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Evaluation of the Clinical Significance of HER2 Amplification by Chromogenic *In Situ* Hybridisation in Patients with Primary Breast Cancer

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Abstract. *Background: The purpose of this study was to assess the clinical relevance of HER2 amplification by a novel chromogenic in situ hybridisation (CISH) technique in patients with primary breast cancer and to determine its relationship with other prognostic markers. Materials and Methods: One hundred and seventy-three breast cancer patients with a mean follow-up duration of 75 months were reanalysed in this retrospective study. Expression of HER2 in tumour tissue samples was assessed by immunohistochemistry (IHC) and CISH. Discrepant cases and tumours presenting a HER2 2+ and 3+ staining with IHC were additionally analysed by fluorescence in situ hybridisation (FISH) to exclude false-positive results. Results: HER2 overexpression and amplification was found in 24.3% and 19.1%, respectively. The clinico-pathological correlations revealed a significant association between positive HER2 status and standard prognostic factors including high tumour grade, large tumour size and absence of steroid hormone receptors. Univariate analysis indicated that HER2 overexpression and amplification were predictive for poor overall (OS) and disease-free survival (DFS). The same effect was also seen in the patient groups with node-negative as well as node-positive breast cancer. By multivariate analysis, HER2 alteration proved to be an indicator of poor prognosis, independent of tumour size, tumour grade, hormone receptor expression, nodal involvement and adjuvant therapy. Conclusion: HER2 expression, as assessed by CISH, is an independent marker for unfavourable prognosis in primary breast cancers.*

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Progress in molecular techniques has resulted in the identification and greater understanding of molecular markers that may have prognostic and predictive value for breast cancer patients. In particular, HER2 gene alteration has been studied intensively in patients with primary breast carcinoma and the determination of HER2 status has become an important tool in the clinical management of these patients.

HER2 amplification/overexpression has been observed in approximately 20-30% of breast cancers. It is associated with a poor clinical outcome and may be predictive of response to certain anticancer therapies (1, 2). Furthermore, a positive HER2 status is a prerequisite eligibility requirement for Herceptin therapy in women with metastatic breast cancer. As a consequence, accurate assessment of HER2 status is essential to ensure that all patients who may benefit from Herceptin are correctly identified (3).

Out of a variety of techniques that have been used in research for the detection of HER2 status (4-6), two methods are now predominant in the routine clinical pathology laboratory: determination of HER2 overexpression by immunohistochemistry (IHC) and HER2 gene amplification by fluorescence *in situ* hybridisation (FISH) (3). Furthermore, two FISH (Inform from Ventana [Tuscon, Arizona, USA] and PathVysion from Vysis [Downers Grove, Illinois, USA]) and two IHC (Dako HercepTest [Glostrup, Denmark] and Pathway Ventana) assays have been approved by the US Food and Drug Administration (FDA) as clinical tests for breast carcinomas. While the FISH procedure has been proven to be reliable and reproducible, a colorimetric or chromogenic modification of this assay would be highly desirable for most practising pathologists. This chromogenic *in situ* hybridisation (CISH) uses conventional peroxidase to generate bright-field gene copy signals and enables the assessment of HER2 gene amplification using regular light microscopes.

Although CISH has been tested with outstanding results for the detection of HER2 alteration (7-12), few data are

