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Proteomic Approach to Gastrointestinal Stromal Tumor Identified Prognostic Biomarkers

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Abstract

Biomarker development is a major research theme in cancer proteomics. Cancer is a genetically and clinically diverse disease, and biomarkers for risk stratification therapy are urgently required. A considerable number of biomarker candidates have been discovered by proteomics, and over the last decade proteomics modalities have been developed to identify promising candidates. Validation studies involving hundreds of samples in independent cohorts is the next challenge to prove the clinical utility of any discovered biomarker candidates. Here, we review our efforts directed toward tissue biomarker development using a proteomics approach. With the aim of developing a prognostic biomarker for gastrointestinal stromal tumor (GIST), we examined the protein expression profiles of primary tumors from 17 GIST patients with different risks of recurrence and prognosis after surgery. Through a comparative study using two-dimensional difference gel electrophoresis and mass spectrometry, we found that overexpression of pfetin was specific to GIST patients with a low risk of metastasis and a favorable prognosis after surgery. Using immunohistochemistry, we examined pfetin expression in 422 additional cases of GIST at four hospitals, and confirmed that GIST patients with pfetin-positive primary tumors had a significantly favorable prognosis in all four cohorts. Moreover, the other research group independently validated the prognostic significance of pfetin in 64 cases of GIST at two hospitals. Pfetin was found to be an independent prognostic factor with significant prognostic utility in all risk classification groups, which are based on tumor size and mitosis status. In addition to pfetin, we also identified DDX39 as a biomarker of unfavorable prognosis using a proteomics approach, and KCTD10 as a marker of favorable prognosis using a knowledge-based approach. Our experience demonstrates the utility of proteomics for biomarker discovery, and the possible clinical application of pfetin for risk stratification therapy in GIST.

Keywords: Gastrointestinal stromal tumor; Prognostic biomarker; Pfetin; Proteomics; Two-dimensional difference gel electrophoresis

Introduction

Cancer is a genetically and clinically diverse disease, and personally optimized therapy is required for optimization of the clinical outcome. Evaluation of prognosis and decision-making for treatments are often based on clinical and pathological observations. However, the clinical outcomes are not always as expected, and molecular biomarkers to complement the present staging system are required. For this purpose, a considerable number of biomarker candidates have been discovered by modern experimental methods at the DNA, RNA and protein levels using biobank resources [1,2]. However, the performance of the discovered biomarker candidates has rarely been confirmed by validation studies, and the clinical significance of the published candidates remains to be clarified [3].

Proteomics is a unique modality in cancer research, and biomarker development is one of the major goals of medical proteomics [4]. Proteomics studies have often employed a relatively small number of samples for discovery purposes, because clinical materials for which well-organized clinical information is available at the initial stage are relatively scarce. As unavoidable confounding factors are often associated with any cancer biomarker study [5,6], the risk of false positive discovery cannot be avoided when thousands of proteins are screened in a small number of samples using modern proteomics modalities. The relative lack of successful validation studies suggests that application of a proteomics approach to biomarker studies may have several drawbacks, raising questions as to whether proteomics is a suitable modality for biomarker discovery. We may need to approach to biomarker research not only expanding capability of proteomics modalities [7].

In this review, we describe our experiences with proteomics for discovering prognostic biomarkers in gastrointestinal stromal tumor (GIST), and our identification of pfetin as one such biomarker [8]. The prognostic utility of pfetin was extensively validated by immunohistochemistry in hundreds of cases from multiple cohorts [8-13]. Our experience suggests that pfetin would be clinically applicable

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for risk stratification therapy in GIST. Moreover, our experience also suggests that proteomics is useful for discovery of biomarkers.

