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Research Article

Electrochemical miRNAs Determination in Formalin-Fixed, Paraffin-Embedded Breast Tumor Tissues Association with HER2 Expression

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Abstract

Whilst breast cancer (BC) management continues to improve, the requirement of novel assays to support the accuracy of HER2 clinical diagnosis remains. Investigation of the potential for miRNAs, recently identified biomarkers of both predictive and prognostic value in BC, to fulfill this role, holds much promise. In this communication we report a new electrochemical strategy, to determine miRNA in formalin-fixed, paraffin-embedded (FFPE) tissue sections from different breast cancer profiles of human patients. We have recently developed a rapid and accurate method to analyze two different target miRNAs in tissue samples. This strategy involves the selective and efficient capture of the DNA/miRNA heteroduplexes, formed by hybridization between the target miRNA and a specific biotinylated DNA probe. This method also includes antibody-conjugated magnetic beads, their labeling with an enzymatic polymer and amperometric transduction using the H_2O_2/hydroquinone (HQ) system at disposable screen-printed carbon electrodes (SPCEs). Quantitative results achieved with the electrochemical bioplatform in the analyzed FFPE tissue samples demonstrated an association of both miRNAs expression with the clinico-pathological features of the specimens. Moreover, the feasibility to identify HER2 tumor subtype proved a similar sensitivity and accuracy as that obtained with fresh-frozen samples.

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ABBREVIATIONS

ASCO: American Society Of Clinical Oncology; BC: Breast Cancer; CAP: College Of American Pathologists; FFPE: Formalin-Fixed, Paraffin-Embedded; HER2: Human Epidermal Growth Factor Receptor 2; HQ: Hydroquinone; IHC: Immunohistochemistry; ISH: *In Situ* Hybridization; LOD: Limit of Detection; PBS: Phosphate-Buffered Saline; RNA_t: Total RNA; Strep: Streptavidin; TNBC: Triple Negative Breast Cancer; SPCE: Screen-Printed Carbon Electrode.

INTRODUCTION

Breast cancer (BC) is the most frequently diagnosed malignancy amongst females worldwide, accounting for 17.81% of all cancer diagnoses and responsible for 10.41% of cancer-related deaths [1].

BC can be currently classified into the following clinically meaningful subgroups: luminal A, luminal B, basal-like/triple-negative, and human epidermal growth factor receptor 2 (HER2)

positive tumors [2]. Overexpression of HER2 (observed in 20-30% BCs) is associated with a relatively poor prognosis and is predictive for therapeutic response.

Currently, all primary BC are semi-quantitatively tested for HER2 mutation using a combination of immunohistochemistry (IHC) and *in situ* hybridization (ISH) and separated into HER2-positive or HER2-negative BC groups.

Regarding current American Society of Clinical Oncology (ASCO) and College of American Pathologists (CAP) guidelines, a tumor should be considered HER2-positive (3+) by IHC if greater than 10% of the cells show uniform strong and complete membrane staining or if there are ≥ 6 HER2 copy number signals per cell by a single-probe ISH assay. Patients not meeting these criteria are considered to have a HER2-negative tumor. However, there is a considerable number (around 20% by Overcast et al., [3]) of BC tested that are considered "borderline" or equivocal by IHC because the circumferential staining of 10% of cells is incomplete or weak or there is less than 10% of tumor cells strong stained. This subgroup need to be tested by an ISH assay (reflex ISH studies), which, in turn, may also have an equivocal result (from 4-10% [3,4]). Therefore, assessing HER2 status in FFPE tissue, albeit crucial for patient management is not always straightforward with the current available laboratory tools.