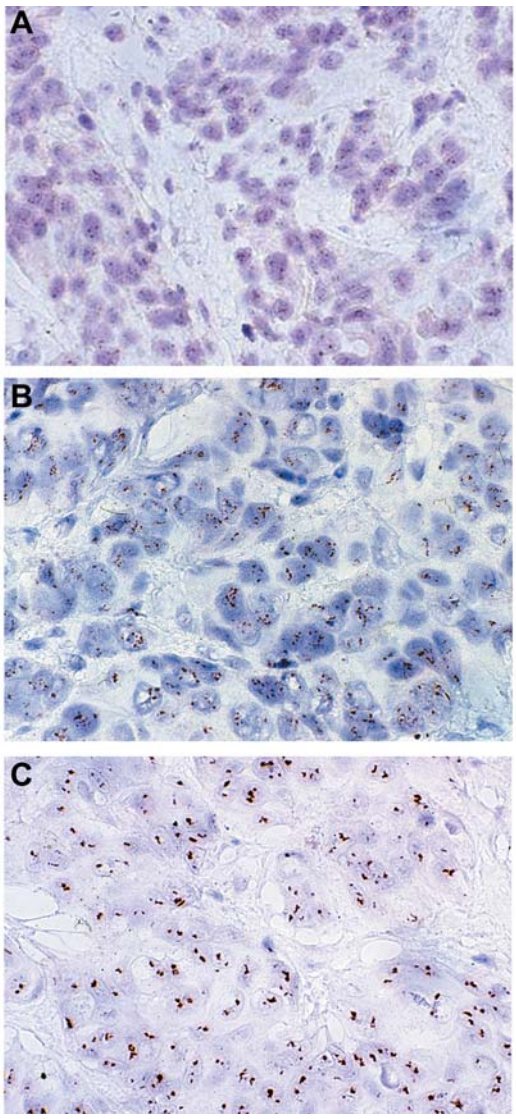

Figure 1. *Non-amplified tumour showing one to four clearly defined signals per nucleus (A). Amplification of HER2 by CISH seen as either individual scattered brown reaction products (B) or numerous signals arranged in clusters (C) in the majority of the nuclei. Counterstained with hematoxylin and magnification x405 (A-C).*

currently available to determine the clinical importance and utility of this method in the management of patients with cancer (13). Clinical validation with outcome data is essential to strengthen the use of CISH for HER2 testing. Therefore, the objective of our study was to evaluate the prognostic impact of HER2 alteration in patients with primary breast cancer detected by a recently established CISH assay (11). Results obtained by CISH and IHC were compared with clinico-pathological parameters. Finally, the prognostic value of HER2 amplification detected by CISH in patients with breast cancer was investigated in univariate and multivariate analyses.

Materials and Methods

The study population was comprised of 173 patients with primary breast cancer as described in our previously published study (11).

Patient population. We analysed archival formalin-fixed and paraffin-embedded tumour specimens from 173 patients with primary invasive breast cancer diagnosed between 1993 and 1999 at the Landes kliniken Salzburg, Austria. Tumour tissue was routinely processed for (immuno) histological analysis. The mean age of the patients was 49.4 years, ranging from 24 to 83 years. All patients had undergone either modified radical mastectomy with axillary dissection or breast-conserving surgery with axillary dissection. The patients had not received any preoperative chemotherapy or endocrine therapy. Altogether, 85 (49.1%) patients received hormone therapy (*i.e.* Tamoxifen), 64 (37.0%) were treated with chemotherapy (cyclophosphamide, methotrexate and fluorouracil) and 24 (13.9%) had both chemotherapy and hormone therapy.

Histological classification was performed according to the World Health Organisation guidelines and tumour staging according to the TNM System of the International Union against Cancer. The total of 173 cases examined was comprised of 123 (71.1%) infiltrating ductal carcinomas (IDC), 27 (15.6%) infiltrating lobular carcinomas (ILC), 8 (4.6%) mixed infiltrating ductal and lobular carcinomas, 10 (5.8%) tubular carcinomas, 3 (1.7%) mucinous carcinomas and 2 (1.2%) medullary carcinomas. Histological grading was assessed according to Bloom and Richardson criteria. Twelve tumours (6.9%) were of grade 1, 116 (67.1%) of grade 2 and 45 (26.0%) of grade 3. Eighty-seven women (50.3%) were lymph node-negative and 86 (49.7%) were lymph node-positive. Menopausal status was known for 170 of the 173 patients, with 125 (72.3%) premenopausal and 45 (26.0%) postmenopausal women. Hormone receptor status was routinely determined with a standard streptavidin-biotin-peroxidase method and was available for 170 of the 173 cases. Eleven cases were estrogen receptor (ER)-negative (6.4%), 159 (91.9%) were ER-positive. Regarding the progesterone receptor status, 22 cases (12.7%) were negative and 148 (85.5%) were positive.

