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Determination of the *Her-2/neu* gene amplification status in cytologic breast cancer specimens using automated silver enhanced in situ hybridization (SISH)

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**Running Title:** *Her-2 SISH in breast cancer cytology*

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Abstract

Silver enhanced in situ hybridization (SISH) is an emerging tool for the determination of the Her-2/neu amplification status in breast cancer. SISH is technically comparable to fluorescence in situ hybridization (FISH) but does not require a fluorescence microscope for its interpretation. While recent studies on histological evaluations of SISH are promising, we aimed to evaluate its performance on 71 cytologic breast cancer specimens with the new combined Her-2/Chr.17 probe. Her-2/neu status as routinely determined by FISH was available for all patients. We found SISH signals in cytologic cell blocks and smear specimen easy to evaluate in most cases. Small numbers of tumor cells and difficulties in identifying tumor cells in lymphocyte-rich backgrounds were limiting factors. Her-2/neu status, as determined by Her-2/Chr17 SISH, was basically identical to the results of the corresponding FISH. The discrepancies were mainly due to the heterogeneity of Her-2/neu amplification in the tumor tissue. Inter-observer agreement for the SISH evaluation was high (kappa value: 0.972).

We conclude that Her-2/Chr17 SISH is a useful and accurate method for the evaluation of the Her-2/neu gene amplification status in cytologic breast cancer specimens, particularly in metastatic breast cancer lesions. The advantages of signal permanency and bright field microscopic result interpretation make this technique an attractive alternative to the current FISH-based gold standard.

Key words: Her-2; SISH; FISH; breast cancer; cytology.
Introduction

Silver enhanced in situ hybridization (SISH) is a recently introduced new bright-field in situ-hybridization technique for the detection of DNA-signals, mainly used for cancer genotyping. The technique is based on enzymatic metallography and metallic silver deposition (7). Resulting silver complex deposits are stable over time and furthermore allow surgical pathologist to assess the signal with conventional bright-field microscopes instead of fluorescence microscopes necessary for fluorescence in situ hybridization (FISH). Until now, the SISH technique has mainly been promoted for the assessment of the Her-2/neu amplification status in breast cancer patients. The most recent SISH version allows for a combined detection of Her-2/neu and chromosome 17 including a chromogenic detection for the latter one.

The identification of invasive breast carcinomas with amplification of the human epidermal growth receptor 2 gene (ERBB2 or Her-2/neu) has, like the assessment of the hormone receptor status, become an essential standard procedure for the assessment of newly diagnosed breast cancers (6, 8). This is mainly due to the availability of effective treatment of tumors with over-expression of Her-2 with humanized anti-Her-2 antibodies. Over-expression of Her-2/neu is found in approximately 15% of breast cancers and it is only in these patients that the above mentioned therapy is beneficial and cost-effective (1, 5, 11, 15). The additional costs for an anti-Her-2 antibody therapy have been estimated between 40,000 and 50,000 US$ (equaling 25,000-35,000 €) (10). Therefore a high reliability and robustness in the assessment of the Her-2/neu amplification status is mandatory.
Fine needle aspirations (FNA) or body cavity fluids containing breast cancer cells are common in cytological routine work. The role of FNA in the diagnosis of metastatic or disseminated disease is undisputed, while the diagnosis of the primary tumor is more controversial, being well established only in few specialized centers (12, 16). Requests for testing of Her-2/neu amplification status on cytological specimens, mainly in the setting of a metastatic disease, have been increasing constantly over the last few years.

According to the last ASCO Guidelines, two different ways of testing are commonly used to determine the Her-2 status in surgical pathology specimens (19). They comprise the detection of the Her-2 protein and/or of the Her-2/neu gene amplification status by immunohistochemistry or by fluorescence, chromogenic or silver labeled in situ hybridization methods (FISH, CISH, SISH). Due to insufficiencies of the immunohistochemistry in terms of inter-observer and inter-laboratory variability, the in situ hybridization method, particularly the FISH analysis is currently regarded as the gold standard (17). However, the need for special microscopic equipment and the rather quick fading of fluorochromes over time are relevant disadvantages of the FISH technique.

