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Expression of c-erbB-2 in Breast Cancer Cell Lines as Experimental Receptor of Magnetic Nanoparticles *

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Abstract— **High mortality in breast cancer is associated to a late diagnosis and therapy of the disease. Our research group is working on the development of an innovative technology to promote selective ablation of breast cancer in early stages by the use of high frequency magnetic fields assisted by magnetic nanoparticles. The concept behind the technical proposal is to increase the electrical conductivity of tumoral tissue by the use of bioconjugated "magnetic nanoparticle-monoclonal antibody". Is expected that bioconjugated recognizes specific genes on the surface of cancer cells. The aim of this study was to evaluate the expression of the *c-erbB-2 gene* and c-erbB-2 antigen in breast cancer cells type BT-474, MCF-7 and MDA-MB-231, as previous step to propose the c-erbB-2 protein as receptor of magnetic nanoparticles. The results suggest that the elected breast cancer cell lines show well-differentiated relative expression of the elected gen and antigen, and sems suitable for experimental evaluation of selective targeting by magnetic nanoparticles.**

I. INTRODUCTION

Cancer is a leading cause of death worldwide. Of the 58 million deaths that occurred worldwide in 2005, 7.6 million (13%) were due to cancer and of these deaths, over 70% occurred in developing countries. In Mexico; oncological diseases represent the second leading cause of death in the general population. In particular Breast Cancer (BC) is the leading cause of female mortality.[1],[2],[3],[4]and [5].

The *c-erbB-2* gene is a member of the *erbB*-like oncogene family, this gene is amplified from 2 to greater than 20-fold in 30% of human primary breast cancers and this alteration is associated with disease behavior [6], [7]. Approximately 25% of primary breast and ovarian tumors overexpress the c-erbB-2 protein. [8].

PCR-based assays allow quantitative determination of *c-erbB-2* amplification (Q-RT-PCR) is not routinely used, however has predominantly been used to detect mRNA in peripheral blood and bone marrow samples [9]. The Q-RT-PCR method has shown high concordance with Immunohistochemistry (IHC) and fluorescent in situ hybridization (FISH), which are currently the most commonly used methods to assess c-erbB-2 status in breast tumours. [10], [11], further flow cytometry method is used to detect the level of c-erbB-2 antigen expression in breast cancer cells *in vitro*. [12].

Nanomedicine has shown an important growing in the war against cancer in the last two decades. Early detection and selective therapies are the main limitations of the current cancer treatments. Several techniques at molecular scale have been focused on early detection and treatment of the disease by identification and selective targeting of cancer cells.

Nanomaterials particularly magnetic nanoparticles (MNPs), are increasingly being considered for a number of biomedical applications due to their inherent ultra fine size, biocompatibility and superparamagnetic properties. The functional properties of the MNPs can be tailored for specific biological functions such as drug delivery, hyperthermia or magnetic targeting, magnetic resonance imaging (MRI), cell labeling and sorting, and immunoassays. [13].

In 2005, De Nardo developed bio-reactive probes by coupling ferromagnetic nanoparticles and chimeric monoclonal antibodies. They used alternating magnetic fields in a mouse model to induce ablative toxic effect on HBT3477 cells of breast cancer. Their results showed the potential value of the technique as ablative therapy against cancer [14].

Johng et al. (2006) developed a novel application of cobalt ferrite nanoparticles (50 nm) coupled to rhodamine isothiocyanate (RITC). Nanoparticles were coated with a biocompatible silica layer and polyethylene glycol, and infused in rats through an incision near the tail vein and lumbar lymph nodes. To ensure the nanoparticles migration a magnet was placed just above the lymphatic vessels. Confocal and electronic transmission microscopies were used to observe the nanoparticles location in vivo [15].

