Full Length Research Paper

Heterogeneity of GST enzymatic activity before and after treatment in patients with breast cancer: pilot study

Fernando Mejia Sanchez[1], Marisol Rodríguez Albarrán[2], J. Amado López Arriaga[1] and Julieta Castillo Cadena[1]*

[1]Centro de Investigación en Ciencias Médicas, Universidad Autónoma del Estado de México, Jesús Carranza No. 205, Col. Universidad, Toluca de Lerdo, México. C.P. 50130.
[2]Facultad de Química, Universidad Autónoma del Estado de México. Paseo Colón esq Paseo Tollocan S/N. Toluca de Lerdo, México. C.P. 50100.

Exposure to anticancer agents such as cyclophosphamide and adriamycin promote the expression and synthesis of gene products whose function is cell protection, such as the family of isoenzymes of glutathione S-transferase, involved in Phase II detoxification of xenobiotics by glutathione conjugation. Response to the treatment is uncertain and different for each patient. Our objective was to determine total GST and GSTT1 enzyme activity induced by the treatment in women with breast cancer and the variability of the response. In 22 women with breast cancer, the total GST and GSTT1 enzymatic activity before and after treatment. The magnitude of enzymatic activity was different in each patient before and after treatment. The value of the median of total GST enzyme activity before treatment was 2.425 µmol/min/mL and after 3.253 µmol/min/mL, it was significantly different, p<0.05. For GSTT1 the median values of enzymatic activity before and after treatment were 0.015 and 0.021 µmol/min/mL, respectively, it was significantly different, p<0.05. Adriamycin and cyclophosphamide induce the expression of GST isoenzymes by increasing the enzymatic activity after treatment. The heterogeneity of enzymatic activity as a treatment response shall be considered for its prescription.

Keywords: Total GST enzyme activity, GSTT1 enzyme activity, breast cancer, adriamycin, cyclophosphamide

INTRODUCTION

Cancer arises from the accumulation of genetic and epigenetic alterations. Amplification of protooncogenes and chromosomal material loss can cause loss in cell cycle control, avoidance of DNA repair and apoptosis, allowing uncontrolled cell replication (Meza et al., 2006).

Breast cancer is a serious threat to the health of women worldwide, each year more than 10 million women are diagnosed with this condition and about 7 million die directly or indirectly due to this neoplasia. In 2005, 4,206 deaths were reported in Mexico from breast cancer, this means that 12 Mexican die every day. It is estimated for 2020 an increase of close to 16,500 new cases (Knaul et al., 2009; Rodríguez et al., 2014).

Glutathione S-transferase (GST),consists of several genes encoding a group of isozymes involved in the

*Corresponding Author E-mail: jcastilloc@uaemex.mx

Phase II of xenobiotics metabolism, these enzymes are in the microsomal fraction and protecting the cell from oxidative. The expression of these enzymes is induced under conditions of oxidative stress. The detoxification mechanism is conjugation of xenobiotic with reduced glutathione (GSH), transforming the toxic agents in water-soluble products and easy removal compounds of the cell. In mammals the most studies classes are Alpha, Mu, Pi, Theta, Omega and Zeta. (Hayes et al., 2005; Castillo et al., 2007; Kiran et al., 2010; Guo et al., 2010; Soto et al., 2011; Mejia et al., 2013).

Cyclophosphamide and adriamycin therapy as chemotherapeutic agents is one of the most widely used to treat breast cancer (Pemble et al., 1994). The cyclophosphamide is an antineoplastic that belongs to the family of alkylating agents, it possesses immunosuppressive properties, depressor of the bone marrow and myelopoiesis, as it forms alkyl adducts and nucleophiles in DNA. To reach its cytotoxic effect, it needs to be activated by the hepatic microsomal enzymatic complex. Adriamycin belongs to the family of anthracyclines, depressors of the bone marrow and cause cell toxicity (Garibay et al., 2015).