Clinical outcome. Information on disease-free period (DFS) and overall survival (OS) was obtained from Tumour Registry records. Disease-free survival was defined as the interval between time of diagnosis and the first recurrence or metastasis. The overall survival was defined as death due to all causes. Duration of follow-up was evaluated as the time elapsed between time of diagnosis and the most recent clinical observation, being 19th January 2001. The mean follow-up time was 75.0±2.0 months (range 11-96 months). At the end of the follow-up period, 149 (86.1%) patients were still alive, 34 (19.7%) women had relapsed after 38.1±4.5 months and 24 (13.9%) patients had died after 37.3±4.9 months.

CISH. The protocol was performed as previously described (11). Briefly, 4-μm-thick, archival formalin-fixed paraffin-embedded sections were deparaffinised with xylene, hydrated and pretreated with enzyme and heat. Denaturation was carried out at 95˚C, followed by hybridisation at 37˚C overnight using a digoxigenin-labeled Her2 probe (Zymed, South San Francisco, California, USA). After hybridisation, stringency washes and a blocking step were performed. Signals were detected using mouse anti-digoxigenin (Boehringer Mannheim, Mannheim, Germany), biotinylated goat anti-mouse and horseradish-streptavidin-biotin

Table I. *Comparison between HER2 expression as assessed by CISH and IHC in 171 breast carcinomas.*

	IHC		
	0 or 1+	2+	3+
CISH			
no amplification	131 (76.6%)	5 (2.9%)	2 (1.2%)
amplification		11 (6.4%)	22 (12.9%)

Insufficient material was left to investigate two out of 173 cases with CISH.

complex (Dako StreptABComplex/HRP Duet kit; Glostrup, Denmark) and followed by development with diaminobenzidine (DAB). Slides were counterstained with hematoxylin.

A positive and a negative control sample (breast tumours with and without known HER2 amplification) were included in every hybridisation batch. The CISH sections were evaluated using a x40 dry objective. Hybridisation signals from at least 60 tumour cells were scored to assess oncogene copy number. The probe displayed a single distinct small dark brown signal at the location of each copy of the HER2 gene. A non-amplified gene copy number was defined as one to five signals per nucleus (Figure 1A). Amplification was defined as six or more signals per nucleus (Figure 1B) or when gene copy clusters were seen in >50% of cancer cells (Figure 1C).

Interpretation was performed independently by two of the authors (N.D. and C.H-K.), who were blinded to the clinical and pathological data.

IHC (HercepTest). HER2 overexpression was assessed using the Dako HercepTest kit. Staining and scoring were performed according to the protocol described in the manufacturer's guidelines.

Fluorescence in situ hybridisation (FISH). For the PathVysion assay, the HER2 DNA probe kit and the paraffin wax pre-treatment reagent kit were purchased from Vysis. The FISH assay was performed according to the manufacturer's recommended protocol.

In each specimen, at least 60 cells were counted for both HER2 gene and chromosome 17 centromere signals under oil immersion at x1000 magnification using the recommended filters. Specimens with a signal ratio of less than 2.0 were designated as non-amplified and 2.0 or greater as amplified.

Statistics. All statistical analyses were performed using the SPSS statistical package (SPSS Inc., Chicago, IL, USA). Associations of HER2 status with other clinico-pathological factors were calculated with the Chi-square test. Fisher's exact test was used when a number lower than 5 appeared in one cell of the contingency table. A p value of 0.05 or less was considered to be statistically significant. Probabilities of disease-free survival (DFS) and overall survival (OS) were calculated by the Kaplan-Meier method; statistical differences between survival curves were carried out with the log rank test. To determine the relative predictive strength of the prognostic variables, a Cox proportional hazard regression model was used. The statistical significance was evaluated using the Wald test and results were displayed as relative risk and respective 95% confidence intervals for each category. The multivariate Cox regression model was based on all of the prognostic factors that were significant in the univariate analyses at the 5% level of significance.