Natarajan et al. (2008) described the development of a breast cancer targeting chimeric L6 (ChL6) monoclonal antibody (Bioprobe) linked to various nanoparticles (NPs): 20 nm superparamagnetic iron oxide (SPIO), 30 nm nanoferrite particles (NFP´s) and 100 nm NFP to produce radioimmunoparticles (RINP´s), as well as the feasibility of delivering thermo ablation to cancer cells. They evaluated the targeting and pharmacokinetics of the RINP´s in HBT 3477 breast cancer cells lines and the use of athymic Balb/c nu/nu mice. The *in vivo* tumor targeting and *in vitro* specific energy absorption rate (SAR) were compared for the various NP´s to predict thermal dose deliverable at the cancer tissues by various alternating manetic fields (AMF) amplitudes. The results suggest that is possible to develop optimal nanoparticles for future NP-AMF thermal breast cancer therapy [16].

Zhang et al. (2009) performed a study to evaluate the cellular uptake behavior of F3-peptide conjugated dextran

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coated iron oxide nanoparticles with normal and tumoral cells by cell-surface charge Z potential measurements. They incubated normal breast ephitelial cells (MCF-10A) and cancer ephitelial cells (MCF-7) with F3 peptide conjugated dextran coated iron oxide nanoparticles. This bioconjugate binds to nucleolin that is located in the cytoplasm and surface of MCF-7. Confocal microscopy analysis was used to visualize the nanoparticles into the tumoral cells. Further determine the significant and sensitive changes in Z potential in the different cells lines. The results suggest that the surface Z potential measurements are a promising approach in studying cellular interaction of nanomaterials and describing their interactions between different cells and specific nanoparticles as a valuable biological characteristic. [17].

Acharya et al. (2009) used polymeric nanoparticles with rapamycin as anticancer drug and coupled to a monoclonal antibody directed against receptor of epidermal growth factor (EGFR) expressed in breast cancer cells. The anticancer effect of the formulation of rapamycin alone, rapamycin-nanoparticles and rapamycin-nanoparticles bioconjugated to the anti-EGFR antibody were evaluated by cytotoxicity in vitro studies in MCF-7 breast cancer cells. Their results showed the possibility to create bioconjugates nanoparticle-antibody [18].

Hathaway et al. (2011) showed the feasibility of detecting breast cancer cells labeled with magnetic nanoparticles using ultrasensitive magnetic field sensors. They characterized by flow cytometry HER2 (c-erbB-2) expression in breast cancer cell lines. Also employs ferrite nanoparticles bound to anti-Her2 antibody incubated with breast cancer cells that express at different proportions to the Her2 marker. The results suggest that the antibody magnetic nanoparticles conjugated represents a promising alternative for the detection of breast cancer *in vitro* and *in vivo.* [12].

Our research group is working on the development of an innovative technology to promote selective ablation of breast cancer in early stages by the use of high frequency magnetic fields assisted by magnetic nanoparticles. The concept behind the technical proposal is to increase the electrical conductivity of tumoral tissue by the use of bioconjugated "magnetic nanoparticle-monoclonal antibody". Is expected that bioconjugated recognizes specific antigen on the surface of cancer cells. The aim of this study was to evaluate the expression of the *c-erbB-2* gene and c-erbB-2 antigen in breast cancer cells Lines BT-474, MCF-7 and MDA-MB-231 as previous step to propose the c-erbB-2 protein as experimental receptor of magnetic nanoparticles coupled to specific Monoclonal antibody (Mab) anti- c-erbB-2 antigen.

II. METHODOLOGY

A. Experimental Design

Breast cancer cell lines: BT-474, MCF-7 and MDA-MB-231 were cultivated in specific conditions described in subsection below. Typification of the expression of the *c-erbB-2* gene by RT Q-PCR and expression of the c-erbB-2 antigen by Flow Cytometry were developed. Relative expressions of the *c-erbB2* gene were estimated between cell lines as well as the fluorescence change in cells without Mab anti-c-erbB2 antigen.

