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Short Communication

Investigation of HER-2 status, treatment response and survival analysis in cervical cancer patients

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Summary

Anti-HER2 therapy has shown benefit in breast and gastric cancers that over-express HER2; therefore, it is critical to determine whether HER2 is over-expressed and amplified in cervical cancer with current FDA-approved tests, as previous works have reported frequencies of over-expression varying from 0% to 77%. We analyzed 100 consecutive patients starting in January 2013. Patients were previously untreated and diagnosed with locally advanced cervical cancer. They received chemoradiation as definitive treatment. We analyzed their primary tumors with HercepTest and HER-2/neu FISH probe, observing a strong adherence to the testing and reporting recommendations for HER2 testing in breast cancer by the American Society of Clinical Oncology. Among 100 patients, accrued 88 had both tests performed. We found 26 (29.5%) FISH-positive cases. HercepTest scores rating 0+, 1+, 2+, and 3+ were observed in 65.3%, 11.5%, 7.6% and 15.3% in the 26 FISH-positive. There was no concordance between the results of both tests using Cohen´s Kappa statistics, neither correlation between FISH status with age, FIGO stage, and response. FISH-positive patients had a non-statistical significant trend for worst 4-year survival. The HER2 protein is over-expressed in only a small subset of invasive cervical cancer as analyzed by Hercept test. Unexpectedly, HER2 gene amplification occurred in around 29.5% of cases. We stress that analyzed HER2 status with the validated methods for the procedures and reports used for breast cancer. Our results must be seen with caution and must encourage further testing in a higher number of patients and different populations to confirm our findings.

Key words: Cervical cancer; HER2; Immunohistochemistry; FISH.

Introduction

Cervical carcinoma, while highly curable in early stages with local therapies, cisplatin-based chemoradiation for locally-advanced disease fails to cure at least 15% to 45% of these patients (bulky IB to IVA). For advanced disease, cisplatin doublets yield a median survival of around 12-13 months. Bevacizumab added to cisplatin, or non-cisplatin doublet increases median survival for four months (from 13 to 17 months), but still without significant impact on a 3-year survival rate. Hence, new treatments are urgently needed [1].

HER2 (also known as c-erbB-2) is a transmembrane receptor protein with tyrosine kinase activity that is over-expressed in some solid tumors. Its over-expression and prognostic significance in breast cancer led to the development of anti-HER2 therapies. Among them, trastuzumab is approved for advanced breast carcinomas overexpressing HER2 and as adjuvant therapy as well [2, 3]. Later, it was also approved for HER2 overexpressing gastric carcinoma [4].

Over-expression of HER2 could be implicated in cervical carcinogenesis [5]. HER2 is over-expressed in cervical cancer in ranges from 0% to 77% of cases. Most likely, the wide variability may be due to the methodology issues. Positivity rates using immunohistochemistry (IHC) with no HercepTest (DAKO) are reported as low as 2.5% [6] and as high up to 77% [7], while other authors report rates of 13%, 38%, 43% and 43% [8-11]. Corresponding rates using Hercep Test are 0% [12] and 0.3% [13]. Regarding gene amplification, a study with chromogenic in situ hybridization reported 0.5% of amplification [14] 14% with Southern blot [15] and 0% using FISH [16]. There is only one study using the HER-2/neu FISH probe (Vysis, Inc., Downers Grove, IL) utilizing chromosome 17 centromeric probe as an internal control. Of these 24 cases studied, 23 were informative, and only 2 (8.7%) were amplified [17].

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Preclinical studies indicate that anti-HER2 therapy with trastuzumab could have a role in cervical cancer. HER2-positive primary cell lines derived from tumor biopsies are sensitive to trastuzumab-mediated ADCC (antibody-dependent cytotoxicity), and trastuzumab inhibits their proliferation [18, 19]. Trastuzumab alone, or in combination with other therapies, is effective in mice models harboring HER2-positive cervical cancer cells [20]. Most of the data above discussed regarding the expression status of HER2 in cervical cancer were obtained before the standardization required in breast and gastric cancer. Thus, we wanted to investigate the expression of HER2 using the HercepTest and the amplification status using the "PathVysion HER-2 DNA Probe Kit" (Vysis) (Vysis, Abbott Laboratories. Abbott Park, Illinois, U.S.A) observing a firm adherence to the testing and reporting recommendations for HER2 testing in breast cancer by the ASCO [21].

