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RESEARCH

Measurement of HER2 in Saliva of Women in Risk of Breast Cancer

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Abstract HER2 amplification can be present in ductal carcinoma in situ (DCIS). The aim of the present study was to test the feasibility of measuring soluble HER2 in the saliva of patients at risk of breast cancer towards early diagnosis and prognosis. Women with lesions classified as 4 according to BIRADS and women with spontaneous nipple discharge (NAF) were recruited for this study. Quantification of soluble HER2 in saliva was performed using the enzyme immunoassay ELISA. Median values of HER2 were quantified in saliva of the control groups and in the patient groups. The statistical test nonparametric Mann–Whitney was applied for the evaluation of median differences. Although the medians increased with the severity of the clinical status, no significant difference was found in all possibilities ($p > 0.05$) when comparing the medians among the patients groups. Interestingly, inter-individual HER2 quantity variations in the saliva were detected in this study in some subjects from each group. Considering possible inter-individual variations, research on saliva-based circulating HER2 has to be reinforced to ensure its correct application in diagnosis, treatment and in follow-up of breast cancer patients. Older and current issues surrounding the controversy about the appropriate methods for HER2 evaluation are discussed.

Keywords Circulating HER2 · Saliva · NAF · Breast cancer · Impalpable lesion

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Introduction

Despite screening by mammography, many cases of breast cancer are still diagnosed at a late stage and result in death or expensive palliative treatments. Many aspects contribute to the late stage diagnosis; one of which is misinterpretation of mammography. To distinguish between cancer and benign disease by examination of images is usually not possible when the lesion is small.

BIRADS (Breast Imaging Reporting and Data System) is a radiological classification (from 0 to 6) which aims to standardize mammography reports by taking into account the stage of diagnosis and the recommendation of action. This model was introduced by the American College of Radiology (ACR). A lesion rated as 4 in BIRADS is a lesion meant to be investigated by an invasive procedure since it can be either benign or malignant. Unfortunately, the type 4 lesion leads to biopsies, most of which are unnecessary.

Generally, about 10 % of spontaneous nipple discharge is a consequence of a malignancy [1]. Nevertheless, distinguishing between malignant and benign causes of nipple discharge is complicated. In some situations, it is necessary to perform invasive procedures which are not pleasant for the patients. Spontaneous nipple discharge can be also referred as NAF (Nipple Aspirate Fluid).

For diagnosis saliva has advantages over blood or tissue-based assays because it is easily collected in a non-invasive way. Currently, the quantification of circulating proteins and

hormones (cortisol, dehydroepiandrosterone, progesterone, testosterone, estradiol and estriol) in saliva is feasible. The mechanism of transportation of proteins and ions from serum into salivary gland ducts are not completely known. However, both active and passive pathways of transportation have been suggested [2].

HER2 (also called neu/ErbB2) is typically a tyrosine kinase transmembrane receptor, belonging to the epidermal growth factor family, that can be also be found soluble in plasma or serum as well as in saliva of a subset of breast carcinoma patients [3–7]. HER2 amplification that is detected in about 20 % of breast carcinomas (the HER2 group) predicts a more aggressive clinical course. Fortunately, HER2 target therapy has been developed. Significant benefits were demonstrated with the use of Transtuzumab® (HER2 targeted monoclonal antibody) and other drugs that inhibit the tyrosine kinase pathway [8, 9]. The HER2 group has been characterized according to genetic signatures [10]. At the cellular level, the main characteristic of this group, in addition to HER2 overexpression, is the normal expression of the hormone receptors for progesterone and estrogen. Determination of the HER2 positive tumor still depends on invasive methods conducted by biopsy or surgery, followed by immunohistochemistry (IHC) revelation that is not a reliable method. Therefore, development of tests that look for molecular markers, such as soluble HER2, in body fluids at diagnosis and during follow-up of treatment are desired.

HER2 amplification can be present in ductal carcinoma in situ (DCIS) [11, 12]. Therefore, the aim of the present study was to test the feasibility of measuring soluble HER2 in the saliva of patients at risk of breast cancer towards early diagnosis and prognosis. Women with lesions classified as 4 according to BIRADS and women with spontaneous nipple discharge (NAF) were recruited for this study. Issues surrounding the controversy about the appropriate methods for HER2 evaluation are under discussion.

