Comparison of liquid based cytology and histology for the evaluation of HER-2 status using immunostaining and CISH in breast carcinoma

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Background: HER-2 amplification is an important prognostic biomarker and treatment determinant in breast carcinoma.

Aims: To correlate immunocytochemical (ICC) expression of HER-2 and gene amplification determined by chromogenic in situ hybridisation (CISH) using liquid based cytology (LBC) with immunohistochemistry (IHC) and CISH using histological samples of the same breast carcinomas.

Methods: Frozen sections and cytobrushings of 103 breast carcinomas were analysed. Four techniques were performed on each tumour: two on LBC samples (ICC, and CISH, both graded as positive, indeterminate, or negative) and two on histological samples (IHC and CISH). Two cell lines (MCF-7, negative; BT 474, positive) were used as controls for cytological analysis. A complementary fluorescence in situ hybridisation technique was carried out in histological samples with low amplification (4–10 dots/nucleus).

Results: Interobserver agreement for the four techniques calculated by the $\kappa$ coefficient indicated a substantial agreement. Nine cases failed in cytology because of poor cellularity. Among 94 cases, 19 were amplified; 73, 12, and 9 tumours were scored 0 or 1+, 2+, and 3+, respectively by IHC and 75, 13, and 6, respectively, by ICC. CISH found no amplification in 72 tumours. Correlations between the IHC and CISH results in the histological and cytological samples were always significant.

Conclusions: HER-2 status could be determined in LBC samples and correlated well with reference histological methods using in situ hybridisation. ICC was less reliable because of the presence of the cytoplasmic membrane. However, these results should be confirmed by a large multicentre study.

Breast carcinomas are the most frequent tumours in women. In several countries, mammographic screening allows the early diagnosis of tumours. However, when a tumour is suspected, morphological analysis alone can establish the diagnosis of carcinoma. In this context, guided fine needle aspiration has become increasingly popular for obtaining tissue specimens for the diagnosis of malignant breast diseases.

“The importance of measuring HER-2 gene amplification and its overexpression in breast carcinoma is clearly established”

HER-2/neu, a protooncogene located on chromosome 17q, encodes a transmembrane tyrosine kinase with substantial homology to the epidermal growth factor receptor.1 The importance of measuring HER-2 gene amplification and its overexpression in breast carcinoma is clearly established—it is associated with poor prognosis,1,2 short survival,3 and response to specific treatment with Trastuzumab.4 Different techniques have been used to measure the cellular concentration of HER-2 in biopsies and surgical samples, but currently the most frequently used methods are immunohistochemistry (IHC)5 and in situ hybridisation.6 A large number of studies have compared both methods to evaluate HER-2 status, with advantages and pitfalls for each one. It is now thought that HER-2 determination is most efficient using IHC as the method of choice, with in situ hybridisation being performed for cancers with indeterminate results (2+ score).7 Several in situ hybridisation techniques are available. Fluorescence in situ hybridisation (FISH) can be carried out using either a single colour (HER-2 probe only) or the more frequently used dual colour technique (using HER-2 and chromosome 17 centromere probes simultaneously).8 The main disadvantage of using FISH in clinical diagnostics is the need for fluorescence microscopy, which is not available in most routine diagnostic laboratories. Moreover, the evaluation of FISH requires a modern epifluorescence microscope equipped with high quality immersion objectives and multiband pass fluorescence filters. In addition, because the fluorescent signals fade within a few weeks, the hybridisation results must be recorded with expensive digital cameras. Thus, to overcome these practical limitations, chromogenic in situ hybridisation (CISH) was recently introduced, in which the DNA probe is detected using a simple IHC-like peroxidase reaction.9,10 Evaluation of HER-2 status with fine needle aspiration from primary breast carcinoma is important in clinical practice. Different techniques have been used to measure the cellular concentration of HER-2: enzyme immunoassay,11 immunocytochemistry (ICC),12 and in situ hybridisation.13–15 Recent studies showed that overexpression of HER-2 can be detected successfully in routine conventional cytological specimens from breast cancer and other neoplasms,16 and have shown that FISH can be used successfully in cytological samples to evaluate HER-2 amplification and in situ hybridisation results.17