In this study, we analyzed the usability and reliability of the SISH technique for the determination of the Her-2/neu amplification status in different cytologic breast cancer specimens in comparison to corresponding FISH results.
Material and Methods

Patients and samples

Seventy-one patients diagnosed with breast cancer at the Division of Cytology of the Institute of Surgical Pathology of the University Hospital Zurich, with known Her-2 FISH status, were included in this study. Twenty-three of the specimens originated from 2006 to 2008 and were selected due to their amplified Her-2/neu status in FISH. The remaining 48 specimens were from consecutive patients in 2009. The study was approved by the project review board at our institution and no further ethical approvals were necessary. The median patient age was 62 years (range 29 to 86 years). The cytological samples consisted of 34 (47.9%) FNAs of primary breast tumors, 25 (35.2%) FNAs of metastases (22 lymph nodes and 3 soft tissue masses) and 11 (15.5%) body cavity fluids (9 pleural effusions and 2 abdominal effusions) as well as 1 mammary secretion. For 64 patients histological data on the primary tumor were available: Fifty-eight (90.6%) had invasive ductal carcinomas, four (6.3%) invasive lobular carcinomas and two (3.1%) carcinomas of other histological types. The pT tumor status was pT1: 22 cases (34.4%), pT2: 30 (46.9%), pT3: 8 (12.5%) and pT4: 4 (6.3%) respectively. The histological tumor grade was G1: 2 (3.1%), G2: 18 (28.1%) and G3: 42 (65.6%). For two cases no grading was given due to too small amounts of tumor in the core biopsy specimens. Results of the Her-2/neu FISH analysis were available for all patients. In 47 (66.2%) cases FISH and SISH analyses were performed on biopsies and/or FNAs of exactly the same primary (36 out of 47 cases) or metastatic (6 out of 47 cases) tumor nodules or fluid specimen (5 out of 47 cases). The combinations of specimen preparations for these 47 cases were
as follows: 19 (40.4%) cases: SISH on cell block and FISH on histologic specimen; 11 (23.4%) cases: SISH on smear and FISH on histologic specimen; 16 (34.0%) cases: SISH and FISH on cell block; 1 (2.1%) case: SISH on smear and FISH on cell block.

In the remaining 24 cases the SISH was mostly (79.2%) performed on metastatic deposits while the FISH was done on the primary tumor. In four cases SISH and FISH were performed on different metastatic deposits and in one case the SISH was done on the primary tumor and the FISH on the metastasis. The combinations of specimen preparations for these 24 cases were as follows: 12 (50.0%) cases: SISH on cell block and FISH on histologic specimen; 11 (45.8%) cases: SISH on smear and FISH on histologic specimen; 1 (4.2%) case: SISH and FISH on cell block.

The median time lag between the specimen taken for the FISH and the one taken for SISH analysis was one week. Only in four cases was this time lag more than 8 weeks.

Silver enhanced in situ hybridization (SISH)

SISH was performed according to the manufacturers’ protocol either on paraffin slides from the cytology cell blocks or on Papanicolaou stained direct smears. Cytologic cell blocks were prepared by coagulating the FNA/fluid cell pellets with a mixture of plasma and thrombin and further standard processing of the clot to paraffin blocks. INFORM HER-2 DNA probe was detected with ultraView SISH Detection Kit and INFORM Chromosome 17 probe with the ultraView AP red SISH Detection Kit (780-4332 &780-4331, Ventana Medical Systems, Tucson, AZ, USA). The whole process was completely automated
using Ventana’s Benchmark autostainers. The protocol of the SISH staining has been described before (7). The specimens were briefly counterstained with Ventana Hematoxilin II.

**Fluorescence in situ hybridization (FISH)**

Deparaffinizing, pre-treatment and protease digestion procedures followed the ABBOTT/Vysis PathVysion Her-2 DNA Probe Kit protocol (VP-200 program). Probe mixes were hybridized at 37°C between 14 and 20h, washed in Rapid-Wash-Solution I at 73°C for 5min, Rapid-Wash-Solution II and H2O for 7min, air dried and counterstained with DAPI. A minimum of 20 nuclei of invasive tumor cells were scored using a Olympus BX61 microscope with fluorescence equipment (Olympus Cooperation, Tokyo, Japan).

