Table 3. Intensities of the 17,272, 8,766, 14,779, and 28,080 *m/z* peaks

Peaks (arrays)	Training cohort (n = 142)			Validation cohort (n = 78)		
	Cancer (n = 71)	Healthy (n = 71)	P*	Cancer (n = 33)	Healthy (n = 45)	P*
17,272 *m/z* (CM10 pH 4)	9.49 ± 2.88†	14.6 ± 2.29†	0.0000	9.74 ± 4.22†	14.5 ± 2.29†	0.0000
8,766 *m/z* (CM10 pH 4)	7.65 ± 3.53†	12.1 ± 5.55†	0.0000	7.04 ± 4.39†	13.4 ± 5.81†	0.0000
14,779 *m/z* (H50)	11.8 ± 4.43†	7.85 ± 3.68†	0.0000	10.4 ± 3.85†	6.46 ± 1.63†	0.00000
28,080 *m/z* (CM10 pH 4)	113 ± 36.7†	132 ± 33.5†	0.0022	92.4 ± 24.3†	110 ± 21.6†	0.0078

*Mann-Whitney U test.
†Mean ± SD intensities in arbitrary units.

detected in 1 of the 71 (1.4%) healthy controls, it was not included in the above discriminating data set generated by machine learning because of its low-positive rate in pancreatic cancer patients [19.7% (14 of 71)].

Statistical differences in all four peaks were recognized between the pancreatic cancer patients and the healthy controls (Mann-Whitney U test, $P < 0.0022$; Table 3). The ROC and AUC values of each peak and their combination in the 142 cases of the training cohort are shown in Fig. 4.

The intensity data of the four peaks obtained in each individual were compiled into a single value, the distance from a fixed SVM hyperplane, using the formula described in Materials and Methods and Supplementary Data. When the distance was positive, the individual was classified as having pancreatic cancer and vice versa. This classifier correctly diagnosed 97.2% (69 of 71) of the cancer patients and 94.4% (67 of 71) of the healthy controls in the training cohort (Fig. 5*A*).

Confirmation of the classifier in the first validation cohort. We next validated the discriminating performance of the classifier in a blinded manner using an independent cohort consisting of 78 individuals (NCCH) who had not been included in the training cohort (Table 1). Again, statistically significant differences in the mean intensities of every peak were observed between the 33 pancreatic cancer patients and the 45 healthy controls (Mann-Whitney U test, $P < 0.0078$; Table 3).

The SVM hyperplane determined in the training cohort was applied to the diagnosis of the 78 cases in the validation set. The same SVM hyperplane separated 90.9% (30 of 33) of the pancreatic cancer patients into the positive direction group and 91.1% (41 of 45) of the healthy controls into the negative direction group (Fig. 5*B*). The overall accuracy of the classification was 91.0% (71 of 78) in the validation cohort.

Combination of the surface-enhanced laser desorption/ionization classifier and CA19-9. Overall, the classifier was able to detect 95.2% (99 of 104) of the pancreatic cancer patients in the training and validation cohorts (Table 4). Although the number of cases was small, 83.3% (10 of 12) of stage I and II cases were detected (training and first validation cohorts). No statistically significant differences in detection rates were seen among cases with different tumor locations or different clinical stages (Table 4). To improve the detection rate, we measured plasma CA19-9 levels in all individuals whose residual samples were sufficient (29 pancreatic cancer patients and 39 healthy controls; Table 5). The sensitivity of CA19-9 (cutoff value of 37 units/mL) was 86.2% (25 of 29) and specificity was 94.9% (37 of 39). The SELDI classifier and the CA19-9 level were complementary. Combining CA19-9 and the SELDI classifier detected 100% (29 of 29) of cancer patients, but this combination yielded six false-positive cases [15.4% (6 of 39); Table 5].

Confirmation of the classifier in a second validation cohort obtained at a different institution. Finally, we did a second confirmatory experiment using samples collected prospectively at another institution. In total, 25 plasma samples from pancreatic cancer patients, individuals with other pancreatic diseases, and healthy volunteers were obtained from TMUH and analyzed in a blinded manner. Although the discovery of biomarkers useful for the differential diagnosis of pancreatic diseases was not the primary goal of this study, the classifier was able to discriminate pancreatic cancer patients and individuals with pancreatic tumors/cysts from healthy controls and pancreatitis patients (Table 4; Fig. 6). Four of the six patients with pathologically unproven pancreatic tumors/cysts were classified into the positive direction group. A close follow-up of these patients has been undertaken, because they may have premalignant or preclinical conditions. The SELDI classifier correctly identified 88.9% (8 of 9)

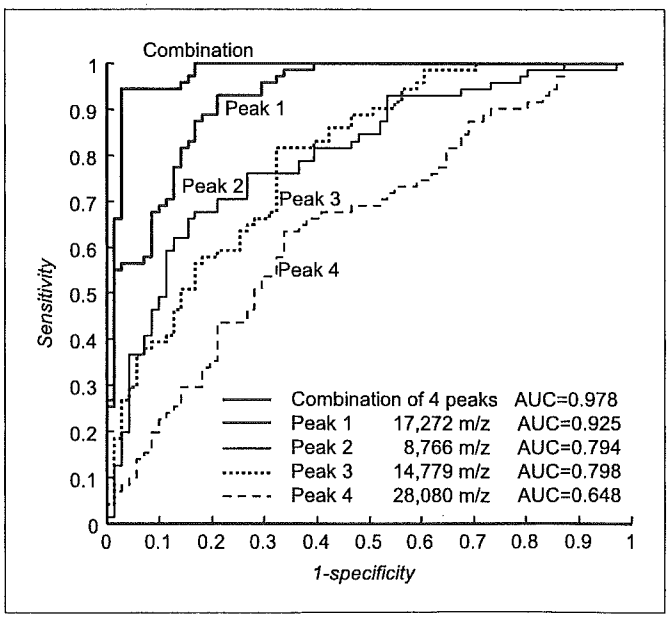

Figure 4. ROC curves and AUC values showing the discriminating capacities of the 17,272, 8,766, 28,080 (CM10 pH 4), and 14,779 (H50) *m/z* peaks individually and in combination.

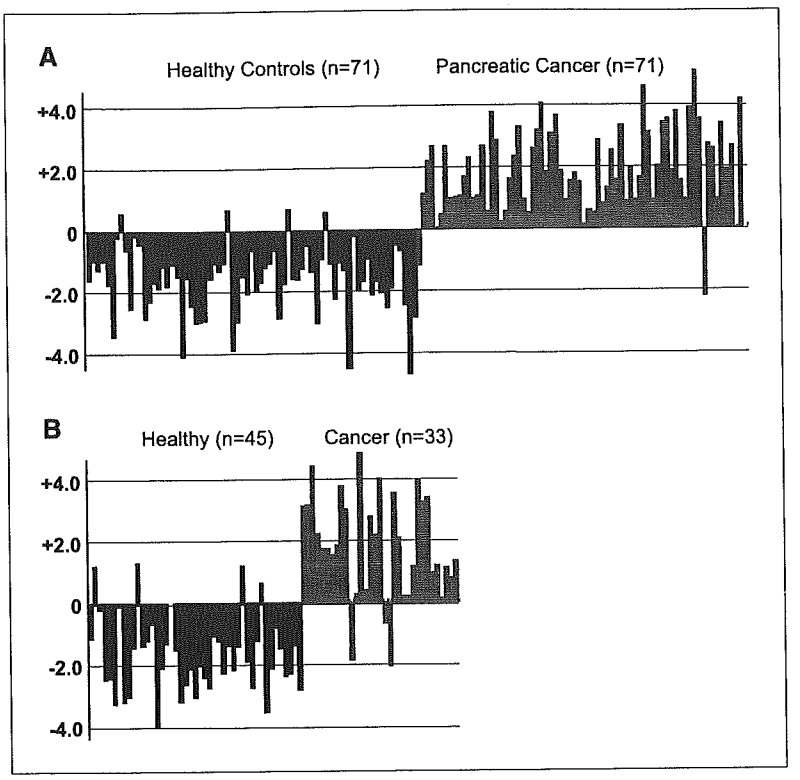

Figure 5. Calculated SVM distances of healthy controls (*black columns*) and pancreatic cancer patients (*gray columns*) in the training (*A*) and first validation (*B*) cohorts. Cases separated into the positive direction from the SVM hyperplane were classified as having "cancer" and those separated into the negative direction were classified as being "healthy."

of the pancreatic cancer patients and 80% (4 of 5) of the healthy controls, whereas the CA19-9 level correctly identified 66.7% (6 of 9) of the pancreatic cancer patients and 100% (5 of 5) of the healthy controls (Fig. 6). Again, in all the pancreatic cancer patients (9 of 9), the SELDI classifier and the CA19-9 level provided complementary results, even in this second validation cohort.

Discussion

Comparative proteomic profiling coupled with a computerized machine learning approach may revolutionize medical practice and cancer diagnosis. We compared the plasma protein profiles of a large number of pancreatic cancer patients and healthy controls with identical age and gender distributions (Table 1) to identify a biomarker for detecting pancreatic cancer patients in a large

Table 4. Diagnostic accuracy of the SELDI classifier

	Training cohort		Validation cohort (NCCH)		Validation cohort (TMUH)	
	No. cases	No. correctly classified samples* (%)	No. cases	No. correctly classified samples* (%)	No. cases	No. correctly classified samples* (%)
Healthy	71	67 (94.4)	45	41 (91.1)	5	4 (80)
Pancreatitis					5	4 (80)
Tumor/cyst†					6	4 (66.6)
Cancer	71	69 (97.2)	33	30 (90.9)	9	8 (88.9)
Cancer location						
Head	34	33 (97.1)	17	14 (82.4)	7	7 (100)
Body or tail	37	36 (97.3)	10	10 (100)	2	1 (50)
Unknown	0	0	6	10 (100)		
Clinical stage						
I	1	0 (0)	1	1 (100)	0	0
II	6	6 (100)	4	3 (75)	0	0
III	10	9 (90)	1	1 (100)	3	3 (100)
IV	54	54 (100)	27	25 (92.6)	6	5 (83.3)

*Number of healthy and chronic pancreatitis cases, considered to be "healthy," and number of pancreatic tumor/cyst and cancer cases given a diagnosis of "cancer."
†Pathologically unproven pancreatic tumor and/or cyst.