In addition, several studies have demonstrated that the HER2 status of a primary tumor may not entirely and accurately reflect the HER2 status of a metastasis from the same tumor when both are evaluated by these IHC/ISH tests. Indeed, an in-depth analysis of the publications related to ErbB2 testing demonstrated that on average, 20% of the ErbB2-negative patients maybe misclassified regarding ErbB2 primary breast tumor status (~2.5 million cases worldwide) and may develop a ErbB2-positive recurrent breast cancer. This significant population of women missed the opportunity to be treated at earlier stages with approved HER2-targeted therapies or to participate in clinical trials with new HER2-targeted therapies.

Several studies have demonstrated the association of miRNA expressions with intrinsic subtypes of BC and resistance to HER2-targeted therapy [5,6]. These findings suggested that the accurate determination of these biomarkers could be combined with the results provided by IHC/ISH to improve the sensitivity/accuracy of HER2 diagnosis and to predict endocrine therapy resistance.

As being one of the most extensively studied non-coding ribonucleic acids, microRNAs (miRNAs), a class of endogenous non-coding RNAs from 19-25 nucleotides in length, have recently emerged as convenient biomarkers for early and reliable breast cancer diagnosis. They have shown tissue-specific expression, correlation with clinico-pathological prognostic indices and dysregulation in breast cancer [7]. These tiny biomarkers have demonstrated their roles to enable disease screening in high-risk patients (as diagnostic biomarkers) and to evaluate several disease parameters (as prognostic biomarkers) [8]. Although lots of efforts have been put for the detection of miRNAs, as predictive and prognostic markers, accurate detection and quantification of miRNAs pose numerous analytical challenges due to their short sequence length, high sequence similarity among family members, low abundance and susceptibility to degradation [9,10].

In this context, electrochemical biosensors are attractive alternatives to conventional methodologies for miRNA determination (Northern blot, microarray and qRT-PCR) in terms of fast response, cost, ease of operation, automation and on-site analysis, meeting better the requirements needed for routine detection [11-16]. Moreover, since the available methodologies for miRNAs detection provide only a limited degree of qualitative data, it would be desirable the exploitation of novel strategies capable to provide accurate quantitative values of miRNAs in a sensitive and straightforward manner [17].

We have recently developed a new attractive electrochemical methodology for miRNAs determination based on the use of magnetic micro carriers, an antibody specific for DNA/RNA heteroduplexes and amperometric detection at disposable electrodes [15]. This amperometric immunosensing approach, which allowed the sensitive and selective determination of any target miRNA was successfully applied to the determination of the endogenous content of two mature miRNAs (miRNA-21 and miRNA-205) in total RNA (RNA_t) extracted from cancer cell lines and human fresh tumor tissues.

Herein, in order to identify whether any of these two miRNAs could be associated with the expression of HER2, we propose to demonstrate the feasibility of this methodology for detecting these target miRNAs in different HER2 profile formalin-fixed, paraffin-embedded (FFPE) breast cancer tissues sections. Our results demonstrate similar analytical performance of the electrochemical platform for both fresh-frozen and FFPE samples. We pursue a good correlation between the concentration of the target mature miRNAs and the HER2 BC status, thus proving the capability of this electrochemical platform to be used as an accurate tool in BC diagnosis

MATERIALS AND METHODS

Apparatus and electrodes

Amperometric measurements were performed with a CHI812B potentiostat (CH Instruments) controlled by software CHI812B. All measurements were carried out at room temperature. The transducers employed were screen-printed carbon electrodes (SPCEs) (DRP-110, DropSens) consisting of a 4-mm diameter carbon working electrode (WE), a carbon counter electrode and an Ag pseudo-reference electrode. A specific cable connector (ref. DRP-CAC, DropSens) acted as an interface between the SPCEs and the potentiostat.

A magnetic separator (DynaMag™-2, Invitrogen Dynal), a constant temperature incubator shaker (Ivymen-Comecta), a Raypa steam sterilizer, a biological safety cabinet (Telstar Biostar), a temperature freezer (New Brunswick Scientific), a refrigerated centrifuge (Sigma 1-15K), and a Stuart SBH130 Analogue Block Heater were also used.