There is evidence showing that the expression of GST genes have a protective effect on the cytotoxicity of chemotherapeutic drugs, due to the fact that changes in GST levels have been linked with resistance to antineoplastic drugs (Soto et al., 2011). The GSTs are of pharmacological and toxicological interest because their expression increases significantly in mammalian tumor cells, so they have been implicated in the resistance of patients to the treatment of different types of cancer (Mejia et al., 2013).

Personalizing cancer therapy is a well established concept, since each patient holds a unique set of variants that influence on the risk, beginning and progression of the disease. For each specific sort and stage of cancer, clinical manifestations vary from individual to individual, showing variations in the tumor's behavior and progression, as well as the variations in the responses to any treatment, largely boosted by a single genome (DNA, RNA and epigenetic) (Uzilov et al., 2016; Avril et al., 2009).

There are very few methods to assess the response of the treatment against cancer. This information is very important as the response degree offers data to forecast the full response with survival (Feldman et al., 1986; Avril et al., 2009). However, there are differences between global responses to treatment, a pathological response or changes in tumor size (Avril et al., 2016).

Regularly, the assessment of the response is performed after two or three cycles, using World Health Organization's system or RECIT system. At present, it is not possible to know whether therapy will be efficacious after the first application of the treatment. The assessment is generally carried out in the middle of treatment, after 4 or 5 months, during which patients

unresponsive to treatment are identified, as well as those who suffer toxicity (Garibay et al., 2015; Avril et al., 2009).

It is necessary to develop a strategy for the therapy and improvement of the clinical methods to assess response to chemotherapy. Especially those which provide the information necessary to select the personalized therapy and obtain an optimum result for patients with cancer (Uzilov et al., 2016; Avril et al., 2009). The aim of the present study is to demonstrate that the total GST and GSTT1 enzyme activity in women with breast cancer increases in the presence of cyclophosphamide - adriamycin and that exits inter-individual variability in the response.

METHODS

Study Group

The study group consisted of 22 women diagnosed with breast cancer at different stages, at the "Centro Oncológico Estatal ISSEMyM" (State Cancer Center), Toluca, Mexico. Participation was by invitation and voluntary, who accepted signed a letter of informed consent according to Mexican policy in the field of health research (2013). A sample of 7 mL of peripheral blood was taken from all the participants before and after receiving the first dose of chemotherapy with Adriamycin/Cyclophosphamide. The blood sample was kept in refrigeration in a heparin vacutainer tube until processing.

Cell lysis

The procedure reported by Pemble et al. (1994) and Zhong et al., (2006)was adopted, which involves placing 1mL of heparinized blood in a 1.5mL tube, then centrifuged at 4,500 rpm for 10 minutes, removing the plasma and leukocytes layer. To hemolyze erythrocytes, 1mL of distilled water was added at 4°C, 700µL of the hemolysate were transferred to a clean tube and added 350µL of K_2HPO_4 20mM (Merk) pH 7.4 and 350µL de EDTA 2mM (Fermont), it kept at 4°C for one hour and finally centrifuged at 4,500 rpm for 10 minutes. The product obtained was the cytosol.

Total GST enzymatic activity

Total GST enzymatic activity was determined according to the method reported by Habig et al., (1974) and Zhong et al., (2006) with modifications proper to this study: make a dilution 1:10 with 100µL of the hemolyzed plasma and K_2HPO_4 0.1M (Merk). Take 100µL of this dilution and make again a dilution 1:10 with K_2HPO_4 0.1M (Merk). Transfer 200µL of this solution to a tube with 1,000µL of K_2HPO_4 0.1 M (Merk) pH 6.5, 100µL of reduced

glutathione (GSH) 1mM (Merk) and 100µL of 1-Chloro-2,4-Dinitrobenzene (CDNB) 1mM (Acrofarma). Mix in vortex and let sit for 3 minutes at room temperature. Finally, read the absorbance in a UV-VIS spectrophotometer at a wavelength of 340 nm against a target prepared under the same conditions.