Materials and Methods

Patients and methods

The files of the first 100 consecutive, histologically confirmed locally advanced cervical cancer patients FIGO (International Federation of Gynecology and Obstetrics) stages IB2-IIB, starting in January 2013, were selected for this study. The criteria for their inclusion were that patients had to be untreated upon admission, to have available biopsies, treatment data, and clinical follow-up after treatment. The Institutional Ethics Committee (rev/10/15) approved the study.

Immunohistochemistry with the HercepTest

HER2 testing was performed with the HercepTest (Dako, North America, Inc. 6392 Via Real, Carpinteria, California 93013, USA) Catalog No. K5204. This test uses a polyclonal antibody (AO485) chosen for its high affinity for HER2. Punch biopsies were fixed in formaldehyde for 24 h and embedded in paraffin. The technique strictly followed the supplier's protocol. The protocol had two stages: a first step consisted of dewaxing in xylene, and rehydration in graded ethanol and finally, PBS. The next step was the incubation with the Antigen Recovery Solution in a water bath at 90-99 °C for 40 min. After incubation, the slides were dried at room temperature and rinsed in washing buffer. The excess buffer removed, and 1-3 drops of the Peroxidase Blocking Solution added for 5 min. Then, a wash with distilled water performed, and the tissue was covered with 1-3 drops of Anti-HER2 antibody and incubated for 30 min. After incubation, the slides were rinsed with wash buffer and incubated again for 30 min with Visual Reagent. The sections were rinsed again with buffer, and the excess removed. Then, the tissue was covered with 1-3 drops of the Substrate-Chromogen (DAB) solution and incubated for 10 min, followed by a wash with distilled water. Then slides were counterstained with Harris's hematoxylin. The tissues were dehydrated to xylene and subsequently mounted with the Entellan resin (Cat. 107961; Merck KGaA, Darmstadt, Germany). The slides were evaluated by two pathologists experienced in HER2 evaluation.

FISH test for HER2

We evaluated HER2 gene amplification with the "PathVysion HER2 DNA Probe Kit" (Vysis, Abbott Laboratories. Abbott Park, Illinois, U.S.A). The kit uses two probes to determine the HER2 copy number of the HER2 gene, orange for HER2, and green for CEP17 (Centromere Enumeration Probe for chromosome 17) that hybridizes with a localized alpha satellite sequence in the centromere of chromosome 17 (17p11.1-q11.1). The technique strictly followed the provider's protocol—preparation of the slides. Sections 2-3 μm thick were incubated at 55 °C for one night before the hybridization. The paraffin removed in three xylene changes for 10 min each. The samples were dehydrated by two changes of ethanol 100 % for 5 min each and then incubated for 20 min in 0.2 N HCl. The slides were washed and subsequently incubated in 2X SSC for 5 min twice. The next step was to incubate the slides for 30 min in a 1M NaSCN solution preheated to 80 °C. The next incubation was performed with 2X SSC for two minutes and incubated with the protease at 37 °C for 30 min., the slides were rinsed with distilled water and then submitted to two changes of 2 X SSC. Following this, the slides were briefly immersed in distilled water and allowed to dry at room temperature, incubated in 10% formalin for 15 min and rinsed again in distilled water. They were allowed to dry at room temperature. Ten μl of the DNA-FISH probe was applied to the selected area and covered with the cover slide. The samples were incubated for 20 hours in a humid chamber at 37 °C protected from light. The post-hybridization washes were performed with 2X SSC for 20 min and then with NP40 0.3% and 0.1% for 5 min each, the samples were allowed to dry and ten μL of DAPI (4,6-diamidino-2-phenylindole) / Antifade was added to the hybridized area and covered with a cover slide. The reading was done by two observers experienced in FISH testing using a MetaSystems Image Analyzer in combination with ISIS software and a Zeiss Axioskop 2 epifluorescence microscope (Carl Zeiss Microscopy, LLC, One Zeiss Drive Thornwood, NY 10594 USA).