Gastrointestinal Stromal Tumor (GIST) and Prognostic Biomarkers

GIST is the most common sarcoma in the gastrointestinal tract, and characterized by frequent mutation and overexpression of c-kit or PDGFR [14,15]. The tyrosine kinase inhibitor, imatinib mesylate, has dramatic inhibitory effects on tumor growth and metastasis in GIST, and benefits a substantial number of patients [16,17]. On the other hand, a significant proportion of GIST patients treated with imatinib have suffered from adverse effects [17-19] and the high cost of imatinib treatment has raised arguments about medical economics [20,21]. Considering that 60% of GIST patients are cured by surgery alone [22], risk stratification therapy has been required to select patients who are suitable for imatinib therapy. A risk classification system based on tumor size and mitotic status has allowed prognostication after treatment, and is used for selection of patients for adjuvant treatments [23]. Tumor origin, c-kit mutation status, and other molecular aberrations have also been considered as prognostic factors [24-27]. However, the prognostic performance of these molecular factors was not validated in the independent sample sets, and further development of prognostic indices for clinical application is required.

Proteomics Approach to Biomarker Discovery in GIST

Biomarker development is a major research theme in proteomics. Protein is the functional translation of the genome, and directly regulates cancer phenotypes. Thus, the proteome can be a rich source of biomarker candidates. Proteomics provides unique data about protein expression status, which may not be obtainable by other approaches. For instance, many lines of evidence have suggested discordance between mRNA and protein, probably because the amount of a protein is determined mainly at the translation step, rather than by the amount of the corresponding mRNA [28]. Post-translational modifications of proteins, localization of proteins, protein-to-protein or protein-to-nucleic acid interaction, and protein activity cannot be predicted accurately by examining DNA sequences or measuring the amounts of mRNA [29]. The malignant features of tumor cells are associated with the aberrant status of these protein characteristics, thus offering the possibility of evaluating the malignant potential of tumor cells by assessing them. Thus, global and direct investigation of proteins by proteomics would be a powerful approach for biomarker discovery.

For this purpose, we have been conducting cancer proteomics studies for discovery of biomarkers, and have used two-dimensional difference gel electrophoresis (2D-DIGE) to create protein expression profiles [30,31]. In 2D-DIGE, protein samples are labeled with fluorescent dyes, mixed together, and separated electrophoretically according to their isoelectric point and molecular weight in polyacrylamide gels. After gel electrophoresis, the separated proteins are observed as protein spots by scanning the gel with a laser scanner. The use of ultra-sensitive fluorescent dye makes it possible to use tiny amounts of samples such as those from laser-microdissected tissues for protein expression profiling [32,33]. Using a large-format gel, it is possible to observe up to 5000 protein spots in a single 2D gel depending on the sample type [33]. Informative protein spots are detected by comparing protein samples with biological and clinical information. Proteins included in the identified spots of interest are determined by mass spectrometry. We developed our original proteomics system based on 2D-DIGE [33], and applied it to cancer proteomics. As is the case of the other proteomics modalities, 2D-DIGE has its own characters and limitations; it cannot visualize all proteins. However, we found that 2D-DIGE is a considerably productive method in biomarker discovery. Firstly, the protein expression level is assessed as fluorescent signals with a wide dynamic range. Secondly, the gel-to-gel variation can be compensated by including the internal standard sample labeled with different fluorescent dye. Thirdly, as protein detection is performed by scanning the gel sandwiched between low-fluorescent glass plates, we can run a large size gel without worrying about gel fragility. As a consequence, we can observe a large number of protein spots in a single 2D gel. Beside advent of novel proteomics modalities in the last decade, 2D-DIGE is still one of the most popular proteomics methods.

Identification of pfetin as a Prognostic Biomarker using a Proteomics Approach

To identify candidate proteins for prognostic biomarkers, we examined the proteome of primary tumor tissues from GIST patients with different pathological and clinical backgrounds [8]. One group of GIST patients were classified as a low-risk group and did not develop metastasis during two years after surgery. The other group were high-risk patients who developed metastasis within one year after surgery. By comparing the protein expression profiles between these two groups, we found 43 protein spots with different intensity, and identified 25 unique gene products corresponding to these 43 protein spots by mass spectrometry.

Among the 25 proteins, we further focused on one protein, pfetin, which was detected in 8 protein spots that showed higher intensity in GIST patients with a favorable prognosis. Using western blotting and immunohistochemistry, we confirmed the correlation between higher expression of pfetin and a favorable prognosis in the GIST cases, which we examined by 2D-DIGE [8]. Pfetin was originally discovered as a unique gene product in the fetal cochlea, during work to identify genes responsible for congenital deafness [34]. Pfetin contains a putative potassium channel domain [34], and is functionally involved in the GAVA b receptor complex [35]. Although several reports have suggested physiologically important functions of pfetin, the molecular background factors linking pfetin expression to favorable prognosis of GIST patients remain to be elucidated.