Reagents and solutions

All reagents used were of the highest available grade. Sodium di-hydrogen phosphate, di-sodium hydrogen phosphate, NaCl and KCl were purchased from Scharlab; hydroquinone (HQ), and

hydrogen peroxide (30%, w/v) were purchased from Sigma-Aldrich.

Protein G-modified magnetic beads (ProtG-MBs, 2.8 μm, 30 mg mL^{-1} Dynabeads Protein G, ThermoFisher Scientific), an anti-DNA-RNA Hybrid [S9.6] Antibody (AbS9.6) (Kerafast, USA) and a streptavidin-horseradish peroxidase (Strep-HRP) conjugate (Roche) were also used.

Buffer solutions, prepared with Milli-Q water (18 MΩ cm at 25^0C), were: phosphate-buffered saline (PBS) consisting of 0.01 M phosphate buffer solution containing 137 mM NaCl and 2.7 mM KCl, pH 7.5 (sterilized after their preparation) and phosphate buffer 0.05 M, pH 6.0. A commercial blocker casein solution (a ready-to-use, PBS solution of 1% w/v purified casein) was purchased from Thermo Scientific.

The DNA and RNA synthetic oligonucleotides (Sigma-Aldrich) used are summarized in Table (1). These four oligonucleotides were dissolved in nuclease free water at 100μM final concentration, aliquoted into smaller volumes and stored at -80°C.

Hybridization procedure

25 μL of 0.05 μM of the corresponding biotinylated antiDNA probe solution and the appropriate amount of the synthetic target or the RNA_t extracted from the biological samples were mixed in a micro-centrifuge tube containing PBS, pH 7.5. The hybridization mixture was shaken at 37°C and 950 rpm for 45 minutes. Control experiments without target miRNA were performed for each hybridization process in order to evaluate the blank signal.

Magnetic beads modification

2.5 μL of ProtG-MBs suspension were transferred into a micro-centrifuge tube and washed twice with 50 μL PBS. The particles were placed in the magnetic concentrator and, after 3 min, in between washings and the supernatant was discarded. Washed MBs were incubated for 45 min at 37°C under continuous stirring (950 rpm) with 25 L of a 2.0 μg mL^{-1} AbS9.6 solution (prepared in PBS, pH 7.5).

After two washing steps with 50μL PBS, AbS9.6-coated MBs were re-suspended in 25 μL of the hybridization mixture solution prepared as indicated in the previous section and incubated during 45 min (950 rpm, 37°C). The b-DNA/miRNA-AbS9.6-MBs were washed twice with 50 μL of the blocker casein solution and, thereafter, re-suspended in 25 μL of a 0.004 U mL^{-1} Strep-HRP solution (prepared also in the blocker casein solution) and incubated for 30 min (37°C, 950 rpm) for enzymatic labeling. The resulting HRP-DNA/miRNA-AbS9.6-MBs were then washed twice again with 50 μL of blocker casein solution. Finally, the modified-MBs were re-suspended in 45 μL of 0.05 M phosphate buffer solution (pH 6.0) to perform the amperometric detection.

Electrochemical measurements

The 45 μL of the modified MBs suspension were magnetically captured onto the WE surface of the SPCE after placing it on a homemade casing of Teflon with an encapsulated neodymium magnet to ensure a reproducible and stable capture of the MBs onto the WE surface [15]. Then, the SPCE/magnet holding block assembly was immersed into an electrochemical cell containing 10 mL of 0.05 M phosphate buffer of pH 6.0 and 1.0 mM HQ (prepared just before starting the electrochemical measurement). Amperometric measurements in stirred solutions were made by applying a detection potential of -0.20 V *vs.* Ag pseudo-reference electrode upon addition of 50 μL of a 0.1 M H_2O_2 solution until the steady-state current was reached (approx. 100 s). The amperometric signals given through the manuscript corresponded to the difference between the steady-state and the background currents and unless otherwise indicated, the presented data corresponded to the average of at least three replicates (confidence intervals calculated for $\alpha = 0.05$).