GSTT1 enzymatic activity

Take 400µL of the previously obtained hemolyzed plasma to a tube containing 400µL of reduced glutathione (GSH) 4mM (Merk), 700 µL of TRIS 20 mM (Sigma) and 200 µL of methylene chloride (CH_2Cl_2) 5mM (Fermont). Incubate at 37°C for an hour. Add 333µL of trichloroacetic acid (CCl_3COOH) at 20% (Merk). Centrifuge at 4,500 rpm for 5 minutes. Take 1 mL of the supernatant and add 500µL of Nash reagent. Incubate at 60°C for 30 minutes. Centrifuge at 7,000 rpm for 5 minutes. Finally, read absorbance in a UV-VIS spectrophotometer at a wavelength of 415 nm against a target prepared under the same conditions.

To quantify enzymatic activity for both trials, the following formula was used:

$$GST\ activity = \frac{A_{340}\ min^{-1} \times 1.4\ mL\ Reaction\ volume}{0.0096\mu mol^{-1}cm^{-1} \times 1000\ mL \times 0.524\ cm \times A} \times D$$

$$= 0.0398 \times A_{340} min^{-1} \times \frac{D}{A} \quad (\mu mol/\ min/\ mL)$$

0.0096 μM^{-1} cm^{-1}: extinction coefficient of GST-DNB
A: Reaction sample volume in mL
$A_{340}\ min^{-1}$: Sample absorbance
D: sample dilution factor

Statistical Analysis

To determine significant differences between total GST and GSTT1 enzyme activity before and after treatment, a comparison analysis was made with Student-Newman-Keuls test. Sigma Stat 3.0 statistical software was used.

RESULTS

Socio-demographic characteristics

The study group consisted of 22 women diagnosed with breast cancer, of which 91.42% had infiltrating ductal and 8.58% infiltrating lobulillar. The age range of participants was 30-66 years with an average of 49.37 years.

Heterogeneity of GST enzymatic activity as a response for the treatment

Results of the enzymatic activity showed a different behavior in each patient. Regarding GST total enzymatic activity, the minimum value before treatment was 0.456
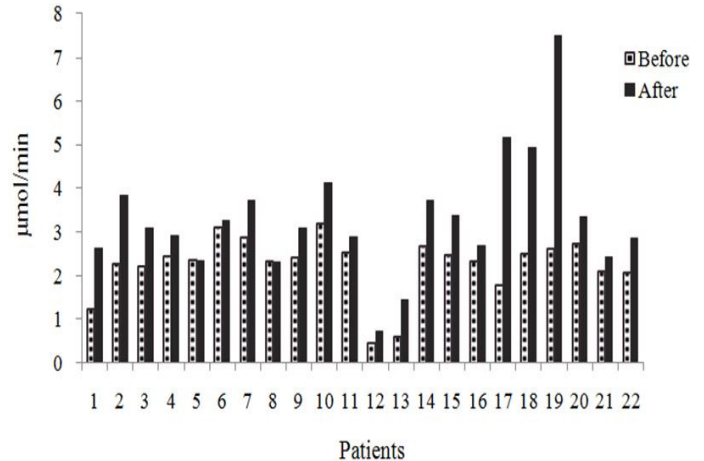

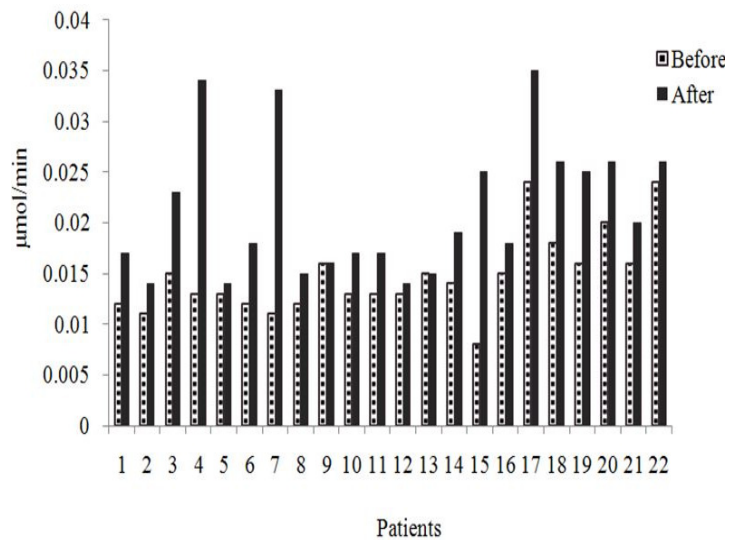**Figure 1.** Heterogeneity of total GST enzymatic activity.

