Short Communication

Issues in applying protein biomarkers identified by proteomics to clinical testing

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Abstract With recent advances in proteomics technology, many new biomarkers have been reported, but only a few have been clinically applied. In this article, we investigated the possibility of clinical application of new tumor marker candidate proteins discovered through proteomic analysis. We have previously reported novel biomarker candidate proteins in serum extracellular vesicles of colorectal cancer patients using targeted proteomic analysis. In order to apply it to clinical tests, we investigated whether it could be reproduced using ELISA, which is currently the mainstream protein measurement method in clinical tests. As a result, although proteomic analysis was able to distinguish between colorectal cancer patients and healthy controls with high accuracy, ELISA was unable to do so. This may be because the sensitivity of MS for measuring trace amounts of proteins is higher than that of ELISA, or because the detection sensitivity of MS or ELISA depends on the state of the protein such as protein localization, stability and post-translational modification. We will discuss why it is difficult to clinically apply biomarkers identified through proteomic analysis, the causes, and solutions.

Key words: mass spectrometry, ELISA, biomarker, clinical testing

Introduction

Cancer is a curable disease if detected and treated early. Therefore, in order to reduce the mortality rate due to cancer, the most important issue is how early cancer can be detected. In recent years, technology for diagnosing early cancer has improved due to the development of CT, MRI, ultrasound, endoscopy, etc. However, all of these clinical tests are highly invasive for patients and expensive, so minimally invasive and low-cost tests are needed. Non-invasive clinical cancer tests include blood tests such as CEA, CA19−9, and AFP for gastrointestinal cancer, PSA for prostate cancer, and fecal occult blood tests for CRC, but all of them result in highly false positive and/or false negative. Thus, there is an urgent need for new blood and urine tests that can diagnose cancer early with high accuracy.

Recent advances in mass spectrometry (MS) have made it possible to identify minute amounts of proteins in body fluids such as blood and urine. Regarding the early detection of cancer, many new tumor marker candidates have been reported, but there is still no biomarker that can replace tumor markers currently used in clinical practice. What is the cause of this? The following are some of the problems that prevent new tumor marker candidates from being used in clinical tests.

1. Candidate tumor markers are often derived from analyses using small-scale samples and cannot be reproduced when validated using large-scale samples.
2. There is no functional support for how the identified tumor marker candidates are related to cancer development, therefore, they are not reliable as clinical tests.
3. It is difficult to reproduce the MS based quantitative

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results of tumor marker candidates using ELISA, which is the current mainstream clinical test.

To address problems 1 and 2 above, other candidate markers are needed that can be replicated in large-scale validation and provide functional support for their association with cancer development. In this report, to verify problem 3, we investigated whether the quantitative results of novel tumor marker candidates obtained by MS could be reproduced by ELISA. We also discuss how new tumor marker candidates obtained by MS can be translated into clinical applications.

Materials and Methods

Sample collection

Plasma samples were collected from cohort 1 between April 2011 and December 2012 (patients with CRC) and October 2017 and July 2018 (healthy controls) at The Cancer Institute Ariake Hospital of Japanese Foundation of Cancer. The protocol was approved by the ethics committees of the National Institute of Biomedical Innovation Health and Nutrition and the Cancer Institute Ariake Hospital of Japanese Foundation of Cancer. Informed consent was obtained from all donors. All experiments were performed in accordance with relevant guidelines and regulations including the Helsinki Declaration of 1964 and later versions.

Antibody

Anti-human primary antibodies are as follows; ANXA3: HPA013398, Atlas Antibodies, ANXA4: 10087-1-AP, Proteintech Group, ANXA11: NB300-875, Novus Biologicals, SLC2A1: 21829-1-AP, Proteintech Group.

Anti-human secondary antibodies are as follows; ANXA3: 11804-1-AP, Proteintech Group, ANXA4: H00000307-M13, Novus Biologicals, ANXA11: 10479-2-AP, Proteintech Group, SLC2A1: 66290-1-Ig, Proteintech Group.

ELISA

A 96-well ELISA plate (Maxisorp, P6991, Nunc) was coated with primary antibodies (50 ng/well) overnight at 4°C. The plate was washed three times with PBST and then blocked in Blocking solution (h-Block-e, BCL-BKHE-01, Beacle, Inc) for 1 h at room temperature. Diluted plasma with PBS were added to each well and incubated for 2 h at room temperature. After three washes with PBST, the plate was incubated with horseradish peroxidase-conjugated secondary antibodies (50 ng/well) for 2 h at room temperature. After washing with PBST, the plate was incubated with 100 μL of freshly prepared BM Blue POD Substrate (11484281001, Roche) for 30 min at room temperature, followed by the addition of a stop solution (0.2 M sulfuric acid). The absorbance was measured at 450 nm using a microplate reader (Bio-Rad).