Table 5. Detection rates with CA19-9, the SELDI classifier, and their combination

	No. cases*	CA19-9, *n* (%)	SELDI, *n* (%)	Combination, *n* (%)
Healthy	39	3 (7.7)	4 (10.3)	6 (15.4)
Cancer	29	25 (86.2)	26 (89.7)	29 (100)
Clinical stage				
I	1	1 (100)	1 (100)	1 (100)
II	3	3 (100)	2 (66.6)	3 (100)
III	1	0 (0)	1 (100)	1 (100)
IV	24	21 (87.5)	22 (91.7)	24 (100)

*Cases whose plasma samples were available for CA19-9 measurement in the NCCH validation cohort.

population composed mainly of healthy individuals. The reproducibility of data obtained using the low-resolution instrument of the ProteinChip system has been a concern, but employing a high-resolution QqTOF instrument was found to significantly improve mass accuracy and minimize day-to-day variations (Fig. 1*B*). The high reproducibility of measurements was confirmed not by using a few high-intensity peaks selected intentionally but rather by using all the peaks detectable in the entire range (intensity and *m/z*) of mass spectra (Fig. 2). We also eliminated fractionation procedures, which increased the number of detectable peaks but significantly decreased reproducibility (Table 2; Fig. 2). A minimal set of four low-molecular weight proteins (Fig. 3) was found to be sufficient for discriminating pancreatic cancer patients with a sensitivity of 97.2% (69 of 71) and a specificity of 94.4% (67 of 71; Fig. 5*A*). This high discriminating capacity was confirmed by LOO cross-validation and ROC analysis (Fig. 4). We confirmed the discriminating capacity of our classifier in two independent validation cohorts (Figs. 5*B* and 6) to eliminate accidental identification of nonbiological/mathematical multivariate classifiers within a closed cohort by overfitting.

We noticed that a peak at 11,516 *m/z* (H50) was detected in 19.4% of the pancreatic cancer patients in the training cohort but in only 1.4% of the healthy controls (the peaks are indicated by a *red arrowhead* in Fig. 3). Tolson et al. (26) reported that an 11.5-kDa protein was detected in 32% of renal cell carcinoma patients but in none of the normal controls. Howard et al. (27) identified 11,682 *m/z* proteins in the sera of lung cancer patients as a diagnostic biomarker using matrix-assisted laser desorption/ionization (MALDI)-TOF-MS. Both groups identified the proteins as fragments of serum amyloid A. Serum amyloid A is an acute-phase reactant and a biomarker for inflammatory disease. The serum amyloid A level is elevated up to 1,000-fold during tissue damage and inflammation and is also increased in patients with various solid tumors and hematopoietic malignancies. However, serum amyloid A has not been recognized as a tumor marker because of its low positive rate (28, 29). Consistently, the 11,516 *m/z* peak was not incorporated into our classifier. The discovery of a single biomarker differing markedly between cancer patients and controls as well as having a high positive rate in cancer patients would be ideal but is perhaps not realistic. Since the discovery of CA19-9 in

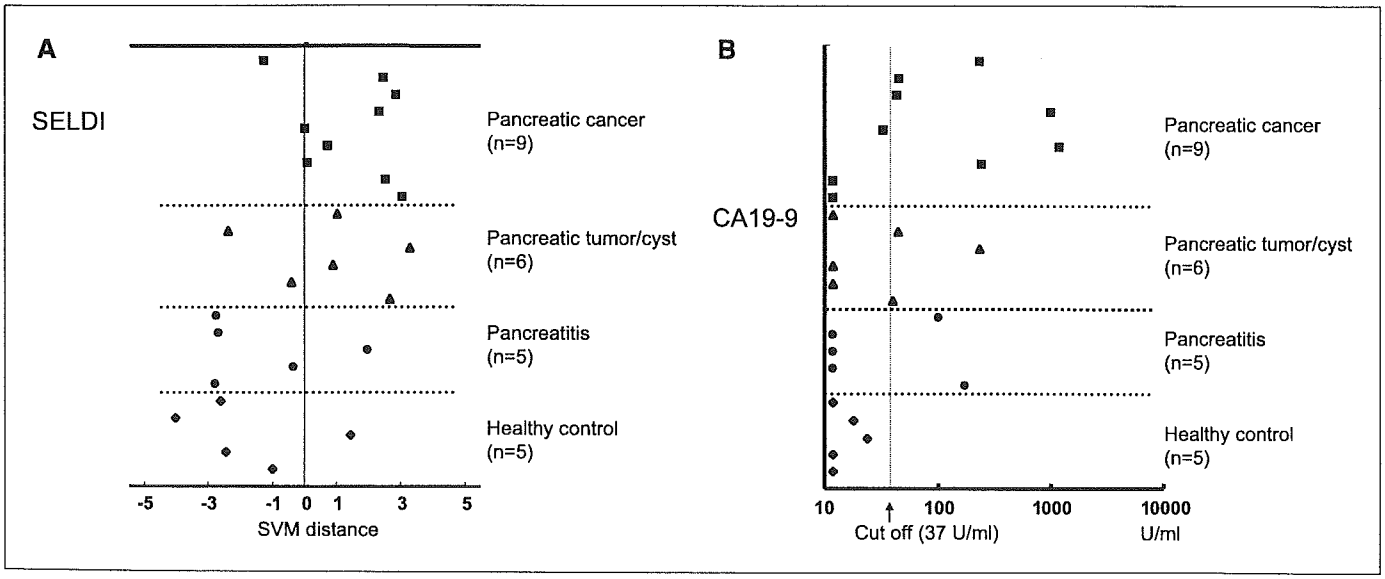

Figure 6. Confirmation in a second cohort treated at a different institution. *A*, calculated SVM distances of nine pancreatic cancer patients, six individuals with pancreatic tumors and/or cysts, five chronic pancreatitis patients, and five healthy controls seen at TMUH. *B*, plasma CA19-9 levels in nine pancreatic cancer patients, six individuals with pancreatic tumors and/or cysts, five chronic pancreatitis patients, and five healthy controls seen at TMUH. The cutoff value was set at 37 units/mL.

1982 (30), no single tumor marker applicable to the clinical diagnosis of pancreatic cancer has been identified. The carcinogenesis of pancreatic cancer is probably mediated via a variety of molecular pathways (2, 31), and multimarker analysis of proteins with different specificities is a realistic alternative to a conventional single biomarker assay.

There are pros and cons to SELDI-MS with high-resolution instruments. Although the primary goal of our study was the development of a bioassay applicable to the detection of pancreatic cancer, attempts to purify proteins from these four low-intensity peaks without contamination by neighboring high-intensity peaks have not been successful to date. However, the high reproducibility of QqTOF-MS warrants direct clinical application of its measurements and does not necessitate the actual protein identification of these peaks. Zhang et al. (12) reported that a set of three peaks, at 3,272, 12,828, and 28,043 *m/z*, could be used to detect early-stage ovarian cancer. The 28,043 *m/z* peak was down-regulated in ovarian cancer patients and was found to be derived from apolipoprotein A1. The relatively abundant 28,080 *m/z* protein identified as one of the peaks down-regulated in pancreatic cancer patients in this study (Table 3; Fig. 3) may be related to apolipoprotein A1. The mass deviation of 0.3% seen in the low-resolution TOF-MS may represent a drift in this region as large as 84 *m/z* (28,080 × 0.003 = 84). At least four peaks were detected between 28,000 and 28,100 *m/z* using the high-resolution QqTOF-MS instrument (Fig. 3). These peaks merged and were detected as a single peak with the low-resolution instrument (data not shown). The intensities of the 8,766, 17,272, and 14,779 *m/z* peaks were one magnitude smaller than that of the 28,080 *m/z* peak (Table 3) and were apparently below the sensitivity of tandem MS. So-called top-down proteomics using Fourier transform (FT)-MS (32) may be necessary to identify the proteins indicated by the low-intensity peaks of our classifier. However, an interface to the SELDI arrays is currently not available for FT-MS.

No significant differences in the detection rates for our classifier were observed among different stages of pancreatic cancer (Table 4). Koomen et al. (33) did plasma protein profiling of pancreatic cancer patients using MALDI-MS and identified a set of eight peaks distinguishing pancreatic cancer patients from controls with a sensitivity of 88% and a specificity of 75%. Protein identification revealed these peaks to be derived mainly from host response proteins. Many low molecular weight proteins detected by SELDI-MS in serum or plasma samples have also been reported to be metabolic products, proteolytic fragments, or peptide hormones. These proteins may not always be attributable to direct secretion or production by cancer cells, instead being the results of host responses in the microenvironment of the tumor (7, 18, 34), such as stromal desmoplastic reactions, inflammation, and angiogenesis. Two of eight pancreatic cancer patients who were classified as having "cancer," but none of normal controls in the TMUH validation cohort, had diabetes (data not shown). This raises the possibility that diabetic conditions, which are often associated with pancreatic cancer patients, also may influence the classifier.

