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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*enezenesulfenyl 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

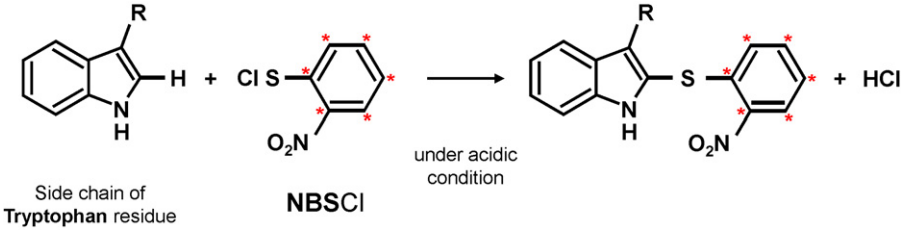

Fig. 1. Structural and reaction formula of the NBS reagent. Asterisk (*) indicates ^{12}C for the light reagent and ^{13}C for the heavy reagent.

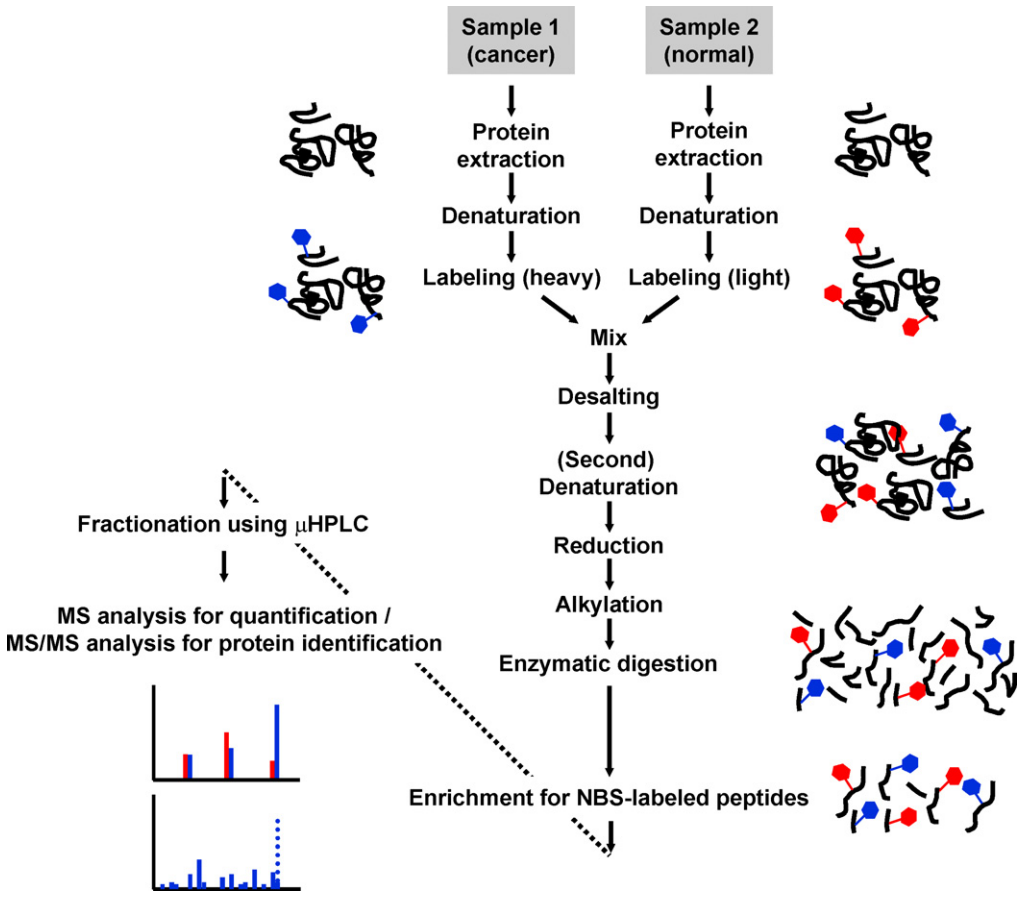

Fig. 2. The NBS method procedure is illustrated. Both proteins and peptides are indicated by black lines. Heavy and light NBS reagents are drawn as blue and red hexagons connected to the peptides with a bar, respectively.

