Chromogenic In Situ Hybridization Is a Reliable Method for Detecting HER2 Gene Status in Breast Cancer

A Multicenter Study Using Conventional Scoring Criteria and the New ASCO/CAP Recommendations

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Abstract

Chromogenic in situ hybridization (CISH) has shown the potential to replace fluorescence in situ hybridization (FISH) to determine HER2 gene status. To validate the reliability of CISH, we used 226 consecutive breast carcinomas from 2 institutions and tested CISH and FISH on the same tumor set simultaneously at different test sites. Besides manufacturers’ scoring criteria, the new American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines were used to interpret HER2 status. The concordance between CISH and FISH for positive and negative results was 98.5% at site A and 98.6% at site B using the manufacturers’ criteria, and 99.0% at site A and 99.1% at site B using the ASCO/CAP criteria. Reproducibility of CISH results was more than 98.0% among 3 sites using the manufacturers’ criteria and 100.0% between 2 sites using the ASCO/CAP criteria. Our results confirm that CISH is reliable for HER2 testing per ASCO/CAP guidelines.

Identification of human epidermal growth factor receptor 2 (HER2) status is important to making therapeutic decisions for patients with breast cancer, both at the time of initial diagnosis and at recurrence. Positive HER2 status (ie, overexpression of the HER2 protein or amplification of the HER2 gene) has been reported in approximately 15% to 20% of women with newly diagnosed breast cancers1,2 and is associated with a poor clinical outcome, resistance to hormone therapy, and resistance to certain types of chemotherapy.3-7 More important, positive HER2 status is a strong predictor of response to anti-HER2 therapies, such as trastuzumab (Herceptin), and, therefore, is a major criterion for patient selection for HER2-targeted therapies.8-15 However, trastuzumab therapy is expensive16 and associated with rare but severe cardiotoxic effects.8,15,17,18 Accurate HER2 testing allows oncologists to administer timely anti-HER2 therapy to patients who may benefit and to spare patients who would not benefit from the costly and potentially harmful regimen.

Various methods of assessing gene amplification, messenger RNA level, and protein expression have been used to determine HER2 status in breast cancer, including Southern, Northern, and Western blots; polymerase chain reaction; and enzyme-linked immunosorbent assay. Immunohistochemical analysis and fluorescence in situ hybridization (FISH) are the 2 methods most widely used to determine HER2 status in the routine diagnostic setting. Each method has advantages and disadvantages. Immunohistochemical analysis measures HER2 protein expression on the cell surface and is easy to conduct in most pathology laboratories. However, the result of immunohistochemical staining is relatively susceptible to variations in tissue fixation and processing. Approximately 20% of HER2 protein tests performed in the local pathology
laboratories of the primary treatment sites proved incorrect when the same specimens were retested at a high-volume, central laboratory. FISH measures the copy number of the HER2 gene, usually in conjunction with the number of chromosome 17 centromere copies. Although this DNA-based assay is more stable and reproducible than immunohistochemical analysis, it is relatively expensive and labor-intensive. In addition, fading of signals produced by the FISH assay is also problematic.

Usually, an HER2 testing algorithm starts with immunohistochemical analysis. Whether a patient is eligible for anti-HER2 therapy depends on whether a clear-cut HER2 status is determined by immunohistochemical analysis. Cases with equivocal immunohistochemical results should be retested and confirmed with FISH.

Because of the disadvantages of FISH, chromogenic in situ hybridization (CISH) has been evaluated as a potential alternative. Like FISH, it measures the degree of HER2 gene amplification but is more straightforward than FISH on scoring. Similar to immunohistochemical analysis, CISH allows detection of HER2 gene copy signals with a conventional peroxidase reaction and enumeration of the HER2 signals with simultaneous histologic examination by ordinary bright-field microscopy. However, the reliability of CISH needs to be validated, ideally with large series of cases and tested at different sites. Concordance between CISH and FISH has been previously evaluated in a number of studies, but most were based on the experience of a single institution with a relatively small number of cases. Intersite reproducibility of CISH has been addressed in only a few studies. Also, these studies used only the scoring criteria established by the manufacturer to define positive and negative HER2 status.