All the pancreatic cancers were detected by complementary use of CA19-9 and/or the SELDI classifier (Table 5). CA19-9 is a tumor marker widely used for the evaluation of therapeutic effects and the detection of pancreatic cancer recurrence but is not considered to be applicable to mass screening (35–38). Ten percent to 15% of humans do not secrete CA19-9 because of their genetic Lewis antigen status (39). The CA19-9 level is often within reference range when pancreatic cancer is still at an early stage and is often elevated in benign biliary and pancreatic diseases. When the cutoff value for CA19-9 was set at 37 units/mL, which is widely used for clinical purposes, the false-positive rate of the combined CA19-9 and SELDI strategy reached 15.4% (Table 5). To increase diagnostic accuracy, the CA19-9 cutoff value may need to be adjusted and the selection of SELDI peaks may need to be further refined.

Early detection seems to be essential for improving the outcomes of pancreatic cancer patients. The SELDI classifier identified in this study has high potential for detecting pancreatic cancers (Tables 4 and 5), but one of the five pancreatitis patients in the TMUH validation cohort was classified into the pancreatic cancer category (Fig. 6). This pancreatitis patient may have a premalignant or preclinical condition and is currently being followed. Alternatively, because inflammatory conditions were not used in training, it is also possible that the classifier may not be entirely specific for the cancer phenotype. Machine learning was done with the training cohort, in which there were no cases with benign pancreatic diseases, because the discovery of biomarkers useful for pancreatic cancer screening in a large population made up mostly of healthy individuals was a primary goal of this study. The final diagnosis of pancreatic cancer is not made solely based on plasma protein profiling. CT, MRI, PET, ultrasound, and endoscopic and/or surgical approaches are employed as well. To evaluate the clinical significance of the biomarkers identified in this study and to refine the selection of biomarkers using a large number of subjects, including patients with pancreatic cancer and other pancreatic diseases, we need to undertake a prospective multi-institutional study.

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腫瘍マーカーと包括的プロテオーム解析
：ペプチドの包括的プロファイリングによる非侵襲的腫瘍マーカー開発法

本田一文*　山田哲司*

はじめに

難治がんの治癒率向上の戦略の一つに，できるだけ早期のがんを効率よく発見し，治療をおこなうということがあげられる．そのためには，非侵襲的に採取できる検体から簡便な方法を使ってがん患者を高精度に選別できるスクリーニング法の開発が急務である．最近，血液成分中に含まれる低分子量領域のペプチドを質量分析装置で網羅的にプロファイルし，疾患を高精度に診断するプロテオミクスプラットフォームが注目を集めている．とくにsurface-enhanced laser desorption/ionization time-of-flight mass spectrometry（SELDI-TOF-MS法）と機械学習法によって卵巣がんの診断マーカー候補を抽出した論文は注目を集め，米国などでは臨床現場への実用化をめざした大規模マーカー探索プロジェクトが進行中である[1]．国立がんセンター研究所 化学療法部・腫瘍プロテオミクスプロジェクトでも，多種類担がん患者血漿・血清に含まれるペプチドプロファイルをSELDI-TOF-MS法で取得し，病態診断マーカーや治療応答性予測マーカー開発を手がけている[2)~4)]．

本稿では，当研究所でおこなわれている質量分析装置を用いた，網羅的ペプチド腫瘍マーカー開発法を紹介する．

1　SELDI-TOF-MS法はどのような原理で，何がわかるのか？

SELDI-TOF-MS法とは，金属表面に官能基が修飾されているプロテインチップ®（サイファージェン社製）と質量分析装置を組み合わせた分析法のことをいう．プロテインチップ®の表面には，蛋白質の性質に従い親和性を有する官能基が修飾されているため，この上に検体をごく微量添加し，官能基の性質に応じて蛋白質を精製することができる．精製された蛋白質は，直接質量分析装置で解析される．解析結果は，横軸が蛋白質の質量電荷比，縦軸がそのイオン化強度の2次元データとして示される（図❶A）．このグラフに表されたピーク1本1本が，ペプチドの質量電荷比とイオン化強度になる．性質の違う官能基をもったプロテインチップ®を用いることにより，より多くのペプチドデータを網羅的に取得することができる[5]．

2　病態診断マーカーをどのように抽出するのか？

質量分析装置から取得されるペプチドプロファイルはすべてデジタルデータである．これをすべて検体ごとに整理統合し，ペプチドプロファイルデータベースを作成する．担がん状態にある患者は，健常人にくらべて血液内に含まれる蛋白質の構成が変化していることが考えられる．そこで，このデータベースのなかから網羅的にペプチドの質量情報を調べ，がん患者と健常人群間の差を抽出する．ここでは，機械学習法とよばれる人工知能などの分野で注目を集めているアルゴリズムを用いて，膨大な情報のなかからがん患者と健常人に特徴的なパターンをコンピュータに学習させる．学習セットには疾患以外には背景が異ならないように，性差などを厳密に調整したものを用意する．一つのペプチド情報からがん患者

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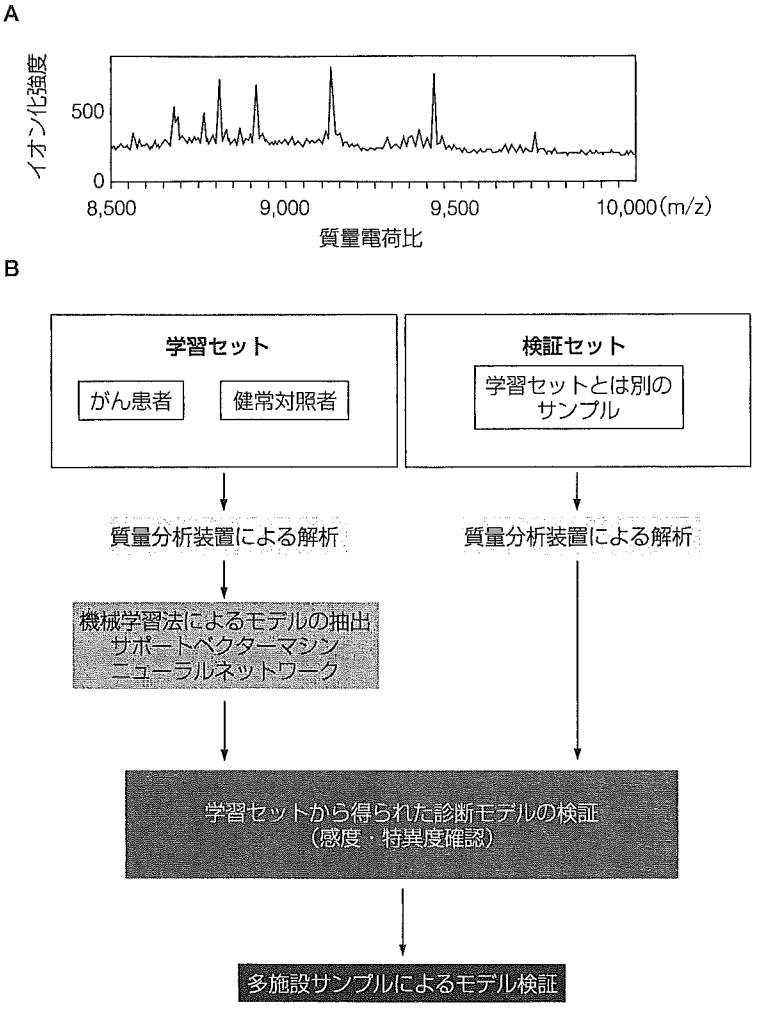

図❶ SELDI-TOF-MS 法によって取得されたピークスペクトル(A)と診断マーカー抽出と検証の流れ(B)
(著者作成)

と健常人が精度よく判別できるマーカーを抽出できれば最もよいが，たとえこのようなものが見つからなかったとしても，複数のペプチド情報を組み合わせて診断することができるマーカーを抽出する．ここで定義された判定基準に従い，学習セットとは別に用意した症例を用いて，その有効性を検証する．検証には，できるだけ多くの施設から同一条件で集められた検体を用いて検証をくり返し，信頼性の高い診断モデルを構築していく(図❶B)．

3 再現性を高めるための工夫とは?

血液中に含まれる微量なペプチドは他に豊富にあるペプチドに隠されて検出されないことが多い．このことを担保するために血液サンプルから豊富な蛋白質を取り除き，液体クロマトグラフィーなどの方法を用いて微量蛋白質を濃縮し検討することが試みられている．この方法は，多検体を処理する場合には処理スピードが遅くなり，さらに再現性の低下などが起こりえる．われわれは再現性の高い実験をおこなうために，実験の前処理はできるだけ簡素化し，そのかわり高い分解能を有する質量分析装置を用いることにより，微量蛋白質の測定を補償するように心がけている．また，正確な質量情報を取得するため，4重極を搭載した直行型質量分析装置を用いて計測をおこなっている[2]．

4　質量分析装置を用いた臨床検査への応用

診断モデルに寄与するペプチドを高度に精製してアミノ酸配列を決定することは可能である．しかしながら，そのピークが単一のペプチドで構成されているかどうかは注意する必要がある．分解能の低い質量分析装置でみたときは1本にみえたピークも，高分解能をもつ質量分析装置でみると，数本のピークで構成されていることがしばしばみられるからである．このような場合，標的ピークを分離同定することは困難なことが多い．最近，ペプチドマーカー候補の多くは，病態によって活性化された消化酵素などにより蛋白質が断片化され，その結果を質量分析装置が質量の変化としてとらえているのではないかということが指摘されはじめた[6]．そうであるならば，酵素抗体法などにより蛋白質の発現量そのものの定量をおこなったとしてもその絶対量には変化がみられず，むしろ正確な質量情報を加味しなければ検査として成り立たない可能性がある．今後は，質量分析装置を用いた臨床検査技術の開発を真剣に考慮する必要があると思われる．