Table II. *Association of HER2 amplification and overexpression by CISH or IHC with clinico-pathological features in 171 primary breast cancers.*

Factor	CISH		IHC	
	Amplified /total	Odds ratio (p value)[a]	Overexpressed[b] /total	Odds ratio (p value)[a]
All tumours	33/171	-	40/171	-
Age				
< 50 years	24/107	1.8	28/107	1.5
≥ 50 years	9/64	(n.s.)	12/64	(n.s.)
Histological type				
IDC	30/130	(n.s.)	37/130	(0.020)
ILC	2/26		2/26	
others	1/15		1/15	
Tumour size				
≤pT1	8/66	2.3	10/66	2.2
≥pT2	25/105	(n.s.)	30/105	(n.s.)
Node status				
negative	17/86	1.1	21/86	1.1
positive	16/85	(n.s.)	19/85	(n.s.)
Recurrence				
No	22/143	3.6	28/143	3.1
Yes	11/28	(0.003)	12/28	(0.008)
Tumour grade				
1 and 2	19/126	2.5	24/126	2.3
3	14/45	(0.019)	16/45	(0.025)
ER				
negative	5/10	4.6	6/10	5.5
positive	28/158	(0.026)[c]	34/158	(0.013)[c]
PR				
negative	8/22	2.8	10/22	3.2
positive	25/146	(0.045)[c]	30/146	(0.011)

ER, estrogen receptor; PR, progesterone receptor; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma.
[a] χ^2 test for trend
[b] Samples with IHC +2 and +3 scores
[c] Fisher's exact test

Results

Comparison of CISH and IHC results. A total of 173 primary breast cancer samples was analysed with CISH and IHC (Table I). Due to non-representative histology, 171 (98.8%) samples were evaluable by CISH and IHC. HER2 staining by IHC was graded as +3 in 25 tumours (14.5%), +2 in 17 tumours (9.8%), and negative (score 0 and +1) in 131 tumours (75.7%). Out of 171 investigated cases, 138 (79.8%) showed no amplification by CISH and 33 cases (19.1%) revealed a HER2 amplification.

Of 171 cases analysed (Table I), 164 (95.9%) cases had identical results for both methods, with 131 (76.6%) tumours negative and 33 (19.3%) amplified/overexpressed. Seven (4.1%) cases were discordant. Out of these, five (2.9%) tumours with weak HER2 protein overexpression were found to be negative by CISH. Two (1.2%) tumours demonstrated strong HER2 overexpression (score +3) but no gene amplification by CISH.

Table III. *Univariate analysis of OS and DFS by various clinico-pathological factors.*

		OS		DFS	
Factor	No.	No. (%)	*P* value	No. (%)	*P* value
Age			n.s.		n.s.
< 50 years	109	13 (86.3)		20 (76.8)	
≥ 50 years	64	11 (79.1)		14 (74.2)	
Histological type			n.s.		
IDC	131	19 (82.4)		28 (72.8)	n.s.
ILC	27	5 (79.3)		6 (76.2)	
others	15	0 (100)		0 (100)	
Tumour size			0.007		0.022
≤pT1	68	3 (89.9)		7 (85.7)	
≥pT2	105	21 (78.7)		27 (69.7)	
Node status			< 0.0001		0.012
negative	87	3 (95.8)		11 (82.0)	
positive	86	21 (70.9)		23 (68.8)	
Tumour grade			n.s.		n.s.
1 and 2	128	16 (85.0)		25 (75.4)	
3	45	8 (79.5)		9 (75.4)	
ER			0.016		0.022
negative	11	4 (63.6)		5 (53.0)	
positive	159	20 (84.3)		29 (76.6)	
PR			0.009		< 0.0001
negative	22	7 (66.1)		11 (47.2)	
positive	148	17 (85.4)		23 (79.4)	
HER2 amplification			<0.0001		< 0.001
non-amplified	138	13 (87.4)		21 (81.6)	
amplified	33	11 (64.7)		12 (51.4)	
HER2 overexpression			<0.0001		< 0.001
no overexpression	131	11 (89.2)		19 (82.5)	
overexpressed (+2 and +3)	42	13 (65.0)		15 (52.1)	
Adjuvant therapy			0.010		< 0.001
hormone therapy	85	5 (91.8)		8 (88.2)	
chemotherapy	64	14 (75.0)		17 (65.5)	
both	24	5 (75.6)		9 (57.5)	

