Clinical Application of Multiple Reaction Monitoring-Mass Spectrometry to Human Epidermal Growth Factor Receptor 2 Measurements as a Potential Diagnostic Tool for Breast Cancer Therapy

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BACKGROUND: Human epidermal growth factor receptor 2 (HER2) is often overexpressed in breast cancer and correlates with a worse prognosis. Thus, the accurate detection of HER2 is crucial for providing the appropriate measures for patients. However, the current techniques used to detect HER2 status, immunohistochemistry and fluorescence in situ hybridization (FISH), have limitations. Specifically, FISH, which is mandatory for arbitrating 2+ cases, is time-consuming and costly. To address this shortcoming, we established a multiple reaction monitoring-mass spectrometry (MRM-MS) assay that improves on existing methods for differentiating HER2 status.

METHODS: We quantified HER2 expression levels in 210 breast cancer formalin-fixed paraffin-embedded (FFPE) tissue samples by MRM-MS. We aimed to improve the accuracy and precision of HER2 quantification by simplifying the sample preparation through predicting the number of FFPE slides required to ensure an adequate amount of protein and using the expression levels of an epithelial cell-specific protein as a normalization factor when measuring HER2 expression levels.

RESULTS: To assess the correlation between MRM-MS and IHC/FISH data, HER2 quantitative data from MRM-MS were divided by the expression levels of junctional adhesion molecule A, an epithelial cell-specific protein, prior to statistical analysis. The normalized HER2 amounts distinguished between HER2 2+/FISH-negative and 2+/FISH-positive groups (AUROC = 0.908), which could not be differentiated by IHC. In addition, all HER2 status were discriminated by MRM-MS.

CONCLUSIONS: This MRM-MS assay yields more accurate HER2 expression levels relative to immunohistochemistry and should help to guide clinicians toward the proper treatment for breast cancer patients, based on their HER2 expression.

Introduction

Human epidermal growth factor receptor 2 (HER2) is a transmembrane protein that can promote the differentiation, development, and survival of cancer cells *(1, 2)*. It is often overexpressed in breast cancer and correlates with a worse prognosis *(1, 3)*. In targeted therapy, anti-HER2 therapy is administered to patients who overexpress HER2 in cancer cells, inhibiting its downstream signaling pathways *(4)*. Thus, accurate detection of HER2 using optimal techniques is crucial for providing the appropriate care to patients.

Immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH), approved by the Food and Drug Administration, are the most widely used methods for assessing HER2 status *(5)*. Although these techniques have been the gold standard for HER2 evaluation, they have limitations *(6)*. The semiquantitative nature and subjectivity of IHC contribute to its high variation and cost, in association with false positive and negative results *(7, 8)*. In addition, FISH, which should be used to verify equivocal HER2 cases *(9, 10)*, has several disadvantages: automated slide stainers are expensive and not always readily available in routine pathology laboratories, and the additional staining is time-consuming and costly *(11, 12)*. Thus, a novel technique that can accurately evaluate whether patients could benefit from

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HER2-targeted therapy than IHC is needed to improve the throughput and economic viability of the overall workflow *(13)*.

Targeted mass spectrometry (MS)-based approaches are emerging as alternatives to IHC, based on their high reproducibility and quantitative nature *(14, 15)*. Multiple reaction monitoring (MRM) has been widely applied to various clinical samples *(16–18)*, including formalin-fixed paraffin-embedded (FFPE) tissue, which has several advantages: plentiful access to vast archives of pathologically characterized clinical samples, and ability to be stored for extended periods without requiring expensive equipment *(19)*.

Three notable studies have previously aimed to quantify HER2 expression levels using FFPE tissues and targeted MS-based approaches *(16, 20, 21)* to stratify HER2 status more accurately and improve the selection of patients for HER2-targeted therapy compared with conventional methods. However, their complex and time-consuming sample preparation procedures make the MRM-MS assay less practical as a clinical assay.

Comparing the expression levels of cellular target proteins by MS-based analysis is predicated on defining the number of cells analyzed per sample. Currently, the total protein or peptide concentrations in each processed sample are used for convenience *(22, 23)* when i) normalizing the amount of specific target proteins, ii) reducing the potential analytical variability that might originate when dramatically different protein amounts are processed, and iii) ensuring an adequate amount of analyte for reliable detection. However, current total protein and peptide measures are poor representations of cell counts, because only a portion of the protein originates from the cells of interest. In addition, the accompanying assays lengthen the overall process and generate inter-experimenter variation. Thus, we designed an alternative protocol to facilitate the clinical application of HER2 quantification by MRM-MS assay. Because HER2 expression is exclusive to the surface of epithelial cells *(24)*, normalizing HER2 using a factor exclusive to epithelial cells in a breast tumor can result in a more accurate stratification of HER2 status. Thus, we applied the quantitative data of an epithelial cell-specific protein as a new normalization factor for calculating HER2 expression levels in an MRM-MS assay.