おわりに

われわれは，質量分析装置を用いた網羅的ペプチドプロファイル法を用いて膵がんの血漿診断マーカーの開発に成功してきた．現在まで膵がんの血漿サンプルは健常対照者や良性膵臓疾患を含めて計245名分の解析が終了しており，4つのペプチドの変化から90%以上の正確さで，膵がんを診断できるモデルを構築している[2]．また，食道がんの術前化学放射線療法に対する治療奏効性予測血清マーカーの開発もおこなった．27例の施術前患者血清を学習セットとして予測モデルを構築し，さらに別の検証セットを用いて15例中14例の奏効性予測に成功している[4]．今後はさらなる基盤整備をおこない，これらマーカーの実用化に向けた大規模多施設共同試験に移りたいと考えている．

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【バイオマーカー探索①】

血液試料の最適な調製法と解析

下重美紀，本田一文，山田哲司

血清・血漿タンパク質には少なくとも10^9以上の発現量格差があるといわれており，アルブミン，イムノグロブリンなどの含有量の多いタンパク質の分離除去やゲル濾過，イオン交換などにより分子量，等電点の違いによる分画，レクチンによる糖タンパク質の精製操作が必要である．金属チップ表面を修飾したプロテインチップ（ProteinChip）は特定の血清・血漿タンパク質を定量的に捕捉・濃縮するのに威力を発揮する．

はじめに

近年の質量分析技術の進歩により，疾患の発症ならびに進行に伴う体液や組織，細胞でのタンパク質やペプチドの発現レベルの変動を高感度かつ網羅的に解析できるようになり，疾患の診断に応用する試みがなされている．われわれは膵癌などの難治性癌患者と健常対照者の血清，血漿のプロテオーム解析を行い，新しい診断マーカーを探索している．本稿では大規模な血液プロテオーム解析において必要な血液検体の要件，血液検体の調製法，質量分析方法，得られたデータの処理などについて，その意義とともに紹介する．

1．試料調製のストラテジー

i）試料調製

研究を始める前に，①十分な検体量や統計学的な解析が可能な症例数があり，②対照となる健常者や良性疾患患者の検体が入手可能であり，③施設の倫理審査を受け，④提供者の同意を得る方法に倫理的な問題がないこと，⑤個人情報が厳密に管理されていることを充分確認することは言うまでもない．検体の処理開始までの取り扱いは，可能なかぎり一定化することにより変動因子を極力取り除く必要がある．特に採血方法（採血管のメーカー・種類），保存条件（温度・期間），凍結融解の回数などは比較する検体群間で一致していることが必須である．さらには採血前の治療の有無，比較する検体群間での年齢や性別の分布などもよく検討しておかなければ，研究そのものが無駄になってしまう可能性がある．

血清，血漿には数千種類以上のタンパク質が含まれていると考えられており，Tirumalaiらの報告によると血漿はその成分の約90％をアルブミンをはじめとする10種類の構成タンパク質で占め，さらに全体の99％までを22種類のタンパク質で構成されている[1]．バイオマーカー候補となりうるタンパク質群は残りのわずか1％に含まれていると考えられている．さらに，タンパク質には少なくとも10^9以上の発現量格差があるといわれており，試料からのアルブミン，イムノグロブリンなどの含有量の多いタンパク質の分離除去が重要である．検体の50％以上を占めるアルブミンは固定化cibacron blue（シバクロンブルー）によってそのリガンドに静電的および，もしくは疎水的に結合させることにより除くことができる（図1，SDS-PAGE lane2）．より特異的に構成タンパク質の除去を行うには，これらを特異的に認識する抗体アフィニティーなどが有効である．最近では同時に6種類（図1，SDS-PAGE lane3）あるいは12種類の高発現タン

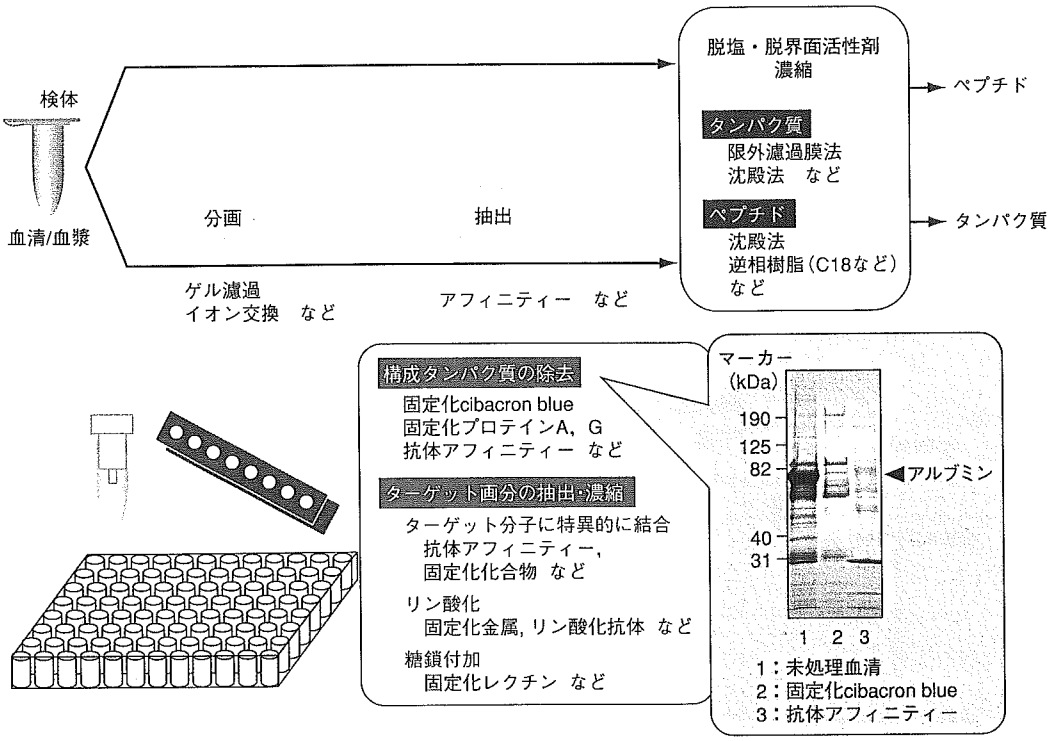

図1●体液試料調製のストラテジー

検体は必要に応じて尿素などで変性後，ゲル濾過，イオン交換などにより分子量，等電点の違いにより分画を行う．さらに，親和性の高い抗体や低分子化合物によるアフィニティーなどにより構成タンパク質の除去〔SDS-PAGE；lane 1：未処理，lane2：固定化cibacron blue，lane3：抗体（6種）アフィニティー〕やターゲット画分の抽出・濃縮により特定タンパク質（群）の抽出を行う．質量解析に供する前には限外濾過膜法，沈殿法などで可能な限り塩および界面活性剤は除く．最近では，分画・抽出などのタンパク質精製ツールがキット化され，スピンカラムやマルチウェルプレートなど微量検体を安定的に処理できる工夫が施されたデバイスとして市販されている

パク質を除くことができるカラムも市販されている．

検体は必要に応じて尿素などで変性後，ゲル濾過，イオン交換などにより分子量，等電点の違いによる分画を行い，低発現量のタンパク質の検出を可能にする．さらに，特定のタンパク質（群）を積極的に捕捉・濃縮するには，親和性の高い抗体や低分子化合物によるアフィニティーが有効である．アルブミン以外の多くの血清・血漿タンパク質は糖鎖付加による翻訳後修飾を受けていることから，特異的な糖残基と可逆的に結合する各種レクチンタンパク質を用いて濃縮・精製ができる．さらに特異性の異なる種々のレクチンを組み合わせて使うことで網羅性を上げることが可能である．

金属チップ表面に化学官能基や固定化された分子を装着し，試料中から特定の性質をもつ分子をチップ上で捕捉・精製するプロテインチップも同様の目的に使用されるものであり，質量分析と組み合わせた実験系は表面エンハンス型レーザー脱離イオン化（surface-enhanced laser desorption/ionization time-of-flight mass spectrometry：SELDI-TOF-MS）法と呼ばれている．

Petricoinらは診断に有用なバイオマーカー検索には，血流中に存在する癌細胞が分泌するタンパク質のみならず，癌の発症および病態の進行に伴う病変局所のプロテアーゼ活性の亢進によって産生されるペプチドの解析も重要であることを指摘している[2]．流血中のペプチドはアルブミンなどのキャリアータンパク質と結合し，腎