Recently, the American Society of Clinical Oncology and the College of American Pathologists (ASCO/CAP) convened an expert panel and released new guidelines to standardize HER2 testing and improve testing accuracy, reproducibility, and the predictive power for response to trastuzumab. A major component of the guidelines was the changes in scoring criteria to define positive and negative HER2 status. The cutoffs for positive status with immunohistochemical and in situ hybridization methods have been elevated and the term “equivocal” has been introduced to classify cases with scores between positive and negative. In this study, we used a large number of breast carcinomas to validate the accuracy of CISH by examining the concordance between CISH and FISH at different test sites, as well as intersite reproducibility of CISH results according to the new ASCO/CAP guidelines.

## Materials and Methods

Paraffin-embedded tissue samples of 226 consecutive cases of surgically resected invasive breast carcinomas were retrospectively obtained from 2 institutions: 110 from The University of Texas M.D. Anderson Cancer Center, Houston (site A), and 116 from the Institute of Medical Technology, University of Tampere, Tampere, Finland (site B). All paraffin blocks were less than 2 years old. HER2 testing was performed using the PathVysion kit for FISH analysis (Vysis, Downers Grove, IL) and the polymer detection kit for CISH analysis (Zymed SPOT-Light HER2 CISH, Invitrogen, Camarillo, CA). We conducted the tests according to the manufacturers’ instructions, using the procedures similar to those reported previously. FISH was performed at site A and site B on duplicate sections of the 226 tumors. CISH was performed at site A, site B, and the Department of Pathology, Invitrogen (site C), on triplicate sections of the 226 tumors. Notably, at sites A and C, all steps of CISH on day 1 and day 2 were conducted manually, and at site B, the steps on day 1 were performed manually, but the steps on day 2 (including stringency wash, signal detection, and hematoxylin counterstain) were conducted with an autostainer 480 (ThermoFisherScientific, Fremont, CA). Immunohistochemical analysis using the HercepTest (DakoCytomation, Carpinteria, CA) was performed at the site that contributed the tumor samples.

The results of each test were interpreted by pathologists at each site who were blinded to the results of HER2 status (the previous status or the status obtained in the present study). The manufacturers’ scoring criteria and the ASCO/CAP recommendations were used to score CISH and FISH results. To standardize the interpretation criteria for HER2 status as determined by the different methods, each interpreter had

### Table 1

<table>
<thead>
<tr>
<th>HER2 Status</th>
<th>Average HER2/CEP17 Ratio (FISH)</th>
<th>Average HER2 Copy Number (CISH)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ASCO/CAP Criteria</td>
<td>Manufacturer’s Criteria</td>
</tr>
<tr>
<td>Nonamplified</td>
<td>&lt;1.8</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td>Equivocal</td>
<td>1.8-2.2</td>
<td>2.0</td>
</tr>
<tr>
<td>Amplified</td>
<td>&gt;2.2</td>
<td>≥2.0</td>
</tr>
</tbody>
</table>

ASCO, American Society of Clinical Oncology; CAP, College of American Pathologists; CISH, chromogenic in situ hybridization; FISH, fluorescence in situ hybridization.
been instructed with a protocol and a PowerPoint (Microsoft, Redmond, WA) tutorial provided by Invitrogen.

In the FISH analysis, at least 20 invasive tumor cells from each sample were scored for nuclear HER2 and CEP17 signals using an Olympus BX61 epifluorescence microscope (Olympus America, Melville, NY) fitted with a SpectrumOrange, SpectrumGreen, and DAPI triple-filter set. If the average HER2/CEP17 ratio was 1.8 to 2.2, another 20 invasive tumor cells were scored and the final ratio of the case was calculated from the total of 40 cells.

In the CISH analysis, at least 30 invasive tumor cells from each sample were scored for average HER2 gene copy number using a light microscope under a 40× dry objective. If the average copy number was 4 to 6 per nucleus, another 30 invasive tumor cells were enumerated and the final average copy number of the case was calculated from the total of 60 cells.