**SISH scoring**

The SISH signals were scored by counting the signals for Her-2 (black) and chromosome 17 (red) according to the manufacturers recommendation in 20 cancer cell nuclei. The Her-2 signals were divided by the chromosome 17 signals. According to the manufacturers manual the threshold for amplification was a value larger than 2.2. Results below 1.8 were considered non-amplified and those between both values equivocal. The evaluations were done independently by two pathologists (FRF and PKB) on conventional Zeiss Axioskope 40 Microscopes with 400x magnification. (Carl Zeiss MicroImaging, Jena, Germany). Both pathologists were blinded to clinicopathologic parameters, previous FISH results and the SISH results of each other.
**Statistical analysis**

Statistical analysis was performed using SPSS, version 18.0. P values < 0.05 were considered significant.
Results

SISH results

Altogether, 50 Her-2 SISH results were obtained from cytologic cell blocks and 23 from direct smears. For two patients the analysis was done on the cell block as well as on the cytologic smear. In these two patients the SISH results were identical on both media for both evaluating pathologists and also consistent with the FISH results.

The SISH signals were easily detectable in almost all cases (Figure 1) and both red and black signals were unchanged in the intensity after 7 months. In comparison to the black Her-2 signals the red chromosome 17 signals appeared generally stronger and bigger. In terms of Her-2/neu amplification status in the SISH analyses, 39 (54.9%) cases were considered non-amplified, 31 (43.7%) were amplified and one (1.4%) was considered equivocal.

Comparison of Her-2 SISH and FISH results (Table 1)

From the 39 cases that were considered non-amplified in the SISH analysis 36 (92.3%) were also non-amplified in the FISH analysis.

Three cases (7.7%) were considered amplified in the FISH analysis. One of the discrepant SISH analyses was applied on a cell block, the other two on cytologic smears. All three samples corresponded to fine needle aspirations of the breast and corresponding FISH was performed on the histologic sample of the same tumor nodule. On review of these cases, in two cases the FISH analysis showed only heterogeneous Her-2/neu amplification with several clearly non-amplified tumor cells. On review of the corresponding SISH
analyses in one of the cases very few definitely amplified cells could be detected. The third case was unequivocally amplified in the histological FISH analysis. However, the smear showed prominent air drying artifacts which were already noted during evaluation and it additionally included cells with polysomy. Nevertheless, also in the informed review no amplified cells could be detected in the SISH in this case.

From the 31 cases that were amplified in the SISH, 30 (96.8%) were also considered amplified in the FISH with one being considered equivocal. The equivocal FISH (histologic sample of the same tumor nodule) was commented to be considered amplified in the report due to heterogeneity.

One case was considered equivocal in the SISH while it was considered amplified in the FISH analysis (both on cell blocks of the same tumor nodule). The repeat of the FISH result showed definitely amplified cells but was also rather heterogeneous.

The inter-observer consistency between the two pathologists independently evaluating the Her-2 SISH was excellent with a kappa value of 0.972. The only discrepant case was considered amplified by one and non-amplified by the other pathologist. The FISH analysis in this case was considered amplified. In the revision of the case on a multiheaded microscope consensus in terms of amplification was reached. However, both SISH and FISH were only just above the positivity limit for this case.
Discussion

In the present study, the \textit{Her-2/neu} amplification status of a series of breast cancer cases was examined in cytology specimens by the recently introduced SISH technique. Results of our study showed high interobserver reproducibility in SISH signal interpretation between two cytologists and a high concordance with FISH results.

Despite being more expensive, the in situ hybridization techniques are considered to be advantageous in terms of reliability for the determination of the predictively important Her-2 status in breast cancer patients in comparison to immunohistochemistry (17). The costs of SISH are comparable to that of FISH. On the Ventana platform, the SISH is completely automated, which ensures consistency and reproducibility of methodology and results. SISH results were available in up to six hours. The major advantage of Her-2 SISH over the current standard FISH is the stability of the signal over time. Furthermore, unlike with FISH, conventional bright-field microscopes are sufficient for evaluation, which is clearly an advantage over time-consuming FISH evaluation sessions.

The first evaluation of Her-2 SISH was performed by Dietel \textit{et al.} on breast cancer tissue samples (7). In the meantime several other groups were able to validate these results on breast cancer tissue (2-4, 9, 18). Like Dietel and colleagues, they found a good reproducibility of the results and high concordance with immunohistochemistry, polymerase chain reaction and with other in situ techniques (including FISH). Our results are strongly consistent with those of the research groups mentioned in terms of reliability and inter-observer consistency. In line with these studies we experienced only a few
equivocal cases. This may be due to the fact that about one third of the cases were selected on the basis of their known positivity.