Figure 2. Heterogeneity of GSTT1 enzymatic activity.

µmol/min/mL, and the maximum 3.293 µmol/min/mL. After treatment, the minimum value was 0.734 µmol/min/mL and the maximum 7.477 µmol/min/mL. In figure 1, the values of the enzymatic activity before and after treatment are displayed. The heterogeneity of the response between each patient is evident.

Regarding GSTT1 enzymatic activity, there was a similar behavior to total GST. The minimal value of the enzymatic activity before treatment was 0.008 µmol/min/mL and maximum 0.024 µmol/min/mL. After treatment the minimum value was 0.014 µmol/min/mL and maximum de 0.035 µmol/min/mL. In figure 2, data for enzymatic activity before and after treatment is displayed.

GST enzyme activity

The values of the total GST enzyme activity were: pre-treatment median 2.425 µmol/min/mL and then 3.253
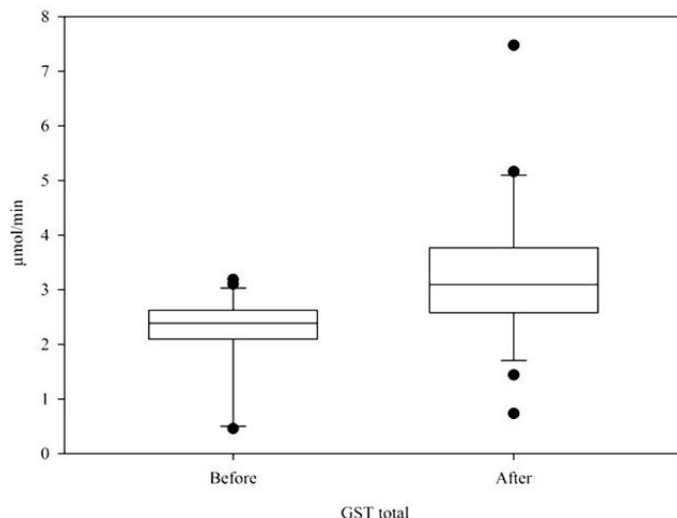
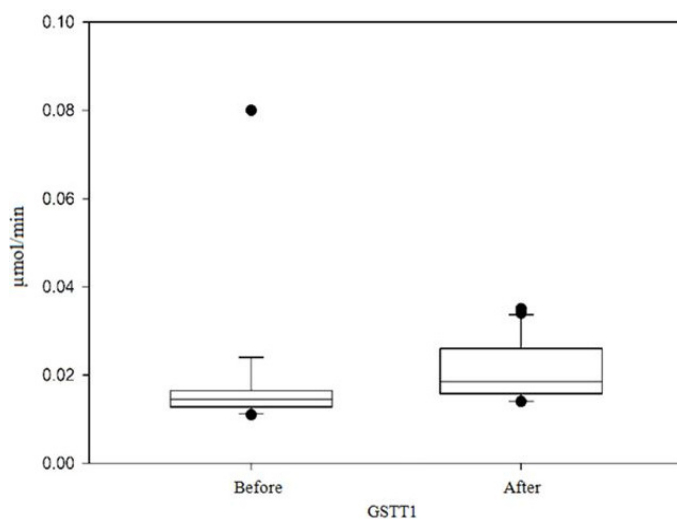**Figure 3**. Results of the enzymatic activity of the total GST.

Figure 4. Results of the enzymatic activity of the total GSTT1.