However, applying the novel protocol is not feasible without addressing the discrepancy between the tumor size and the corresponding tumor content across individual FFPE tissue specimens. To address this problem, we devised a two-part solution: (1) determining the number of FFPE tissue slides, based on the cell count from a single slide, to ensure adequate amounts of protein per analysis; and (2) using the expression level of an epithelial cell-specific protein as a normalization factor when measuring HER2 expression levels.

We aimed to establish a novel MRM-MS assay to determine HER2 status, especially for ambiguous IHC results in FFPE breast cancer samples, by determining an adequate number of FFPE slides per sample to perform a reliable MS analysis and using the expression levels of an epithelial cell-specific protein as a normalization factor.

Materials and Methods

All materials, sample preparation steps, and instrument conditions are detailed in the Supplemental Data.

PATIENTS AND TISSUE SAMPLES

Two hundred ten patients who underwent surgical resection after being diagnosed with invasive ductal carcinoma at Seoul National University Hospital from January 2010 to December 2017 were selected. Those who had received neoadjuvant chemotherapy were excluded. The final cohort was composed of HER2 0 (n = 30), HER2 1+ (n = 30), HER2 2+/FISH-negative (n = 61), HER2 2+/FISH-positive (n = 59), and HER2 3+ cases (n = 30). Pathological and clinical data were reviewed thoroughly and obtained from the electronic medical records system. A summary of the patients and the characteristics of their breast cancer tissues is presented in Table 1. All contents of this study were approved by our institutional review board (Institutional Review Board No. 1709-037-883), and all participants provided written informed consent.

ASSESSMENT OF HER2 STATUS IN BREAST CANCER

The diagnostic algorithm for scoring HER2 was to perform IHC in all cases, supplemented by FISH in equivocal IHC cases (HER2 2+), per the 2007 and 2013 American Society of Clinical Oncology/College of American Pathologists guidelines *(5, 10)*. IHC and FISH were performed on 4 μm tissue sections.

FFPE TISSUE SAMPLE PREPARATION

Tumors marked by pathologists in deparaffinized FFPE tissue sections (10 μm thick) were manually collected and isolated for denaturation using RapiGest (Waters). The denatured proteins were digested with trypsin for 4 h (Supplemental Fig. 1). The sample preparation procedure was followed by removal of RapiGest byproducts, the addition of stable isotope-labeled internal standard (SIS) peptides, online desalting, and MRM-MS analysis (Fig. 1). A detailed protocol can be found in the Supplemental Methods.

PARAMETERS FOR REPRESENTING TUMOR CONTENT

The light-to-heavy peptide peak area ratios (PARs) for HER2 surrogate peptides were used to estimate the amount of HER2 protein. Five categories of normalization factors that represent tumor content were acquired and evaluated to normalize the amount of HER2

Table 1. Demographic and clinical characteristics of the study population in the MRM-MS analysis (n = 210).

	HER2 status				
Group	HER2 0 (n = 30)	HER2 1+ (n = 30)	HER2 2+/ FISH-negative (n = 61)	HER2 2+/ FISH-positive (n = 59)	HER2 3+ (n = 30)
Age (years)					
	53.80 ± 9.57	53.60 ± 11.39	48.62 ± 9.86	54.22 ± 10.95	50.57 ± 11.66
FISH status					
Negative	0	2	61	0	0
Positive	0	0	0	59	20
NA	30	28	0	0	10
Estrogen receptor					
Negative	18	7	4	20	20
Positive	12	23	57	39	10
Progesterone receptor					
Negative	21	7	11	30	24
Positive	9	23	50	29	6
Subtype					
HER2	0	0	0	21	20
Luminal A	10	23	53	0	0
Luminal B	3	0	4	38	10
TNBC	17	7	4	0	0
Nuclear grade					
1	0	1	0	1	0
2	5	15	38	13	5
3	25	14	22	45	25
NA	0	0	1	0	0
Histological grade					
I	1	3	6	1	0
II	4	16	37	25	8
III	25	11	17	33	22
NA	0	0	1	0	0
Tumor size					
<2.0 cm	7	12	28	30	19
2.0–4.9 cm	21	17	32	27	10
≥5.0 cm	2	1	1	2	1