FFPE breast tissues and RNA_t extraction

BC patients signed the corresponding informed consent and the study had the approval of the University Hospital of Getafe´s Ethics Committee. Samples from 15 breast cancer (T) and paired normal adjacent (NT) FFPE tissues were collected at the University Hospital of Getafe (Madrid, Spain) (see Table (2)). HER2 status of all samples was established by IHC and subsequent SISH (silver-based in situ hybridization) when needed following ASCO/CAP recommendations [18]. Five of them (cases 1, 2, 3, 4 and 10) were classified as Luminal A tumors after showing positive estrogen and progesterone receptor (ER and PR) IHC staining and an equivocal (2/3) immunostaining for HER2 with a subsequent SISH analysis that failed to show HER2 amplification (HER2/CEP17 ratio under 2.0 and average HER2 copy number under 4 signals/cell). These 5 cases are, therefore, HER2-negative, and were designed as the group of HER2-equivocal in our study. Another 5 cases (5, 6, 9, 12, 13) had negative immunostaining for ER and PR as well as for HER2. All 5 cases were considered triple-negative BC and included as HER2-negative group in our study. The remaining 5 cases (7, 8, 11, 14 and 15) had variable staining for hormonal receptors but a positive and complete circumferential strong IHC staining for HER2 in more than 10% of tumor cells. These constituted the cases of the HER2-positive study group. For RNA_t extraction, 5 microtome sections from each paraffin tumor block, 6 micron-thick were placed in eppendorf tubes.

RNA_t was extracted from 1 eppendorf tube of each of all these FFPE tissues using the microRNA easy FFPE kit (QIAGEN) following the recommended protocol and eluting the RNA_t with 30 μL of RNase-free water. RNA_t quality and concentration were evaluated by measuring the absorbance with an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). In all cases, the 260/280 and 260/230 absorbance ratio values obtained assess the purity of RNA_t extracted.

Statistical analysis

A one-way analysis of variance was performed to check significant differences between the three analyzed BC subtypes for both target miRNAs, $P < 0.05$ indicates a statistical significance. In case of statistically significant differences between the analyzed BC subtypes, a Bonferroni's multiple range test was used to evaluate whether the obtained mean values were significantly different from each other.

Unless otherwise indicated, the presented data corresponded

Table 1: Oligonucleotides used in this work.

Oligonucleotide	Sequence (5´ - 3´)
b-antiDNA-205 Probe	5´-AGACTCCGGTGGAATGAAGGA-Biotin-3´
Target miRNA-205	5´-UCCUUCAUUCCACCGGAGUCU-3´
b-antiDNA-21 Probe	5´-TCAACATCAGTCTGATAAGCTA-Biotin-3´
Target miRNA-21	5´-UAGCUUAUCAGACUGAUGU-3´

Table 2: HER2 tumor status established by IHC/SISH Assays.

Breast tissue	Subtype
1	Luminal A (HER2 2/3)*
2	Luminal A (HER2 2/3)*
3	Luminal A (HER2 2/3)*
4	Luminal A (HER2 2/3)*
5	TNBC (0/3)
6	TNBC (0/3)
7	HER2 + (3/3)
8	HER2 + (3/3)
9	TNBC (0/3)
10	Luminal A (HER2 2/3)*
11	HER2 + (3/3)
12	TNBC (0/3)
13	TNBC (0/3)
14	HER2 + (3/3)
15	HER2 + (3/3)

Abbreviations: TNBC: Triple Negative Breast Cancer; *: cases equivocal by IHC, tested by SISH.

to the average of at least three replicates and the confidence intervals were calculated for $\alpha = 0.05$.

