RESEARCH ARTICLE

ESR1 and *PGR* Gene Promoter Methylation and Correlations with Estrogen and Progesterone Receptors in Ductal and Lobular Breast Cancer

Alma Delia Medina-Jaime[1], Francianella Reyes-Vargas[2], Victoria Martinez-Gaytan[3], Graciela Zambrano-Galvan[4], Eduardo Portillo-DelCampo[5], Jorge Alberto Burciaga-Nava[6], Miguel Reyes-Romero[6], Antonio Sifuentes-Alvarez[6]*

Abstract

The aim of this work was to analyze methylation of the promoter sites of the *ESR1* and *PGR* genes and to determine correlations with immunohistochemical expression of estrogen and progesterone receptors in ductal and lobular breast cancers. An observational, descriptive, molecular study was conducted on 20 ductal and 20 lobular breast cancer samples with immunohistochemical determination of estrogen and progesterone receptor expression. The methylation analysis of *ESR1* and *PGR* promoter sites was carried-out by methylation-specific PCR. For correlation analysis, Kendall's tau coefficient was determined. Positive correlations were found between estrogen and progesterone receptors, estrogen receptor and unmethylated progesterone receptor, progesterone receptor, and unmethylated progesterone receptor. Negative correlations were found between estrogen receptor and methylated progesterone receptor, progesterone receptor and methylated progesterone receptor, methylated and unmethylated estrogen receptor, and methylated and unmethylated progesterone receptor. The results suggest that methylation of promoter sites of *ESR1* and *PGR* is a relatively uncommon event in ductal and lobular breast cancer, and also suggest that the determination of epigenetic states of *ESR1* and *PGR* could represent an alternative or complement to the histopathological expression analysis.

Keywords: Biomarkers - breast cancer - estrogen receptors - epigenetics - methylation - progesterone receptors

Asian Pac J Cancer Prev, **15 (7)**, 3041-3044

Introduction

Breast cancer is one of the most common cancers with greater than 1,300,000 cases and 450,000 deaths each year worldwide and is the first cause of death by cancer in women of developed countries (Cancer Genome Atlas Network, 2012).

Histopathologically, the ductal and lobular breast tumors are the two main classes, which together give account for more than 80% of the cases.

At molecular level breast cancer is classified in four groups named luminal A and B, which express estrogen (ER) and progesterone (PR) receptors, HER2-positive, which over-expresses receptors for epidermal growth factor, and basal like (triple negative) (Goldhirsch et al., 2013). This classification has therapeutical implications because luminal tumors are responsive to anti-estrogen or aromatase inhibitor therapy, and HER2-positive to monoclonal antibodies, whereas basal-like tumors have only chemotherapy options; the luminal group is the most frequent and varied. Within each breast cancer class there is significant heterogeneity caused by different subsets of genetic and epigenetic alterations.

In the recent years, much of the research in breast cancer has been focused in the characterization and functional analysis of epigenetic alterations (Feinberg et al., 2004; Esteller 2008; Parrella 2010, Baylin et al., 2011). The main epigenetic alterations consist in DNA methylation, histone modifications such as acetylation, methylation and phosphorylation, and translational interference by micro-RNAs.

Aberrant DNA methylation plays a pivotal role in the development of different types of cancer. DNA methylation occurs mainly in cytosines preceding guanines and together they are known as CpG dinucleotides; they are found at high density in the promoter sites in most of the genes, which are named CpG islands. In this way, promoter methylation of genes represents a key event in cancer development because it is translated in gene silencing; so, the study of methylated genes represents the emergence of epigenetic biomarkers (Esteller 2008; Baylin et al., 2011; Heichman et al., 2012).

On the other hand, the role played by ER and PR in the breast cancer development is well known (Radisky et

*[1]Health Services of Durango SSD, [2]Cancer State Center of the Ministry of Health of Durango SSD, [3]UMAE 23 IMSS Monterrey, [4]Faculty of Dentistry UJED, [5]Espana Autonomous University of Durango, [6]Faculty of Medicine and Nutrition UJED, Durango, Mexico *For correspondence: sifual55@hotmail.com*

al., 2007; Allred 2010; Castoria et al., 2010); estrogens drive the cancer development and tumors expressing ER are more differentiated and responsive to treatments based on anti estrogens (i.e. tamoxifen) or aromatase inhibitors (i.e. anastrozole) (Renoir et al., 2013). Likewise, PR also provide useful information in defining subgroups of responders to therapy (Allred et al., 2010; Knutson et al., 2013; Purdie et al., 2014).

