

Detection of *HER2* Amplification Using the SISH Technique in Breast, Colon, Prostate, Lung and Ovarian Carcinoma

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Abstract. *HER2* gene amplification was explored using the silver stain hybridization *in situ* (SISH) technique in colon, prostate, lung, ovarian and breast carcinomas. Clinical pathological features and immunohistochemical (IHC) expression were evaluated for *HER2* in 225 carcinomas. All cases were subjected to SISH investigation. Statistical analysis revealed an association between *HER2* protein expression and gene amplification in breast carcinoma. 14% of colon carcinomas (5 IHC score 0, 1 score 1+ and 1 score 2+), 2% of prostate carcinoma (IHC 2+), 4% of lung carcinomas (IHC 2+) and 16% ovarian carcinomas (IHC 3+) revealed gene amplification. SISH is an advantageous technique for the detection of gene amplification. The use of the SISH technique in breast carcinoma may be an alternative to other *in situ* hybridization (ISH) techniques however more detailed studies seem necessary to detect *HER2* gene amplification in other human malignancies.

The search for reliable new prognostic markers in human malignancies is imperative when they can be associated with target therapies. One of the best examples of this association is the expression of *HER2* receptor in breast tumours in order to utilize monoclonal antibody trastuzumab (1, 2). To date, *HER2* expression in the cell membrane has been studied thoroughly in breast carcinomas and has dramatically modified the understanding of this neoplasm in terms of disease-free and overall survival (3-6).

The *HER2* receptor and other members of the same family, such as EGFR, are now being evaluated in other human neoplasms in order to reveal similar effects of

trastuzumab in non-breast malignancies. *HER2* differs from other members of the same family as it appears to be a preferential partner for receptorial dimerization (7), an event necessary for signal intracellular cascade transduction after ligand-receptor interaction (8). The described dimerization affinity and the absence of a specific ligand (9) favours, with protein cellular overexpression, a spontaneous dimerization and, subsequently, a constitutive transmission of signalling (10). These facts form the basis of the oncogenic relevance of *HER2* and the resultant need to increase the understanding of its significance as a specific target therapy.

This study aimed to investigate *HER2* gene amplification in breast carcinomas as well as in colon, prostate, lung and ovarian tumours using the silver stain hybridization *in situ* technique (SISH). Gene status in these carcinomas has been associated with protein expression of *HER2* and with known specific prognosticators for each single tumour.

Patients and Methods

Patient specimens. Two hundred and twenty-five carcinomas (50 breast, 50 colon, 50 lung, 50 prostate and 25 ovarian carcinomas) were selected randomly from the files of the U.O.C. of Anatomic Pathology ASL Frosinone, Italy, between January 2005 and December 2008. Clinical information was obtained from the existing medical records. The clinical data collected included the patient's name, race, age, the family and patient's cancer history and type of surgery, post-operative treatment, data and site of eventual recurrence, and the patient's current status (alive or deceased). Ethical approval and informed consent were acquired from all eligible patients. Histopathological data included tumour size, histological subtype and grade, evidence of necrosis and stage of disease according to current TNM classification. Control specimens were obtained from patients who underwent surgery for non-neoplastic disease. Representative blocks of the tumour were chosen for immunohistochemical staining and for molecular biology investigations.

Immunohistochemical Procedures and evaluation of *HER2*, Ki-67, oestrogen and progesterone expression. The IHC procedure followed the data sheet protocol for automatized immunohistochemistry with BenchMark®XT Ventana Medical System

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Key Words: SISH, *HER2*, breast, colon, prostate, lung, ovary.

(Ventana). This study used the 4B5 clone for HER2, SP1 for oestrogen receptors, 1E2 for progesterone receptors and Ki-67 for proliferation index Ki-67.