B. Cell Lines Cultures

BT-474, MCF7 and MDA-MB-231 breast cancer cells were acquired from the American Type Culture Collections (rockville, MDA) and seeded in 75 cm2 polystyrene bottles with Hybri-Care (ATCC Catalog No. 46-X), Eagle's Minimum Essential (ATCC Catalog No. 30-2003) and Leibovitz's L-15 (ATCC Catalog No. 30-2008) mediums respectively; reconstituted or suplemented acordingly technical indications of the cells supplier. Every cell type were incubated at 37°C with a CO2 and Oxygen controlled atmosphere.

C. RT Q-PCR of c-erb-B2 expression.

To evaluate the expression of *c-erbB-2* gene three independent biological replicates of cell lines culture was carried out. Total RNA was isolated, quantified, DNase treated and finally, RT Q-PCR was performed using the corresponding cDNA from each sample. All RT Q-PCR assays were analyzed in triplicate. Primers express software for Real-Time PCR ver 3.0 Applied Biosystems was used to design primers for RT Q-PCR. The elected oligonucleotide sequences used as primers for the genes studied are summarized in Table 1.

RNA was reverse transcribed using the GeneAmp RNA PCR Core Kit (Applied Biosystems), according to the manufacturer's protocol. RT Q-PCR was performed in the ABI PRISM 7000 Sequence Detection System (Applied Biosystems) by monitoring in real time the increasing of fluorescence using the SYBR Green PCR Master Mix (Applied Biosystems).

The relative quantification was calculated using CT method, which uses the arithmetic formula $2^{-\Delta\Delta CT}$ [19]. To validate the $2^{-\Delta\Delta CT}$ methods, we verified that amplification efficiency of target gene and the actin endogenous control. Statistically significant differences in gene expression between cell lines were analyzed using one-way analysis of variance with Sigma Stat statistical software ver 2.0 SPSS, Inc.

Table 1. Oligonucleotides used in RTQ-PCR

Name	Sequence (5'-3')
c-erbB-2	F:TGGTGTATGCAGATTGCCAAG R:TCTCGTCAATGTCCAGCAGC
actin	F:CGGGAGATTGTGCGAGATGT R:GGAAGCGTTCATTCCCAATG

D. Flow Cytometry.

Staining procedure: BT-474, MCF7 and MDA-MB-231 cells were harvested with a cell scraper and washed with sterile Phosphate Buffer saline (PBS). Harvested cells were counted by using 0.4% Trypan blue solution on a automatic cell counter (mod. countess, Invitrogen). Each sample consisted of 1×10^6 cells suspended in 200 µL of cold media to which 20 µl of Mouse (monoclonal) Anti-Human p185HER-2 (c-erbB-2) FITC conjugate antibody (Invitrogen

cat. No.AHO0918). The cells were mixed thoroughly and incubated for 15 to 30 minutes in the dark at room temperature (20° to 25°C). Wash with PBS with 0.1% azide, add 0.5 mL of 1% paraformaldehyde, mix thoroughly, and analyzed in a Gallios flow cytometer (Beckman Coulter).

III. Results

A. RT Q-PCR c-erbB-2 expression.

PCR efficiencies were nearly equal between *c-erbB-2* and the endogenous control actin. Single peaks were identified in the melt curve for each gene screened, indicating that unique PCR pure products were produced (data no shown). Fig. 1 shows the relative expression levels of transcripts. The relative expressions of *c-erbB-2* gene in BT-474, MCF7 and MDA-MB-231 cell lines were 94.73, 1.86 and 1.00 respectively.

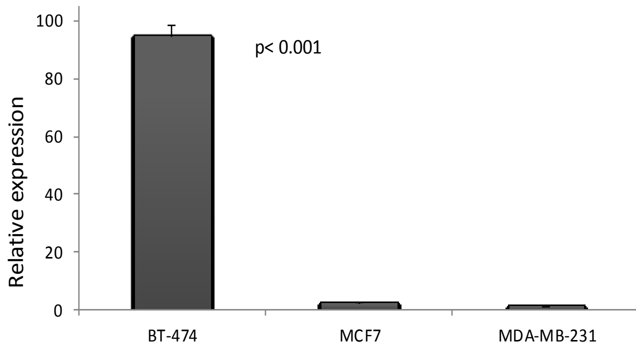

Fig. 1. Relative expression of *c-erbB-2* gene in BT-474, MCF-7 and MDA-MB-231 breast cancer cell lines.