μmol/min/mL after treatment. These values ranged from 0.456 to 3.293 μmol/min/mL before chemotherapy and after chemotherapy values ranged from 0.734 to 7.477 μmol/min/mL. Statistical analysis with Student-Newman-Keuls test showed significant differences between total GST enzyme activity before and after treatment, which showed a significant increase ($p <0.050$). Figure 3 shows these results.

Regarding the results of GSTT1 enzymatic activity, the median value before treatment was 0.015 μmol/min/mL, in a range from 0.008 to 0.024 μmol/min/mL. While median after treatment, it was 0.021 μmol/min/mL, in a range from 0.014 to 0.035 μmol/min/mL. Statistical analysis with Student-Newman-Keuls test showed significant differences between GSTT1 enzyme activity before and after treatment, which showed a significant increase ($p <0.050$). Figure 4 shows these results.

DISCUSSION

Breast cancer is clinically heterogeneous diseases, since histologically similar tumors may present different forecast and response to treatments. Patients with the same histological variety and even the same clinical stage have different responses for the same therapeutic schema and different forecast. Tumors have a different biological behavior, these differences in clinical behavior are due to molecular differences between tumors (Rouzier et al., 2005; Ruvalcaba et al., 2014).

Histopathology is frequently used as the assessment standard for the response to primary chemotherapy in cancer. However, the criteria of histopathological response have limitations, on the other side, there are no methods fully efficient to forecast response to chemotherapy. However, some works have adopted the approach of grouping breast cancers as responsive or non-responsive, define the differences in the genetic expressions between these groups and use such indicator (Rouzier et al., 2005; Avril et al., 2009).This way, there are reports on the assessment of treatment response, namely Ruvalcaba et al., (2006) who reported full pathological responses in 25% out of 360 breast cancer cases treated with chemo-radiotherapy. While Alvarado et al., (2009) studied 112 breast cancer patients treated with neoadjuvant chemotherapy and reported full pathological response only for 29.5%.

This way, it is known that exposure to anticancer agents such as cyclophosphamide and adriamycin promote the expression and synthesis of gene products whose function is cell protection, such as the family of isoenzymes of glutathione S-transferase, which is involved in the metabolism of a xenobiotic variety, among which are chemotherapeutic agents(Davies et al., 2001; Guo et al., 2010; Kiran et al., 2010).

Analyzing the results of the total GST enzyme activity, a significant increase after treatment was identified, this indicates an increase in the levels of GST isozymes at the finish of the first chemotherapy dose compared to baseline values, which may be due the gene activation by the drug at the time they were recognized as substrate, reflecting induction of protein synthesis of this group of isoenzymes. This was demonstrated by Cheng et al., (1997), who studied tumors in 20 patients with ovarian carcinoma. They determined the expression of GSTP1 before and after chemotherapy by means of Western blot and found and increment in the expressions levels of GSTP1 after chemotherapy and associated it with drug resistance in patients with ovarian carcinoma. Similar results were found by Geng et al., (2013) while studying gastric cancer cells undergoing *in vitro* chemotherapy. They determined that cells resistant to cisplatin, 5-fluorouracil and mitomycin C present a significant increment in the expression of GSTP1. In like manner, Jankova et al., (2012) ascertained the association between global survival and the expression of GSPT1 in

104 patients with colon cancer under adjuvant chemotherapy based on 5- fluorouracil and 104 matched controls. They found that patients with low GSTP1 levels were not benefitted from chemotherapy, while those with high levels did improve. On the other side, and by contrast Murphy et al., (1992) studied the enzymatic activity of GST in biopsies of human ovarian tumors, which were taken before and after chemotherapy. The analysis did not show significant differences between the activity of glutathione S-transferase and the distribution of isoenzymes in these groups.

Changes in the expression of glutathione S-transferases are associated with higher resistance to cytotoxic chemical products. The degree of resistance is related with the specificity of the isoenzyme substrate (Schecter et al., 1991). Therefore, it is important to early assess the patient's treatment response in order to guide later decisions.