Abbreviations: CI, confidence interval; CISH, chromogenic in situ hybridisation; FISH, fluorescence in situ hybridisation; ICC, immunocytochemistry; IHC, immunohistochemistry; LBC, liquid based cytology; SSC, standard saline citrate

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Accepted for publication 6 January 2005

compares well with histological analysis. Recently, LBC has been introduced for mammary aspirates. There is a good correlation between LBC and conventional breast fine needle aspirate preparations. Moreover, LBC offers the possibility of adjunctive investigations (ICC and in situ hybridisation) on the same homogeneous material. Therefore, it will be useful to evaluate HER-2 status by this new cytological method because it is already possible using conventional routine cytology specimens.

Thus, the aim of our study was to analyse these two new techniques together: LBC and CISH to evaluate HER-2 status. Consequently, we will correlate these new techniques with conventional techniques: immunohistochemistry (IHC) and CISH using histological samples of the same breast carcinomas. In addition, ICC analysis of HER-2 expression will also be carried out on LBC samples.

MATERIALS AND METHODS

Patients

A prospective study was carried out at two medical centres in Reims, France—the Medical School Hospital and the Cancer Centre (Jean Godinot Institute)—between January 2002 and October 2003. Patients underwent an excisional biopsy, which allowed the diagnosis of invasive breast carcinoma. A frozen section of each tumour was obtained to establish the diagnosis of breast carcinoma and then a cytobrush sample was taken from the fresh tumour and rinsed in ThinPrep transport solution (Preservcyt; Cytyc, Boxborough, Massachusetts, USA). The histological and cytological samples were gathered together and analysis took place one week to three months after surgery. The stained slides were analysed microscopically by two independent observers (HS and PB). The degree of interobserver agreement was measured using the $\kappa$ coefficient (0, no agreement; 1, perfect agreement).

Histology

Tissue specimens

Formalin fixed samples were obtained from patients with breast carcinoma. For each patient, one block containing the central part of the tumour was selected for IHC and in situ hybridisation. Tumour stage and histological grade were determined for each patient.

Immunohistochemistry

Sections (4 µm thick) were dewaxed and rehydrated in graded alcohols. Antigen retrieval was achieved by heating slides in a water bath at 98°C for 10 minutes in citrate buffer (pH 6.0). After cooling the sections to room temperature, endogenous peroxidases were quenched with 0.3% H$_2$O$_2$ in water. Monoclonal antibody NCL-CB11 from Novocastra Laboratories (Newcastle upon Tyne, UK) was applied at a dilution of 1/500 and incubated for 30 minutes at room temperature. Immunohistochemical staining was performed using the streptavidin biotin kit (LSAB II; Dako, Glostrup, Denmark). The peroxidase was visualised using 3-amino-9-ethylcarbazol as chromogen. The slides were rinsed with water, counterstained with haematoxylin, and mounted.

IHC scoring was based on the membrane immunoreactivity: no staining or membrane staining in less than 10% of the tumour cells was considered negative (0); faint/barely perceptible membrane staining in more than 10% of the tumour cells was defined as 1$^+$; weak to moderate complete membrane staining in more than 10% of the tumour cells was defined as 2$^+$; and strong complete membrane staining in more than 10% of the tumour cells was defined as 3$^+$. As described previously, the results were divided into three categories: negative or without overexpression (0 or 1$^+$), positive with overexpression (3$^+$) (fig 1D), and indeterminate (2$^+$). All histological tests were evaluated without knowledge of the cytological result.

Figure 1 Immunohistochemistry in invasive ductal carcinoma. HER-2 is characterised by membranous staining. Sections were counterstained with haematoxylin. (A, B) No overexpression of HER-2 (0 and 1$^+$, respectively); (C) intermediate positivity (2$^+$); (D) overexpression (3$^+$).
CISH and FISH
Probes
The HER2 gene status was determined using the digoxigenin labelled SPOT-Light® HER2 DNA probe (Zymed, South San Francisco, California, USA). A biotin labelled chromosome 17 centromere probe (Zymed) was used for those tumours with six to 10 HER2 gene copies/nucleus.

