS and MS/MS analyses are illustrated. Blue and red lines indicate peaks of heavy and light NBS reagent-labeled peptides, respectively.

heavy and light labeled peptides from a C18 column by RP-LC [24]. The latter two issues were reported problems [35,36] in studies utilizing the original ICAT reagent [37]. Both the simple structure of the NBS reagent and the use of ^{13}C instead of deuterium as a stable isotope element led to solution of these problems.[1] Thus, we have developed a novel method that includes all of the basic characteristics needed for quantitative proteome analysis. We called this method the "NBS method", referring to the abbreviation of the central reagent.

3. Method optimization and improvement

Although the basic methodology was developed as mentioned in the former section, additional refinements were needed to optimize it for practical use, especially in the case of biologically derived samples consisting of complex protein mixtures. The main problems to be addressed were loss of sample, generation of by-products (molecules with mass increases of 57 Da; we assume that this was due to an unexpected alkylation (carbamidomethylation) of a side chain other than the cysteine SH group [25]), and contamination of eluted fractions with unlabeled peptides. We reviewed the entire protocol and decided to optimize the denaturing conditions and the enrichment step [25]. First of all, we used urea or guanidine hydrochloride for the denaturation step, instead of the original protocol's SDS denaturation, because they are compatible with trypsin digestion at relatively high concentrations and can be removed easily. Use of these denaturants at high concentrations is advantageous to keep proteins, including hydrophobic and membrane proteins, soluble and to avoid aggregation and/or proteolysis. Next, we used a phenyl resin instead of a Sephadex LH-20 to enrich labeled peptides, because the NBS-labeled tryptophan side chain is aromatic as well as hydrophobic, and π-electron interactions between the NBS-indole ring moiety and phenyl groups in the media should increase the specificity of the binding.

We then examined which condition is best suited for each step as well as influences of various conditions on downstream steps [25]. We finally established one optimum condition that provided several improvements: almost 100% labeling in less than 10 min, suppression of by-products (+57 Da), at least 80–90% recovery of the labeled peptides with better separation from unlabeled peptides, more accurate quantitation, and reduction of the total operation time [25]. As a result, there was minimal sample loss during the NBS reaction procedure so the sensitivity was preserved. In addition, use of the phenyl column resulted in a somewhat chromatographic separation of labeled peptides, as described below. Comparison of the original and improved protocols showed there was more than a five-fold increase in the number of observed NBS-labeled paired peaks using the improved protocol [25].

4. Discovery of a selective matrix for NBS-labeled peptides

In the NBS method, MS/MS analysis is indispensable for the identification of proteins, and we often use MALDI-IT-TOF-type MS only for this purpose. However, we had a problem here in detecting NBS-labeled peptides by this type of MS. It was generally recommended for this instrument to use a cool matrix such as 2,5-dihydroxybenzoic acid (DHB), to avoid decay of ionized molecules during ion trapping. However, DHB was found to be incompatible with the detection of NBS-labeled peptides [26]. Therefore, we searched for another cool matrix that would be suitable for detection of NBS-labeled peptides by MALDI-IT-TOF MS. After screening benzoic acid derivatives, we found two matrices, α-cyano-3-hydroxycinnamic acid (a structural isomer of conventionally used CHCA (α-cyano-4-hydroxycinnamic acid)) and 3-hydroxy-4-nitrobenzoic acid (HNBA), which satisfied our requirements [26] (Fig. 4). We then made the fortuitous discovery that the HNBA matrix has an additional special property of selectively detecting NBS-labeled peptides in mixtures of labeled and unlabeled peptides [26]. The mechanism behind this selectivity is that the sensitivity of the HNBA matrix in detecting labeled peptides is similar to that of CHCA, whereas its sensitivity for detecting unlabeled peptides is greatly decreased, compared to CHCA. Thus, the HNBA matrix preferentially detects labeled peptides, for example from samples containing unlabeled impurities. This matrix possesses yet another favorable property, in that it suppresses fragmentations (mainly −16 and −32 Da species that result from detachment of oxygen(s)) [26], which were known to occur during the MALDI-TOF MS measurement of nitrobenzene compounds [38,39]. We investigated the mechanisms underlying these phenomena and found that various nitrobenzene compounds showed a similar effect, although the detection sensitivities of these matrices were much lower than that of HNBA [26]. We defined a "selectivity index" to indicate the size ranges over which labeled peptides are detected preferentially over unlabeled peptides by comparing results obtained using a given matrix to results obtained using a conventional CHCA matrix [26]. The selectivity index for the HNBA matrix was as high as 10. Interestingly, that of the original matrix for MALDI-IT-TOF MS, DHB, is about 0.1.