SISH technique. Automated SISH of consecutive slides from the same paraffin blocks as for hematoxylin-eosin (H&E) were stained according to the manufacturer's protocols for the INFORM HER2 DNA and chromosome 17 probes. Both probes were labelled with dinitrophenol (DNP) and optimally formulated for use with the ultraView SISH Detection Kit and accessory reagents on Ventana's Benchmark®XT (Ventana Medical System, Inc., Tucson, AZ, USA) series of automated slide stainers. The HER2 DNA probe was denatured at 95°C for 4 or 8 min, for sections from surgical intervent and for section from biopsy, respectively, and hybridisation was performed at 52°C for 2 h. After hybridization, appropriate stringency washes (3 times at 72°C) were performed. The chromosome 17 probe was denatured at 95°C for 12 min and hybridization was performed at 44°C for 2 h. After hybridization, appropriate stringency washes (3 times at 59°C) were performed. The HER2 and chromosome 17 DNP-labelled probes were visualised using the rabbit anti-DNP primary antibody (Ventana) and the ultraView SISH Detection Kit. The detection kit contains a goat anti-rabbit antibody conjugated to horseradish peroxidase (HRP) utilised as the chromogenic enzyme. The chemistry of the SISH reaction, briefly described, is driven by the sequential addition of silver A (silver acetate), silver B (hydroquinone) and silver C (H₂O₂). Here, the silver ions (Ag⁺) are reduced by hydroquinone to metallic silver atoms (Ag). This reaction is fuelled by the substrate for HRP, hydrogen peroxide (silver C). The silver precipitation is deposited in the nuclei and a single copy of the HER2 gene is visualised as a black dot. The specimen is then counterstained with Ventana Hematoxilin II for interpretation by light microscopy.

Scoring of HER2, Ki-67, oestrogen and progesterone immunoreactivity. Immunohistochemistry evaluation of HER2 with 4B5 was undertaken using ASCO/CAP 2007 guidelines (11), integrated with the DAKO recommendation (12): each sample was placed into one of four categories (0, 1+, 2+, 3+). Tumours with complete absence of staining or with incomplete membranous staining in less than 10% neoplastic cells were scored as 0; those with weak, incomplete membranous staining in more than 10% neoplastic cells or with complete but inconspicuous membranous staining were classified as 1+; complete weak to moderate membranous staining in more than 10% of neoplastic cells or strong membranous staining in less than 30% were classified as 2+; strong and complete membranous staining in greater than 30% of the tumour cells were classified as 3+.

Oestrogen and progesterone immunoreactivity was considered positive when more than 10% of neoplastic cells revealed nuclear staining.

Proliferative index, evaluated with Ki-67, was classified as low (<10%), medium (from 10% to 25%) and high (>25%).

Two independent observers scored each slide, discrepancies were discussed, and a consensus score was provided.

Scoring of SISH gene amplification. HER2 gene amplification status was classified according to the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP 2007) guidelines (11), applying the following criteria in 20 cells for each case: 1. Negativity for HER2 gene amplification was defined as a HER2/Chr17 ratio less than 1.4; 2. Equivocal for HER2 gene

amplification was defined as a HER2/Chr17 ratio between 1.4 and 4; 3. Positivity for HER2 gene amplification was defined as a HER2/Chr17 ratio greater than 4.

For the equivocal cases, additional counts were made in another 20 to 80 cells for each case, with the following criteria: Negativity for HER2 gene amplification was defined as a HER2/Chr17 ratio less than 1.8; Equivocal for HER2 gene amplification was defined as a HER2/Chr17 ratio between 1.8 and 2.2; Positivity for HER2 gene amplification was defined as a HER2/Chr17 ratio greater than 2.2. Two independent observers scored each slide, discrepancies were discussed, and a consensus score provided.

Statistical analysis. Cut-off levels were determined according to positive and negative immunohistochemical and gene amplification expressions. All data were tested for association with clinico-pathological features using the chi-square test or the Fisher's exact test when appropriate. The primary statistical outcome was HER2 amplification and HER2 expression from morphological features. Univariate and multivariate analysis were conducted using the Cox regression model. All analyses were conducted using the StatXact 4 for Windows (Cytel Software Corporation, Cambridge, MA, USA).