RESULTS

The strategy we recently developed by our group for selective and rapid miRNAs quantification [15] has been employed for the determination of the target mature miRNAs in FFPE breast cancer tissues. This strategy relies on the efficient hybridization of each target miRNA with a corresponding specific biotinylated DNA probe, the selective capture of the resulting b-DNA/miRNA heteroduplexes with a specific DNA-RNA antibody immobilized in an oriented way on Prot G-MBs and labeling of the captured biotinylated DNA/miRNA heteroduplexes with Strep-HRP conjugate (Figure 1). Upon magnetically capturing of the modified MBs onto the WE of a SPCE, amperometric detection at – 0.20 V (*vs* the Ag pseudo reference electrode) was carried out by measuring the catalytic reduction current of added H_2O_2 as enzyme substrate, using hydroquinone (HQ) as redox mediator [19]. The current, related to the amount of HRP immobilized on the surface (reactions involved are also shown in Figure (1)), is proportional to the number of b-DNA/miRNA duplexes captured onto the MBs and, hence, to the concentration of the target mature miRNA in the sample under analysis.

The main goal of this communication was to demonstrate the usefulness of this electrochemical methodology for the fast, simple and reliable determination of mature miRNAs in FFPE breast tissues sections, since none of the previously reported electrochemical biosensing methodologies have been applied to the analysis of this type of samples. For such purpose two relevant miRNAs, miRNA-21 and miRNA-205, associated with BC and linked to resistance to HER2-targeted therapies [6,20-23], were selected. MiRNA-205 is a new oncosuppressor in breast cancer able to interfere with the proliferative pathway mediated by HER receptor family and involved in cell fate and miRNA-21, a highly prevalent malignancy biomarker up-regulated in BC.

A key variable in miRNA determination is the quantity of RNA_t extracted from FFPE breast tissue sections. At this respect, we compared two different approaches: 1.0µg of RNA_t per each of the miRNA to be determined and, the only use of 1.0µg amount of RNA_t using a sequential approach. In this later case, the extracted RNA_t was firstly supplemented with the b-antiDNA-205 probe which selectively hybridized with miRNA-205. After capturing the b-antiDNA-205/miRNA-205 duplex by the AbS9.6-MBs, the remaining RNA_t extract was supplemented with the b-antiDNA-21 and a new batch of AbS9.6-MBs was used for the selective capture of the b-antiDNA-21/miRNA-21. Figure (2) shows the lack of significant differences in the amperometric signals provided for both target miRNAs by using the above mentioned protocols. This constitutes a very relevant and practical result, considering the limited amount of RNA_t extracted from clinical samples.

The endogenous content of mature miRNA-21 and miRNA-205 was determined in raw total RNA (RNA_t) extracted from 15 different human breast cancers (T) and their paired normal adjacent (NT) FFPE breast tissues (5 from each of the 3 different HER-2 expression profiles: HER2+(3/3), Equivocal (2/3) and HER2-(0-1/3), see detailed information in Table (2)). As it is shown in Figure (3), and in agreement with previous reports, miRNA-205 was down-regulated in T tissues [24,25] whereas miRNA-21 was highly up-regulated in breast T tissues compared to the matched NT breast samples [26]. It is worth mentioning that no significant differences were found for measurements made in NT FFPE human breast tissues for any of the two miRNAs described. Therefore, a single amperometric response of these samples for each miRNA is included in the figures.

Since no apparent matrix effect was observed when ≤ 1 µg of extracted RNA_t was analyzed, quantification of target miRNAs in these complex biological samples was accomplished simply by interpolation of the amperometric signals measured in the extracted RNA_t into the calibration graph constructed with each miRNA standard solutions. The slope values of the linear calibration plots constructed for miRNA-21 in the presence of 1.0 µg RNA_t extracted from NT (9,070±504) nA nM^{-1} and T (10,256±606) nA nM^{-1} were not significantly different than that obtained with miRNA-21 standard solutions (9,548±211) nA nM^{-1}. Final results for the determination of both miRNAs in FFPE breast tissues are summarized in Table (3) and displayed in Figure (4).