FISH and CISH procedure
FISH and CISH were performed on 4 μm thick tissue sections mounted on Superfrost Plus microscope slides (Fisher, Pittsburgh, Pennsylvania, USA). The slides were baked overnight at 38°C and then dewaxed twice for 10 minutes in xylene, for five minutes in ethanol, and three times in water. Tissue sections were placed in a ceramic coplin jar heated at 95 ˚C for 10 minutes in a bath water and then pretreatment kit; Zymed) and loosely capped. They were kept at 95°C for 10 minutes in a bath and then washed immediately with deionised water. Enzyme digestion was carried out by covering the section with prewarmed 37°C pepsin (SPOT-Light tissue pretreatment kit; Zymed) and incubating at 37°C for 10 minutes. The slides were then washed with deionised water, dehydrated with graded ethanol, and air dried. Next, 10 μl of ready to use chromosome 17 centromere probe was applied to the centre of the 20 x 20 mm coverslip. After sealing the edges of the coverslips with rubber cement, the tissue sections and probes were denatured at 95°C for six minutes in a water bath and for one minute on crushed ice. Hybridisation was carried out in a drying oven at 37°C overnight. Stringent washing was carried out with 0.5× standard saline citrate (SSC) at 75°C for five minutes.

CISH
Endogenous peroxidase activity was blocked in 3% H2O2 for 10 minutes. Non-specific background was eliminated by incubating the tissue sections in CAS-Block (Zymed). This was followed by incubation with fluorescein isothiocyanate conjugated mouse anti-digoxigenin antibody for 45 minutes at room temperature and then incubation with horseradish peroxidase conjugated goat anti-fluorescin isothiocyanate antibody for 45 minutes at room temperature. Development was carried out with diaminobenzidine for 20 minutes. Tissue sections were counterstained with haematoxylin, dehydrated, and coverslipped. Positive controls were included in each staining run. A case of infiltrating ductal carcinoma, positive for the HER-2/neu oncoprotein by IHC analysis and amplified by CISH, was used as a positive control and non-neoplastic breast tissue was used as a negative control. In most cases, non-neoplastic elements served as internal controls. The CISH sections were evaluated using a microscope with a ×40 dry objective (Zeiss axioplan; Carl Zeiss AG, Jena, Germany). As described by Tanner et al,11 amplification was defined as six or more signals/nucleus in more than 50% of the cancer cells, or when a large gene copy cluster was seen.11 Unaltered gene copy was defined as 1–5 copies/nucleus. In tumours with a borderline copy number count (6–10 copies/nucleus in more than 50% of cells), an adjacent serial section was hybridised with a chromosome 17 centromere probe (Zymed) for comparison.

FISH
In these cases, the biotin labelled chromosome 17 centromere probe was detected with sequential incubation with alexa red conjugated streptavidin for 45 minutes at room temperature. The mounting medium containing DAPI (Vectashield; Vector Laboratories, Burlingame, California, USA) was removed by washing with phosphate buffered saline and Tween 20.

The FISH results were evaluated using a microscope (Zeiss aplanopan; Carl Zeiss AG) equipped with a ×100 oil immersion objective and DAPI/rhodamine filter sets (Carl Zeiss AG). HER-2 amplification was determined as the ratio of HER-2 CISH to chromosome 17 centromere FISH signal counts. Ratios < 2 denoted no amplification, and those between 2 and 5 denoted low level amplification.

In our study, true amplification was found in two situations: high level amplification was defined as greater than 10 discrete copies/nucleus detected by CISH in histology and low level amplification was defined as a HER-2 to chromosome 17 centromere signal count ratio between 2 and 5.

Cytology
For each case, two thin layer samples were prepared using the ThinPrep technique (Cytyc) and post-fixed in absolute ethanol for 24 hours. Two breast cancer cells lines fixed in Preservcyt were used as positive (BT 47416) and negative (MCF-717) controls for HER-2 amplification.