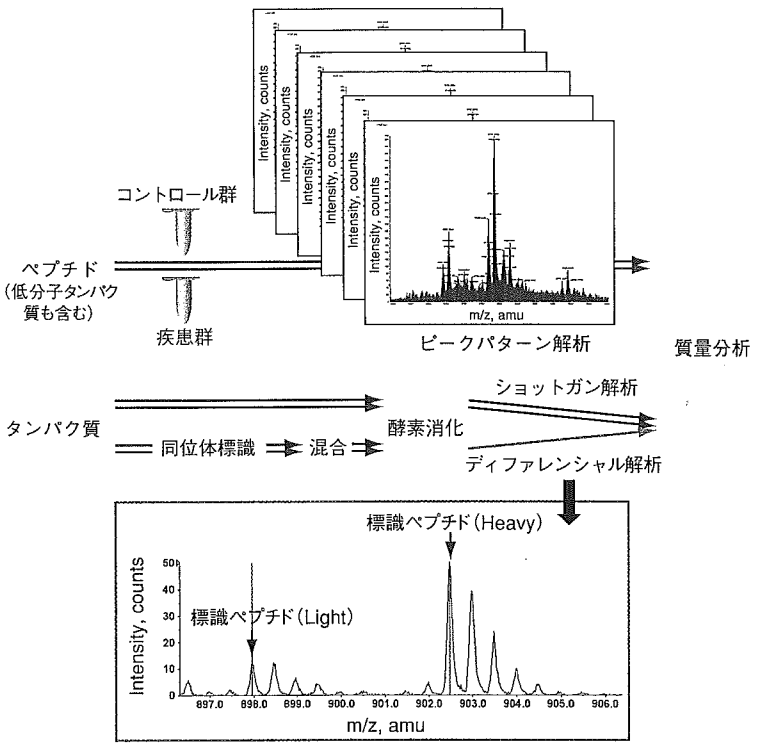

図2 ●体液試料の質量分析

質量分析による定量解析には，SELDI-TOF-MSやMALDI（matrix-assisted laser desorption ionization）-MSのように低分子タンパク質を含むペプチドをそのまま解析する方法に加えて，タンパク質をトリプシンなどの基質特異性の高い酵素で消化し，ペプチドとして物理化学的な性状を均一にして解析するショットガン法がある．ショットガン法ではICAT（isotope-coded affinity tag）などの*in vitro*での安定同位体タンパク質標識法を応用することで，ディファレンシャル解析が可能である

のクリアランスを回避していると考えられ，アルブミン結合ペプチドの検索もバイオマーカー候補を捕捉・濃縮する方法として有用であると考えられる．

ii）血液試料の質量分析

サンプルを質量解析に供する前には可能なかぎり塩および界面活性剤は除く必要がある（図1）．最近では，分画・抽出などのタンパク質精製ツールがキット化され，スピンカラムやマルチウェルプレートなど微量検体を安定的に処理できる工夫が施されたデバイスとして市販されており便利である[3]．

質量分析による定量解析には，SELDI-TOF-MSやMALDI（matrix-assisted laser desorption ionization）-MSのようにタンパク質・ペプチドをそのまま解析する方法に加えて，先にトリプシンなどの基質特異性の高い酵素で消化し，ペプチドとして物理化学的な性状を均一にして解析するショットガン法がある（図2）．ショットガンはICAT（isotope-coded affinity tag），iTRAQなどの*in vitro*での安定同位体タンパク質標識法を応用することで，比較定量が可能である．ICAT法では比較したい2検体を，分子量の異なる^{13}C（Heavy）もしくは^{12}C（Light）の同位体を含有する試薬で標識し，酵素消化後，標識部に導入されたビオチンタグで精製することで検体の複雑性を下げ，図2に示すようにペプチドの質量スペクトルから，由来するタンパク質の発現量を比較することができる．

われわれは無標識の検体を低流速（50～200 nl/min）でかつ長時間流速が一定の液体クロマトグラフィーで分離し，質量分析器で定量し，ペプチドをその質量/価数（m/z）と量を二（ないし三）次元画像として表す2D-ICAL-MS（two dimensional Image converted analysis of nano-flow liquid chromatography and mass spectrometry）法を開発した（日本ヒトプロテオーム機構第3回大会要旨集）．

2. マーカー分子の絞込みやバリデーション

プロテオーム解析では莫大な量の情報が発生するため，生物学的，臨床的意味のある情報を抽出するためには，バイオインフォマティクスの手段を用いる必要がある．われわれの研究室で行っている機械学習法の手順を示す（図3）．年齢，性別，採血法，採血時期に偏りがないような疾患群とコントロール群のサンプルを学習セットとして用意する．このセットの質量解析を行い，得られたスペクトルからピーク情報を定量的に検出する．単一のピークのみから最終判別をすることは判別率が十分でないことが多いため，人工知能などに応用される機械学習アルゴリズム〔サポートベクタマシン（線形，非

線形），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〕を用いて，癌と正常を区別するマルチマーカーからなるデーターセット（分類器）を抽出する．

しかし，この段階では数学的に強制的に疾患群とコントロール群を分類しただけに過ぎず，生物学的な意味のない分類をしただけの可能性がある．そこで，学習セットにおける分類結果が正しいかどうかを，検証セット（臨床情報が未知の全く別の検体）を用いて評価する．検証セットを盲検してはじめて，学習セットの分類器が診断的意義のあるものであると確認できる．このような流れでマーカー分子の絞り込みやバリデーションを行うことにより，その後に臨床情報未知検体サンプルが持ち込まれたとしても高い判別率で癌か否かを判定することが可能である．

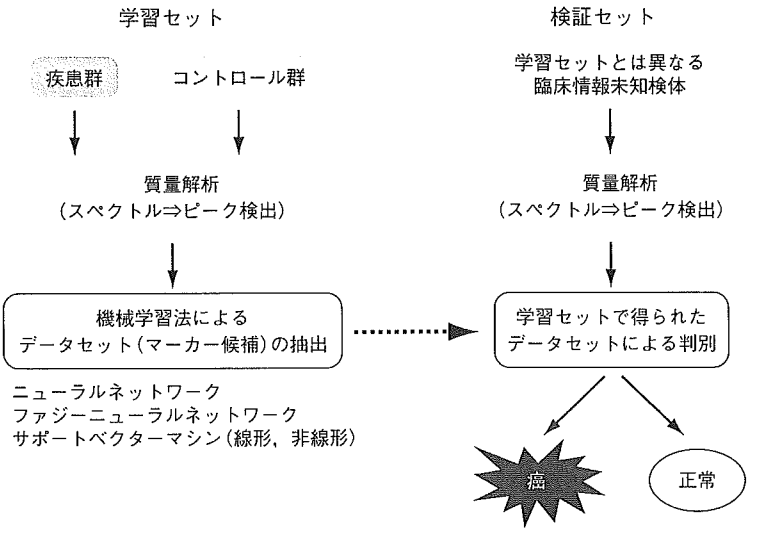

図3●マーカー分子の絞り込みやバリデーション

年齢，性別，採血法，採血法時期に偏りがないような疾患群とコントロール群のサンプルを学習セットとして用意する．このセットの質量解析を行い，得られたスペクトルからピーク情報を定量的に検出する．次に，機械学習アルゴリズム〔サポートベクタマシン（線形，非線形），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〕を用いて，癌と正常を区別するマルチマーカーからなるデーターセット（分類器）を抽出する．そこで，学習セットにおける分類結果が正しいかどうかを，検証セット（臨床情報が未知の全く別の検体）を用いて評価する

3．応用例および課題

われわれの研究室ではSELDI-TOF MS法を用いたアプローチで微量の検体を再現性よくハイスループットに解析することによって，有効な癌のマーカー候補があがってきているので紹介する．腎腫瘍の9割を占める腎細胞癌の早期診断マーカーを検索することを目的とした検討[4]では，解析に供した血清では構成タンパクの除去は行わず，陰イオン交換により分画した．さらに各分画を疎水性，金属親和性そしてイオン交換の各プロテインチップ上で特定の性質をもつ分子の捕捉，精製を行い，サイファージェン社の飛行時間型質量分析計であるPBS Ⅱcを用いて分析を行った．検出ピークは未分画では数百であったものが，分画操作により約3,000に増加していた．これらのピークを図3に示す機械学習法により解析を行ったところ，2つのピークを組み合わせることにより，感度，特異度共に良好に腎細胞癌を判別できるマーカーピークを得ることができた．さらにマーカー検索の再現性，安定性を向上させるためには，質量分析の精度を向上させることが必要であると考えられたので，高分解能型四重極飛行時間型（quadropole time-of flight：QTOF）質量分析計を用い，膵臓癌の血漿腫瘍マーカーの探索を行った[5]．血漿サンプルを未分画でプロテインチップに捕捉・濃縮し，質量分析を行い，得られたピークを機械学習法により解析を行ったところ，感度，特異度ともに高い値を有する4つのピークを検出した．このマーカーピークは既存の腫瘍マーカーであるCA19-9と相補的で，両者の組み合わせにより膵臓癌患者の100％の検出が可能であった．膵臓癌は早期診断が非常に困難であるために，この血漿腫瘍マーカー開発は多くの期待が寄せられており，実用化を進めている．

バイオマーカー検索アプローチは，癌の診断だけでなく，個別化治療選択ための診断法開発にも用いられる．われわれは進行食道癌の術前化学放射線療法（5-フルオロウラシル＋シスプラチン＋放射線照射）の奏効性を左右しうるマーカー分子の検索を行った[6]．化学放射線療法前

に採取した血清を各種プロテインチップ上での捕捉，精製後，高分解能質量分析計にて分析を行った．得られたピークの解析結果から化学放射線療法の奏効性を予測することが可能な4つのピークを抽出することに成功している．

おわりに

質量解析によるプロテオーム解析は，機械およびアプリケーションの技術開発が先行して，実体が何年か遅れてついてきている現状がある．その要因として，生体内で発現しているタンパク質を発現量格差がある状態にもかかわらず高感度な質量分析計で網羅的という言葉とともに一網打尽に分析できるかのような幻想を抱いていたことがある．しかし最近は試料調製の重要性がさまざまな場面で認識されはじめてきている．今後，試料調製技術のさらなる進展により臨床に役立つバイオマーカー検索に役立てたいと考えている．

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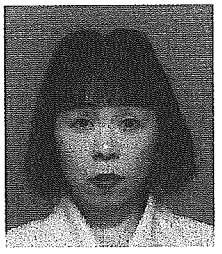