Insufficient material was left to investigate two out of 173 cases with CISH

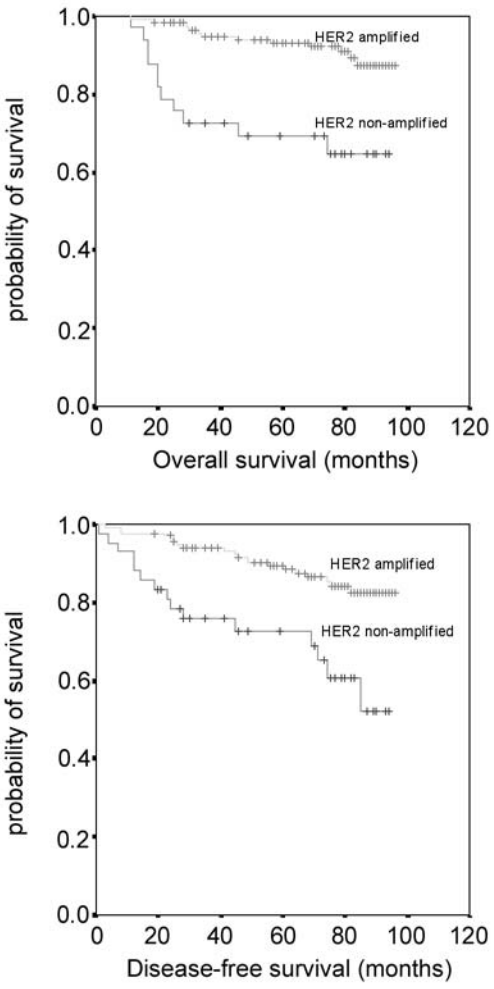

Figure 2. *Association of HER2 amplification with patient prognosis in breast cancer (Kaplan-Meier method and log rank test). HER2 amplification by CISH is significantly related to death (A, p<0.0001) and recurrence (B, p<0.001).*

Discrepant cases between CISH and HercepTest (7/171) and all IHC-positive cases (score +2 and +3), a total of 42 cases, were analysed with the FISH (PathVysion) assay. FISH was successful in 38 out of 42 cases; 2 cases had insufficient tumour present on the slides and two other cases could not be scored because of high background and low signal intensity, despite several attempts. Among 25 IHC-positive tumours (score of +3), 2 cases showed no gene amplification by FISH and by CISH. Concordance between CISH and FISH was 100% for the 38 cases analysed.

Correlation between HER2 alteration and clinico-pathological parameters. The relationships between clinico-pathological parameters and HER2 status determined by CISH and IHC are outlined in Table II. HER2 alteration significantly correlated with a higher tumour grade (p=0.019 for CISH, p=0.025 for IHC). Patients with recurrent tumours were more likely to have HER2-positive tumours (p=0.003 for CISH, p=0.008 for IHC). Negative ER and PR status were significantly associated with HER2 overexpression (p=0.006 and 0.011, respectively) and amplification (p=0.013 and 0.034, respectively).

HER2 overexpression was also seen more frequently in IDC than in ILC (p=0.020). A similar trend could be detected with CISH, although this trend did not reach statistical significance. No significant relationships were found between HER2 status determined by both methods and age, tumour size and node status.

HER2 expression in univariate and multivariate analysis of survival. The overall (OS) and disease-free survival (DFS) for all patients were 83.2% and 75.4%, respectively. OS and DFS were investigated in order to evaluate the prognostic value of

Table IV. *Results of multivariate Cox regression analysis for OS and DFS.*

Variable	OS			DFS		
	RR	95% CI	*P* value	RR	95% CI	*P* value
HER2 status by IHC						
negative	1.0 (referent)		< 0.0001	1.0 (referent)		0.014
positive	5.5	2.2-14.0		2.6	1.2-5.7	
Node status						
negative	1.0 (referent)		< 0.001	1.0 (referent)		0.047
positive	10.1	2.7-37.5		2.3	1.0-5.2	
PR						
negative	–	–	–	1.0 (referent)		0.006
positive				0.3	0.1-0.7	
HER2 status by CISH						
negative	1.0 (referent)		0.001	1.0 (referent)		0.025
positive	4.5	1.8-11.3		2.5	1.1-5.7	
Node status						
negative	1.0 (referent)		0.001	1.0 (referent)		0.033
positive	8.5	2.4-30.4		2.5	1.1-5.6	
PR						
negative	–	–	–	1.0 (referent)		0.008
positive				0.3	0.1-0.7	