Immunocytochemistry
After post-fixation of the cytological slides and antigen unmasking for 10 minutes at 98°C in citrate buffer (pH 6.0), blocking of endogenous peroxidase was carried out in 3% hydrogen peroxide for 10 minutes. The dilutions and incubation times of the primary antibody and the secondary reagents were the same as for IHC. Immunopositivity was scored quantitatively on cytological samples as for the histological samples. The evaluation of HER-2 amplification was carried out in at least 100 cells.

CISH
After post-fixation, the slides were air dried and placed in CISH pretreatment buffer (SPOT-Light tissue pretreatment kit; Zymed) at 75°C for 10 minutes, followed by washing in 2× SSC and incubation at 37°C for five minutes in prewarmed pepsin at 37°C (SPOT-Light tissue pretreatment kit; Zymed). The slides were then washed with 2× SSC and 10 μl of the HER-2 probe solution was placed on the centre of a 20 x 20 mm coverslip. After sealing the edges of the coverslips with rubber cement, the tissue sections and probes were denatured at 80°C for three minutes. Hybridisation was carried out at 37°C overnight. The dilutions and incubation times of the secondary reagents were the same as in CISH for histology. The ICC and CISH techniques were done in batches of 10 slides with appropriate positive and negative controls. The evaluation of amplification was carried out on at least 100 cells.

Statistical analysis
The χ² test was used to evaluate the correlations between IHC, ICC, and CISH in histological and cytological samples. A p value < 0.05 was considered to be significant. All the tests were performed with the Stat View program (Abacus Concepts, Berkeley, California, USA).

The analytical performance of ICC and CISH in cytological samples was also analysed for specificity, sensitivity, and negative and positive predictive values.

RESULTS
In total, 103 cases were assessed by IHC and ICC for HER-2 protein expression and by CISH in histological and in cytological samples for gene amplification. CISH cytology and/or ICC analysis failed in nine cases; five because of a lack of cells, and four because of inadequate digestion despite using several thin layer preparations and an optimised protocol. Therefore, four results (IHC and CISH in
histological samples; ICC and CISH in cytological samples) are available for 94 cases.

Clinical features of the patient population
The patients’ ages ranged from 28 to 84 years with a median of 58.88 years (table 1). Most tumours (78 of 94) were pure invasive ductal carcinomas, whereas 14 were lobular carcinomas and two were mucinous carcinomas. Among the 94 carcinomas, 24 were poorly differentiated (Bloom 3), 48 were moderately differentiated (Bloom 2), and 22 were well differentiated (Bloom 1).

Histology
Interobserver agreement for the two techniques calculated by generalised $k$ values indicated a substantial agreement (0.68 to 0.80).

Using IHC, among 94 cases, 73 were negative or without overexpression of HER-2 (0 or 1+) (fig 1A, B), 12 were indeterminate (2+) (fig 1C), and nine were positive with HER-2 overexpression (3+) (fig 2D).

With CISH, 72 tumours showed no amplification, with 1–5 gene copies/nucleus (fig 2A) and 11 showed high amplification (fig 2C). Eleven tumours had 6–10 copies of the HER-2

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Figure 2   Examples of chromogenic in situ hybridisation for the HER-2 oncogene and fluorescence in situ hybridisation (FISH) for the chromosome 17 centromere in breast cancer. (A) A tumour with one or two clearly identifiable copies of the HER-2/neu gene (no amplification). (B) This tumour contained six to 10 copies of HER-2/neu gene/nucleus in more than 50% of the cells. (C) Typical high amplification of the HER-2/neu gene appears as a peroxidase positive cluster of gene copies. In these tumours, the FISH technique for the chromosome 17 centromere produced two types of results: (D) two spots showing low amplification, and (E) six to 10 copies/nucleus corresponding to aneuploidy and no amplification.
gene/nucleus in more than 50% of cells (fig 2B). In these cases, FISH analysis showed low amplification in eight cases, with a HER-2 to chromosome 17 centromere signal count ratio between 2 and 5 (fig 2D) and three cases of false amplification with a ratio under 2 (fig 2E). In summary, HER-2 was amplified in 19 of 94 cases, 11 had high level and eight had low level amplification. All amplified cases were ductal carcinomas; nine were poorly differentiated (Bloom 3), four moderately differentiated (Bloom 2), and six were well differentiated (Bloom 1).