A one-way analysis of variance was performed for each miRNA using data presented in Table (3). The ANOVA test showed statistically significant difference between the three analyzed BC subtypes for both miRNA-21 and miRNA-205 ($P < 0.05$). A Bonferroni's multiple range test was used to evaluate

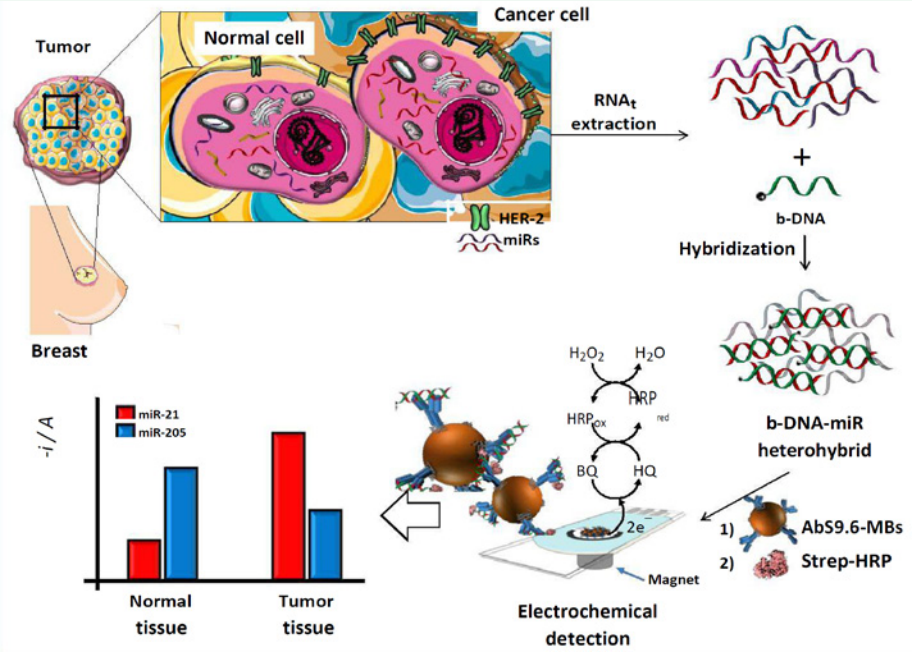

Figure 1 Schematic illustration of the electrochemical approach used for the determination of target mature miRNAs in FFPE breast tissue sections. Relative sizes of the components are not drawn at real scale in order to visualize all of them.

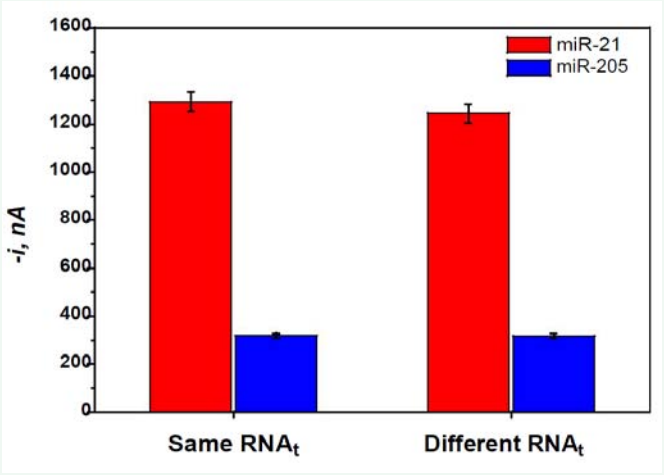

Figure 2 Comparison of the amperometric responses obtained with the electrochemical bioplatform for both target miRNAs using the same 1.0 μg RNA_t extract (a) or two independent 1.0 μg RNA_t extracts (b) from an FFPE HER2+ breast cancer sample. (Error bars estimated as triple of the standard deviation (n=3)).

whether the obtained mean values were significantly different from each other. A statistically significant difference at the 95.0% confidence level among HER2+ and HER2- tumor groups was seen for miRNA-21. Significant differences in miRNA-205 expression were found between the HER2+ and the equivocal group.