Materials and Methods

Patients and Controls

All women involved in the study were adults, living in Rio de Janeiro, Brazil, and gave their informed consent. Saliva samples were collected between 2008 and 2009. Women included in this study had no previous history of breast cancer, were not pregnant or lactating for the previous 6 months before saliva collection.

Four groups of women were analyzed: the negative control group; the group with spontaneous nipple discharge; the group with impalpable lesions classified as 4 according to BIRADS that were submitted to investigative biopsy; and as positive controls a group of breast carcinoma patients positive for HER2 (3+) by IHC (DAKO antibody), Fig. 1.

Saliva samples of HER2 positive patients were collected before treatment. The histopathologic classification of tumor types was based on the World Health Organization (WHO). More details about these 4 groups are shown at Table 1. An example of a ductal ectasia that caused the nipple discharge is shown in Fig. 2. Figure 3 shows a papiloma that was found in one of the cases with nipple discharge, coexistent with ductal ectasia.

The subjects were required to abstain from eating, drinking, smoking or using oral hygiene products for at least 1 h before saliva collection. One milliliter of saliva was collected from each subject in a sterile flask. Saliva samples were subjected to centrifugation at 14.000 g for 10 min. Two microliter of 0.5 M EDTA and 1 μL of 10 mM PMSF were added to the supernatants and stored at −70 °C until analysis.

HER2 Quantification by ELISA

Quantification of soluble HER2 in saliva was performed using the enzyme immunoassay ELISA kit sp185 HER2 ELISA, IB49607 from IBL America according to the manufacturer's instructions. All experiments were duplicated. One hundred microliter of saliva were used without dilution in the ELISA assay. HER2 concentration was determined in U/mL and in U/mg of total protein.

Quantification of total protein in the saliva was performed using bicinchoninic acid method (Pierce Chemicol Co., No. cat.23225) according to the manufacturer's instructions. 25μL aliquots of diluted saliva (1:2) were added in a microplate. Absorbance was measured in an ELISA reader (Molecular Devices SpectraMax 190) with SoftMax Pro 4.3 SL. Total protein concentrations were determined using a standard curve made with BSA with varying concentrations from 0 to 2 mg/mL.

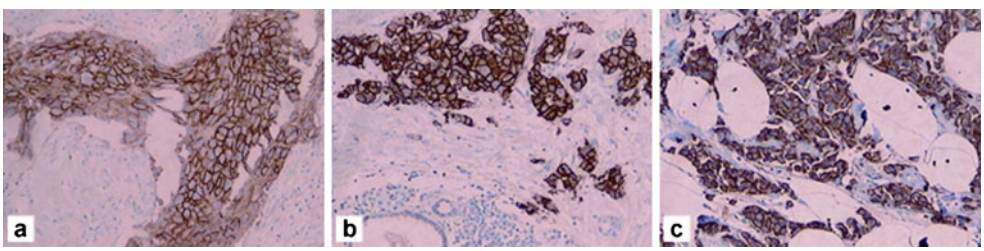

Fig. 1 Showing the HER2 immuhistochemistry of the 3 positive cases (**a**, **b**, **c**). Magnification: **a** – 100X; **b** – 100X; **c** – 200X

Table 1 Clinical characteristics of groups analyzed for HER2 in the saliva

Groups	N	Age average (years)	Hospital	Clinical characterization
Control	11	52.5	HUGG	Women with no breast dysfunction.
Nipple discharge	28	50.2	HUGG	Women with mammary duct ectasia. One of them also showed a papiloma.
Impalpable lesions – BIRADS 4	7	61	INCA	After investigation, tumors from 2 women were negative for malignancy. The others were classified as DCI (1 case grade I, 2 cases grade II and 2 cases grade III). All confirmed breast carcinoma tumors were HER2 negative.
HER2	3	63.6	INCA	HER2 positive patients. Tumors from 2 patients were classified as DCI grades I and II, while the third case was classified as DLI+CDIS.

HUGG Hospital Universitário Gafree e Guinle, Rio de Janeiro, *INCA* Instituto Nacional de Câncer, Rio de Janeiro, *DCI* Invasive Ductal Carcinoma, *DLI* Invasive Ductal Lobular Carcinoma, *CDIS* Ductal Carcinoma in situ

Statistical Analysis

Values of Median and Standard deviation were calculated. The statistical test nonparametric Mann–Whitney was applied for the evaluation of median differences, two by two, among the 3 patients groups versus the controls' and among the patients' groups themselves. The data was analyzed in PASW statistical program, version 18. In all statistical tests the level of 5 % of significance was considered ($p<0.05$).