Evaluation of HercepTest and FISH

Protein expression and gene copy number scores were registered using ASCO guidelines [21].

HER2 immunohistochemistry

Score 0+: no stain or faint, incomplete membrane stain in not more than 10% within the cells. Score 1+: weak and incomplete membrane staining in more than 10% of the tumor surface. Score 2+: complete intense membrane staining in less than 10% of the invasive tumor cells or weak/moderate heterogeneous incomplete staining in more than 10% of the invasive tumor cells. Score 3+: intense complete homogenous membrane staining in more than 10% of the invasive tumor cells.

Evaluation of HER2 gene copy signals by FISH

Positive status was defined either as an average HER2 gene copy numbers of 6 at cases with a HER2/CEP17 ratio of < 2 or a HER2/CEP17 ratio of 2 or more independently of the average gene copy number. Negative status was defined as an average gene copy number of < 4 with a HER2/CEP17 ratio of < 2. Equivocal cases were defined as an average gene copy number of at least 4 and < 6 with a HER2/CEP17 ratio of < 2.

Statistical analysis

Concordance between FISH and HER2 results was calculated with the Cohen's Kappa; the association among patient's characteristics, response, and treatment factors with FISH status with chi-square or t-student for categorical and non-categorical variables. Survival curves were constructed with the Kaplan-Meier method and compared with the log-rank test. Statistical analysis was performed using SPSS (version 20; IBM Corp., Armonk, NY, USA).

Table 1. — *Clinical and pathological characteristics of patients.*

Variable	Number (%)
Age. Median (IR)	48 42-58.5
ECOG	
0	53 (60.2)
1	35 (39.8)
Histology	
Squamous	54 (61.4)
Adenocarcinoma	32 (36.4)
Other	2 (2.3)
FIGO stage	
IB2	6 (6.8)
IIA	3 (3.4)
IIB	58 (65.9)
IIIA	2 (2.2)
IIIB	19 (21.5)

Results

Patients

A total of 100 consecutive files were selected starting in January 2013. Figure 1 shows the diagram for case selection. We report a total of 88 patients who had both IHC and FISH analyses. The median age was 48 years; most were squamous histology, had an ECOG (Eastern Cooperative Oncology Group) status of 0, and were staged as IIB (Table 1). All patients were submitted to concurrent chemoradiation using weekly cisplatin in all but one; 42% and 31.8% of patients received six and five weekly applications, respectively. All but one completed external beam radiation (EBRT) and eight patients also received extended-field radiotherapy. Brachytherapy (Brachy) was performed in 84 (95.5%) patients. After completing therapy, complete response (CR) was registered when patients had no clinical and cytological evidence of disease in the third month. Any clinical, cytological, or pathological residual disease was designated as a non-complete response (NCR).

HER2 expression and gene amplification

As shown in Table 2, among 88 patients, 26 (29.5%) had gene amplification, whereas 62 (70.4%) were negative. All but two FISH-positive cases had a ratio > 3 and < 4; all others had a ratio between > 2 and < 3. Regarding HercepTest results, almost two-thirds (56 patients, 63.6%) were rated as 0+ score, 19 (21.5%) score 1+, 7 (7.9%) score 2+ and 6 (6.8%) scored 3+. Figure 2 shows representative cases for IHC 3+ and FISH-positive cases.