Estrogen receptor-α (ER-α) is encoded by the *ESR1* gene, located in chromosome 6; its transcription is under control of seven promoters, two of them, named A and B contain CpG islands. In breast cancer, the estrogenic signaling pathway is involved in diverse grade. The immunohistochemical determination of ER-α is the conventional method and the gold standard, although it is accepted that there are unsolved questions regarding heterogeneity in staining methods and arbitrary criteria for interpreting the results, which can result in specificity with up to 20% of false negatives (Allred 2010; Hammond et al., 2010). The PR is encoded in the *PGR* gene located in chromosome 11, under control by two promoters, named A and B, which as A and B *ESR1* promoters, contain CpG islands. Immunohistochemical determination of PR involves the same problems of staining and interpretation depicted above for ER-α (Allred 2010; Hammond et al., 2010).

The determination of *ESR1* and *PGR* promoter methylation has scantly been studied (Lapidus et al., 1998; Mc Cormack et al., 2007; Gaudet et al., 2009; Ramezani et al., 2012), with results not definitive to date. Methylation analysis could represent an early biomarker and also an alternative or complement to the ER and PR immunohistochemical determination in breast cancer, with potential for be determined in circulating DNA (Sturgeon et al., 2012), which could be of help with diverse purposes in clinical stages.

The aim of this work was to analyze the methylation status of promoters containing CpG islands in the *ESR1* and *PGR* genes, and to determine their correlation with the immunohistochemical determination of ER and PR in ductal and lobular breast cancer.

Materials and Methods

A correlational, observational, descriptive molecular study was conducted in excisional biopsies obtained with therapeutic aim from women resident of the north of Mexico. Twenty ductal (18 infiltrating, and 2 *in situ*) and 20 lobular infiltrating breast cancer cases, with immunohistochemical determination of ER and PR qualitatively determined as positive or negative, were studied. Written informed consent was obtained.

DNA extraction

DNA was purified from a 100-300 mg tissue sample by homogenization with 1 ml of DNAzol solution (Invitrogen, Carlsbad, CA), followed by centrifugation at 10,000xg by 5 min; the supernatant was mixed with 0.5 mL absolute ethanol and the precipitated DNA was collected by brief centrifugation, washed twice with 70% ethanol and finally resuspended in water. The DNA quantification was done by absorbance at 260nm and integrity assessed by agarose gel electrophoresis and ethidium bromide staining.

Bisulfite modification and methylation analysis

Qualitative analysis of the *ESR1* and *PGR* promoters was done by methylation specific PCR (MSP) (Herman et al., 2001), which employs bisulfate-modified DNA; 1 μg of DNA was modified with the IMPRINT DNA Modification Kit (MOD50, Sigma Aldrich, St. Louis, MO) according to the manufacturer's protocol. The modified DNA was stored at -36˚C until its analysis within the next 20 days. For MSP analysis, primers for CpG islands in *ESR1* and *PGR* were selected according to previous reports (Lapidus et al., 1998; Sasaki et al., 2001). MSP was performed using 1µl of the modified DNA in a final reaction volume of 25µl, containing 12.5µl of GoTaq Master Mix (Promega Inc., Madison, WI, USA), 0.5µl of each forward and reverse primers for unmethylated (UM) and methylated (M) promoters (Table 1), and 10.5µl of water. Primers were synthesized by Integrated DNA Technologies (Coralville, IA). The PCR amplification consisted of 35 cycles of denaturation at 95˚C for 45 sec, annealing at 55˚C for 45 sec and extension at 72˚C for 45 sec. The amplification products were analyzed by agarose gel electrophoresis with ethidium bromide staining.

Statistical analysis

Descriptive statistics and Kendall's tau correlation coefficient were analyzed with the on-line Free Statistics Software (Wessa, 2011).

Results

Age group

The median age of the entire sample was 54 years, with an inter-quartile range between 47 and 60.75 years; in the ductal carcinoma group, the median age was 53 years with an inter-quartile range between 47 and 66 years; in the lobular carcinoma group, the median age was 54.5 years with an inter-quartile range between 48 and 60 years.