Results

Table 2 shows the median values of HER2 that were quantified in saliva of the control groups and in the patient's groups. Although the medians increased with the severity of the clinical status, no significant difference was found in all possibilities ($p>0.05$) when comparing the medians (in U/mL and in U/mg of total protein) among the patients groups.

Discussion

Measurement of HER2 in breast cancer patients and the resulting clinical information represent a challenge since its discovery in the 80's. In a cohort of 189 primary tumors, HER2 was found to be amplified from 2- to greater than 20-fold in 30 % of the tumors [13]. Correlation of gene amplification with several disease parameters was evaluated. HER2 gene overexpression was found be a significant predictor of both overall survival and time to relapse in patients with breast cancer, retaining its significance even when adjustments were made for other known prognostic factors. Moreover, HER2 amplification showed greater prognostic value than most known prognostic factors, including hormonal-receptor status, in lymph node-positive disease. However, in about the same time, many publications produced contradictory results about HER2 real value.

HER2 amplification was assayed by Southern Blot method in 362 tumors from patients with primary breast cancer (185 node-positive patients and 177 node-negative patients) [14]. The overall amplification rate was 33 % (30 % for node-negative patients; 31 % for patients with 1–3 positive nodes; 40 % for patients with >3 positive nodes). Gene copy number was not found associated with axillary lymph node status, steroid receptor status, or patient age but was weakly correlated with the size of the primary tumor. Amplification of the HER2 gene was not correlated with either disease-free or overall survival in univariate or multivariate analyses. The results were unambiguously negative for patients with node-negative disease. Thus, the authors concluded that HER-2 amplification would only be of marginal utility as a prognostic factor for predicting clinical outcome. Schroeter et al. [15] performed a retrospective study of HER2 amplification applying IHC in 276 breast cancer samples from 253

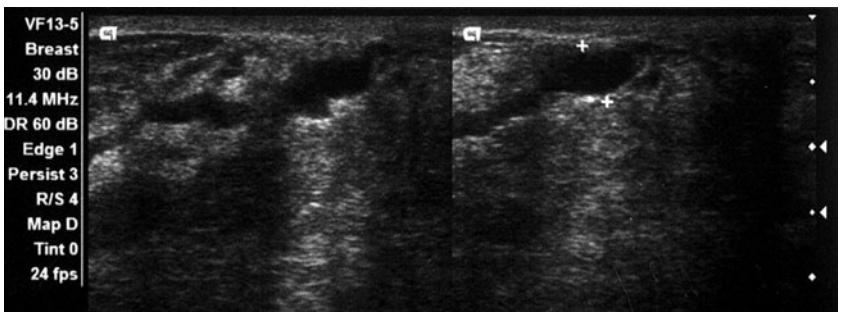

Fig. 2 Duct ectasia detected by ultrasonography

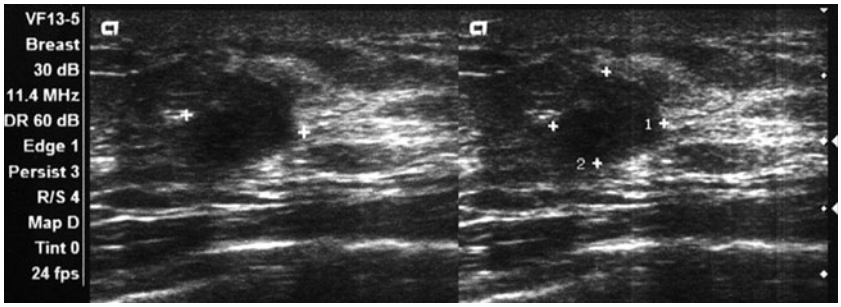

Fig. 3 Papiloma found in one the patients with nipple discharge and duct ectasia, detected by ultrasonography

patients. The follow-up period was 7 and 12 years. This study concluded that there was a significant difference in prognosis the first years after diagnosis, but this difference seemed to vanish in a longer follow-up period of 12 years. In another study, the prognostic value of HER2 amplification was also investigated in 230 node negative breast cancers by IHC [16]. Patients follow up was at least 7 years after primary treatment. Positive immunostaining was observed in 20.9 % of cases, whereas strong diffuse positivity was recorded only in 8.7 % of cases. A significant association of HER2 expression to prognosis was observed only for cases showing a strong diffuse immunostaining, but such association was no longer statistically significant at multivariate analysis adjusting for other prognostic factors. The authors concluded that HER2 expression is of no value to predict the clinical course of node negative patients.