Extensive Validation Study

We started an immunohistochemical validation study to examine the correlation between higher expression of pfetin and favorable prognosis. First, we examined pfetin expression in 210 additional cases of GIST at the National Cancer Center Hospital [8]. We used a polyclonal antibody kindly provided by Prof. C.C. Morton, who originally cloned pfetin gene [34]. Immunohistochemistry revealed that there was a significant difference in overall survival between 171 GIST patients with pfetin-positive primary tumors and 39 with pfetin-negative primary tumors; the 5-year metastasis-free survival rate was significantly higher in the pfetin-positive than in the negative group overall (93.9% versus 36.2%, $P<0.0001$) [8]. These observations led us to continue the validation study.

For our mmunohistochemical validation study, we created an original monoclonal antibody against pfetin using *in-vitro*-translated recombinant pfetin. Initially, we confirmed that the reactivity of the original monoclonal antibody was equivalent to that of the polyclonal antibody using immunohistochemistry and Western blotting. Then, we

examined 100 newly enrolled cases of GIST treated at Niigata University Hospital [9]. Immunohistochemical validation was successful in these 100 cases; the GIST patients with pfetin-positive primary tumors had a significantly better outcome than those with pfetin-negative tumors. We continued our validation using 40 additional GIST cases treated at Juntendo Shizuoka Hospital [10] and 72 GIST cases treated at Juntendo University Hospital [11]. Pfetin was proven to have prognostic utility in these two GIST cohorts. Thus, we confirmed the prognostic utility of pfetin in a total of 371 GITS cases at 4 hospitals using our original antibody. When we stratified these 371 patients according to risk classification, we found that pfetin retained its prognostic value in all three risk classification groups [36]. It was noteworthy that even when the patients were grouped as being of low or intermediate risk, patients with a poor outcome were significantly characterized as being pfetin-negative by immunohistochemistry.

This series of validation studies was performed in our laboratory; we received formalin-fixed, paraffin-embedded tissue sections and stained them with anti-pfetin antibody. Another research group has also confirmed the prognostic value of pfetin. Using a commercially available antibody for immunohistochemistry, Hasegawa et al. examined the expression of pfetin in 64 cases of GIST treated at Sapporo Medical University Hospital and Sunagawa Memorial Hospital, and confirmed that pfetin was a prognostic factor [13]. Our results were also reproduced when another commercial antibody against pfetin was employed [13,37].

Immunohistochemical studies demonstrated that pfetin expression was not observed in Cajal cells [8,13]. Moreover, pfetin expression was unique to GIST among the other sarcomas [13]. Although these observations may suggest possible roles of pfetin in GIST, the significances of unique pfetin expression in the etiology of GIST are not clear yet.