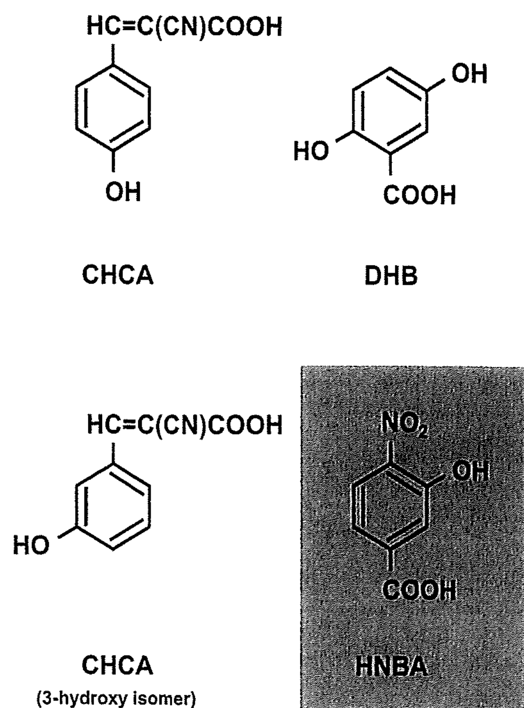

Fig. 4. Structural formulas of the four matrices, CHCA, DHB, CHCA (3-hydroxy isomer) and HNBA.

As mentioned above, the HNBA matrix has quite unique and favorable features for the detection of NBS-labeled peptides. However, it is less usable due to unstable signal detection and rapid signal decay, compared to conventionally used CHCA [26]. These drawbacks were compensated by the combined use of HNBA and CHCA: the usability was greatly increased with a minor loss of selectivity [26]. Surprisingly, in addition to this, the sensitivity in detecting labeled peptides was increased about four-fold [26]. The idea of using two matrices as a co-matrix was very simple, but the practical and beneficial effects have become significant in this

[1] In the case of the ICAT reagent, these issues were already solved by the use of ^{13}C and by removal of the cleavable tag introduced into the improved reagent, cICAT [36].