Flow Cytometry c-erbB-2 Expression.

The flow cytometric profiles with relative fluorescence intesity percentage of the expression of the antigen c-erbB2 are shown in figure 2. The histograms on the left column show breast cancer cells without monoclonal antibody (negative control). The BT-474 breast cancer cell line overexpress c-erbB-2 surface antigen (23.7%), followed by MCF7 cell line (4.7%). The MDA-MB-231 breast cancer cell line not express the surface antigen (Figure 2 A, B and C).

IV. Discussion

Using RT Q-PCR to evaluate the expression of gene *c-erbB-2* in three cell lines, we confirmed high expression of *c-erbB-2* in BT-474 followed by MCF7 and MDA-MB-231, similar to reported by others authors [20], [21], [22] and [23]. Such observations were well correlated by the expression evaluated by Flow Cytometry where receptor staining proved to be specific to the c-erbB-2 antigen.

The observations suggest the pertinence to propose the elected cell lines to study the concept of selective targeting of breast cancer cells by MNPs with superparamagnetic propierties (high saturation magnetization), coated with a polysaccharide matrix, labeled with Fluorescein Isotiocianate (FITC) fluorophore, and conjugated through covalent binding to anti-c-erbB-2/c-neu IgG rabbit monoclonal antibody.

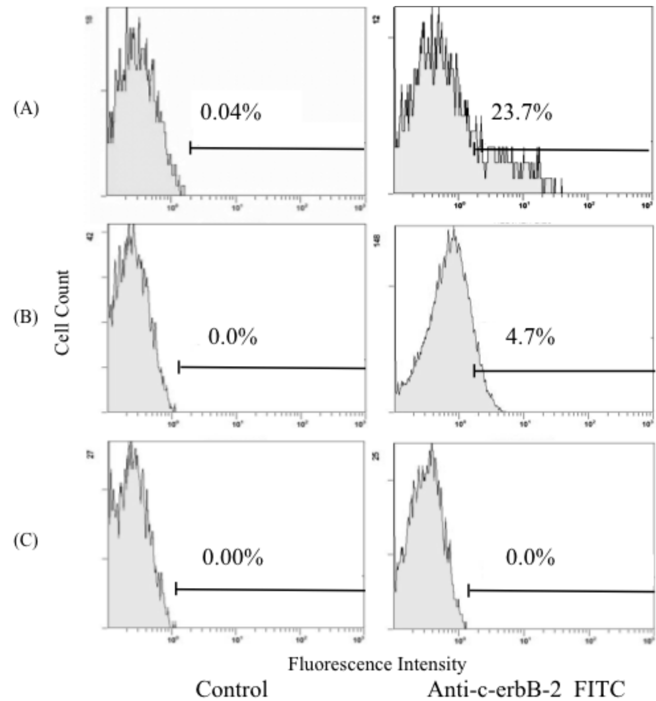

Fig. 2. Flow cytometry typification of the expression of c-erbB-2 antigen in breast cancer cells type: (A) BT-474, (B) MCF7 and (C) MDA-MB-231. Left column show breast cancer cells without monoclonal antibody (negative control).