Commercialization of our Monoclonal Antibody against pfetin

Our original monoclonal antibody was extensively used for immunohistochemical studies of pfetin in GIST. To facilitate validation studies of pfetin, we decided to commercialize our anti-pfetin monoclonal antibody and contracted Medical and Biological Laboratories (anti-pfetin mAb code: D348-3, Ina, Nagano, Japan) for this purpose. We purified the antibody on a large scale from the supernatant of a hybridoma, followed by immunological characterization (Figure 1). Firstly, we confirmed that the new antibody distinguished the GIST cases with different prognosis and pfetin expression by SDS-PAGE/western blotting (Figure 1A). Samples 1-3 were obtained from the patients who did not have metastasis for more than two years observation period. Samples 4-6 were derived from the GIST patients who had metastasis within one year after surgery. We compared 2D-PAGE/western blotting images between the previous and newly purified antibody against pfetin (Figure 1B). The images of 2D-PAGE/western blotting are equivalent between these two antibodies. Pfetin was observed in multiple protein spots with different molecular weight in 2D-PAGE/western blotting. Reflecting these observations, pfetin was detected in double bands in SDS-PAGE/western blotting. We evaluated the prognostic performance of the antibody in the 71 GIST cases that had been examined in our previous studies (Figure 2, Supplementary Table 1) [11]. We performed immunohistochemical examination according to our previous report [11], and confirmed that the newly purified antibody clearly distinguish the pfetin positive case from the pfetin negative one (Figure 2A). Among the 71 cases examined, 46 were pfetin-positive, and the 5-year disease-free survival (DFS) rate was 95.65%. In contrast, the 5-year DFS rate for the 25 pfetin-negative GIST cases was 48.99% (Figure 2B). Multivariate analysis resulted in identification of pfetin as an independent prognostic factor ($P<0.05$, Table 1). We found that risk classification was also an independent prognostic factor ($P<0.05$, Table 1). When the GIST patients were stratified according to the risk classification, The DFS analysis showed a trend toward longer survival on the pfetin positive arm than on the pfetin negative arm in low risk (n=50) and high risk groups (n=15). The number of cases in the intermediate risk classification was too small for analysis (n=6). These observations indicated that the newly purified antibody against pfetin is useful for immunohistochemical study.

Other Biomarkers Discovered by Proteomics and a Knowledge-based Approach

To increase the number of proteins that can be revealed by 2D-DIGE, we developed a large-format electrophoresis device [33] and applied it for investigation of GIST [38]. As a consequence, the number of observed protein spots was increased from 1513 to 2250 [8,38]. We found that an ATP-dependent RNA helicase, DDX39, was upregulated in primary tumors of patients who developed metastasis within one year after surgery [38]. DXX39 was originally discovered as a novel growth-associated RNA helicase [39], and its overexpression was reported in lung squamous cell carcinoma [40]. DDX39 contributes to global genome integrity and protection of telomere structure [41]. Besides these data suggesting the contribution of DDX39 to the malignant

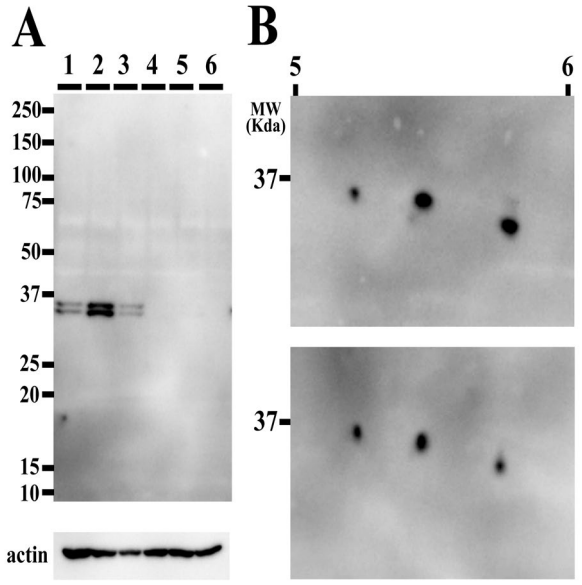

Figure 1: Western blotting evaluation of the newly purified monoclonal antibody against pfetin. A. SDS-PAGE/western blotting exhibited that the antibody distinguished pfetin positive (case 1-3) and negative (case 4-6) cases. The positive and negative pfetin expression of these cases were examined in our previous studies [11]. B. Western blotting with 2D-PAGE separation using previously purified antibody (upper panel), and newly purified one (lower panel). Western blotting was achieved according to our previous report [9]. 5 and 50 micrograms of proteins were separated by SDS-PAGE and 2D-PAGE, respectively. The first separation in 2D-PAGE was performed using 24 cm length IPG DryStrip gel (pI ranges between 4 and 7, GE), and the second separation was done by a home-made SDS-PAGE gel according to our previous report [9]. This study was approved by the ethical committee of National Cancer Center and Juntendo University.