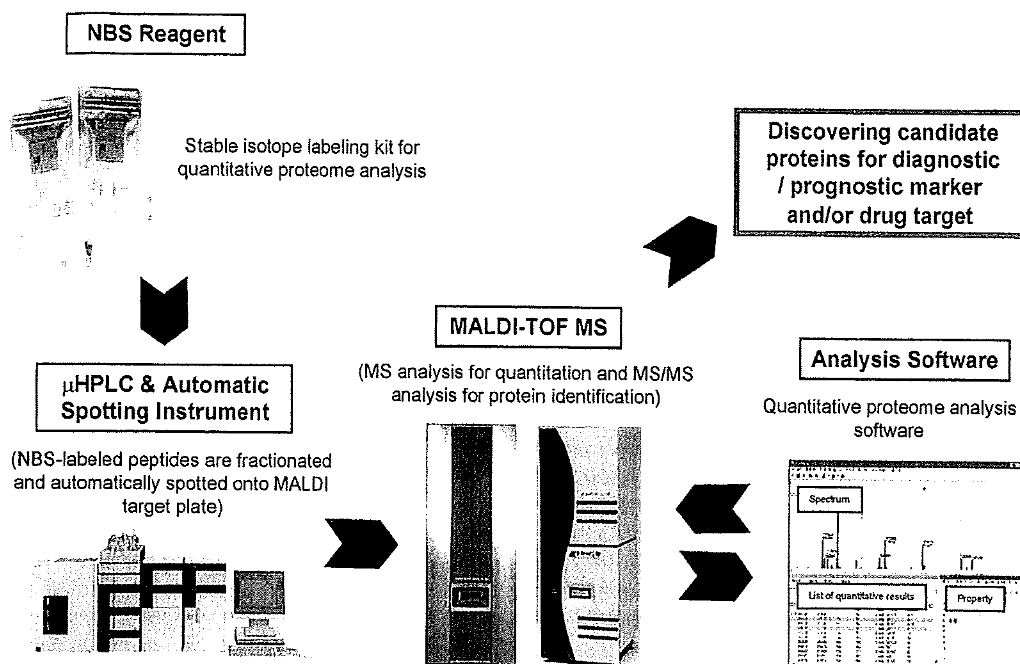

Fig. 5. Total analytical system using the NBS method.

case. In conclusion, discovery of HNBA matrix and its incorporation into an HNBA–CHCA co-matrix system increased the usability and detection sensitivity of target signals, thus increasing the dynamic range of the NBS detection method.

5. Establishment of an analytical system

Aside from the improvement and refinement of the NBS method, construction of an analytical system is also essential to our goal. Although the NBS method reduces much of the sample complexity, typical eluates from the enrichment step still contain many thousands of peptides, and fractionation by HPLC is indispensable for wider coverage and accurate quantitation. Systematic quantitation and protein identification are also desirable.

As mentioned above, the NBS method can be applied to both ESI-MS and MALDI-TOF MS. ESI-MS can be connected "on-line" with HPLC and run cooperatively and automatically. Recent advances in ESI-MS (with regard to both hardware and software) have resulted in fast scan speeds and an improved duty cycle, which can maximize the higher-resolution separation of peptides achieved by HPLC. MALDI-TOF MS can also be connected "off-line" with HPLC, but a droplet spotted onto a well of the MALDI plate is corresponding to a number of ESI-MS scans. From this point of view, LC/on-line ESI-MS-based analysis is superior to LC/off-line MALDI-MS-based analysis. However, in ESI-MS analysis, it is still the case that only part of the eluate is applied to MS or MS/MS analysis, and decisions as to which peaks should be analyzed by MS/MS are made during the continuous flow from HPLC. Sprayed samples cannot be measured again. On the other hand, in MALDI MS analysis, MS/MS analyses can be performed after the HPLC separation and MS analysis, by referring to the quantitation result from MS analysis. In addition, data can be accumulated using a target plate upon which all eluates from HPLC are deposited. This should result in higher sensitivity for protein identification. Here, we preserved all of the advantages of MALDI MS analysis, including the existence of the HNBA matrix, and constructed an LC/off-line MALDI-TOF MS system [27] (Fig. 5). First, an NBS-labeled peptide sample was applied to μHPLC and separated on a capillary ODS (C18) column. Each fraction (drop) of eluate was automatically deposited onto a MALDI target plate by a spotting apparatus, followed by automatic MS analysis. Next, the NBS-labeled paired peaks with significant differences in their intensities were selected and subjected to MS/MS analysis to identify their sequences and parent proteins. Operational conditions throughout these steps were optimized to construct an efficient system. For example, an eluate from a phenyl column was divided into three fractions and each was separately applied to an ODS column [27]. Both ODS and phenyl columns belong to the same reversed-phase but they exploit different interactions. Our results showed that only 10–20% of the peaks overlapped between neighboring fractions. Thus, a simple fractionation on a phenyl column increased the total number of peptides detected. Analytical software was also developed and incorporated into this system. This enables selection of all paired peaks with 6 m/z (and 12 m/z) differences for quantitation, and it is also easy to filter peaks with relatively large differences in their peak intensities. In this way, a total analytical system was constructed and then validated using rat and mouse sera [27]. Three sets of rat and mice sera, each paired samples from normal and diseased animals, were examined in order to evaluate this new analytical system. In all three experiments, 1000–2000 paired peaks were detected, and 32 pairs were selected by the software as differentially expressed protein tags with more than three-fold differences in expression [27]. Less than 100 paired peaks were detected without HPLC separation, clearly demonstrating that the system functions effectively for global proteome analysis [27].