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1998年東京医科歯科大学大学院博士課程を修了後，タンパク質精製，分子細胞生物学を中心に研究に従事し，2002年より質量分析技術に取り組んでいる．2004年8月より現職．癌の早期診断・病態診断に有用な腫瘍マーカーの開発を見出すことを目的とした研究を行っている．

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Efficacy of gemtuzumab ozogamicin on ATRA- and arsenic-resistant acute promyelocytic leukemia (APL) cells

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Acute promyelocytic leukemia (APL) cells express a considerable level of CD33, which is a target of gemtuzumab ozogamicin (GO), and a significantly lower level of P-glycoprotein (P-gp). In this study, we examined whether GO was effective on all-*trans* retinoic acid (ATRA)- or arsenic trioxide (ATO)-resistant APL cells. Cells used were an APL cell line in which P-gp was undetectable (NB4), ATRA-resistant NB4 (NB4/RA), NB4 and NB4/RA that had been transfected with MDR-1 cDNA (NB4/MDR and NB4/RA/MDR, respectively), ATO-resistant NB4 (NB4/As) and blast cells from eight patients with clinically ATRA-resistant APL including two patients with ATRA- and ATO-resistant APL. The efficacy of GO was analyzed by ^{3}H-thymidine incorporation, the dye exclusion test and cell cycle distribution. GO suppressed the growth of NB4, NB4/RA and NB4/As cells in a dose-dependent manner. GO increased the percentage of hypodiploid cells significantly in NB4, NB4/RA and NB4/As cells, and by a limited degree in NB4/MDR and NB4/RA/MDR cells. Similar results were obtained using blast cells from the patients with APL. GO is effective against ATRA- or ATO-resistant APL cells that do not express P-gp, and the mechanism of resistance to GO is not related to the mechanism of resistance to ATRA or ATO in APL cells.
Leukemia (2005) **0**, 000–000. doi:10.1038/sj.leu.2403807
Keywords: acute promyelocytic leukemia; gemtuzumab ozogamicin (Mylotarg); all-*trans* retinoic acid (ATRA); arsenic trioxide and drug resistance

Introduction

Recently, gemtuzumab ozogamicin (GO, Mylotarg™), a calicheamicin-conjugated humanized anti-CD33 monoclonal antibody (mAb), has been introduced for the treatment of acute myeloid leukemia (AML).[1] However, the clinical outcome after the treatment with GO was negatively associated with P-glycoprotein (P-gp) function in AML.[2] In our previous studies, we found that resistance to GO was mainly mediated by P-gp.[3,4] Acute promyelocytic leukemia (APL) cells express a considerable level of CD33 antigen and a significantly lower level of P-gp compared with other types of AML.[5] Therefore, GO may become established as a successful treatment for APL. In fact, GO has been introduced with the clinical efficacy in the treatment of APL.[6,7] However, *in vitro* efficacy of GO on APL cells as well as all-*trans* retinoic acid (ATRA)- and arsenic trioxide (ATO)-resistant ones has not been studied well. Moreover, drug interaction among ATRA, ATO and GO, and the mechanisms of resistance of APL cells to them remain unclear.[8]

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Materials and methods

Cells

The cell lines used were a human APL cell line, NB4, which was kindly provided by Dr M Lanotte (Hospital Saint-Louis, Paris, France);[9] ATRA-resistant NB4 (NB4/RA) cells; NB4 and NB4/RA cells transfected with MDR-1 cDNA (NB4/MDR and NB4/RA/MDR, respectively); and ATO-resistant NB4 (NB4/As) cells. The NB4/RA and NB4/As cells were obtained by culturing NB4 cells with gradually increasing concentrations of ATRA and ATO, respectively.[10] mdr-1 messenger RNA (mRNA) was not detectable in NB4, NB4/RA or NB4/As cells by RT-PCR.[11,12] NB4/MDR and NB4/RA/MDR cells had detectable mdr-1 mRNA, but did not have detectable MDR-related protein (MRP) mRNA or lung-resistant protein (LRP) mRNA. Blasts were collected from four patients with APL at diagnosis, six patients with clinically ATRA-resistant but ATO-sensitive APL and two patients with clinically ATRA- and ATO-resistant APL.

Flow cytometric analysis for CD33 and Pgp expression

For evaluation of CD33 expression, cells were stained with phycoerythrin (PE)-conjugated anti-CD33 mAb (Becton Dickinson Immuno-cytometry Systems, San Jose, CA, USA), according to the manufacturer's instructions. For P-gp analysis, cells were incubated with biotinylated MRK16 (Fab') mouse mAb or a subclass-matched control mAb, and stained with streptavidine-Per CP (Becton Dickinson Immuno-cytometry Systems) as previously described.[11] Over 10 000 events were analyzed with the Epics XL flow cytometer (Beckman Coulter, Fullerton, CA, USA). APL cells obtained from the patients were also gated by CD-45 staining.[4]

Humanized anti-CD33 mAb and GO

GO consists of three essential parts: an antibody a cytotoxic agent, and a linker. The antibody, humanized IgG4 (hP67.6), targets the CD33 antigen. The cytotoxic agent is *N*-acetyl (NAc)-γ calicheamicin dimethyl hydrazide (DMH), a derivative of calicheamicin antitumor antibiotics.[1,2] GO, humanized non-conjugated anti-CD33 mAb (hP67.6) and free NAc-γ calicheamicin DMH were kindly provided by the Wyeth Research Division of Wyeth Pharmaceuticals Inc. (Philadelphia, PA, USA). The amount of GO used in an experiment was determined based on the concentration of NAc-γ calicheamicin DMH bound to the antibody. One microgram of GO contains 27.1 ng of NAc-γ calicheamicin DMH, and approximately 97% of a GO molecule is composed of the linker and antibody.

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^{3}H-Thymidine (^{3}H-TdR) incorporation analysis for assessment of cell proliferation

Cells were plated in a 96-well microplate (BD Biosciences, Billerica, MA, USA) at 2×10^5 cells per well in the presence or absence of GO containing 5, 10 or 100 ng/ml NAc-γ calicheamicin DMH or the respective concentration of hP67.6, in 100 μl of RPMI 1640 medium containing 10% fetal calf serum (FCS) and 1 μCi of ^{3}H-TdR. The detailed method was described in our previous papers.[3] The level of ^{3}H-TdR incorporation upon incubation with GO was compared with that upon incubation with hP67.6. The analysis was repeated five times.

Dye exclusion test with propidium iodide staining

After incubation of cells with GO or hP67.6. for the indicated period of time, cells were stained with 0.2 μg/ml propidium iodide (PI) (Sigma, Saint Louis, MO, USA) solution and counted. The numbers of dye-stained (dead) and unstained (living) cells both decreased and the amount of debris rapidly increased, making it difficult to evaluate the cell viability properly. Therefore, viable cells were evaluated. The viable cell count (/ml) after incubation with GO was compared with that after incubation with hP67.6. The analysis was repeated five times.

Cell cycle distributions

The cell cycle distribution was analyzed by flow cytometry with PI staining. The detailed method was described in our previous papers.[3,4] Cell cycle distribution could be analyzed after incubation with 10 or 100 ng/ml of GO for 24 or 48 h. GO temporarily arrests NB4 cells at the G2/M phase, and increases the percentage of hypodiploid cells, by which we evaluated the effect of GO.[3,4] Then, the GO-sensitive cells rapidly change to debris. The analysis was performed in triplicate.

Results

Flow cytometric analysis of CD33 and P-gp expression on NB4 cells and its sublines

The amount of CD33 expressed on the cells did not significantly differ among NB4, NB4/RA, NB4/MDR, NB4/RA/MDR and NB4/As cells. P-gp was not expressed on NB4, NB4/RA or NB4/As cells, in agreement with previous reports.[11,12] Equivalent levels of P-gp were expressed on NB4/MDR and NB4/RA/MDR cells.

^{3}H-TdR incorporation into NB4 cells and its sublines

Upon 72-h incubation with GO containing 5, 10 or 100 ng/ml of NAc-γ calicheamicin DMH, the level of ^{3}H-TdR incorporation into NB4 cells and its sublines decreased in a dose-dependent manner (Figure 1a). In each cell line, the level of ^{3}H-TdR incorporation was lower than that in the same cell line that had been incubated with the corresponding concentration of hP67.6. Upon incubation with GO containing 100 ng/ml NAc-γ calicheamicin DMH, there were significant differences in the level of ^{3}H-TdR incorporation between NB4 and NB4/MDR cells at 48 and 72 h ($P<0.01$ each), and between NB4/RA and NB4/RA/MDR cells at 48 and 72 h ($P<0.01$ each) (Figure 1b). There were no significant differences in the level of ^{3}H-TdR incorporation between NB4 and NB4/RA cells, or between NB4/MDR and NB4/RA/MDR cells at any time point. ^{3}H-TdR incorporation in NB4 cells upon 72-h incubation with GO containing 10 ng/ml NAc-γ calicheamicin DMH corresponded with that with 1000 ng/ml free NAc-γ calicheamicin DMH, which corresponded to a concentration of NAc-γ calicheamicin DMH that was approximately 100 times greater than that in GO. The rate did not change between P-gp-negative and positive NB4 cells.