CONCLUSIONS

In conclusion, the results of this study show that adriamycin and cyclophosphamide induce the expression of genes of the different classes of GST family, by increasing the enzymatic activity after treatment in women with breast cancer.

The response to treatment in patients with some type of neoplasia has been and remains uncertain, therefore, any attempt to visualize it opportunely, it is important.

ACKNOWLEDGEMENTS

REFERENCES

Alvarado MA, Arrieta O, Gamboa VC, Saavedra PD, Morales BR, Bargallo RE, Lara MF (2009). Concurrent chemo-radiotherapy following neoadjuvant chemotherapy in locally advanced breast cancer. Radiat. Oncol. 4: 24.

Avril N, Sassen S, Roylance R (2009). Response to therapy in breast cancer. J. Nucl. Med. 50:55-63.

Avril S, Muzic RF, Plecha D, Traughber BJ, Vinayak S, Avril N (2016). ¹⁸F-FDG PET/CT for monitoring of treatment response in breast cancer. J. Nucl. Med. 57: 34S-39S.

Castillo JC, Contreras GS, Poblano BR, Posadas GR, Ramírez GJ (2007). Actividad en la enzima Glutatión S-transferasa T1 en floricultores expuestos a plaguicidas. Bioquímia. 32: 138.

Cheng X, Kigawa J, Minagawa Y, Kanamori Y, Itamochi H, Okada M,Terakawa N (1997). Glutathione S-transferase-pi expression and glutathione concentration in ovarian carcinoma before and after chemotherapy. Cancer. 79: 521-527.

Davies SM, Robison LL, Buckley JD, Tjoa T, Woods WG, Radloff GA, Perentesis JP (2001). Glutation S-transferase polymorphisms and outcome of chemotherapy in childhood acute myeloid leukemia. J. Clin. Oncol. 19: 1279-1287.

Feldman LD, Hortobagyi GN, Buzdar AU, Ames FC, Blumenschein GR (1986). Pathological assessment of response to induction chemotherapy in breast cancer. Cancer Res. 46: 2578-81.

Garibay GJ, Mejia SF, Ramírez SJE, Flores MMV, Castillo CJ (2015). Genotoxic and cytotoxic damage by cyclophosphamide and adriamycin as a response to treatment in breast cancer patients: Pilot study. J. Cancer. Ther. 6: 163-168.

Geng M, Wang L, Chen X, Cao R, Li P (2013). The association between chemosensitivity and Pgp, GST-π and Topo II expression in gastric cancer. Diagn. Pathol. 10: 198.

Guo X, Zeng Y, Deng H, Liao J, Zheng Y, Li J, O'Brien SJ (2010). Genetic polymorphisms of CYP2E1, GSTP1, NQO1 and MPO and the risk of naso-pharyngeal carcinoma in a chinese population of southern China. BMC Res. Notes. 27: 212-321.

Habig WH, Pabst MJ, Jakoby WB (1974). Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249: 7130-7139.

Hayes JD, Flanagan JU, Jowsey IR (2005). Glutathione transferases. Ann. Rev. Pharmacol. Toxicol. 45: 51-88.

http://dof.gob.mx/nota_detalle.php?codigo=5284148&fecha=04/01/2013 . Accessed 22 May 2017.

Jankova L, Robertson G, Chan C, Tan KL, Kohonen-Corish M, Fung CL, Clarke C, Lin BP, Molloy M, Chapuis PH, Bokey L, Dent OF, Clarke SJ (2012). Glutathione S-transferase Pi expression predicts response to adjuvant chemotherapy for stage C colon cancer: a matched historical control study. BMC Cancer. 28: 1-9.

Kiran B, Karkucak M, Ozan H, Yakut T, Ozerkan K, Sag S, Ture M (2010). GST (GSTM1, GSTT1 and GSTP1) polymorphisms in the genetic susceptibility of Turkish patients to cervical cancer. J. Gynecol. Oncol. 21: 169-173.