In conclusion, we have constructed an analytical system suitable for the NBS method with off-line LC–MALDI-TOF MS. Using this system, even low-intensity peaks from proteins with a relatively low abundance may be identified and analyzed, for example when they are differentially expressed in two samples.

6. Applications to clinical samples

Our final purpose in developing this quantitative proteome analysis system is to discover novel biomarkers (and drug target proteins), because there is certainly a need to find practical biomarkers for clinical uses, such as early disease detection, diagnosis, prognosis, imaging and so on. There are still no diagnostic markers for many diseases [40], and in other cases specific

Table 2
Summary of the two application studies using human clinical samples.

	Analyzed specimens	Detected paired peaks per specimen	Differential paired peaks selected for MS/MS analysis	Identified peptides	Identified proteins	Reported earlier	Novel
Colorectal carcinoma (CRC)	12	~5000	320	138	128	30	**98**
Renal cell carcinoma (RCC)	14	6000–7000	225	108	92	24[a]	**68**

Identified proteins are classified as "Reported earlier" and "Novel", and the numbers of the latter are highlighted in bold.
[a] Includes two proteins which are discordant in their up/down-regulated states with our results [40].

biomarkers exist but are effective only in advanced disease cases [41]. Here, we applied the analytical system described above to surgically resected specimens from colorectal carcinoma (CRC) [41] and renal cell carcinoma (RCC) [40], and the protein expression profiles of cancerous and normal parts were compared. The results are summarized in Table 2. In both cases, about 200–300 paired peaks were selected as having significantly different expression levels, and as occurring with sufficient frequency among patients, and roughly 100 of these peaks were identified [40,41]. About 20–30% of the identified proteins had been reported in earlier studies, and the remaining 70–80% were newly found to be associated with the corresponding cancer [40,41]. The results showed that this analytical system is reliable as well as quite useful to discover novel biomarker proteins. Compared to earlier proteome studies using a 2D-gel method, higher molecular weight proteins and basic proteins were predominantly identified in our method. More precisely, 17 proteins with molecular weight (MW) larger than 100 kDa and six proteins with p*I* > 10 were identified among 128 proteins in our CRC analysis [41], whereas only two proteins with MW > 100 kDa and no proteins with p*I* > 10 among 168 proteins were reported in two earlier CRC studies using a 2D-gel method [42,43] (Fig. 6). This illustrates the advantage of our method compared to earlier methods. Several proteins were further verified and validated by Western blotting, RT-PCR and immunohistochemical (IHC) staining.