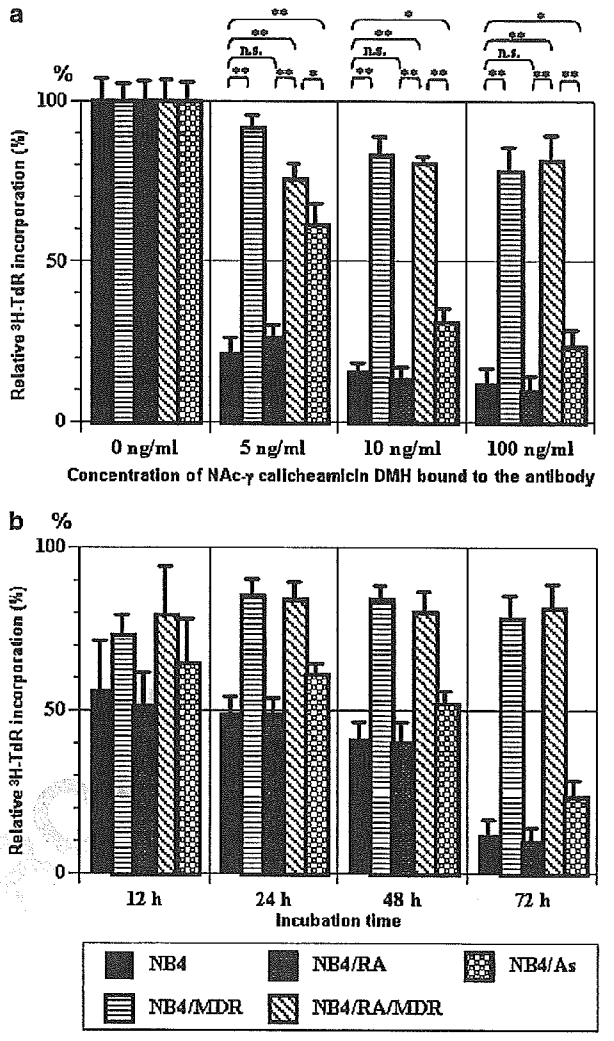

Figure 1 (a) ^{3}H-TdR incorporation by NB4 cells and its sublines after incubation with GO containing 5, 10 or 100 ng/ml NAc-γ calicheamicin DMH or with the respective concentrations of hP67.6 for 72 h. At 72 h, the amount of incorporated ^{3}H-TdR (cpm) was determined by liquid scintillation counting. In (a, b) the amount of incorporated ^{3}H-TdR are expressed as the ratio (%) of incorporated ^{3}H-TdR after incubation with GO to that after incubation with the respective concentration of hP67.6, normalized to 100%. The data from five independent experiments are expressed as mean values of the % response ± standard deviation (s.d.). At 72 h incubation with hP67.6, the mean level of ^{3}H-TdR incorporation by NB4 cells was 57 000 (53 200–62 300). Statistical significance was calculated by Student's *t*-test. $^*P<0.05$, $^{**}P<0.01$. (b) Time course of ^{3}H-TdR incorporation by NB4 cells and its sublines. Cells were incubated in medium with GO containing 100 ng/ml NAc-γ calicheamicin DMH or the respective concentration of hP67.6 for 12, 24, 48 or 72 h.

Viable cell count analysis by flow cytometry

By the incubation with GO containing 5, 10 or 100 ng/ml of NAc-γ calicheamicin DMH for 72 h, the viable cell counts of NB4 cells and its sublines decreased in a dose-dependent manner (Figure 2a). Figure 2c shows their cell counts upon 24-, 48- or 72-h incubation of these cell lines with GO containing 100 ng/ml NAc-γ calicheamicin DMH or the respective concentration of hP67.6. GO similarly decreased the number of NB4 and NB4/RA cells. GO also decreased the count of NB4/As cells. GO slightly reduced the counts of NB4/MDR and NB4/RA/MDR cells. The counts of NB4 cells and its sublines upon 72-h incubation with GO containing 10 ng/ml NAc-γ calicheamicin DMH corresponded with those with 1000 ng/ml free NAc-γ calicheamicin DMH, respectively.

The combination of ATRA and GO reduced the count of NB4 cells by a greater degree than GO or ATRA alone ($P=0.019$ and $P<0.01$, respectively), but did not reduce the count of NB4/RA cells by a significantly greater degree than GO or ATRA alone (Figure 2b). Upon incubation with GO and ATO, the counts of NB4 and NB4/RA cells were less than those upon incubation with GO ($P<0.01$ each) or ATO alone ($P<0.01$ each).

Cell cycle distribution

The increase percentage in the number of hypodiploid cells on cell cycle distribution upon incubation with GO containing 10 or 100 ng/ml of NAc-γ calicheamicin DMH for 48 h is summarized in Figure 3. The percentage of hypodiploid cells in the NB4, NB4/RA and NB4/As cells was increased upon 12-h incubation with GO, and it was the highest upon 48-h incubation. Beyond 48 h, it was difficult to evaluate the proportion of hypodiploid cells accurately because these GO-sensitive cells transformed into debris, as reported previously (Figure 4).[3] Upon incubation with GO, the percentage of hypodiploid cells in the NB4/MDR and NB4/RA/MDR cells were significantly less than those in the NB4 and NB4/RA cells, respectively ($P<0.01$ each). The addition of ATRA to GO further increased the percentage of hypodiploid cells in NB4 cells ($P=0.043$), but not in NB4/RA cells ($P=0.97$). Upon incubation with GO and ATO, the percentage of hypodiploid cells in NB4 and NB4/RA cells were slightly higher than those upon incubation with GO alone ($P=0.091$ and 0.082, respectively).

Similar results were obtained using blast cells derived from the patients with APL (Table 1). Upon incubation with GO for 48 or 60 h, the hypodiploid portion considerably increased in APL cells that had been obtained from not only the four cases at diagnosis, but also five ATRA-resistant and two ATRA- and ATO-resistant cases. Two patients, whose APL relapsed after achieving complete remission (CR) by ATRA and receiving postremission chemotherapy, were treated according to the Japanese phase 1 and 2 study of GO. They were resistant to re-induction therapy by ATRA, but achieved CR and CR without platelets recovery (CRp) after treatment with GO, respectively.

Discussion

Recently, GO with or without ATRA was introduced for the treatment of APL, and the clinical efficacy of these therapies has been reported in newly diagnosed or relapsed patients with APL.[6,7] There are two basic reasons that support the clinical application of GO on APL. One is that large amounts of CD33 are commonly expressed on the surface of APL cells. Therefore,

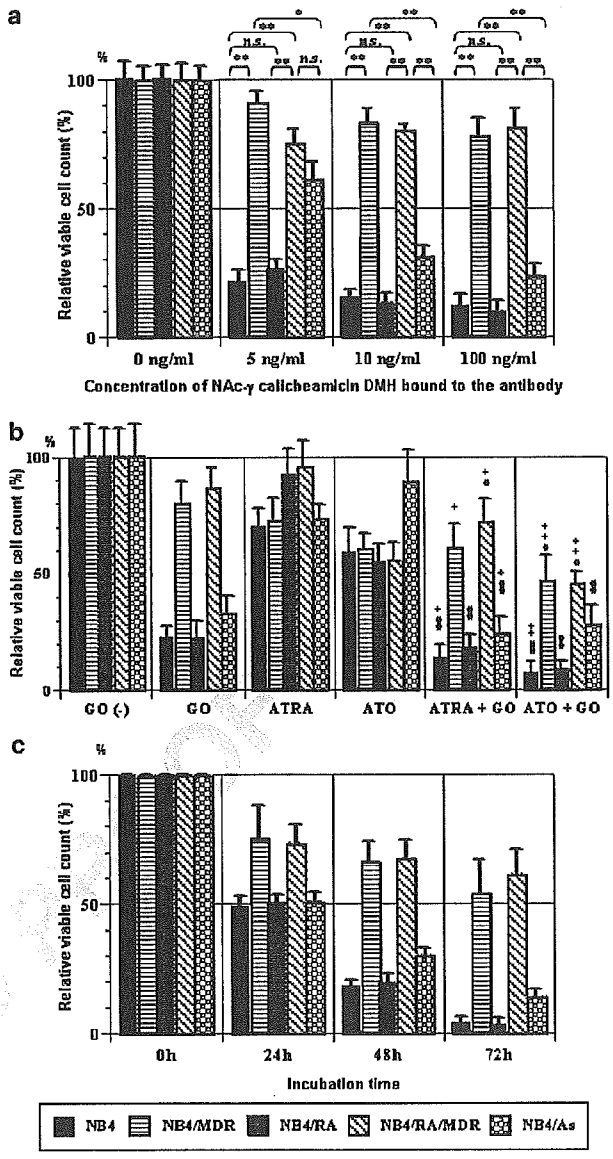

Figure 2 (a) Viable cell counts of NB4 cells and its sublines after incubation with GO containing 5, 10 or 100 ng/ml NAc-γ calicheamicin DMH or with the respective concentrations of hP67.6 for 72 h. The cell counts are expressed as the ratio of the cell counts after incubation with GO to that after incubation with the respective concentration of hP67.6, normalized to 100%. The data from five independent experiments are expressed as mean values of the % count±s.d. Statistical significance was calculated by Student's *t*-test. $^{*}P<0.05$, $^{**}P<0.01$. (b) Viable cell counts of the five cell lines upon incubation with GO containing 5 ng/ml NAc-γ calicheamicin DMH with or without ATRA or ATO for 72 h. The ratio of the cell count after incubation with the agents to that after incubation in the absence of both agents was calculated. Statistical significance was calculated by Student's *t*-test. $^{*}P<0.05$, $^{**}P<0.01$ comparing GO+ATRA or GO+ATO *vs* ATRA alone or ATO alone, respectively. $^{+}P<0.05$, $^{++}P<0.01$ comparing GO+ATRA or GO+ATO *vs* GO alone. (c) Viable cell counts of the five cell lines upon incubation with GO containing 100 ng/ml NAc-γ calicheamicin DMH, or with the respective concentration of hP67.6 for up to 72 h. The ratio (%) of the cell count after incubation with GO to that after incubation with the respective concentration of hP67.6 was calculated.