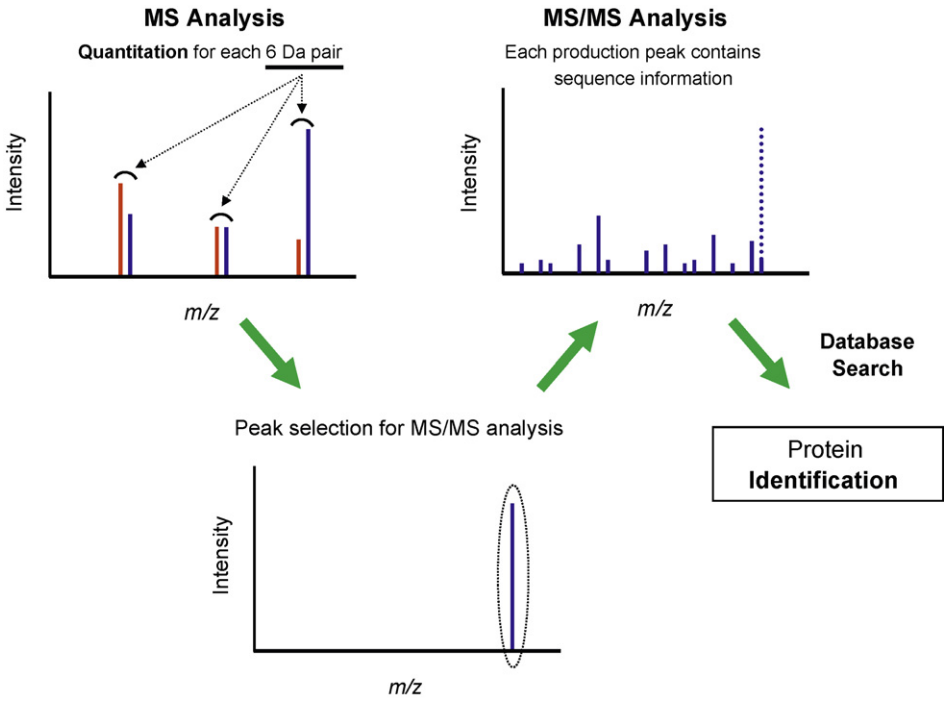

Fig. 3. MS and MS/MS analyses are illustrated. Blue and red lines indicate peaks of heavy and light NBS reagent-labeled peptides, respectively.

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.

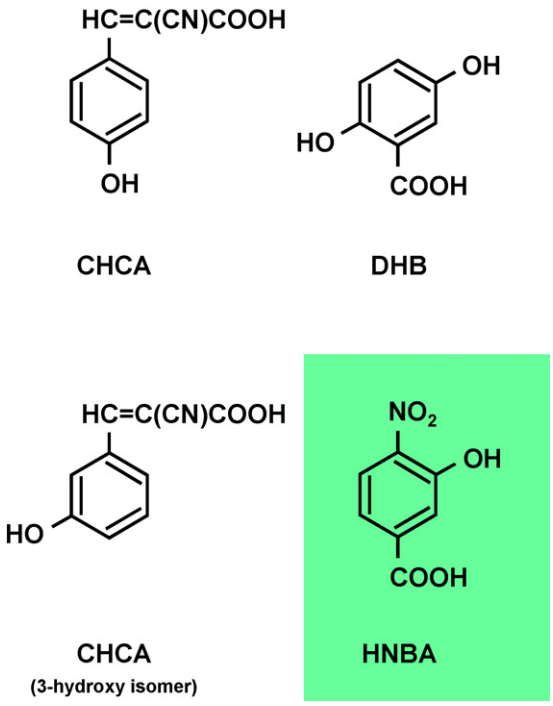

Fig. 4. Structural formulas of the four matrices, CHCA, DHB, CHCA (3-hydroxy isomer) and HNBA.

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].