DISCUSSION

The results provided by the biosensing platform are in agreement with the function of miRNA-205 as tumor suppressor [21] and of miRNA-21 as an oncogene [27] in BC and with the negatively regulation of miRNA-205 by HER2 overexpression [28]. Results reported by other authors indicated that miRNA-21 expression is upregulated and that its function is increased in HER2+ breast cancer cells and in tumors with drug resistance to trastuzumab [5,29]. It seems that its sole expression can be used to further screen trastuzumab-chemotherapy-resistant HER2+ breast cancer patients [23]. In this sense it is worth to mention that none of our HER2+ BC cases had neoadyuvant anti-HER2 treatment; therefore, the analyzed tissues were always obtained previously to any chemotherapy treatment of the patient.

Moreover, the quantitative results provided by the employed electrochemical platform are in good agreement with the ranges reported by other authors using completely different methodologies and fresh-frozen samples. For instance, Xu et al.,

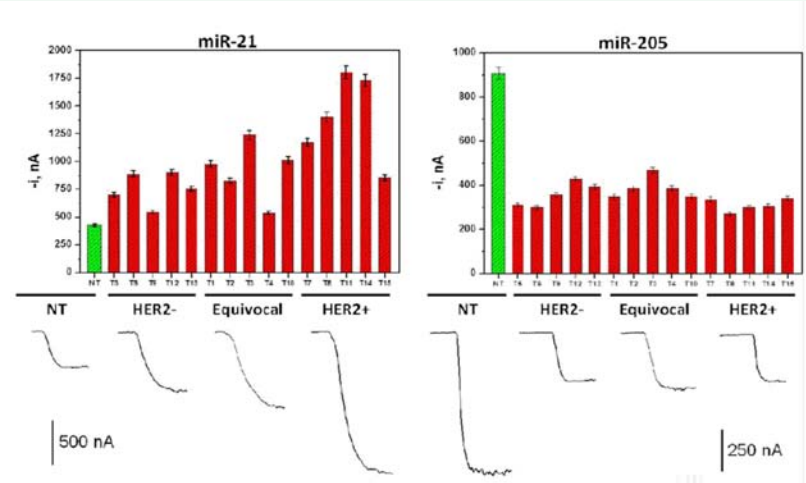

Figure 3 Amperometric responses obtained with the electrochemical bioplatform for mature miRNA-21 (left) and miRNA-205 (right) in 1.0 μ g raw RNA_t extracted from FFPE human breast tissues. Amperometric traces recorded with the electrochemical biosensor for miRNA-21 (left) and miRNA-205 (right) in representative samples. (Error bars estimated as triple of the standard deviation (n=3)).

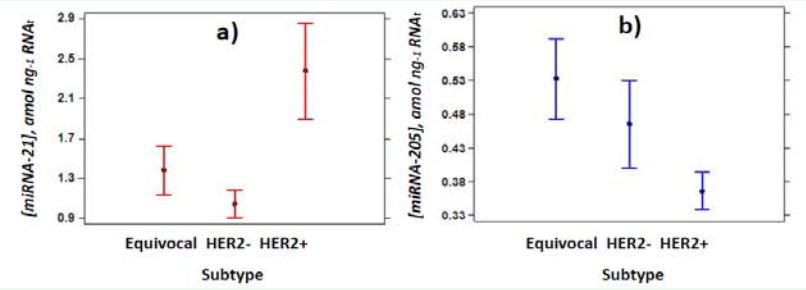

Figure 4 Mean values and standard errors for both biomarkers in the three different HER2 subtypes.

Table 3: Mean concentration of target mature miRNAs (expressed in amol per ng of RNA_t) in FFPE breast tissues.