Knaul FM, Nigenda G, Lozano R, Arreola OH, Langer MD, Frenk J (2009). Cáncer de mama en México: una prioridad apremiante.Salud Pública Méx. 51: 335-230.

Mejia F, Cadena CJ, Sánchez MJC (2013). Development and application in Mexican of a method for the Identification of polymorphisms of GSTP1. J. Med. Med. Sci. 4: 287-290.

Meza JJ, Montaño LA, Aguayo GA (2006). Bases moleculares del cáncer. Rev. Invest. Clin. 58: 56-70.

Murphy D, McGown AT, Hall A, Cattan A, Crowther D, Fox BW (1992). Glutathione S-transferase activity and isoenzyme distribution in ovarian tumour biopsies taken before or after cytotoxic chemotherapy. Br J. Cancer. 66: 937–942.

Pemble S, Schroeder KR, Spencer SR, Meyer DJ, Hallier E, Bolt HM, Ketterer B, Taylor JB (1994). Human glutathione S-transferase Theta (GSTT1): cDNA cloning and the characterization of a genetic polymorphism. Biochem. J. 300: 271-276.

Rodríguez M, Mejia F, Lecourtois M, Domínguez V, Castillo J (2014). Influence of GSTT1, GSTM1 and GSTP1 polymorphisms on the development of breast cancer. J. Cancer Ther. 5: 552-559.

Rouzier R, Perou CM, Symmans WF, Ibrahim N, Cristofanilli M, Anderson K, Hess KR, Stec J, Ayers M, Wagner P, Morandi P, Fan C, Rabiul I, Ross JS, Hortobagyi GN, Pusztai L (2005). Breast cancer molecular subtypes respond differently to preoperative chemotherapy. Clin. Cancer Res. 11: 5678-5685.

Ruvalcaba LE, Robles VC, Poitevin CA, Chávez MM, Gamboa VC, Vilar CD (2006). Complications after breast cancer surgery in patients treated with concomitant preoperative chemoradiation: a case-control analysis. Breast Cancer Res. Treat. 95: 147-152.

Ruvalcaba LE, Barreda ZLE, García ON, Morales VF, Bautista PV, Maffuz AA, Rodríguez CS (2014). Respuesta a la quimioterapia neoadyuvante (QTneo) según los subtipos intrínsecos determinados por inmunohistoquímica (IHQ) en pacientes con cáncer de mama. Gac. Med. Mex. 150: 161-170.

Schecter RL, Woo A, Duong M, Batist G (1991). In vivo and in vitro mechanisms of drug resistance in a rat mammary carcinoma model.Cancer Res. 51:1434-42.

Soto O, Cabrera GP, Téllez TG, Barrera FJL, Juárez RA, Castillo CJ (2011). Relationship of polymorphisms of Glutathione S-Transferase GSTT1 and GSTM1 with the response to chemotherapy in Mexican women with advanced breast cancer. J. Cancer Ther. 2: 354-361.

Uzilov AV, Ding W, Fink MY, Antipin Y, Brohl AS, Davis C, Lau CY, Pandya C, Shah H, Kasai Y, Powell J, Micchelli M, Castellanos R, Zhang Z, Linderman M, Kinoshita Y, Zweig M, Raustad K, Cheung K, Castillo D, Wooten M, Bourzgui I, Newman LC, Deikus G, Mathew B, Zhu J, Glicksberg BS, Moe AS, Liao J, Edelmann L, Dudley JT, Maki RG, Kasarskis A, Holcombe RF, Mahajan M, Hao K, Reva B, Longtine J, Starcevic D, Sebra R, Donovan MJ, Li S, Schadt EE, Chen R (2016). Development and clinical application of an integrative genomic approach to personalized cancer therapy. Genome Med. 8: 62.

Zhong SL, Zhou SF, Chen X, Chan SY, Chan E, Ng KY, Huang M (2006). Relationship between genotype and enzyme activity of glutathione S-transferases M1 and P1 in Chinese. Eur. J. Pharm. Sci. 28: 77-85.