Liquid based cytology
Interobserver agreement for the two techniques calculated by generalised $\kappa$ values indicated a substantial agreement (0.71 to 0.75).

Immunocytochemical analysis
Among 94 cases, 75 had an ICC score of 0 or 1+ (fig 3C, D). None of these cases showed gene amplification, and 72 were negative (0, 1+) and three indeterminate (2+) by IHC (table 2).

Thirteen cases were categorised as indeterminate (2+) (fig 3E). Six showed low and seven high gene amplification (table 3), eight were indeterminate with IHC, four positive, and one negative (table 2).

Six cases overexpressed HER-2 (3+) (fig 3F). One of these cases had low and five had high gene amplification. One was indeterminate and five were positive (3+) by IHC (table 2).

CISH
The HER-2 gene was amplified in 11 of the 94 cases (fig 4E). Two of these cases were indeterminate by IHC and nine were positive. All these cases were amplified in the histological samples, 10 had low and one had high amplification.

In addition, 11 cases showed low amplification (fig 4D). However, in the histological samples, eight showed low amplification and three were aneuploid. Four were negative and seven were indeterminate by IHC.

Finally, 72 tumours were not amplified (fig 4C) by CISH in either the cytological or histological samples. Sixty nine of these cases were negative with IHC and three were indeterminate.

Comparison between histological and cytological analyses
We correlated the results obtained from both the histological and cytological techniques. There was a significant correlation between the IHC results and the techniques used on the cytological samples (ICC and CISH) ($\chi^2$ test, $p < 0.01$; table 2).

Figure 3 Immunocytochemistry for the HER-2 protein in liquid based cytology samples from patients with breast cancer. (A) Negative control (MCF7), (B) positive control (BT474), (C) sample with no expression (0), (D) sample with no expression (+1), (E) sample with indeterminate expression (2+), and (F) sample with overexpression of the protein.
There was also a significant correlation between the CISH results using histological samples and the techniques used on the cytological samples (ICC and CISH) (χ² test, p < 0.01; Table 3).

When CISH for the HER-2 probe together with FISH for the chromosome 17 centromere using histological samples is taken as the standard testing method, the positive predictive value of positive ICC scores (≥3+) was 100% and the negative predictive value of negative ICC scores (0 or 1+) was 100%. The sensitivity and specificity of ICC was also calculated. The analyses were performed with or without the tumour group that had indeterminate (2+ score) results. The sensitivity, with ≥3+ only was 31% (95% confidence interval (CI), 21.6% to 40.3%), and with ≥3+ and ≥2+ it was 100%; specificity was always 100%. The same comparisons were done with CISH using cytological samples, with a limit of more than six copies of the HER-2 gene/nucleus. The sensitivity was 100%, the specificity 96% (95% CI, 92% to 99.9%), the positive predictive value was 86.4% (95% CI, 79% to 93%), and the negative predictive value was 100%.

**DISCUSSION**

Conventional fine needle aspiration is often now complemented by LBC aspiration. When a series of excised palpable breast masses was analysed with these two techniques, LBC provided greater cellularity and better nuclear detail than conventional smears and was as sensitive in identifying the tumorous cells as conventional fine needle aspiration. However, LBC is less specific, although this observation was not significant. In our study, we did not carry out breast aspiration because the cytobrush technique produced optimal results in our breast surgical specimens. Moreover, a preliminary study found no difference between cytobrush and aspiration samples in the morphology of the cells, only in the amount of material obtained and the number of tumorous cells isolated. However, our study shows clearly that in breast carcinoma there is a highly significant correlation between the reference technique—i.e., in situ hybridisation using histological samples—and the analysis of HER-2 status in LBC samples, with the same problem of discriminating between high grade in situ and invasive carcinoma.