HercepTest scores rating 0, 1+, 2+, and 3+ were observed in (17) 65.3%, (3) 11.53%, (2) 7.69% and (4) 15.3% of the 26 FISH-positive patients. We grouped Cohen´s Kappa values under three conditions: grouping HercepTest negative (0+) or positive (1+, 2+, and 3+) versus FISH negative or positive. In this case, the Kappa value was 0.068 (no agreement). When grouped HercepTest negative (0+ and 1+) or positive (2+ and 3+), the Kappa value was 0.148 (poor agreement). There was also poor agreement (Kappa value 0.227) when HercepTest grouped negative (0+, 1+, 2+) or positive (3+). These data indicate that there was no concordance between these tests. As there were only 6 cases of HercepTest 3+, we analyzed the correlation between histology with FISH status only. Among the 62 squamous cell carcinoma cases, 31% were FISH positive, whereas 25% were positive in adenocarcinoma histology ($x^2 p$ = 0.590). There was no correlation with age or FIGO stage.

HER2 status and response and survival

Among the 88 patients, 79 (80%) had a complete response (CR), and 18 (20%) did not. There was no statistically significant association between response with HER2 status either by HercepTest protein expression (taking as positive scores 2+ and 3+), x^2 p = 0.980, or gene amplification by FISH, $x^2 p$ = 0.0774. A comparison of clinicopathological characteristics of the patients according to the FISH results is shown in Table 3. At a median follow-up time of 40 months (44-56), overall survival (OS) rates were non-statistically significant different. However, there was a trend for better survival in FISH-negative patients (Figure 3). The median OS was not reached in both groups. The 4-year survival probability in the FISH-positive patients was 58%, whereas it was 76% in the FISH- negative. HR: 0.79 (95% CI: 0.79 - 4.0), p = 0.23. 4-year-PFS curves (not shown) were 58% *vs.* 52% HR 1.73 95% CI: 0.63-2.4), p = 0.429.

Discussion

The results of this study show that among 88 patients analyzed for both tests, 6 (6.8%) and 26 (29.5%) of patients that were scored 3+ for HER2 protein expression and posi-