Results and Discussion

We have previously reported that four proteins, annexin A3, A4, A11 and solute carrier family 2, facilitated glucose transporter member 1 (SLC2A1), were significantly increased in patients with early-stage CRC than in healthy controls by targeted proteomic analysis of serum EVs[1). Receiver operating characteristic (ROC) analysis to examine how accurately the four proteins could distinguish between patients with early-stage CRC and healthy controls showed a very high area under the curve of over 0.91, indicating that the four proteins have a very high ability to identify patients with early-stage CRC. Furthermore, the sensitivity of the four proteins for identifying patients with early-stage CRC were much higher than CEA, which is currently used clinically as a tumor marker for CRC.

Therefore, we investigated whether the quantitative results of these tumor marker candidate proteins could be reproduced by ELISA. In ELISA, it would be complicated and impractical to include the steps of purifying EVs from blood and extracting proteins, so we used sandwich ELISA to measure plasma itself rather than purified EVs. First, we quantified ANXA3, 4, 11, SLC2A1 by sandwich ELISA using 0, 0.04, 0.2, 1, 5, 10 μL of pooled plasma obtained from healthy controls (HC) and CRC patients (CC) (Fig. 1). As a result, for ANXA3 and 11 among the above four proteins, a plasma volume-dependent increase in protein quantification values was observed. Then, we quantified ANXA3 and 11 by ELISA using individual samples from healthy controls (HC) and CRC patients with or without metastasis (CC_m, CC_n) (Fig. 2). As a result, no significant difference was observed between healthy controls and CRC patients. There are several possible reasons why ELISA cannot reproduce the quantitative results of tumor marker candidate proteins obtained through proteomic analysis. First, EV proteins were quantified by MS for discovery and verification of these tumor marker candidates, whereas plasma proteins were directly quantified by ELISA. ELISA may be measuring these tumor marker can-

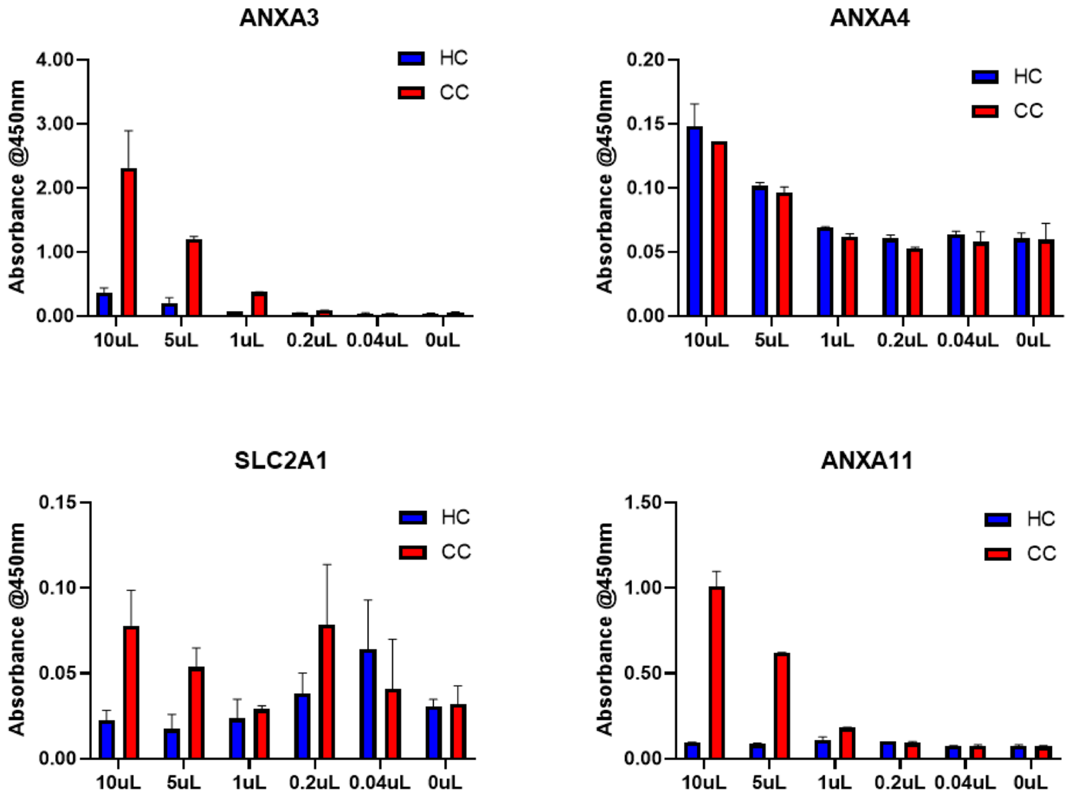

Fig. 1. Quantification of tumor marker candidate proteins for CRC by sandwich ELISA using pooled plasma of CRC patients.
Tumor marker candidate proteins, ANXA3, 4, 11, SLC2A1, were quantified by sandwich ELISA using 0, 0.04, 0.2, 1, 5, 10 μL of pooled plasma obtained from healthy controls (HC) and CRC patients (CC). Data represent the mean ±SD of three independent experiments.