In a practical view; the findings allow us to propose the c-erbB-2 protein as experimental target in the evaluated cell lines to increase its electrical conductivity of tumoral tissue by the use of bioconjugated "magnetic nanoparticle-monoclonal antibody" and induce selectively hypertermy by magnetic fields. The biophysical fundament is based on the composed electrical conductivity (σ) as a function of the electrical properties of the cell membrane and the effect of MNPs. The induction of currents in conductive materials by oscillating magnetic fields is explained on the basis of the Faraday induction law, which formulated in terms of the Maxwell's differential equation, is expressed as follows:

$$\Delta \times E = -\partial B / \partial t \tag{1}$$

A magnetic field **B** induces a time varying electromotive potential **E** in a conductive medium. This electric potential induces currents in the medium (eddy currents). According to the law of charges conservation, a current density **J** induced in a conductor is directly proportional to the electromotive potential **E** and the electrical conductivity σ of the material:

$$J = \sigma E \tag{2}$$

The eq. 2 supports that the higher conductivity of the material, the greater the energy that the material will absorb.

In this sense, and accordingly to the basic principle of physiological thermoregulation, which is supported by the first law of thermodynamics, in the normal state the energy generated in one body by external forces will be balanced as the outside heat so that the storage of energy will be minimal. We assume that the electrical properties of tumoral tissue with MNPs will be modified so that their composite electrical conductivity will increases, this increase will allow that the influence of magnetic fields reflect a selective induction of currents in the tumoral region, the electric field intensity generated by such currents will be dissipated as heat. Besides the exposure of cells to alternating magnetic fields results in the deposition of heat through the induction of Eddy currents in cells that is dependent upon the field frecuency, amplitude, and duration of exposure.

The findings allow us encourage further studies in order to confirm experimentally the technical feasibility to use the c-erbB-2 as possible target for the use of superparamagnetic nanoparticles in the studied breast cancer lines.

V. Conclusion

The breast cancer cell types: BT-474, MCF7 and MDA-MB-231 show well-differentiated relative expression of the gene *c-erbB-2* characterized by RT Q-PCR, and the surface antigen by Flow Cytometry. The studied cell lines are suitable for experimental evaluation of selective targeting by magnetic nanoparticles coupled to specific Mab anti c-erbB-2.

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References

[1] Kircher, MF, Mahmood, U, King RS, Weissleder R. and Josephson, L. (2003). A multimodal nanoparticle for preoperative magnetic resonance imaging and intraoperative optical brain tumor delineation. Cancer Res.63, 8122–8125

[2] Neuwalt EA. et al (2004). Imaging of iron oxide nanoparticles with MR and light microscopy in patients with malignant brain tumors. Neuropathol. Appl. Neurobiol. 5, 456–471.

[3] Nam, J. M. & Mirkin, C. A. (2004) Bio-barcode-based DNA detection with PCR-like sensitivity. J. Am. Chem. Soc. 126, 5932–5933.

[4] Dirección General de Epidemiología; SSA; Sistema Epidemiológico y Estadístico de las Defunciones, 2001.

[5] Dirección General de Epidemiología; SSA; Compendio de Cáncer 2000.

[6] Slamon DJ, Clark GM, Wong SG, Levine WJ, Ullrich A, McGuire WL. (1987) Human breast Cancer: Correlation of relapse and survival with amplification of the HER-2/neuoncogene. Science. 235:177-82.

[7] Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, et al. (1989). Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science. 244:707-12.

[8] Khosravi SP, Pérez Manga G. (2006). La relevancia clínica de la sobreexpresión de HER-2 en el cáncer de mama. An Med Interna (Madrid). 103- 104).

[9] Pawlowski V, Revillion F, Hornez L et al. (2000). A real-time one-step reverse transcriptase-polymerase chain reaction method to quantify c-erbB-2 expression in human breast cancer. Cancer Detect Prev. 24:212–223.

[10] Lehmann-Che J, Amira-Bouhidel F, Turpin E, Antoine M, Soliman H, Legres L, Bocquet C, Bernoud R, Flandre E, Varna M, de Roquancourt A, Plassa LF, Giacchetti S, Espié M, de Bazelaire C, Cahen-Doidy L, Bourstyn E, Janin A, de Thé H, Bertheau P. (2011). Immunohistochemical and molecular analyses of HER2 status in breast cancers are highly concordant and complementary approaches. Br J Cancer.104(11): 1739-46.