FISH, fluorescence in situ hybridization.

protein, as follows: (1) tumor area (μm2), (2) total cell count, (3) total protein amount (μg), (4) total peptide amount (μg), and (5) light-to-heavy peptide PARs for surrogate peptides from 10 epithelial cell-specific proteins and 20 housekeeping proteins, as assessed by MRM-MS analysis. The following formula was used to normalize the light-to-heavy peptide PARs for HER2 surrogate peptides.

$$\frac{\text{Peak area ratio for HER2 surrogate peptide}}{\text{Normalization factor}}$$

MRM-MS ASSAY

All MRM-MS analyses were performed on an Agilent 6490 triple quadrupole mass spectrometer with a Jetstream electrospray source, equipped with a 1260 capillary liquid chromatography system (Agilent

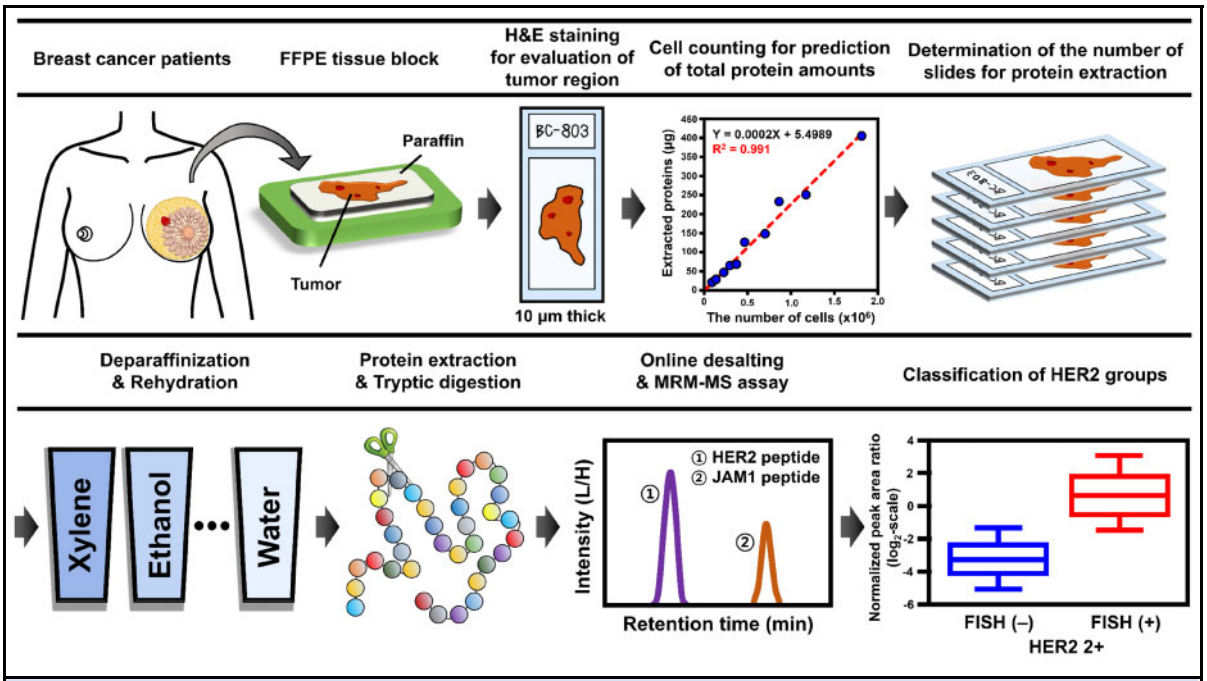

Fig. 1. Schematic of overall procedure, from sample preparation to MRM-MS assay, for determining HER2 status. Four- and 10-μm-thick formalin-fixed paraffin-embedded (FFPE) tissue sections per sample were used for hematoxylin and eosin staining and protein extraction, respectively. Hematoxylin and eosin-stained images were reviewed by pathologists to identify and assess tumor regions. After determining the number of FFPE slides based on the tumor cell counts in a single slide to ensure an adequate amount of protein, FFPE tissue sections were deparaffinized and rehydrated. The resulting tryptic peptides were analyzed by MRM-MS assay for measuring HER2 expression levels.