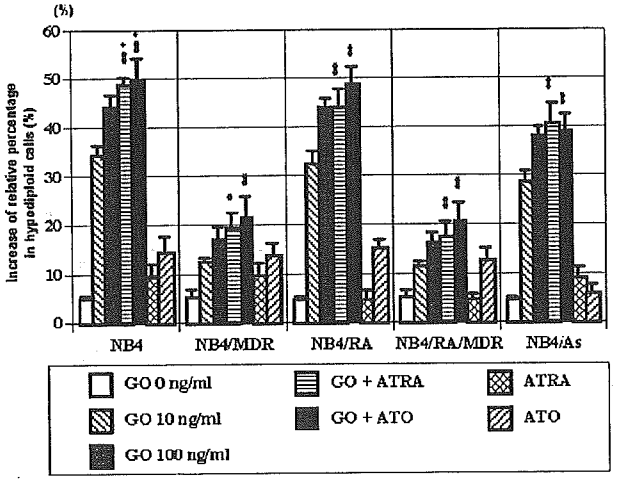

Figure 3 Percentage of hypodiploid cells in cell cycle distribution patterns upon 48-h incubation with GO containing 10 or 100 ng/ml of NAc-γ calicheamicin DMH or with the respective concentrations of hP67.6. Cells were also incubated with GO containing 100 ng/ml of NAc-γ calicheamicin DMH in combination with 10^{-6} M ATRA or 10^{-6} M ATO. Each bar represents the mean ± s.d. of three experiments. $^{*}P<0.05$, $^{**}P<0.01$ comparing GO + ATRA or GO + ATO *vs* ATRA alone or ATO alone, respectively. $^{+}P<0.05$, $^{++}P<0.01$ comparing GO + ATRA or GO + ATO *vs* GO alone.

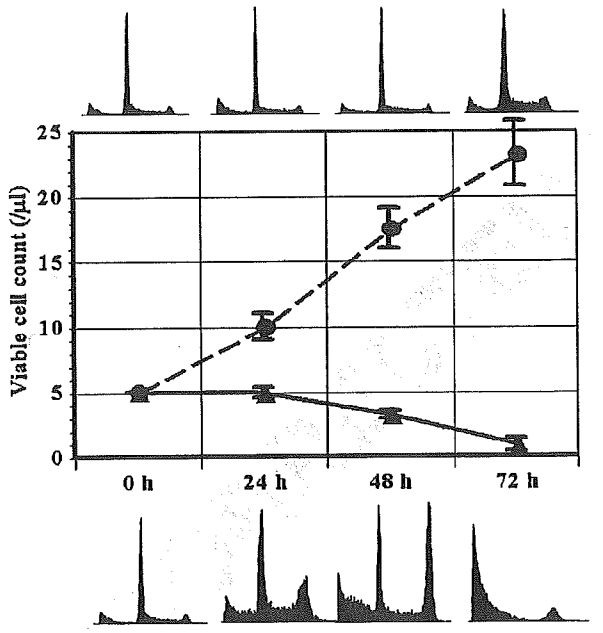

Figure 4 The cell growth curve and cell cycle distributions of NB4 cells upon 24-, 48- and 72-h incubation with GO containing 10 ng/ml NAc-γ calicheamicin DMH (continuous line and lower distribution patterns, respectively) or with the respective concentrations of hP67.6 (dotted line and upper distribution patterns, respectively).

several different anti-CD33 mAbs have been used for the treatment of APL, and notable results have been reported, especially in the use of GO.[6,7] Another reason is that a low level of P-gp is expressed on APL cells.[5] GO is sometimes not effective in some other subtypes of AML because the detached calicheamicin derivative is pumped out by P-gp.[3,4] Therefore, this mechanism of resistance to GO is not theoretically applicable to APL cells.

In this study, GO showed antiproliferative and cytocidal effects on ATRA-resistant NB4 cells as well as NB4 cells. We previously demonstrated that MDR modifiers, PSC833 and MS209, had no effect on ATRA-resistance in APL, which indicated that P-gp has a limited role in ATRA-resistance.[11] Intracellular ATRA concentration was not influenced by P-gp.[11] Clinical evidence, including our reports, also supported the independence of P-gp and ATRA-resistance.[13] Taking these data into consideration, GO is predictably effective on ATRA-resistant APL unless P-gp is expressed.

In the previous report, the combination of GO and ATRA was given to some patients with APL. In a study conducted in the US, GO was administered with ATRA to 19 patients with untreated APL.[6] The CR rate was 16/19 (84%), and 14 became PCR-negative. In relapsed APL, Lo-Coco *et al*[7] reported 14 cases of patients who achieved molecular remission after treatment with GO among 16 relapsed APL cases. However, there has been no *in vitro* study to explain these clinical efficacies. We performed the present study using NB4 and its drug-resistant sublines in an attempt to elucidate the mechanisms of GO. In APL, the drug resistance, which has been studied and discussed previously, might be built up by multiple causes and procedures.[8,13] Further studies on APL should be performed from many directions. It is also important to determine the optimal dosage of these drugs as well as the optimal timing of their administration.

GO also showed efficacy on ATO resistant NB4 cells, which do not express P-gp. The cellular glutathione and MRP levels are reported with their relationship to ATOresistance.[10,14] Walter *et al*[15] reported that MRP1 might attenuate the susceptibility to GO, although by a smaller degree than P-gp. We could not find an obvious relationship between MRP1 and GO resistance.[16] Our data suggest that the MRP and the cellular glutathione levels play limited roles, while P-gp plays a major role in GO resistance.

GO showed antiproliferative and cytocidal effects on APLs that do not express P-gp (Figure 4). GO increased the percentage of hypodiploid cells (Figure 3) while it inhibited cell proliferation in the early phase (Figures 1 and 2). After undergoing these changes, GO-sensitive cells rapidly collapsed into debris. The time-lag and variation of the effect of GO on APL cells might be explained by differences in the level of CD33 expression on the cells, and the length of time required for binding, and internalization of GO and detachment of calicheamicin from GO. Alternatively, GO could have various different actions against cells. Apoptosis, which is one of the main mechanisms of GO, did not explain all of the observed morphological changes of GO-treated cells in our previous study using videomicroscopy.[3] However, analysis of the changes in cell cycle distribution could be a valuable test for analyzing the susceptibility of AML cells to GO. It has a high degree of usability for samples derived from cases that contain different phenotypes.

We confirmed the antileukemia effect of GO on APL in an *in vitro* study using an APL cell line and its ATRA- and ATO-resistant sublines. GO seems to be promising for the treatment of not only untreated but also relapsed APL. A larger clinical study of GO for the treatment of relapsed and refractory APL is needed. The results of such study may suggest how GO should be integrated into the management of APL.

Table 1 Background of the patients and *in vitro* effect of GO on the cell cycle

Case no.	Sex	Age (year)	t(15;17)[a]	PML/RARα[b]	Status	Blast (%) in bone marrow	CD33 (%)	P-gp (%)	% increase in hypodiploid cells by GO[c]		Clinical response to ATRA	Clinical response to ATO	Clinical response to GO
									48 h	60 h			
DIAG-1	M	63	(+)	(+)	At diagnosis	92.2	99	3.7	13.2	22.7	CR	NT	NT
DIAG-2	F	21	(+)	(+)	At diagnosis	88.0	97.9	1.8	17.5	25.3	CR	NT	NT
DIAG-3	M	55	(+)	(+)	At diagnosis	67.6	97.8	3.5	12.1	17.9	CR	NT	NT
DIAG-4	M	51	(+)	(+)	At diagnosis	68.1	88.3	4.1	18.3	35.8	CR	NT	NT
ATRA-1	F	36	(+)	(+)	2nd relapse	20.2	98.7	1.8	10.7	18.4	NR	CR	NT
ATRA-2	M	57	(+)	(+)	2nd relapse	83.2	89.8	9.1	6.6	8.1	NR	CR	NT
ATRA-3	F	38	(+)	(+)	2nd relapse	77.2	94.6	1.4	16.9	27.9	NR	CR	NT
ATRA-4	F	21	(+)	(+)	2nd relapse	94.8	97.8	0.1	32.4	43.5	NR	CR	NT
ATRA-5	M	51	(−)	(+)	3rd relapse	30.8	97.9	4.4	9.6	12.8	NR	NT	CR
ATRA-6	M	48	(+)	(+)	3rd relapse	86.0	97.1	4.2	13.8	25.6	NR	NT	CRp
ATO-1	M	50	(+)	(+)	3rd relapse	78.0	83.6	11.6	10.8	13.4	NR	NR	NT
ATO-2	F	38	(+)	(+)	2nd relapse	38.7	85.4	4.5	9.5	15.3	NR	NR	NT

[a]Karyotype was analyzed by G-banding.
[b]PML/RARα was analyzed by RT-PCR or FISH.
[c]Defined as the difference in the percentage of hypodiploid cells between samples that had been incubated with nonconjugated anti-CD33 mAb or GO.
NT, not treated by ATO or GO *in vivo*; NR, no response; CR, complete remission; CRp, CR without platelets recovery.
After treatment with GO, the percentage of hypodiploid cells considerably increased in APL cells that had been obtained not only from the four patients at diagnosis but also from several ATRA-resistant patients and ATRA-and-ATO-resistant patients. Two patients, whose APL relapsed after achieving CR by ATRA and receiving postremission chemotherapy, were treated according to the Japanese phase 1 or 2 study of GO. They were resistant to reinduction therapy by ATRA, but achieved CR and CRp by treatment with GO, respectively. The coefficient of correlation (r) between the percentage of P-gp expression and the increase in hypodiploid portion was 0.60 and 0.65 upon 48- and 60-h incubation with GO, respectively.

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