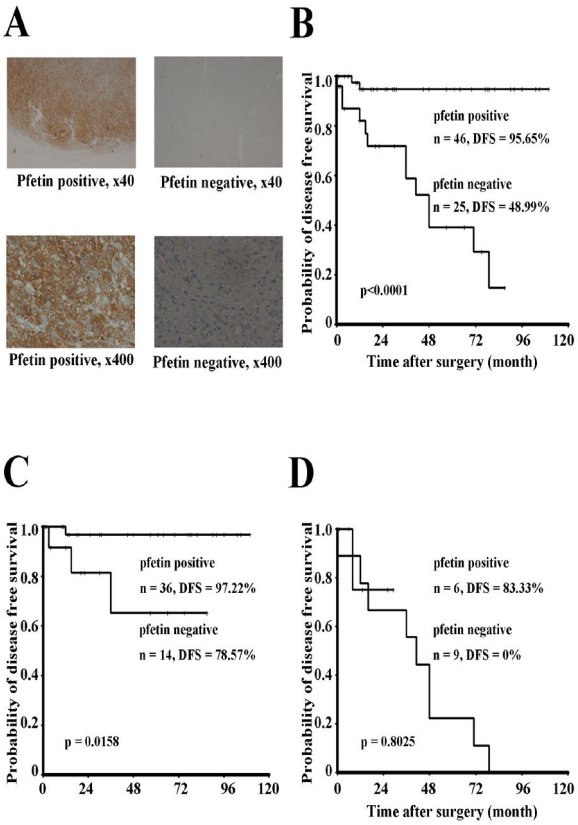

Figure 2: Immunohistochemical validation of the newly purified monoclonal antibody against pfetin. A. Immunohistochemical images of pfetin in GIST. B. GIST patients stratified according to the results of immunohistochemistry had significantly different outcomes after surgery. GIST patients in low risk classification (C) and those in high risk classification (D). Immunohistochemistry was achieved according to our previous reports [9-11].

potential of tumor cells, there was no indication that DDX39 might be associated with GIST until our investigation. We confirmed the prognostic value of DDX39 in 72 cases of GIST. Immunohistochemistry revealed that there was a significant difference in disease-free survival between 51 GIST patients who had primary tumors weakly expressing DDX39 and 21 whose primary tumors strongly expressed DDX39; the 5-year disease-free survival rate was significantly higher in the DDX39-weak than in the DDX39-strong group (90.2% versus 52.4%; P=0.0037) [38]. Thus, integration of immunohistochemical data for pfetin and DDX39 appeared to be promising. The 5-year disease-free survival rate of the GIST patients with pfetin-positive and DDX39-weak primary tumors was 100%, while that of patients with pfetin-negative and DDX39-strong primary tumors was 0% [11]. These results will be further confirmed by examining additional cases of GIST.

Recently, a novel association between GIST and a unique transcription factor, ETV1, was revealed by meta-transcriptome analysis; ETV1 was commonly included in gene expression signatures of GIST [42]. ETV1 expression was unique to GIST, and *in vitro* and *in vivo* experiments revealed that it contributed to the proliferation of GIST cells, and induced tumor growth in xenograft models. ETV1 promoted the signal transduction pathway of MPKAP kinase 2, whose overexpression was associated with a shorter survival period in GIST patients. Although ETV1 plays a key role in GIST, its expression level was not associated with clinical outcome [42]. To explore the molecular background factors underlying poor clinical outcome in GIST cases, we investigated proteins regulated by ETV1. Although ETV1 itself was not a prognostic biomarker, as ETV1 is a transcription factor unique to GIST, we hypothesized that there should be prognostic biomarkers among genes whose expression is regulated by ETV1. According to a previous report, silencing of ETV1 resulted in a variable gene expression pattern, and on the basis of the data, we focused on one protein, KCTD10 [42]. KCTD10 belongs to the same gene family as pfetin (KCTD12). KCTD10 interacted with proliferating cell nuclear antigen and contributed to cell proliferation [43,44]. These results suggested that KCTD10 might play a role in worsening the prognosis of GIST patients, and thus be a predictive biomarker. Firstly, using immunohistochemistry, we confirmed that ETV1 was not a prognostic biomarker in our study cohort [12], being consistent with the previous report [42]. We performed immunohistochemical examination of KCTD10 in 72 GIST cases, and found that it was a candidate biomarker for favorable prognosis; the disease-free survival rate was 88.5% in patients with KCTD10-positive tumors and 55.8% in those with KCTD10-negative tumors (p<0.0001) [12]. While these results were contrary to our expectation [42], the prognostic utility of KCTD10 and its molecular backgrounds would be worth exploring in newly enrolled GIST patients.