Technologies). Skyline (MacCoss Lab) was used to process all raw MRM-MS data files *(25)*. A detailed protocol can be found in the Supplemental Methods.

All data have been deposited to Panorama Public *(26)* and ProteomeXchange ID (PXD017691) *(27)*.

Results

EQUATION FOR PREDICTING PROTEIN AMOUNTS EXTRACTED FROM INDIVIDUAL SAMPLES

The amounts of proteins extracted from a single tissue section unpredictably differ between individual samples because the tumor area and protein content, even in cases with the same tumor area, vary between individuals. This variation can eventually translate into errors in the quantitative MRM-MS assay. In addition, the extracted protein amounts may be insufficient for the MRM-MS assay because the number of slides required to obtain adequate initial protein amounts can be difficult to predict. Conversely, the excessive use of FFPE tissue slides can result in the unnecessary waste of samples. Thus, a guideline was needed for how many FFPE tissue sections from each sample were required to obtain the required amounts of extracted proteins prior to the sample preparation procedures.

Based on the assumption that the amount of extracted protein is proportional to the number of cells, we constructed a calibration curve showing the extracted protein amounts versus tumor cell counts using 30 FFPE slides representative of the 210 samples used in this study (Supplemental Figs. 2). The curve of extracted proteins versus tumor cell counts was linear (Fig. 2), with a regression coefficient of 0.991, showing that the quality of this calibration curve was sufficient to yield reliable results between cell counts and extracted proteins. In addition, all coefficient of variation values for the amounts of proteins that were extracted from the 3 consecutive FFPE tissue sections were $< 20\%$ in each individual (Supplemental Table 1), confirming that the relation between estimated protein amounts and cell counts in a tumor area was proportional. After counting the total number of cells in tumor areas of 210 individual samples using Aperio *(28)*, we converted the estimated cell counts into protein amounts per the equation ($Y = 0.0002X + 5.4989$) generated by the calibration curve. The number of FFPE tissue sections

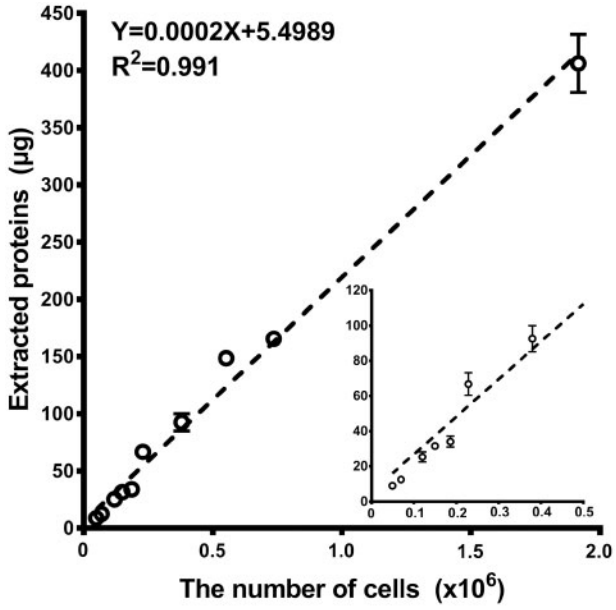

Fig. 2. Calibration curve, constructed from plotting the extracted protein amount against the total cell count in the tumor area. A total of 30 formalin-fixed paraffin-embedded (FFPE) slides from 10 individual samples were used to construct the calibration curve. Error bars represent SD values for the extracted proteins from the 3 consecutive tissue sections. The smaller curve in the inset is a magnification of the low-concentration region.

necessary to obtain a minimum of 150 μg of proteins was determined to prepare samples for MRM-MS assay, and the required amount of protein was obtained from 87.6% of samples (Supplemental Table 2). A detailed description of the generation of the calibration curve of extracted proteins versus tumor cell counts can be found in the Supplemental Data.

TARGET CANDIDATE SELECTION AND MRM-MS ASSAY DEVELOPMENT

To determine the best estimation of HER2 expression levels, 46 epithelial cell-specific proteins and housekeeping proteins were identified as potential candidates for the normalization of the amount of HER2 protein that was determined in each analysis by MRM-MS, based on data-mining from previous reports and public databases *(24, 29)* (Supplemental Table 3). A total of 37 proteins (62 tryptic peptides) among the initial 46 proteins and HER2 protein were reproducibly detected using the semiquantitative MRM-MS assay. Subsequently, the final 55 surrogate peptides were confirmed to have interference-free transitions *(30)* (Supplemental Table 4).