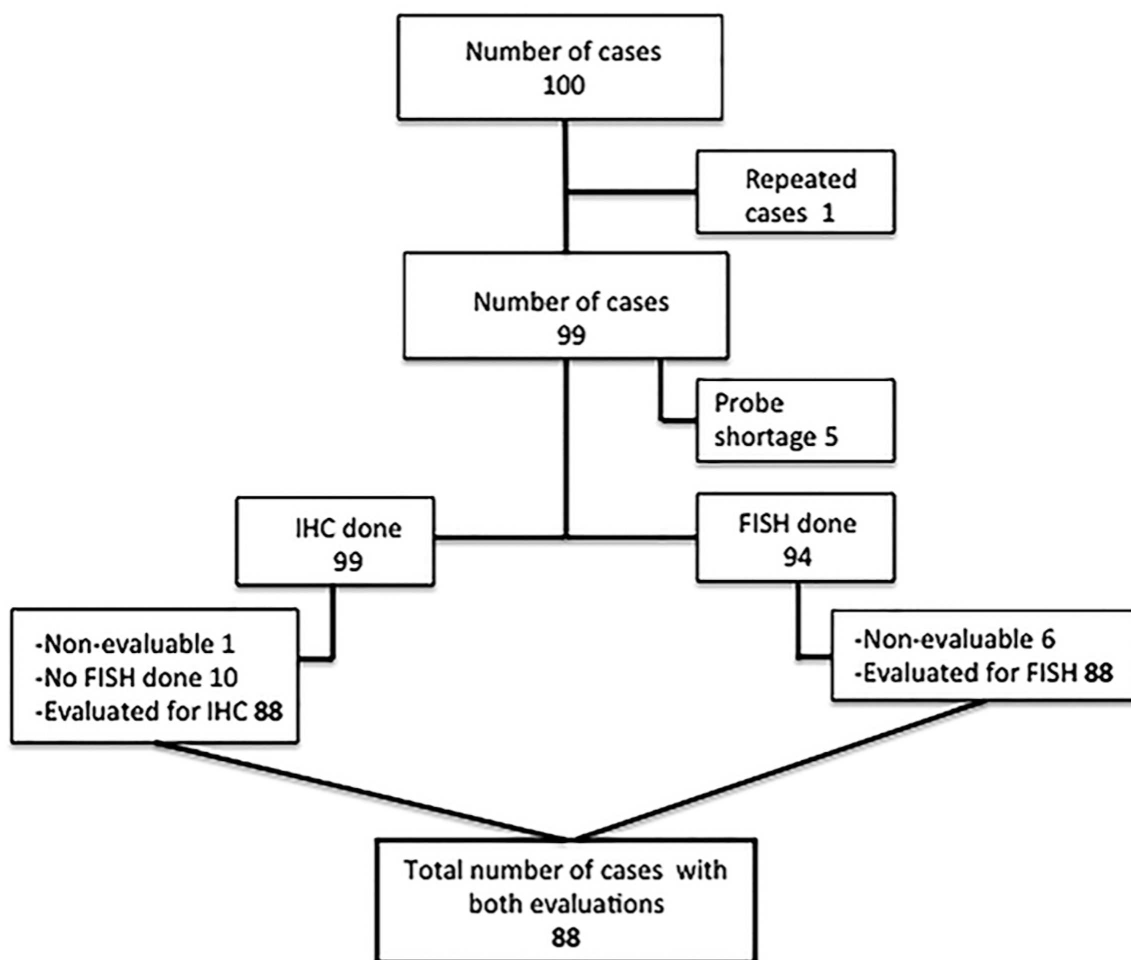

Figure 1. — Flow diagram of study patients. The diagram shows that FISH was done in 94 samples but only 88 were informative. Regarding IHC although this was performed in the 99 samples, for the final analysis we considered only 88 samples, which corresponds to the same samples that had an evaluable result for FISH.

Table 2. — *Results of FISH and HER2 testing in the 88 patients.*

HercepTest score	HercepTest 88 patients	FISH-negative 62 patients	FISH-positive 26 patients
0	56 (63.6%)	39 (62.9%)	17 (65.3%)
1+	19 (21.5%)	16 (25.8%)	3 (11.53%)
2+	7 (7.9%)	5 (8.06%)	2 (7.69%)
3+	6 (6.8%)	2 (3.22%)	4 (15.3%)

tive for HER2 gene amplification, respectively. There was a poor concordance between protein expression and gene amplification. Moreover, there was no association between patient's characteristics and treatment response with FISH status, though there was a trend for better survival in FISH-negative patients.

Because of the antitumor efficacy of anti-HER2 therapies in breast and gastric cancer [21, 4], there is a growing interest in determining which other epithelial cancers, including cervical carcinoma, would express this receptor to then testing anti-HER2 agents. Regarding cervical cancer, the HER2 receptor may participate in cervical carcinogenesis and progression [5, 22]. Besides, trastuzumab and lapatinib have a robust antitumor effect in mice xenografted with a human cervical tumor having HER2 gene amplification and HER2 protein overexpression [23]. Thus, there is a window of opportunity of increasing the drug armamentarium against cervical cancer that overexpresses HER2. As far as we know, there are no reports on the simultaneous use of FDA-approved HercepTest and the PathVysion HER2 DNA Probe Kit to analyze the expression in primary cervical tumors. Our results are exciting and encourage further analysis because if results confirmed, as much as a third of patients would be candidates for anti-HER2 therapy.