In the immunohistochemical analysis, HER2 expression was scored as 0 (no staining), 1+ (weak and incomplete membrane staining), 2+ (strong, complete membrane staining in ≤30% of tumor cells or weak/moderate heterogeneous complete membrane staining in ≥10% of tumor cells), and 3+ (strong, complete, homogeneous membrane staining in >30% of tumor cells). HER2 status was considered negative or non-overexpression if the immunohistochemical score was 0 or 1+, equivocal if the score was 2+, and positive if the score was 3+.

The reliability of the CISH was assessed by comparing CISH results with the corresponding FISH results at sites A and B and by evaluating the reproducibility of CISH results between sites. Concordance and the Cohen κ coefficient with corresponding 95% confidence intervals (CIs) were used to evaluate intermethod and intersite agreement. The relationship between the κ value and the level of agreement was proposed by Landis and Koch35: 0 to 0.20, slight agreement; 0.21 to 0.40, fair agreement; 0.41 to 0.60, moderate agreement; 0.61 to 0.80, substantial agreement; and 0.81 to 1.00, almost perfect agreement. The data analysis was performed by a statistical consultant at a company (StatServ Consulting, Chino Hills, CA).

This study was conducted with the approval of the M. D. Anderson Institutional Review Board and the review board of the University of Tampere.

Results

Of the 226 breast carcinomas, the histologic type was ductal in 175, lobular in 26, and other in 25. The success rate of FISH was 96.5% at site A (218/226) and 97.8% at site B (221/226); the success rate of CISH was 91.6% at site A (207/226), 98.7% at site B (223/226), and 99.1% at site C (224/226). Factors that contributed to the failure of a test included limited or no invasive carcinoma cells in the section, poor nuclear resolution, high background staining, and persistent autofluorescence of the FISH result.

When using the manufacturers’ scoring criteria, the concordance between CISH and FISH at site A was 98.5% (95% CI, 95.7%-99.7%) with a Cohen κ coefficient of 0.9463; at site B, the concordance was 98.6% (95% CI, 96.1%-99.7%) with a Cohen κ coefficient of 0.9518. Table 2 shows the excellent reproducibility of CISH results between sites, with a concordance of 99.0% between sites A and B, 98.6% between sites B and C, and 98.1% between sites A and C. Overall, HER2 amplification as determined by FISH was found in 16.8% of cases (34/202) at site A and 17.2% of cases (38/221) at site B. HER2 amplification as determined by CISH was found in 16.3% of cases (33/202) at site A and 16.7% of cases (37/221) at site B. Table 3 shows the concordance between CISH and FISH results at different test sites and the reproducibility of CISH results between sites when we used the scoring criteria recommended in the ASCO/CAP guidelines. When a 3-category

Table 2

Intersite Reproducibility of CISH Results Using the Manufacturer’s Scoring Criteria

<table>
<thead>
<tr>
<th>Site*</th>
<th>Concordance (95% CI)</th>
<th>Cohen κ Coefficient (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A and B</td>
<td>99.0% (96.5%-99.9%)</td>
<td>0.97 (0.918-1.000)</td>
</tr>
<tr>
<td>B and C</td>
<td>98.6% (96.1%-99.7%)</td>
<td>0.95 (0.856-1.000)</td>
</tr>
<tr>
<td>A and C</td>
<td>98.1% (95.1%-99.5%)</td>
<td>0.93 (0.861-0.998)</td>
</tr>
</tbody>
</table>

CI, confidence interval; CISH, chromogenic in situ hybridization.

* Site A, The University of Texas M.D. Anderson Cancer Center, Houston; site B, Institute of Medical Technology, University of Tampere, Tampere, Finland; site C, Invitrogen, Carlsbad, CA.