Results

Breast carcinomas revealed HER2 overexpression with score 3+ in 9 out of 50 cases (18%) and with score 2+ in 14 cases (28%) (Figure 1). Twenty-seven cases were negative (score 1+ in 14 cases and score 0 in 13 cases).

All score 3+ cases and 2 out of the 14 score 2+ cases revealed gene amplification on SISH (22%). One other case was equivocal and no gene amplification was present when re-evaluated with fluorescence *in situ* hybridization (FISH). No case with score 1+ or 0 on immunohistochemistry revealed gene amplification. Statistical analysis between the two methods revealed a statistical association ($p=0.0001$). Moreover, statistical associations were revealed between gene amplification and negative receptor status ($p=0.0001$), poorly differentiated carcinomas ($p=0.0003$) and high proliferative index ($p=0.014$).

Colon cancer revealed no cases of HER2 score 3+; in 1 out of 5 (10%) 2+ cases, HER2 was amplified. Interestingly, in 6 out of 45 (13.3%) immunohistochemically negative cases (5 score 0, 1 score 1+), HER2 was amplified (Figure 2).

Prostate carcinoma revealed only 4 cases with score 2+; all the others revealed score 0 by means of immunohistochemistry. Among these 4 cases, one revealed gene amplification.

Lung carcinoma revealed 9 cases with score 2+ (8 adenocarcinomas and 1 squamous cell carcinoma). In two out of 8 adenocarcinomas, HER2 was amplified. No small cell carcinoma (out of 10) revealed immunohistochemical over-expression or gene amplification.

Finally the results revealed overexpression of HER2 in 4 ovarian epithelial surface carcinomas. (score 3+). All four cases revealed gene amplification ($p=0.0001$). However, no case was associated with neoplastic histotype or grade of differentiation. No other case revealed HER2 expression or gene amplification.