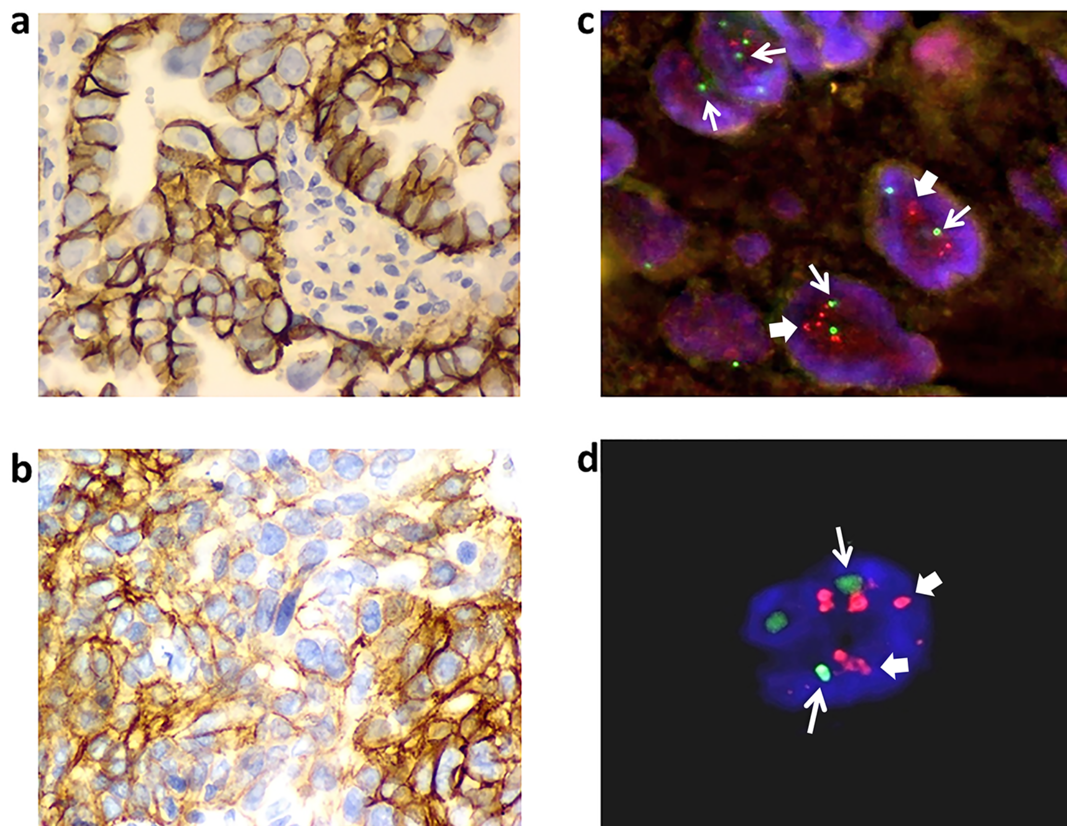

Figure 2. — Representative photomicrographs of HercepTest 3+ and FISH+. Pictures at left side show (a) Adenocarcinoma. Glandular spaces lined by columnar cells. The cells show strong complete membrane and basolateral membranous staining. 40X. (b) Squamous cell carcinoma. Nest of cohesive cells with strong and complete membranous staining. 40X. (c) and (d) at right side show to amplified cases. Thick arrows point to orange HER2 signal. thin arrows point to the green centromere signal.

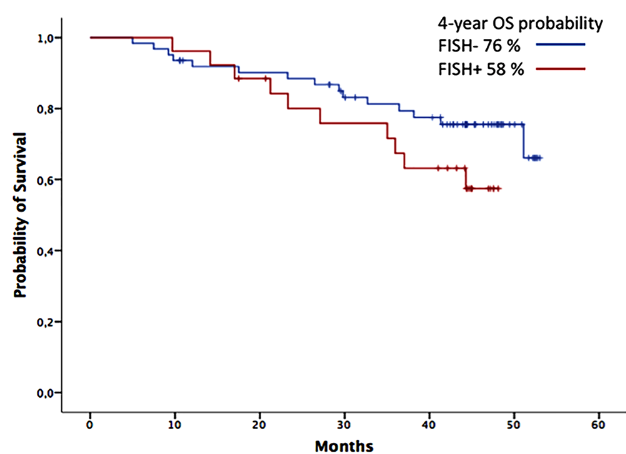

Figure 3. — At a median follow-up time of 40 months (44-56), overall survival rates (OS) were non-statistically significant different, though there was a trend for better survival in the FISH-negative patients (**blue line**). HR: 0.79 (95% CI: 0.79 - 4.0), p = 0.23.