A different conclusion was reached when the association of HER2 overexpression and the risk of recurrence in node-negative breast cancers was tested by another group [17] applying IHC. The cohort was composed of 105 patients who experienced recurrent disease, matched with 105 women with no recurrence (disease-free controls). The risk of developing recurrent disease in node-negative women with any level of HER-2 amplification was 3.0 times more in relation to the controls, while the group of patients with high amplification had a risk of recurrence 9.5 times greater than the controls. These results demonstrated that HER2 was an independent indicator of increased risk of developing recurrent disease in women with node-negative breast cancer. The authors observed that although frozen tissue is optimal for analysis, formalin-fixed, paraffin-embedded tissue can yield meaningful results when reagents of sufficient sensitivity and specificity are used. The need of scoring in conjunction with computerized image analysis was stressed.

Today, it is generally accepted that there is a significant correlation between HER2 overexpression and poor prognosis in patients with node-positive breast cancer. HER2 status predicts response to therapy and, therefore, guides treatment decision-making. However, measuring of HER2 continues to challenge. Currently, the gold standard for measuring HER2 is by IHC that is based on a semi-quantitative eye-scored counting and is expressed in numbers from 0 to 3+. Scoring 0 and 1+ are considered negative; 2+ is weak positive and the tissue should be submitted to FISH (Fluorescence in situ hybridization) for confirmation [18]; 3+ is considered positive. Disagreements or variations in HER2 scoring among pathology laboratories have been reported indicating that the actual scoring tests to determine HER2 status show pitfalls [19, 20]. This problem can results in a great impact on the treatment of some breast cancer patients, creating situations in which patients requiring target therapy may not receive it and the contrary maybe also happing. At least in part, the pitfalls in HER2 measurement are due to *difficult-to-intepret* methodological approaches. This issue is far from ending.

More problems concerning HER2 measurement were revealed when intra-individual variations were described at diagnosis and at recurrence. A retrospective tissue-based study that investigated the correlation of intra-individual HER2 status between primary breast cancers and corresponding recurrences in a population derived cohort, has concluded that in 10 % of the patients the HER2 status had changed, from positive to negative and vice versa [21]. And, in a significant number of patients (56.9 %) the nodal metastasis showed discordance in comparison to the primary tumors for both HER2 scoring and for other breast cancer molecular markers [22]. This heterogeneity might account for a significant proportion of treatment failure.

Concerns about the HER2 measurement emphasize the need for research in HER2 measurement in saliva as an alternative or complementary method. However, our results do not totally corroborate with that. HER2 medians (Table 2) increased from the control group, passing through

Table 2 HER2 quantification in the saliva of the four groups assayed for HER2 in the saliva

Groups	Median U/mL (SD)	Median U/mg of total protein (SD)
Control	37.38 (129.48)	28.97 (63.63)
Nipple discharge	43.15 (113.04)	26.17 (106.74)
Impalpable lesions	149.9 (111.36)	101.97 (56.17)
HER2	296.48 (163.15)	122.01 (63.02)

SD Standard Deviation

the risk groups (nipple discharge and impalpable lesions) up to HER2 group, with the exception of control and nipple discharge groups (U/mg of proteins) which medians are slightly inverted. However, the comparative statistical analysis of the medians, in either U/mL and or in U/mg of total protein, has not identified significant differences. Each group (control, nipple discharge, impalpable lesions and HER2) showed major internal variations that were derived from some subjects who contributed to the broad standard deviations found (Table 2). For example, in the control group, 2 subjects showed HER2 quantities in the saliva much higher than in the HER2 positive group. And after 3 years of follow up, none of these 2 subjects shows a sign of carrying breast cancer lesions. For unknown reasons, inter-individual HER2 quantity variations in the saliva were detected in this study in some subjects of each group. In conclusion, considering possible inter-individual variations, research on saliva-based circulating HER2 has to be reinforced to ensure its correct application in diagnosis, treatment and in follow-up of breast cancer patients.

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