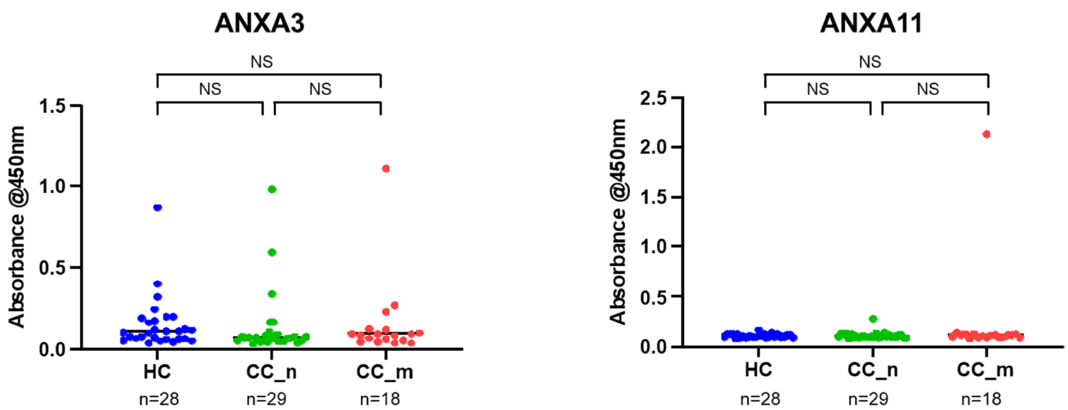

Fig. 2. Quantification of tumor marker candidate proteins for CRC by sandwich ELISA using individual plasma of CRC patients.
Tumor marker candidate proteins, ANXA3 and 11 were quantified by sandwich ELISA using 5 μL of individual plasma obtained from healthy controls (HC) and CRC patients (CC). CC_n: CRC without metastasis, CC_m: CRC with metastasis. NS: Not significant.

didate proteins present outside of EVs, which may lead to the results showing no quantitative difference between cancer patients and healthy controls. On the other hand, measuring proteins in EVs using ELISA is difficult because of problems such as the complicated pretreatment method and antibody reactivity to the denatured EV proteins during the pretreatment. Second, whether or not the reason ELISA did not reproduce the MS results in this study was due to EV purification, the detection sensitivity of MS or ELISA depends on the state of the protein such as protein stability (degradation), protein truncation and post-translational modification. If target proteins were degraded or post-translationally modified in the blood, the recognition protein sequence by two antibodies used for sandwich ELISA is critical for detection rate. In this case, MS has an advantage for detection of target proteins.

As mentioned above, it is clear that it is not easy to reproduce the quantitative results of proteins identified by proteomic analysis using ELISA. If this is the case, it is reasonable to consider that it would be better to use MS in clinical tests to quantify candidate marker proteins identified by proteomic analysis. However, at present, there are several obstacles to using MS in clinical testing. First, handling MS is complicated and requires skill. Second, it takes time to measure multiple samples due to poor throughput of MS. Third, it is difficult to automate preprocessing and data analysis[2]. In addition, handling of the clinical samples as laboratory examination or external quality assessment (EQA) is difficult, and the third-party certification of mass spectrometers is needed in Japan. We believe that the biggest problem is the poor reproducibility of MS analysis. Clinical test requires consistent results regardless of when, where, and who performs the test, but current MS-based protein quantification assays can vary depending on time, location, operator, and type of instrument. Various efforts have been made to overcome this problem. Recently, a joint study between Taiwan and the United States attempted to measure thyroglobulin, a marker of thyroid cancer recurrence, using LC-MS. It has been reported that for MS to be used as a clinical test, the following requirements must be met[3].

- Establishment of standard operating procedures (SOPs)
- Staff training and competency assessment including MS technical specialists for instrumentation operation and troubleshooting
- Maintaining strict consistency within the workspace and hardware instrumentation
- Preparation of calibrators and quality control samples
- Evaluation of quantification performance including within-day repeatability, between-day reproducibility, lowest quantifiable concentration
- Proficiency testing through interlaboratory comparison testing to assess individual laboratory performance, ensuring competence and quality assurance

Conclusion

Recent advances in MS research have led to the identification of many new tumor marker candidate proteins present in body fluids such as blood and urine. However, these results are difficult to reproduce by ELISA, which has hindered the development of new tumor marker tests. On the other hand, although some attempts to use MS in clinical tests have been reported, they are still in the development stage and many challenges remain to be overcome. However, due to the high accuracy of MS and the power of multimarker analysis, we may see a time in the future when MS will be used as a clinical test to measure many biomarkers, contributing to the early detection of various diseases.

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Conflict of Interest

The authors declare that they have no competing interests.

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