The original tumor site of GIST was highly correlated with prognosis; the clinical course of small-intestinal GIST is more aggressive than that of gastric GIST [45]. We examined differences in protein expression profiles between tumor tissues derived from the stomach and those from the small intestine [46]. A proteomics approach using 2D-DIGE identified proteins showing differences in expression between GIST

Variable	Number of cases	pfetin positive	pfetin negative	Correlation (pfetin) χ^2 *P* value	Disease-free survival		Multivariate analysis of disease-free survival by Cox regression		
					Rate (%)	Log-rank (*P* value)	*P* value	Relative risk	95% confidence interval
Age									
<60	34	20	14	0.224	76.47	0.4463			
≥60	37	26	11		81.08				
Sex									
F	26	19	7	0.197	88.46	0.2402			
M	45	27	18		73.33				
Site									
Stomach	52	33	19	0.571	78.85				
Small intestine	16	13	3		81.25	0.7867			
Other	3	0	3		66.67				
Histology									
Spindle	62	42	20	0.225	80.65				
Epithelioid	7	3	4		71.43	0.9707			
Mixed	2	1	1		50				
Size (cm)									
<5	44	33	11	0.006	88.64	0.0028	0.356	0.54	0.146-1.999
5–15	24	13	11		70.83				
≥15	3	0	3		0				
Necrosis									
Present	16	9	7	0.299	75	0.4581			
Absent	55	37	18		80				
Risk classification[a]									
Low	50	36	14	0.028	92	<0.0001	0.028	2.696	1.116-6.514
Intermediate	6	4	2		83.33				
High	15	6	9		33.33				
Recurrence/ Metastasis									
Present	15	2	13	<0.0001					
Absent	56	44	12						
pfetin									
Positive	46	46	0		95.65	<0.0001	0.015	0.126	0.024-0.664
Negative	25	0	25		48				

[a]Risk classification accrding to Miettinen's risk classification

Table 1: Univariate and Multivariate analysis and the relationship between clinicopathologic variables and pfetin expression of the 71 GIST cases.

primary tumor tissues obtained from the esophagus and stomach. These included prohibitin, pigment epithelium-derived factor, and alpha actinin-4, which are associated with various malignancies, and thus their roles in GIST are quite intriguing.

Conclusion

Using a proteomics approach, we explored prognostic biomarkers in GIST. To our knowledge, pfetin is the most successful tissue biomarker to have been discovered by proteomics and validated by immunohistochemistry. Several challenging issues remain to be addressed. Firstly, it should be confirmed whether a therapeutic strategy based on testing for pfetin would be beneficial for GIST patients. It would be particularly interesting to know whether pfetin-negative GIST patients classified as being at low or intermediate risk would benefit from therapy using imatinib or possible drugs. The correlation between pfetin expression and resistance to treatments should be investigated for better clinical application of pfetin. To answer this question, a prospective clinical study may be necessary. Secondly, the molecular backgrounds associated with pfetin expression and its prevalence in patients with a favorable outcome should be elucidated. Pfetin expression is unique to GIST among other sarcomas [13], and the unique molecular mechanisms regulating pfetin expression in GIST remain to be explored. Pfetin may play tumor-suppressive roles in GIST according to its pattern of expression. Studies of pfetin function would be worth pursuing in order to develop novel therapeutic applications for GIST. Thirdly, our proteomic studies suggested the presence of multiple prognostic biomarkers such as pfetin, DDX39 and KCTD10 in GIST. The associations of these biomarker proteins, as well as identification of additional ones, may provide clues to further understanding the malignant features of GIST cells.

Our experience of GIST proteomics suggests that proteomics is a powerful tool for biomarker discovery. The proteome is a highly complex group of molecules, and each proteomics modality allows observation of only part of the proteome. Thus, it is also challenging to utilize other proteomics modalities to address the issue of prognostic biomarkers in GIST.

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