[11] Cuadros M, Talavera P, López FJ, Garcia Pérez I, Blanco A, Concha A. (2010). Real-time RT-PCR analysis for evaluating the Her2/neu status in breast cancer. Pathobiology. 77(1): 38-45.

[12] Hathaway HJ, Butler K S, Adolphi NL, Lovato DM, Belfon R, Fegan D, Monson TC, Trujillo JE, Tessier TE, Bryant HC, Huber D L, Larson RS and Flynn ER. (2011). Detection of breast cancer cells using targeted magnetic nanoparticles and ultra-sensitive magnetic field sensors. Breast Cancer Research. 13:R108.

[13] Yallapua MM, Othmanb FS, Curtis TE, Guptaa KB, Jaggia M,. and Chauhana CS. (2011). Multi-functional Magnetic Nanoparticles for Magnetic Resonance Imaging and Cancer Therapy. Biomaterials. March ; 32(7): 1890–1905.

[14] DeNardo SJ, DeNardo G, Miers LA.(2005). Development of Tumor Targeting Bioprobes (111 In-Chimeric L6 Monoclonal Antibody Nanoparticles) for Alternating Magnetic Field Cancer Therapy. Clin Cancer Res. 11(19 7087s Suppl).

[15] Johng HM, Yoo SJ, Yoon JT, Shin SH. et. al. (2007).Use of Magnetic Nanoparticles to visualize Theadlike Structures Inside Lymphatic Vessels of Rats". eCAM; 4 (1) 77-82.

[16] Natarajan A, Gruettner C, Ivkov R, DeNardo LG, G. Mirick G, Yuan A, Foreman A, and DeNardo SJ. (2008). NanoFerrite Particle Based Radioimmunonanoparticles: Binding affinity and in vivo pharmacokinetics. Bioconjug Chem. June ; 19(6): 1211–1218.

[17] Zhang Y, Yang M, Park JH, Syngelin J, Ma H, Sailor MJ, Ruoslahti E, Ozkan M, Ozkan C. (2009). A Surface-Charge Study on Cellular-Uptake Behavior of F3- Peptide-Conjugated Iron Oxide Nanoparticles. Small. September ; 5(17): 1990–1996.

[18] Acharya S, Dilnawaz F, Sahoo SK.(2009). Targeted Epidermal Growth Factor Receptor Nanoparticle Bioconjugates for Breast Cancer Therapy. Biomaterials. Oct ; 30(29):5737-50. Epub. Jul 23.

[19] Livak KJ, Schmittgen TD. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta CT) Method. Methods. 25:402-408.

[20] Sahin O, Fröhlich H, Löbke C, Korf W, Burmester S, Majety M, Mattern J, Schupp I, Chaouiya C, Thieffry D, Poustka A, Wiemann S, Beissbarth T and Arlt D. (2009). Modeling ERBB receptor-regulated G1/S transition to find novel targets for de novo trastuzumab resistance BMC Syst Biol.1;3:1.

[21] Bunn Jr., PA; Helfrich B; Soriano, AF, et al. (2001). Expression of Her-2/neu in human lung cancer cell lines by immunohistochemistry and fluorescence in situ hybridization and its relationship to in vitro cytotoxicity by trastuzumab and chemotherapeutic agents. Clin Cancer Res.7:3239-3250.

[22] Konecny G; Pauletti G; Pegram M; et al. (2003). Quantitative association between Her-2/neu and steroid hormone receptors in hormone receptor-positive primary breast cancer. J Nat Cancer Inst. 95(2): 142-153.

[23] Larson JS; Goodman LJ; Tan Y; Defazio-Eli L; et al. (2010). Analytical validation of a highly quantitative, sensitive, accurate and reproducible assay (HERmark ®) for the measurement of Her2 total protein and Her2 homodimers in FFPE breast cancer tumor specimens. Pathol Res Inter.1-14.