Table 3

Reliability of CISH for Detecting HER2 Gene Status in 226 Breast Carcinomas Obtained Consecutively From Two Institutions*

<table>
<thead>
<tr>
<th>ASCO/CAP criteria (amplified, equivocal, nonamplified)</th>
<th>Site A</th>
<th>Site B</th>
<th>Reproducibility Between Site A and Site B on CISH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cohen κ coefficient</td>
<td>0.8365</td>
<td>0.8768</td>
<td>0.9502</td>
</tr>
<tr>
<td>ASCO/CAP criteria (amplified, nonamplified)</td>
<td>0.9616</td>
<td>0.9666</td>
<td>1.0000</td>
</tr>
</tbody>
</table>

ASCO, American Society of Clinical Oncology; CAP, College of American Pathologists; CISH, chromogenic in situ hybridization; FISH, fluorescence in situ hybridization.* Site A, The University of Texas M.D. Anderson Cancer Center, Houston; site B, Institute of Medical Technology, University of Tampere, Tampere, Finland.
criterion (amplified, equivocal, and nonamplified) was used for comparison (Table 1), the concordance between CISH and FISH results was 95.0% at site A and 96.4% at site B, and the reproducibility of CISH results between sites was 98.5%. When the 2-category criterion defined in the ASCO/CAP guidelines for amplified and nonamplified status was used for comparison (Table 1), we observed near-perfect concordance between CISH and FISH: 99.0% at site A and 99.1% at site B (Table 3) [Image 4], [Table 5], [Image 1], and [Image 2].

We observed discrepant HER2 status between CISH and FISH in 4 cases, 2 at each test site (Tables 4 and 5) [Table 6]. Of the 4 cases, 3 showed negative HER2 status by CISH but positive status by FISH. However, the FISH score for all 3 cases was just above the cutoff for positive status as defined by the ASCO/CAP guidelines (2.30, 2.38, and 2.52; Table 6). Of note, 2 of the discrepant cases (1 at each site) were derived from the same tumor, which was tested and scored simultaneously at each site using both CISH and FISH; its corresponding immunohistochemical score was equivocal (2+) [Image 3]. In the third case, the discrepancy (negative by CISH and positive by FISH) occurred only at site B, and the corresponding immunohistochemical score was 0 (Image 3). In the fourth case, the discrepancy occurred only at site A; the average HER2 copy number in CISH was 8.67 (positive) and the average HER2/CEP17 ratio in FISH was 1.55 (negative). The corresponding immunohistochemical score for this case was 3+ (positive) (Image 3). None of the 4 discrepant cases showed polysomy of chromosome 17 by FISH. Significant heterogeneity of signal distribution was not identified in these cases. However, in 1 discrepant case (case 1 at site B), only approximately 25% of tumor cells demonstrated visible CISH signals, and this phenomenon was observed at both sites.

**Table 4**
Comparison of HER2 Status (Amplified or Nonamplified Based on ASCO/CAP Guidelines) Between CISH and FISH Performed at Site A*

<table>
<thead>
<tr>
<th>CISH</th>
<th>FISH</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplified (&gt;6)</td>
<td>30</td>
<td>31</td>
</tr>
<tr>
<td>Nonamplified (&lt;4)</td>
<td>162</td>
<td>163</td>
</tr>
<tr>
<td>Total</td>
<td>194</td>
<td></td>
</tr>
</tbody>
</table>

ASCO, American Society of Clinical Oncology; CAP, College of American Pathologists; CI, confidence interval; CISH, chromogenic in situ hybridization; FISH, fluorescence in situ hybridization. *Concordance, 99.0% (95% CI, 96.3%-99.9%); Cohen κ coefficient, 0.9616 (95% CI, 0.9087-1.0000). Cases excluded from analyses were as follows: 24 with missing CISH or FISH results and 8 with equivocal results in CISH or FISH. Site A, The University of Texas M.D. Anderson Cancer Center, Houston.

**Table 5**
Comparison of HER2 Status (Amplified or Nonamplified Based on ASCO/CAP Guidelines) Between CISH and FISH Performed at Site B

<table>
<thead>
<tr>
<th>CISH</th>
<th>FISH</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplified (&gt;6)</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Nonamplified (&lt;4)</td>
<td>178</td>
<td>180</td>
</tr>
<tr>
<td>Total</td>
<td>215</td>
<td></td>
</tr>
</tbody>
</table>

ASCO, American Society of Clinical Oncology; CAP, College of American Pathologists; CI, confidence interval; CISH, chromogenic in situ hybridization; FISH, fluorescence in situ hybridization. *Concordance, 99.1% (95% CI, 96.7%-99.9%); Cohen κ coefficient, 0.9666 (95% CI, 0.9206-1.0000). Cases excluded from analyses were as follows: 5 with missing CISH or FISH results and 6 with equivocal results in CISH or FISH. Site B, Institute of Medical Technology, University of Tampere, Tampere, Finland.