A general matter of concern regarding the SISH technology could be that in contrast to FISH results, which were often analyzed by one or a small group of people in an institution, SISH signals would theoretically be readable by any surgical pathologist during routine work. This restriction implied in the FISH analysis, which is also recommended by national and international breast cancer working groups, is advantageous in terms of inter-observer consistency. Therefore, although the consistency in our study was high, specialization and division of work, as it is common practice in most breast centers, should also be applied for SISH analyses.

In contrast to previous studies, our study assessed the usability of SISH in cytologic specimens. The use of hormone receptor analyses as well as other immunohistochemical stains on cytologic specimen has already been demonstrated long ago (13). Recently, Nassar et al. evaluated new monoclonal antibodies against hormone receptors and Her-2 on cell blocks (14). Our results demonstrate for the first time that Her-2/neu amplification status assessments with SISH can be performed with high reliability on cytologic specimens. The rare discrepancies were mostly due to the intratumoral heterogeneity among the cells, an inherent tumor characteristic.

Many specimens in most modern cytologic laboratories are nowadays preferentially prepared and evaluated simultaneously on both direct smears and cell blocks, allowing for the analysis of different aspects of the examined
tissue. However, the examiner interpreting study results must not forget the inherent important biological differences between three dimensional direct smears of intact cells and two dimensional cuts of the tissue. During cutting of the specimen for histological processing, substantial parts of cells are discarded and will not be appreciated upon later microscopic analysis. This truncation effect must be taken into account in the quantitative studies pertaining to the content of the nucleus, such as counting signals of an in situ hybridization reaction. The consideration of the cell geometry plays an important role in evaluating chromosome polysomy, as shown by Bubendorf for EGFR FISH, where the cut off values established for histology specimen must not be applied when interpreting signals on cytology specimens from non small cell lung cancer (20). The effect of the sample type (smear vs. paraffin cut) on evaluating gene amplification is less pronounced and could not be observed in our study group.

An important biological point to be addressed is the inability to distinguish invasive carcinoma cells from in situ carcinoma when interpreting signals on cytology specimens from primary tumors. Therefore careful selection of the samples which are suitable for submission to SISH is crucial to the optimization of results. No such problem exists for metastatic lesions, as pleural effusions or lymph node metastases.

The major practical limitation of SISH technique on cytological specimen was the presence of too few tumor cells on some scarcely cellular samples and the fact that some cells on the smear might also get lost during processing. Another difficulty faced in this study was reliable distinction of cancer cells from non-malignant epithelial cells and lymphocytes in faintly counterstained
SISH specimens. The comparison with the hematoxylin/eosin or Papanicolaou stain was very helpful in these cases. Air drying and other artifacts (which are more common in cytologic than histologic samples) can also hinder correct SISH evaluation. It was also notable that the cases with discrepantly non-amplified SISH results were at least one year old or older and consisted of direct smears and not cell block specimens. This fact suggests a diminished sensitivity of the SISH after longer storage times of direct smears and confirms the value of preserving parts of cytologic samples in the form of cell blocks for later ancillary studies. However, our review of these cases concludes that heterogeneity of the tumor in terms of Her-2/neu gene amplification is the main factor leading to discrepancies, especially when analyzing small amounts of cancer cells..

We conclude that the determination of the Her-2/neu gene amplification status in cytologic breast cancer specimens using automated silver enhanced in situ hybridization (SISH) is a reliable and consistent technique, well suited for routine application in surgical pathology, if the evaluation of Her-2 status is indicated.

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Figure legend

Figure 1 SISH signals in cytologic breast cancer specimens. Cell block (A/B) and cytologic smear specimens (C/D) without (A/C) and with (B/D) amplification of the Her-2/neu gene status. The red chromosome 17 signals appear larger than the black Her-2 signals as best seen in the non-amplified probes. In the amplified samples the Her-2 signals dominate the picture with multiple accumulated intranuclear black signals (Original magnification 650x). Insets show the corresponding FISH.

A – Cell block of a pleural effusion in a patient (80 year old) with a history of breast cancer. Both SISH and FISH were performed on the same specimens.

B – SISH on the cell block of the fine needle biopsy of the breast tumor in a 67 year old patient. FISH was performed on the tumor which was resected one week later.

C and D – In both cases SISH on the direct smear of the FNA of the lymph node metastasis. FISH was in both cases performed on the core biopsy of the breast tumor taken at the same time. C: 45 year old patient and D: 69 year old patient.