RR, relative risk of death; CI 95%, confidence interval of relative risk

HER2 status for breast cancer progression. As shown in Table III, univariate analysis focusing on OS revealed nodal involvement ($p<0.0001$), tumour size ($p=0.007$), hormone receptor status (for ER $p=0.016$, for PR $p=0.009$), adjuvant therapy ($p=0.010$) as well as HER2 status (for CISH $p<0.0001$, for IHC $p<0.0001$) to be significant prognostic factors in primary breast cancer. Univariate analysis for DFS showed nodal involvement ($p=0.012$), tumour size ($p=0.022$), hormone receptor status (for ER $p=0.022$, for PR $p<0.0001$), adjuvant therapy ($p<0.001$) as well as HER2 status (for CISH $p<0.001$, for IHC $p<0.001$) to be significant prognostic factors in primary breast cancer. Patient age at diagnosis, tumour grade and histological type did not affect either OS or DFS. Figure 2 illustrates the statistically significant difference in both OS and DFS between patients with tumours showing HER2 amplification and those whose tumours did not. To exclude the possibility that treatment regimes influence the prognostic analysis for HER2, we also analysed the imbalance of adjuvant treatment in the HER2-negative and –positive cohorts, but no significant difference could be found (data not shown). When survival was stratified for lymph node status, HER2 positivity retained the prognostic impact in the patient groups with node-negative as well as node-positive breast cancer (data not shown).

Multivariate Cox regression analysis focusing on OS included tumour size, nodal involvement, hormone receptor status, adjuvant therapy and HER2 gene alteration determined by CISH and IHC and identified HER2 status and nodal involvement as independent prognostic factors (Table IV). In detail, the risk of death for HER2 overexpressing patients was 5.5 times higher than the HER2-negative group ($p<0.0001$). Similarly, patients with HER2 amplified tumours had a 4.5 times higher risk of death than the patient group with HER2 non-amplified tumours ($p=0.001$). Focusing on DFS, nodal involvement, HER2 status and PR were shown to be significant independent prognostic factors.

Discussion

The present study was performed to assess the prognostic value of CISH in detecting HER2 oncogene amplification in archival human breast carcinoma. Up to this time, only a few studies on the CISH technique for detecting HER2 in breast cancer in the diagnostic pathology literature exist, but few of them evaluate the prognostic value of this method (7-10, 12, 13).

In our previously published study (11), we evaluated CISH results in comparison with IHC and FISH and found them to be highly comparable. Moreover, since CISH is a method that combines the advantages of both IHC analysis and FISH, it may be an alternative in terms of reliability and practicability to routinely assess HER2 status.

In the present study, we re-evaluated the prognostic significance of HER2 detected by IHC and CISH. The clinico-pathological correlations were essentially the same, revealing a significant association between positive HER2 status and standard prognostic factors including high tumour grade, large tumour size and absence of hormone receptors and this is, in general, consistent with previous studies (14). Furthermore, the frequency of HER2-positive cases was clearly higher in IDC (23.1% for CISH, 28.5% for IHC) than in ILC (7.7%). These findings are in line with other studies that have shown

that this histopathological type of carcinoma exhibits the highest HER2 expression compared to other types (14).

Univariate analysis for OS and DFS confirmed the significance of known clinico-pathological parameters, including tumour size and lymph node status, hormone receptor status, adjuvant therapy and HER2 status. Both the IHC and the CISH assay predicted patient survival in univariate and multivariate analysis, confirming that HER2 amplification and overexpression are correlated with poor OS and shorter DFS independently of other variables (1). Furthermore, univariate analysis stratified by lymph node status demonstrated that HER2 alteration was a statistically significant prognostic factor in both lymph node-positive and lymph node-negative patients. These findings are in agreement with several studies (15, 16), although others have shown a positive correlation in node-positive breast tumours only (17-19).

In conclusion, these results add important data to the recent literature suggesting that HER2 amplification as detected by CISH could be used as an additional prognostic marker in breast cancer. This study confirms recent promising results that CISH may provide an interesting alternative in oncogene screening studies on archival materials and, ultimately, in clinical diagnostics as a more simple and economic technique that allows direct identification of the cells showing gene amplification. Larger comparative studies are needed to evaluate the interobserver reproducibility of this assay and its relevance to clinical therapeutic outcome and, particularly, its portability to automated instrumentation.

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