**Image 1**
Representative chromogenic in situ hybridization tests showing negative/nonamplified HER2 status (A) and positive/amplified HER2 status (B) in breast carcinomas (A and B, ×100).
Overall, when using ASCO/CAP scoring criteria, HER2 amplification was found in 16.0% of cases (31/194) at site A and 17.2% of cases (37/215) at site B by FISH and in 16.0% of cases (31/194) at site A and 16.3% of cases (35/215) at site B by CISH. In addition, perfect intersite reproducibility (100.0%) was observed on CISH results between sites A and B.

**Discussion**

HER2 testing is a standard of care in the management of patients with breast cancer, especially in determining a patient’s eligibility for trastuzumab therapy. In combination with or in sequence after chemotherapy, trastuzumab therapy

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**Table 6**

<table>
<thead>
<tr>
<th>Site†</th>
<th>Case No.</th>
<th>CISH/FISH</th>
<th>Immunohistochemical Result</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative (&lt;2)/ Positive (&gt;2.2)</td>
<td>Positive (&gt;6)/ Negative (&lt;1.8)</td>
<td></td>
</tr>
<tr>
<td>A/1</td>
<td>1.77/2.38</td>
<td>8.67/1.55</td>
<td>2+</td>
</tr>
<tr>
<td>A/2</td>
<td>8.67/1.55</td>
<td>1.77/2.38</td>
<td>3+</td>
</tr>
<tr>
<td>B/1</td>
<td>1.80/2.30</td>
<td>6.87/1.55</td>
<td>0</td>
</tr>
<tr>
<td>B/2</td>
<td>2.77/2.52</td>
<td>0.67/1.55</td>
<td>2+</td>
</tr>
</tbody>
</table>

CISH, chromogenic in situ hybridization; FISH, fluorescence in situ hybridization.

*Case 1 at site A and case 2 at site B were derived from the same tumor.

† Site A, The University of Texas M.D. Anderson Cancer Center, Houston; site B, Institute of Medical Technology, University of Tampere, Tampere, Finland.

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**Image 2** Representative fluorescence in situ hybridization tests showing negative/nonamplified HER2 status (A) and positive/amplified HER2 status (B) in breast carcinomas (A and B, ×1,000).

**Image 3** Representative immunohistochemical staining for HER2 showing immunohistochemical scores of 0 (A), 2+ (B), and 3+ (C) in breast carcinomas (A-C, ×400).
has been shown to improve patients’ clinical outcome in metastatic\cite{8,14,15} and adjuvant settings.\cite{9,10,12} Recently, the incorporation of trastuzumab into neoadjuvant therapy has shown promising results and was found to be associated with a significantly increased pathologic complete response rate.\cite{36-39}

HER2 status may be also important in selecting patients for treatment with lapatinib, a small-molecule dual HER1/HER2 tyrosine kinase inhibitor.\cite{40} Because of the increasing number of patients whose eligibility for anti-HER2 therapy needs to be determined, a reliable and straightforward test for HER status is highly desirable.

CISH has been gaining in popularity, mainly because of the simplicity and ease of scoring as compared with FISH. With the availability of an autostainer that expedites the stringency wash, signal detection, and hematoxylin counterstain steps, CISH may receive wide acceptance. Although a number of studies have previously evaluated the reliability of CISH, these were mostly based on the experience of a single institution with a relatively small number of cases. The reported overall agreement between CISH and FISH was 84% to 100%.\cite{22-28} Strengths of our present study include its large sample and the simultaneous determination and scoring of HER2 status on the same set of tumors at different test sites.