CISH on ThinPrep processed fine needle breast aspirates has been used previously to evaluate oestrogen and progesterone receptor numbers and showed a good correlation with the reference technique of enzyme immunoassay. This technique can be performed on samples that have been preserved for several days in Preservcyt with a good correlation with newly prepared specimens. In our study, the preservation of the cells was also adequate. We were able to perform ICC up to three months after fixation with optimal cellular reproducibility. However, in nine cases the number of cells was too low to perform all the techniques, in particular ICC. The number of cases with indeterminate expression of the HER-2 protein (2+) using ICC and cytological samples was slightly higher than when using IHC and histological samples (13 versus 12). Because the presence of the cytoplasmic membrane results in dilution of the signal, overexpression of this protein was better determined in histological sections than in cytological samples (nine versus six). In contrast, however, automated image analysis can be applied to cytological samples, so that a quantitative signal can be obtained.
"Our study shows clearly that in breast carcinoma there is a highly significant correlation between the reference technique—in situ hybridisation using histological samples—and the analysis of HER-2 status in liquid based cytology samples."

In situ hybridisation of LBC samples is a recently developed technique, which was first used in cervical cytology, in particular to detect human papillomaviruses.25 A recent study reported that in situ hybridisation and ICC could be performed on the same cervical cytology sample.26 FISH can be used for the detection of aneuploidy in other types of cytological samples, such as LBC urine samples.27 In cytological samples, in situ hybridisation performed on whole nuclei provides the most accurate estimation of gene and chromosome copy numbers because nuclear truncation artifacts are avoided, resulting in accurate gene to chromosome ratios.28 Moreover, truncation might result in a considerable underestimation of gene copy. Indeed, in our study, one case showed low amplification in the histological sample but more than 10 copies of the gene in the cytological sample. Nonetheless, globally there was a good correlation between in situ hybridisation using histological and cytological samples.

FISH measures the number of gene copies in cancer cell nuclei. When dual colour hybridisation probes (using HER-2 and chromosome 17 centromere probes simultaneously) are used, it enables true HER-2 amplification to be distinguished from chromosomal aneuploidy. However, this double hybridisation is useful only for cases with poor amplification (six to 10 spots/nucleus)—only 11 tumours in our study. In most of these cases (eight of 11), the tumours had true low amplification and only three cases showed false amplification. In the rare cases where it was necessary, we used FISH to determine the number of copies of chromosome 17 in the tumour cell nuclei, but only in the histological sections because of the lack of cells in the cytological samples. CISH uses a simple IHC-like peroxidase reaction and seems to be a good alternative to FISH. Previous studies have clearly shown that CISH can provide a very useful, accurate, and practical alternative for determination of HER-2 status in archival formalin fixed breast cancer samples.11 29 30 Our present study shows that CISH alone or associated with FISH (in rare cases with six to 10 copies of the HER-2 gene) can provide a good evaluation of HER-2 status in histological or LBC samples.

In conclusion, LBC enables an accurate evaluation of HER-2 amplification to be made, particularly when using in situ hybridisation, because in contrast to histology it is performed on whole nuclei. ICC was less reliable because of the presence of the entire cytoplasmic membrane. LBC of breast fine needle aspirates could also be used to evaluate HER-2 status as a guide to the usefulness of treatment with Trastuzumab.
HER-2 status with liquid based cytology

Take home messages

- HER-2 status could be determined in liquid based cytology (LBC) samples and correlated well with the reference histological methods (immunohistochemistry and in situ hybridisation) using chromogenic in situ hybridisation
- Immunocytochemistry on LBC samples was less reliable because of the presence of the cytoplasmic membrane
- These results should be confirmed by a large multicentre study

ACKNOWLEDGEMENTS

We gratefully acknowledge Cytyc, Boxhorough, Massachusetts, USA for supplying the ThinPrep kits without charge. We thank the Pol Bouin laboratory personnel and particularly Mrs Boutens and Mrs Evvard for their support and assistance. Supported by Programme Hospitalier de Recherche Clinique (PHRC regional 2001) from the French Health Ministry and by SA Neully-sur-seine France.

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