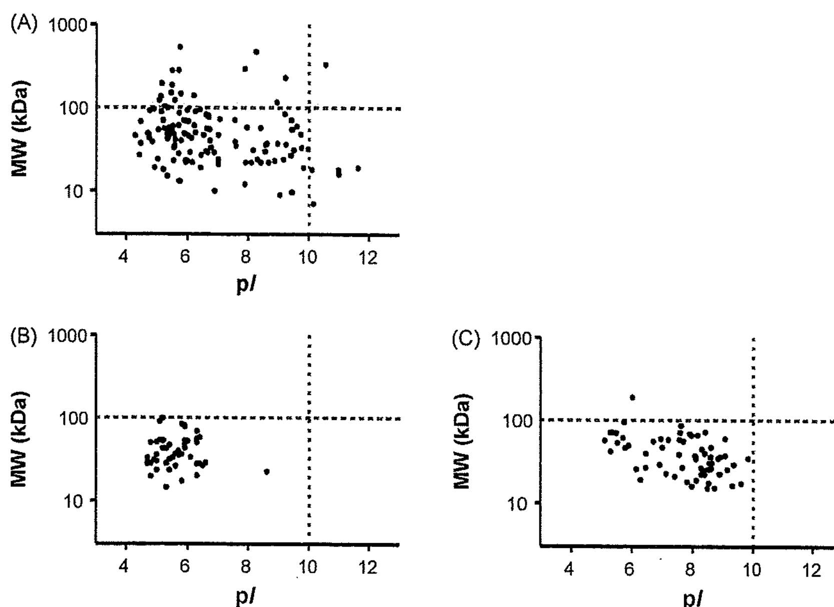

Fig. 6. Proteins identified by each method were plotted according to their predicted p*I* and molecular weight (MW). (A) NBS method [41], (B, C) 2D-gel method [42,43]. *y*-axis is on a logarithmic scale. Areas corresponding to MW larger than 100 kDa and/or p*I* > 10 are highlighted in yellow.

Heavy-labeled

	Sprot	Decoy	False discovery rate
Peptide matches above identity threshold	43	0	0.00 %
Peptide matches above homology or identity threshold	46	0	0.00 %

Light-labeled

	Sprot	Decoy	False discovery rate
Peptide matches above identity threshold	65	0	0.00 %
Peptide matches above homology or identity threshold	66	0	0.00 %

Fig. 7. Confirmation of the reliability of protein identification. The data used for protein identification were divided into two groups (72 for light-labeled and 66 for heavy-labeled) and analyzed again by MASCOT MS/MS Ions Search using both the usual SwissProt database and a decoy database. Search parameters used are as follows: trypsin digestion allowing up to 1 missed cleavage, fixed modifications of NBS (W) (or NBS:13C(6) (W)) and carbamidomethyl (C), variable modifications of oxidation (M), peptide tolerance 0.3 Da, MS/MS tolerance of 0.5 Da, and restriction to peptides with sequences containing one or more tryptophan residues.

Good reproducibility of this system was verified in the above CRC study; correlation coefficients between the first and second experiments were over 0.95 for all 12 specimens [41]. In addition, reduction of analysis complexity by avoiding redundant identification of peptides from the same protein was demonstrated; the number of identified peptides was very close to the number of identified proteins (Table 2). Such accuracy and simplification of analysis are the essence of this method. In terms of protein identification, one may suspect some of the search results, because most of the proteins were identified by only one peptide hit. However, the reliability of the protein identification was confirmed by the absence of false-positive identification ($p < 0.05$) using a decoy database (Fig. 7).

Some differences between the results obtained from the NBS proteome analysis and Western blotting analysis were attributed primarily to differences between the methods [41]; the NBS method is quite precise but only detects tagged peptides derived from whole proteins, whereas Western blotting detects entire proteins, but with less resolution and specificity, depending on the properties of the antibodies utilized. It is necessary to combine NBS analysis with IHC staining, as NBS analysis alone provides no information about the localization of detected proteins. Therefore, combination of the NBS method with other complementary analyses is needed to extract the maximum amount of information from the obtained data. It is important to fractionate samples before NBS analysis to increase the number and dynamic range of detected peptides [41]. This approach has been described in a paper on serum glycoproteome profiling in lung cancer [44]. Here, utilization of the NBS method combined with immunodepletion of six abundant proteins and lectin column selection led to successful detection of interleukin-12 (IL-12), which is an extremely low-abundant protein in serum. The NBS method has also been effectively used in combination with the regular 2-DE method to find drug-responsive proteins using a breast cancer cell line [45]. In this report, fine discrimination and accurate quantitation of two proteins that co-migrated as one spot were demonstrated by using the NBS method. When a conventional 2-DE/MS method was used, it was difficult to evaluate this spot as two proteins and thus the ratio of protein abundance was also reported incorrectly.