CISH results showed a high level of concordance with corresponding FISH results at each site when the manufacturers’ scoring criteria or the ASCO/CAP scoring recommendations were used. When the 3-category criterion was used, the concordance between CISH and FISH was 95.0% at site A and 96.4% at site B. Since the new ASCO/CAP guidelines mandate that a new test should demonstrate a high (≥95%) concordance with another validated test for positive (amplified) and negative (nonamplified) results before the new test is routinely used, we compared CISH with FISH using the 2-category (amplified and nonamplified) criterion. The resulting concordance was excellent: 99.0% at site A and 99.1% at site B, which exceeds the minimum concordance rate mandated by the ASCO/CAP guidelines.

Intersite reproducibility is another important parameter to address the reliability of CISH results. In previously published studies, the observers were usually from the same institution and evaluated the same CISH-stained slides.\cite{29} High reproducibility of CISH results at multiple laboratories has been reported by 2 groups,\cite{30,31} but only the manufacturers’ scoring criteria were used. In our study, the CISH staining was performed simultaneously at 3 test sites using the same set of tumors obtained consecutively from 2 institutions and scored by pathologists at each site; therefore, variability in technical performance and interpretation at different sites has been taken into consideration. We observed strong paired agreement in CISH results among the 3 test sites when the manufacturers’ scoring criterion was used (98.1%, 98.6%, and 99.0%) and between sites A and B when the ASCO/CAP 3-category criterion was used (98.5%) and the ASCO/CAP 2-category criterion was used (100.0%). These results indicate that CISH can be used as a practical alternative to FISH in an HER2 testing algorithm.

Discordance in HER2 status between CISH and FISH was observed, 2 cases at each test site. Several factors might be responsible for the discrepancy. Of the 3 cases negative by CISH but positive by FISH, all showed low-level amplification by FISH (scores of 2.30, 2.38, and 2.52; Table 6). The technical challenges of scoring cases with a low level of HER2 amplification have been previously described.\cite{40} Interestingly, 1 tumor had discordant HER2 status results at both test sites, and its immunohistochemical score was equivocal (2+), likely representing a “true” difficulty in the interpretation. In the other 2 cases, the discrepant result was identified only at 1 test site, indicating that technical variation and/or interobserver scoring variation might be the cause.

Intratumoral heterogeneity in HER2 status can occur but is rare.\cite{41,42} The difference in the foci selected for scoring could have contributed to the discordance. Although we did not encounter significant heterogeneity in the cases examined, we noticed that in 1 of the 4 discrepant cases, only approximately 25% of the tumor cells demonstrated visible CISH signals. This observation was reported at both sites for that case, indicating that this might be related to an intrinsic factor of the tumor block.

Previous studies indicated that polysomy of chromosome 17, which is not uncommon in HER2 testing, may contribute to a discrepancy between CISH and FISH results.\cite{43,44} This is because the CISH method, unlike FISH, usually does not assess the internal control CEP17. Although the HER2 copy number in most polysomy nonamplified cases is 3 to 5 per nucleus, it can occasionally reach the positive cutoff for CISH.\cite{27,29,31} As a result, these cases might be erroneously interpreted as gene amplification when, in fact, no actual amplification is present with FISH in which the final status is based on the HER2/CEP17 ratio. To overcome this drawback, some investigators have advocated that CEP17 testing with CISH on adjacent tissue sections should be included in cases that show equivocal results or low amplification.\cite{28,29,45}

In our study, we did not identify such cases. The only case showing HER2 amplification by CISH but nonamplification by FISH did not demonstrate polysomy of chromosome 17, and the corresponding immunohistochemical study showed positive HER2 status (3+). We believe that other preanalytic or analytic variables\cite{32,33} might be responsible for the discrepancy.

Silver in situ hybridization is another bright-field in situ hybridization method that showed high concordance with FISH when using the ASCO/CAP scoring criteria.\cite{46} It is a fully automated technique and seems to be suited for routine application in pathology laboratories if validated.
Overall, the present study showed a very high correlation between CISH and FISH results when using manufacturers’ and ASCO/CAP scoring criteria at different test sites. The concordance using a 2-category criterion achieves the high level of concordance required by the ASCO/CAP guidelines. This study also showed excellent reproducibility of CISH results between test sites. These findings support the notion that CISH is a reliable alternative to FISH for HER2 testing.

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