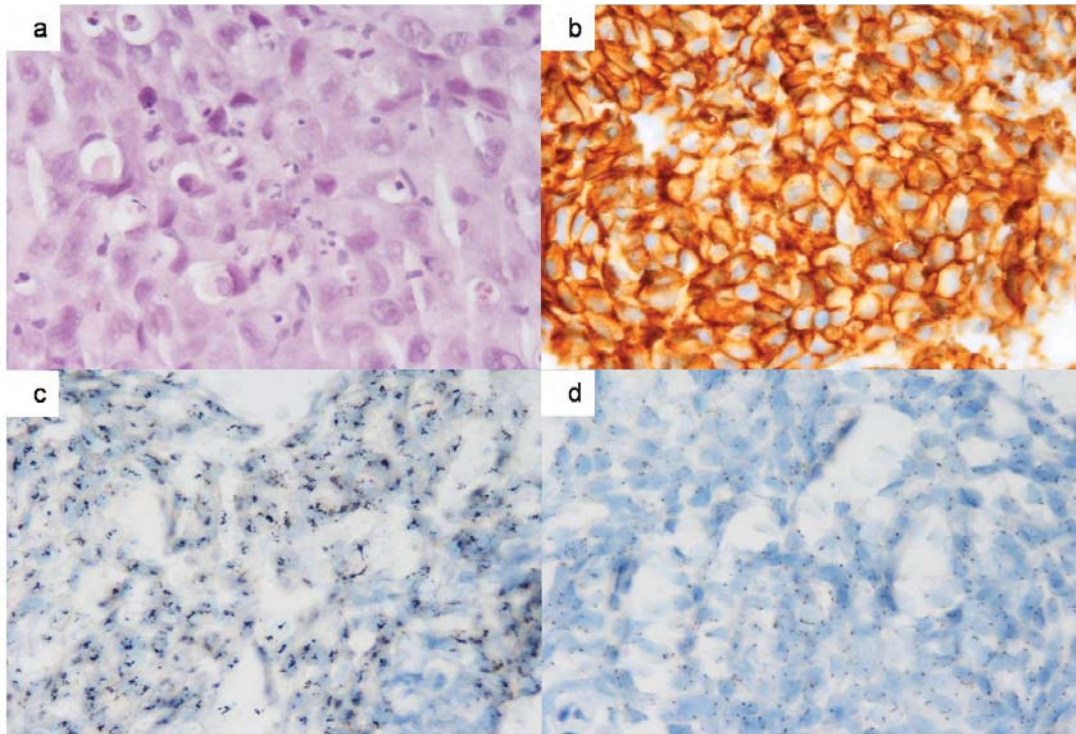


Figure 1. Poorly differentiated infiltrating duct breast carcinoma with *HER2* protein expression and gene amplification. a: H-E, $\times 400$; b: IHC, *HER2* score 3+, $\times 400$; 1c: SISH, *HER2*, $\times 200$; 1d: SISH, Ch17, $\times 200$.

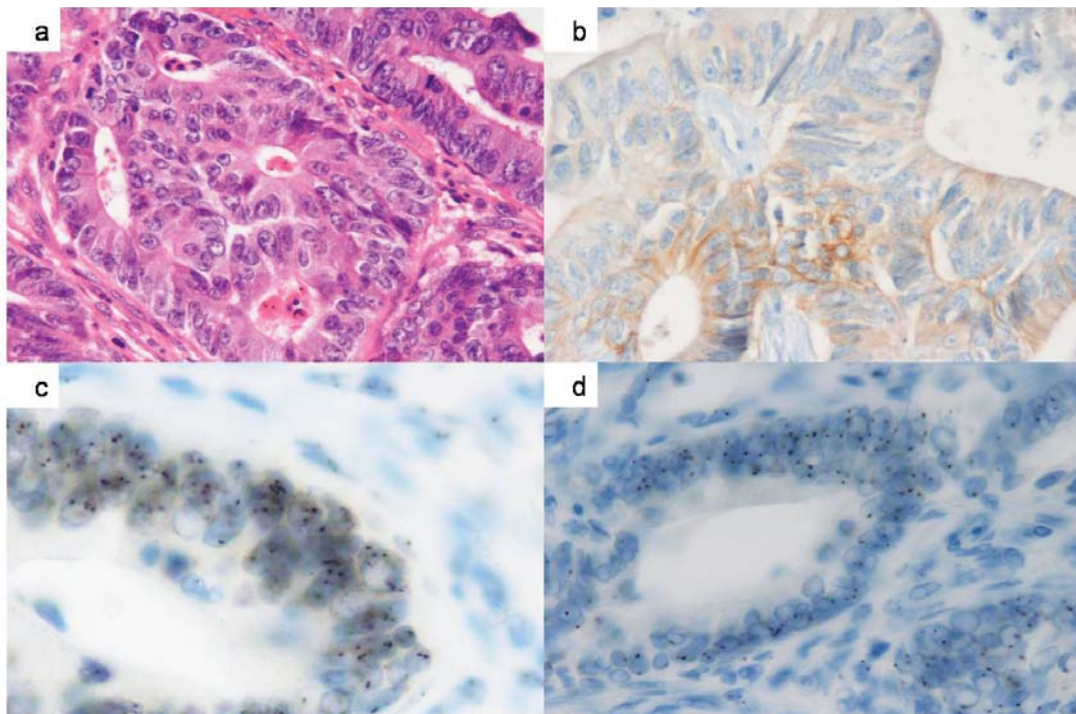


Figure 2. Moderately differentiated colon carcinoma without *HER2* protein expression and with gene amplification. a: H-E, $\times 400$; b: IHC, *HER2* score 1+, $\times 400$; 2c: SISH, *HER2*, $\times 600$; 2d: SISH, Ch17, $\times 400$.

Discussion

Surgery, radiotherapy and chemotherapy have improved the life quality and survival rates in many cancer cases, making the search for reliable prognostic markers of specific cancers imperative.