The 6.8% of 3+ positive HercepTest we found in this study can be within the range of solid neoplasias other than breast carcinoma [24]. However, the high HER2 gene amplification found by FISH is certainly unexpected. HPV may promote the amplification of the Carbamyl-P synthetase, Aspartate transcarbamylase, Dihydro-orotase, CAD gene under certain conditions [25]. Known mechanisms facilitating gene amplification are common chromosomal fragile sites, defects in DNA replication and telomere dysfunction all these affected by HPV-E6/E7 oncoproteins [26, 27, 28]. Nonetheless, these are general mechanisms of gene amplification. Detailed mechanistic studies are needed to determine whether viral oncoproteins preferentially, could induce HER2 gene amplification.

On the other hand, it is at least intriguing that among 20 (77%) out of 26 FISH+ patients had zero or 1+ IHC expression while only 6 (23%) had 2+ or 3+. Our results do not follow the pattern of concordance observed in breast cancer [29, 30], which cannot be attributed to the technique, as we performed and analyzed the assays with close adherence to current guidelines. Our results in some aspects are similar to gastric cancer [31]. However, gene activity is the result of complex dynamics between the transcription rate of the DNA template, the stability of the mRNA, the translation efficiency of the transcript, and the degradation of the protein [32]. Hence further mechanistic studies are needed to explain such discordant results.

Table 3. — *Patient characteristics and treatment in according to FISH status.*

Variable	**FISH - N (%)**	**FISH + N (%)**	***p*-value**
Age. Median, (IR)	46 42-56.5	48 43-58.5	$p = 0.861$
ECOG			
0	38	17	$p = 0.717$
1	24	9	
Histology			
Squamous	43	19	$p = 0.713$
Adenocarcinoma	18	6	
Other	1	1	
FIGO stage			
IB2	4	1	$p = 0.814$
IIA	2	1	
IIB	37	16	
IIIA	2	0	
IIIB	21	8	
Cisplatin cycles			
5 – 6	44	22	$p = 0.764$
< 5	18	4	
Completed EBRT			
Yes	61	26	$p = 0.480$
No	1	0	
Completed Brachy			
Yes	59	25	$p = 0.729$
No	3	1	
Radiation treatment time (days)	52 (41-79)	54 (42-76)	$p = 0.954$
Received extended field radiation			
Yes	5	3	$p = 0.543$
No	59	21	

Within the limited sample size of our study analyzing HER2 expression in cervical cancer using validated methods of analysis, we found no association of FISH status with age, ECOG, histology, and FIGO stage. It is still unclear whether or not its expression correlates with response or prognosis. A study found a negative correlation between the over-expression of HER2 with radiation response [33]. However, we found no relationship between response to chemoradiation and HER2 status (either HercepTest 2+ or 3+ or FISH status). The prognostic value of HER2 expression is still controversial. Its over-expression correlates with either better [34], worst [35, 36] or does not correlate [37, 38] with survival. Here we show in Table 3 that the patient's characteristics and treatment factors were well-balanced regarding the FISH status. Despite this, FISH-positive patients have a non-statistically significant trend for more reduced survival, suggesting that HER2 amplification could portend a more aggressive tumor behavior, however, the study was not aimed to demonstrate its prognostic value upon survival. Therefore, the prognostic role of HER2 in cervical cancer remains to be confirmed.

Conclusions

We demonstrate that HER2 protein is over-expressed in a small subset of invasive cervical cancer and that unexpectedly, gene amplification is frequent, occurring in 29.5% of cases. We stress that did the analysis using validated methods for the procedures and reports used for breast cancer. Our results must be seen with caution but must encourage further testing in a higher number of patients and different populations. Ideally, studies should include HER2 gene mutation testing as activating mutations have been reported in cervical cancer [39] and could also be targeted [40]. The confirmation of our findings should be followed by clinical trials testing anti-HER2 therapy in invasive cervical cancer.

Authors' contributions

All the authors' substantially contributed to the experimental work, in the analysis and interpretation of the results as well as in the preparation and/or critical review of the present manuscript. Each author has approved the manuscript version that will be sent for the editor's consideration.

Ethics approval and consent to participate

The study was approved by the Instituto Nacional de Cancerologia Ethics Committee (rev/10/15).

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Conflict of Interest

The authors declare no conflict of interest.

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