Reverse calibration curves of the surrogate peptides of HER2 and junctional adhesion molecule 1 (JAM1) were generated for an FFPE tissue sample to confirm the suitability of the peptides for the MRM-MS assay (Supplemental Fig. 3, Supplemental Table 5). The calibration points shown in Supplemental Fig. 3 were adjusted based on the purity of the unpurified SIS peptides (Supplemental Fig. 4). To validate the analytical method, the stability and reproducibility of surrogate peptides of HER2 and JAM1 were evaluated according to Clinical Proteomic Tumor Analysis Consortium guidelines *(31)* (Supplemental Figs 5 and 6, Supplemental Tables 6 and 7). A detailed description of the process of target candidate selection can be found in the Supplemental Data.

MEASUREMENT OF SURROGATE PEPTIDES FOR ESTIMATION OF HER2 EXPRESSION LEVELS

The workflow for determining HER2 status by the MRM-MS assay is depicted in Fig. 1. The reviewed hematoxylin and eosin images, which are marked with bold lines that delineate the tumor area, were used to estimate the total cell count for the tumor area using the nuclear counting algorithm in Aperio *(28)*. The number of slides necessary to extract a minimum of 150 μg of proteins per sample was calculated from the calibration curve in Fig. 2. A total of 55 surrogate peptides–6 HER2 peptides and 49 candidate peptides for normalization (19 surrogate peptides from 10 epithelial cell-specific proteins

and 30 surrogate peptides from 20 housekeeping proteins)–were quantified by MRM-MS analysis to determine HER2 status. The entire list of proteins and surrogate peptide sequences for the MRM-MS assay is detailed in Supplemental Table 4. By Spearman rank correlation analysis, all 6 HER2 surrogate peptides correlated positively and significantly with each other (Supplemental Fig. 7).

AGREEMENT BETWEEN MRM-MS DATA AND IHC/FISH DATA

Five categories of normalization factors for the normalization of the light-to-heavy peptide PARs for HER2 surrogate peptides were measured and compared: tumor area (μm^2), total cell count, total protein amount (μg), total peptide amount (μg), and light-to-heavy peptide PARs for the surrogate peptides of 10 epithelial cell-specific proteins and 20 housekeeping proteins from the MRM-MS assay. This generated 5 types of normalized quantitative data for HER2 surrogate peptides, which were then compared with IHC and FISH data on 210 individual samples. The light-to-heavy peptide PARs for HER2 surrogate peptides in 210 samples before and after normalization and the measured values of the normalization factors are shown in Supplemental Tables 8 and 9.

To select the normalization factor that best represents the number of tumor cells, which in turn determines HER2 expression levels used to discriminate between equivocal HER2 subgroups, area under the receiver operating curve (AUROC) values were calculated using each normalized value of 120 HER2 2+ samples (Supplemental Table 9). A total of 318 AUROC values were generated when considering 53 normalization factors and 6 HER2 surrogate peptides (Supplemental Table 10). When the 318 combinations were arranged according to decreasing AUROC values, the combination between the light-to-heavy peptide PAR for the HER2 surrogate peptide (VLQGLPR) and that for the JAM1 surrogate peptide (VTFLPTGITFK) showed the highest AUROC value of 0.908 (95% confidence interval [CI], 0.842–0.953), followed by AUROC values of 0.810 (95% CI, 0.728–0.875) for total cell count, 0.802 (95% CI, 0.719–0.869) for total peptide amount (μg), 0.777 (95% CI, 0.692–0.848) for total protein amount (μg), and 0.771 (95% CI, 0.685–0.842) for tumor area (μm^2) (Fig. 3).

Additionally, a single HER2 surrogate peptide (VLQGLPR) was superior to the average of HER2 surrogate peptides when discerning equivocal HER2 subgroups (Supplemental Fig. 8). Single- and multi-marker analyses using logistic regression were performed to determine the best predictive model *(32)* (Supplemental Table 11). The AUROC values for the best predictive models defined by both single- and multi-marker analyses, which were 0.891 and 0.899, respectively, were not significantly different ($P = 0.8432$,