Pool of samples from	miRNA-21			miRNA-205		
5 different patients	NT	T	T/NT	NT	T	NT/T
HER 2-	0.34	1.05	3.1	1.36	0.47	2.7
Equivocal		1.39	4.1		0.53	2.4
HER2+		2.38	7.0		0.37	3.4

reported miRNA-21 levels of (0.62-8.85)x10^8 and (0.22-1.79)x 10^9 copies per mg of tissue in normal and tumor breast tissues, respectively [17]. Considering the common yield of RNA_t extracted from the samples (1 μg per mg of tissue) these ranges should correspond to (0.1-1.5) and (0.4-3.0) amol per ng RNA_t, which include results provided by the electrochemical bioplatform. It is also worth mentioning that the results found in these FFPE tissue samples are similar to the concentration ranges obtained with T fresh-frozen samples using the same electrochemical platform ((1.6-3.9) amol miRNA-21 per ng RNA_t) [15]. We therefore support that this methodology provides similar sensitivity and accuracy for mature miRNAs determination in both, FFPE and fresh-frozen tissue samples.

These results highlight, for the first time, the capability of this electrochemical biosensor to perform accurate miRNA determination in FFPE which is of great interest considering the multiple benefits of using FFPE tissue samples in clinical research for the diagnosis and therapeutic follow-up of a wide range of cancers and other human pathologies (inflammation, immune-related diseases, etc.). FFPE is the standard method of tissue processing used in Pathology Departments for cancer diagnosis. Working with FFPE samples opens up to a huge potential of novel candidate miRNA as diagnostic or prognostic cancer biomarkers. There is a vast amount of FFPE tissue samples housed in hospitals, clinics, and research facilities, available to be studied which would allow a quicker advance research in disease diagnostics, outcomes, and therapies.

Although the relatively small number of samples analyzed in this work offers little statistical strength, we consider these preliminary results sufficient, as a proof of concept, to outline the

feasibility and potential applicability of this recently developed electrochemical approach to perform quantitative determination of any target miRNA in FFPE tissues. These data certainly serve to pursue the potential applicability of this electrochemical platform, from this exploratory research phase to a larger epidemiological validation study aimed in the reliable assessment of HER2 status of breast cancer patients.

In this context it is important to remark that, in comparison with the state of the art qRT-PCR, the electrochemical bioplatform allows the target miRNA determination to be carried out directly in raw RNA_t extracted from FFPE tissues (without reverse transcription into cDNA) which implies a lower cost and a shorter analysis time. Moreover, the use of simple, portable and cost-effective instrumentation suitable to perform multiplexed detection required by electrochemical transduction, can be claimed as an important practical advantage in the implementation of user-friendly and affordable devices to perform routine and decentralized analysis.

CONCLUSION

In this work, a recently developed electrochemical immunosensing approach for miRNAs determination was applied to the detection of miRNA-21 and miRNA-205 in FFPE breast cancer tissues with different HER2 status by IHC/ISH. Exploratory phase data with 15 FFPE samples revealed results in agreement with the IHC/FISH clinical procedures and with the quantitative ranges established by other authors and showed strong consistency with the results presented using the same electrochemical platform in fresh-frozen tumor breast tissue samples. Results demonstrated also that mean concentration values for both miRNAs vary depending on the HER2 status. A statistical comparison showed significant differences between HER2-positive and HER2-negative tumors for miRNA-21 and between HER2-positive and HER2-equivocal samples for miRNA-205. These findings highlight the potential of this platform to provide complementary data to other tests in the identification of HER2-subtypes patients in just 2 h by determining both miRNAs concentrations. The sensitivity, simplicity and compatibility with the analysis in FFPE samples, makes of this rapid electrochemical methodology a promising tool for high-throughput and multi-miRNAs bioanalysis. Finally, the proof of concept obtained with this electrochemical bioplatform allow to move forward from the exploratory phase onto the validation phase with a larger cohort, under the strict supervision of breast cancer specialized pathologist.

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