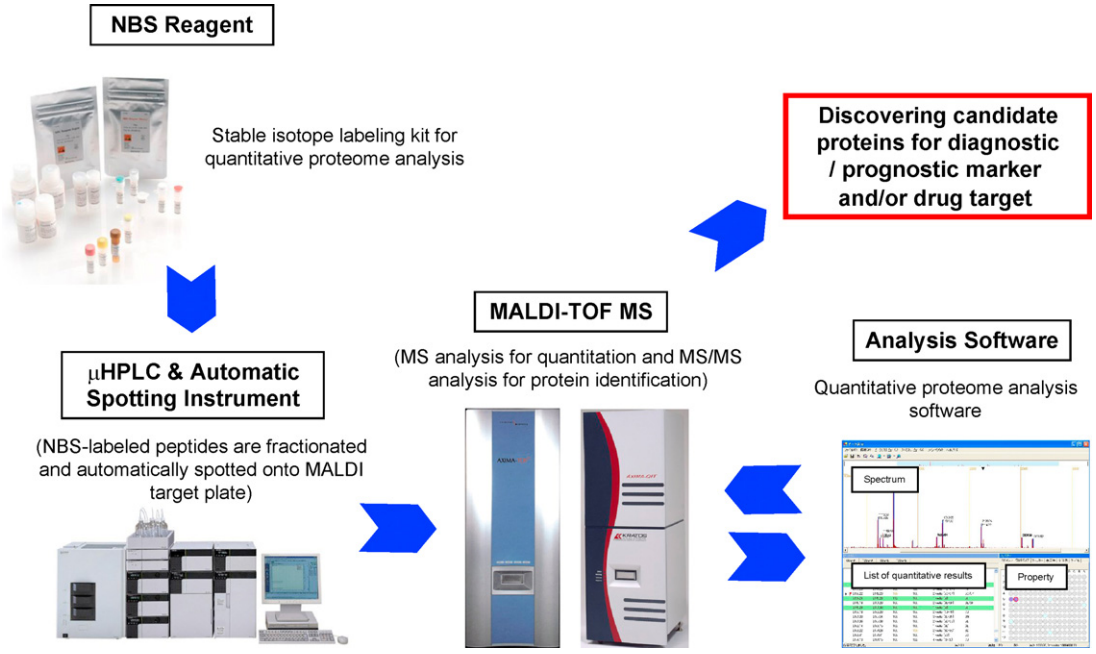

Fig. 5. Total analytical system using the NBS method.

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.

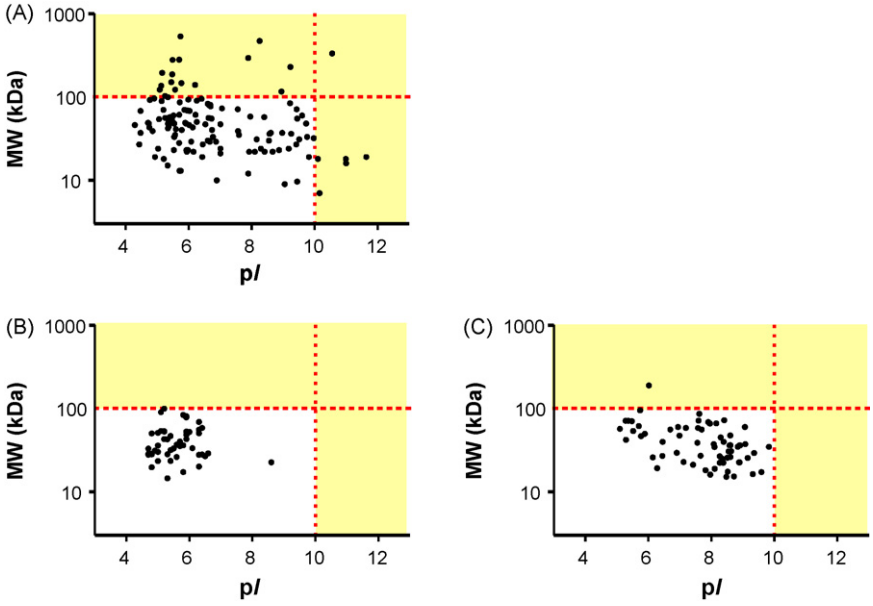

Fig. 6. Proteins identified by each method were plotted according to their predicted p*I* and molecular weight (MW). (A) NBS method [41], (B, C) 2D-gel method [42,43]. *y*-axis is on a logarithmic scale. Areas corresponding to MW larger than 100 kDa and/or p*I* > 10 are highlighted in yellow.

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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