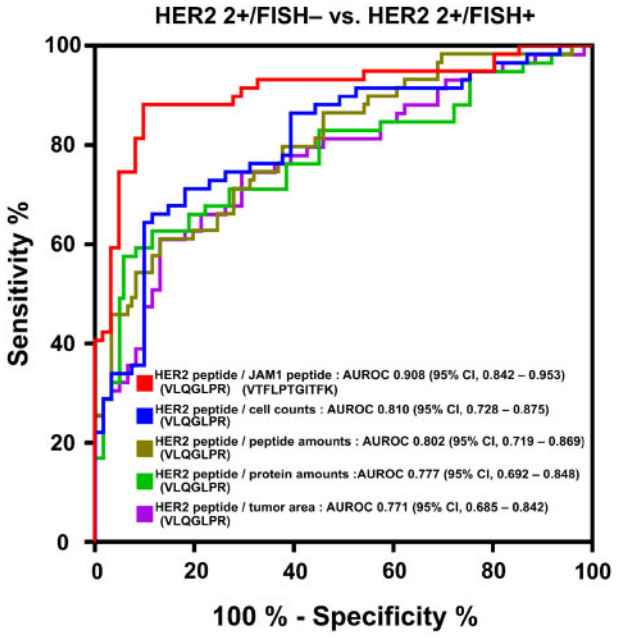

Fig. 3. Area under the receiver operating curve (AUROC) with respect to normalized quantified data on HER2 peptide in HER2 2+/FISH-negative versus HER2 2+/FISH-positive. Five types of normalized quantitative data for HER2 surrogate peptides were used to generate the AUROC curve.

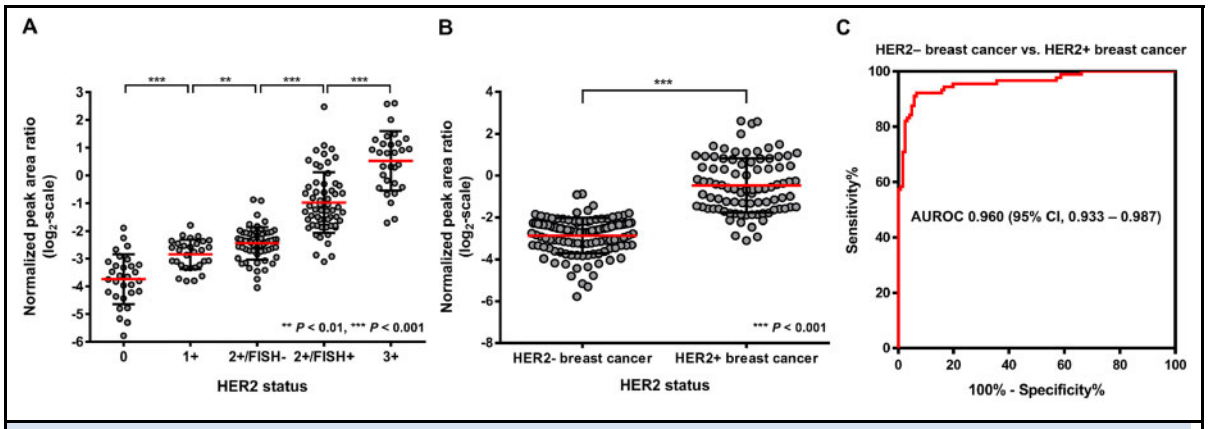

Fig. 4. Scatter dot plots and an area under the receiver operating curve (AUROC) of the light-to-heavy peptide peak area ratios for a HER2 surrogate peptide normalized by those for a JAM1 surrogate peptide plotted against IHC/FISH score. In scatter dot plots a, b) and an AUROC curve c), the light-to-heavy peptide peak area ratios for the HER2 surrogate peptide (VLQGLPR), normalized by those for the JAM1 surrogate peptide (VTFLPTGITFK), with the highest AUROC value used to determine whether the data generated by MRM-MS assay followed the tendencies in the IHC and FISH data. Top bars: Mann-Whitney U test; $**P < 0.01$; $***P < 0.001$.

DeLong test). A detailed description of the correlation between MRM-MS data and IHC/FISH data can be found in the Supplemental Data.