Targeted therapy with trastuzumab has dramatically changed the natural history of breast carcinomas in terms of clinical and prognostic outcome, since patients who overexpress HER2 or have its gene amplified may benefit from this approach (3-6).

The aim of this study was to investigate the expression and gene amplification of HER2 by means of immunohistochemistry and using a new technique, SISH. SISH is a chromogenic hybridization *in situ* technique using silver, and is used both in breast carcinomas and in other common human malignancies (colon, prostate, lung and ovary). The aim of this study was to assess the reliability of SISH as a new investigative technique and to determine whether different human tumours other than breast may benefit of target therapy with trastuzumab.

Breast carcinomas in this study revealed data in accordance with that already published in the literature in terms of percentage of HER2 overexpression and associations with other common prognostic markers (4, 13, 14). Moreover the SISH technique, like FISH in other studies (15, 16), has more accurately defined stage 2+ cases by categorizing them into coded groups (amplified vs. non-amplified), thus avoiding subjecting patients to unnecessarily toxic pharmacological therapy.

Statistical associations among HER2 and other prognostic indicators considered in this study corroborate the finding that HER2 may be used as an independent prognostic factor (17). Starting from this hypothesis it has been supposed to use SISH in immunohistochemistry negative scored cases in presence of high differentiation grade, high proliferation index and negativity for oestrogen/progesterone receptors.

Overexpression of EGFR and the KRAS mutation have been extensively studied in colon carcinomas (18, 19) while few data have associated HER2 status with discordant results (5 to 50% of overexpression) (20, 21).

Data from this study revealed a high discordance between HER2 overexpression and gene amplification (12% compared to 4% reported in literature for breast carcinomas). Moreover, these results revealed no association between gene amplification and morphological markers of tumoural aggressiveness (grade, stage, *etc.*).

In order to explain these data, the common mechanism of HER2 expression and the resistance mechanisms commonly reported in literature (PTEN, IGF-IR, p95HER2) (22-24) were considered but with no significant results.

It may be supposed, however, that a recently studied membranous mucin, MUC4, masks the antibody-binding epitope of HER2, leading to diminished trastuzumab binding (25, 26).

Published studies on prostate carcinoma reveal *HER2* amplification in 13% of cases (27) in advanced disease (28). The numbers of cases in this study are too few to draw firm conclusions; however, studies that include a larger numbers of cases and a stricter selection of patients, as reported in literature, may identify patients that are suitable for trastuzumab therapy.

Lung carcinomas have been studied extensively in terms of EGFR (29). Some recent papers however have revealed HER2 overexpression in 16% of lung adenocarcinomas (30). The presented study agrees with the findings in the literature, although a greater number and a better stratification of patients is necessary for firm conclusions to be drawn. Improved new therapies may be developed if the correlation between HER2 and other prognostic indicators such as EGFR is better understood. Finally, from this study it is hypothesized that biases due to cell crushing in small cell lung tumors may interfere with IHC and ISH techniques.

HER2 expression is a subject of current investigation in ovarian carcinomas but with conflicting results (31, 32). In all the cases in the present study, similar to breast carcinoma, HER2 overexpression was confirmed by gene amplification, suggesting that efforts to study HER2 in this malignancy should be continued. The discrepancy between *HER2* gene amplification and the immunohistochemical protein expression in colon carcinoma should be addressed in more detailed studies.

In conclusion, SISH is a reliable, inexpensive and time-efficient technique by which to detect *HER2* gene amplifications in human malignancies other than breast carcinoma.

In order to use the SISH technique, experienced pathologists should be trained to detect gene amplification correctly. More data and a larger number of cases associated with a better knowledge of oncogenic mechanisms for *HER2* amplification are necessary to properly identify patients to be submitted to this targeted pharmacological approach

Disclosure/Conflict of Interest

Ramieri Maria Teresa, Murari Riccardo, Botti Claudio, Pica Emanuela, Zotti Giancarlo and Alo Piero Luigi have no conflict of interest to declare.

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Received February 5, 2010

Revised March 31, 2010

Accepted March 31, 2010