7. Conclusions and future aspects

Compared to other quantitative proteome methods, the NBS method has a unique aspect of tryptophan tagging, which is combined with an analytical system and optimized to detect less abundant proteins. We believe that this system has potential to discover novel disease-related proteins; this has already been achieved to a certain degree, as described in the previous section. However, the proteins identified here are just biomarker "candidates". They are now under evaluation for possible use as clinical diagnostic markers, using sera from both cancer patients and healthy volunteers. There are numerous such disease-related candidates awaiting further validation [46]. We must consider by what means and how to validate these biomarker candidates and determine how they will be used. Detailed analysis of each protein identified is needed as well. ELISA (enzyme-linked immunosorbent assay) is one of the most promising and powerful techniques used to screen for biomarker availability [47]. It has been used widely and intensively, but it is time-consuming and costly to establish reliable systems. Development of a novel technique with both sensitivity and specificity, either alone or in combination with other techniques, could offer valuable shortcuts. For example, a multiplex protein detection method with high sensitivity and specificity was used for biomarker validation: it combined oligonucleotide primer-tagged antibodies with real-time PCR and DNA manipulation techniques [48]. Another promising MS measuring technique is multiple reaction monitoring (MRM); this has been increasingly used for data validation and is now being applied to clinical diagnoses [49,50]. The utilization of MS for these types of applications, as well as for biomarker candidate discovery, will open up new possibilities for clinical applications.

In any case, our primary goal is to discover practical biomarkers for clinical applications from the lists of candidate proteins identified using the NBS method. Achievement of this goal will constitute an important contribution to human welfare.

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RESEARCH ARTICLE

A new approach for detecting C-terminal amidation of proteins and peptides by mass spectrometry in conjunction with chemical derivatization

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We describe a mass spectrometric method for distinguishing between free and modified forms of the C-terminal carboxyl group of peptides and proteins, in combination with chemical approaches for the isolation of C-terminal peptides and site-specific derivatization of the C-terminal carboxyl group. This method could most advantageously be exploited to discriminate between peptides having C-terminal carboxyl groups in the free (COOH) and amide ($CONH_2$) forms by increasing their mass difference from 1 to 14 Da by selectively converting the free carboxyl group into methylamide ($CONHCH_3$). This method has been proven to be applicable to peptides containing aspartic and glutamic acids, because all the carboxyl groups except the C-terminal one are inert to derivatization, according to oxazolone chemistry. The efficiency of the method is illustrated by a comparison of the peaks of processed peptides obtained from a mixture of adrenomedullin, calcitonin, and BSA. Among these components of the mixture, only the C-terminal peptide of BSA exhibited the mass shift of 13 Da upon treatment, eventually unambiguously validating the C-terminal amide structures of adrenomedullin and calcitonin. The possibility of extending this method for the analysis of C-terminal PTMs is also discussed.

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1 Introduction

One of the most important subjects in proteomics is to identify PTMs characterizing a majority of mature proteins, which are biological entities having various cellular functions that are not anticipated by genomic information. Owing to its rapidly improving performance, mass spectrometry has been the method of choice for protein/peptide analysis. The development of experimental techniques for protein sequencing by mass spectrometry has led to the discovery of a variety of PTMs occurring at the N-termini of proteins, as well as in amino acid side chains. For example, it has been estimated that 30–80% of mature proteins in eukaryotes are subjected to N-terminal acetylation [1, 2]. In contrast, the C-terminal carboxyl group is usually free from such modifications with a very few exceptions, which include amidation in relatively small peptide hormones [3] and derivatization with glycosyl phosphatidylinositol for anchoring the modified proteins to cell membranes [4–6]. In particular, C-terminal amidation is very difficult to detect because the mass difference between the carboxyl and carboxamide groups is only 1 Da. A most unfortunate situation could arise when a protein has asparagine (Asn) or

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Abbreviations: Asn, asparagine; **Gln,** glutamine; **LysC,** lysyl endopeptidase; **MDPNA,** methanediphosphonic acid

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