To determine whether the data that were generated by MRM-MS matched well with the IHC and FISH scores, the light-to-heavy peptide PARs for the HER2 surrogate peptide (VLQGLPR) that was normalized by those for the JAM1 surrogate peptide (VTFLPTGITFK) in 210 samples were examined (Supplemental Table 9). By Mann–Whitney U test, significant differences were found in 5 HER2 groups, and particularly the MRM-MS data distinguished between HER2 2+/FISH-negative and HER2 2+/FISH-positive groups ($P < 0.001$), which could not be differentiated by IHC (Fig. 4A). The MRM-MS assay also distinguished HER2-negative from HER2-positive breast cancer, which would be expected to benefit from HER2-targeted therapy ($P < 0.001$) (Fig. 4B). An AUROC analysis was conducted to further assess the ability of the MRM-MS assay to distinguish between HER2-negative and HER2-positive breast cancer. An optimal cutoff value of 0.2635 ($\log_2$-scaled normalized PAR $= -1.9241$) was defined as the value that provided the highest levels of clinical sensitivity and specificity, as evidenced by the proximity of this value to the top left corner of the curve, correlating with an AUROC value of 0.960 (95% CI, 0.933–0.987) (Fig. 4C).

Discussion

In this study, we adopted MS-based targeted proteomics, which has become the preferred method for biomarker studies of various human samples due to its high analytical sensitivity, reproducibility, accuracy, and precision *(33)*, by complementing the limitations of conventional techniques for determining HER2 status: semiquantitative scores, high interobserver variability, and extra labor required by additional staining to arbitrate equivocal HER2 cases *(34, 35)*. This report details the development of a clinical MRM-MS assay with FFPE breast cancer tissues that can overcome the aforementioned limitations of conventional methods by stratifying HER2 status more simply and precisely.

Two previous representative studies aimed to determine the HER2 status more accurately with FFPE tissues using a targeted MS-based approach *(21, 36)*, and our study complements these studies. A more recent study analyzed 40 individual samples, which was a substantially smaller cohort than ours. Notably, the creation of an aptamer-peptide probe entailed in the previous study is complex and laborious *(36)*. The complexity of the sample preparation procedures described in both studies renders them impractical for use as clinical assays. In contrast, rather than controlling the total protein and peptide amounts within the workflow by directly measuring these components, we simplified the workflow by estimating the number of slides upfront that would be required to ensure a sufficient amount of extracted protein for each analysis and by using the expression levels of an epithelial cell-specific protein as a normalization factor for measuring HER2 expression levels. As such, our method has the potential to save time and costs as part of an overall workflow in determining

HER2 status, which may accelerate the clinical adoption of similar MRM-MS assays.

We compared 5 categories of normalization factors to select the most suitable alternative to conventional normalization methods in sample preparation *(22, 23)*. Consistent with our hypothesis, the light-to-heavy peptide PAR for a surrogate peptide of JAM1, a breast epithelial cell protein, was found to be the most suitable normalization factor for measuring HER2 expression levels, followed by total cell count, total peptide amount (μg), total protein amount (μg), and tumor area (μm^2) (Fig. 3, Supplemental Table 10). Tumor area had the worst performance because it merely provided indirect values of tumor content. Specifically, larger tumor sizes do not necessarily represent greater tumor content, because tumor cell densities differ substantially between samples. Similarly, aside from JAM1, all examined normalization factors reflect both tumor cells and nontumor cells (macrophages, fibroblasts, and lymphocytes), which likely lower their normalization performances.

Two notable aspects of our study differentiate it from earlier efforts to quantify HER2 expression levels by mass spectrometry: (1) simplifying the overall workflow by predicting the protein amounts that can be extracted from each FFPE specimen and by using an epithelial cell-specific protein as a normalization factor for quantifying HER2 expression levels *(24)*; and (2) potentially reducing the number of equivocal HER2 cases, which account for 18% of all newly diagnosed breast cancers *(37)*. As a result of the superior accuracy of MRM-MS assay relative to IHC reducing the number of equivocal cases requiring FISH assessment, we estimate that our MRM-MS/FISH workflow would reduce the mean analytical time by 1 hour and reduce the mean cost per analysis 3.5-fold relative to an IHC/FISH workflow *(37–39)* (Supplemental Table 12). When using normalized HER2 quantitative data, 106 (88.33%) of 120 equivocal HER2 cases were correctly classified (Supplemental Tables 13 and 14).

However, several challenges remain to be addressed. To implement our developed MRM-MS assay in clinical practice, an absolute cutoff value for normalized HER2 quantitative data should be established by using purified SIS peptides in a future study. In addition, variable cell counts and the poor correlation observed between cell counts and extracted protein amounts across all 210 samples were attributed to variations in tumor sizes and cell densities in FFPE tissues and the imprecision of the manual scraping procedure, respectively. Thus, compared with the calibration curve in Fig. 2, the correlation between the cell counts and extracted protein amounts was lower in 210 samples because the proteins were extracted from multiple slides, which further compounded variations in extracted protein amounts. However, we do not believe that this poor correlation adversely affected the sample preparation procedure. The required amount of protein (>150 μg) was obtained from most of the samples, using the linear

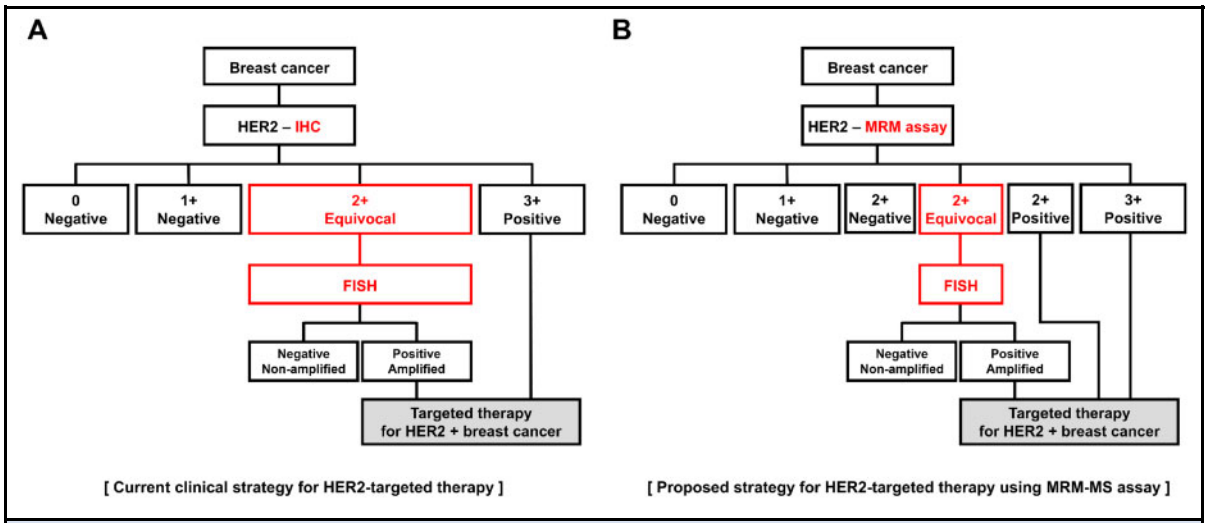

Fig. 5. Conventional diagnostic strategy using immunohistochemistry (IHC) and the proposed strategy using MRM-MS assay for HER2-targeted therapy. a) The conventional diagnostic strategy for HER2 scoring entails performing IHC in all cases, supplemented by fluorescence in situ hybridization (FISH) in equivocal IHC cases (HER2 2+). b) The novel application of MRM-MS assay to breast cancer patients discriminates between equivocal HER2 subgroups (HER2 2+/FISH-negative and HER2 2+/FISH-positive), reducing the number of cases that require ancillary FISH tests.

regression equation in Fig. 2 (Supplemental Table 2). In addition, variations in the extracted protein amounts across samples were likely reduced by determining the number of FFPE slides to use for each sample, based on the equation in Fig. 2, which provides a clear guideline for sample use, as opposed to previous subjective decisions. A possible solution is to increase the precision in excising the tumor area using laser-capture microdissection, which would likely improve the correlation between the extracted protein amounts and the cell counts by limiting the obtained tissue to the exact tumor area and also reduce the required labor costs compared with manually scraping the FFPE slides with a scalpel *(40)*.

In summary, our MRM-MS assay, which distinguishes between equivocal HER2 subgroups, can potentially decrease the time and costs required for the diagnosis of breast cancer patients by reducing the number of cases that require ancillary FISH tests (Fig. 5). In addition, the simplified assay procedure can reduce the barriers to entry for the clinical application of the MRM-MS assay. The proposed protocol would provide clinicians with valuable diagnostic information and facilitate the proper treatment for breast cancer patients.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations HER2, human epidermal growth factor receptor 2; FISH, fluorescence in situ hybridization; MRM-MS, multiple reaction monitoring-mass spectrometry; FFPE, formalin-fixed paraffin-embedded; JAM1, junctional adhesion molecule A; IHC, immunohistochemistry; SIS, stable isotope-labeled internal standard; PAR, peak area ratio; LC, liquid chromatography; AUROC, area under the receiver operating curve; CI, confidence interval

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