Estrogen and progesterone receptors by immunohistochemistry

The proportion of positivity for ER and PR in the sample was 0.72 and 0.80 respectively, with a variable degree of positivity going from 10 to 100% (not shown). The frequency of ER and PR by histological type is shown in Table 2.

Methylation of ESR1 and PGR promoters

Table 1. ESR1 and PR Primers for MSP

Promoter	Sense (5'-3')	Antisense (5'-3')
UM-*ESR1**	GGTGTATTTGGATAGTAGTAAGTTTGT	CCATAAAAAAACCAATCTAACCA
M-*ESR1**	GTGTATTTGGATAGTAGTAAGTTCGTC	CGTAAA AAAAACCGATCTAACCG
UM-*PGR***	TGATTGTTGTTTGTAGTATG	CAACAATTTAATAACACACA
M-*PGR***	TGATTGTCGTTCGTAGTACG	CGACAATTTAATAACACGCG

*Lapidus et al., 1998; **Sasaki et al., 2001

For *ESR1*, the promoter was found mainly unmethylated in a proportion of 0.85. For *PGR*, the promoter was found as for *ESR1*, mainly unmethylated in a proportion of 0.95. Patterns and proportions by histological type are presented in Tables 3 and 4.

Correlation of ESR1 and PGR promoter methylation status with the presence of ER and PR in ductal breast carcinoma

A positive correlation was observed between: ER and PR, ER and UM-*PGR*, PR and UM-*PGR*. A negative correlation was observed between: ER and M-*PGR*, PR and M-*PGR*, UM-*ESR1* and M-*ESR1*, UM-*PGR* and M-*PGR*. Correlation matrix is shown in Figure 1.

Table 2. Proportion of Positive Samples for Estrogen (ER) and Progesterone (PR) Receptors by Histological Type n/N (1)

	ER	PR
Ductal	14/20 (0.70)	14/20 (0.70)
Lobular	15/20 (0.75)	18/20 (0.90)
Total	29/40 (0.72)	32/40 (0.80)

Table 3. *ESR1* And *PGR* Methylation Promoter Status and Immunohistochemical Determination of Estrogen (ER) and Progesterone (PR) Receptors, by Sample and Histological Type Rectangles Indicate Positivity

Sample	Histological Type	UM-ESR1	M-ESR1	UM-PGR	M-PGR	ER	PR
1	Ductal	+	+	+	-	+	+
2	Ductal	+	-	+	-	+	+
3	Ductal	+	+	+	-	+	+
4	Ductal	+	-	+	-	-	-
5	Ductal	+	-	+	-	+	+
6	Ductal	+	-	+	-	+	+
7	Ductal	+	-	-	+	-	-
8	Ductal	-	+	+	-	+	+
9	Ductal	+	+	+	-	+	+
10	Ductal	-	+	+	-	+	+
11	Ductal	+	-	+	-	+	+
12	Ductal	+	-	+	+	-	-
13	Ductal	+	-	+	-	+	+
14	Ductal	+	-	+	-	+	+
15	Ductal	+	-	+	-	+	+
16	Ductal	+	-	+	+	-	-
17	Ductal	+	-	-	+	-	-
18	Ductal	+	-	+	-	+	+
19	Ductal	+	-	+	-	+	+
20	Ductal	+	-	+	+	-	-
21	Lobular	+	-	+	-	+	+
22	Lobular	-	+	+	-	+	+
23	Lobular	+	-	+	-	+	+
24	Lobular	+	-	+	+	-	-
25	Lobular	+	+	+	-	+	+
26	Lobular	+	-	+	-	+	+
27	Lobular	+	-	+	-	+	+
28	Lobular	+	-	+	-	-	+
29	Lobular	-	+	+	-	-	+
30	Lobular	+	-	+	-	+	+
31	Lobular	+	-	+	-	+	+
32	Lobular	-	-	+	+	+	+
33	Lobular	+	+	+	-	+	+
34	Lobular	+	-	+	-	-	-
35	Lobular	+	-	+	-	+	+
36	Lobular	+	-	+	-	+	+
37	Lobular	+	+	+	-	+	+
38	Lobular	+	-	+	-	+	+
39	Lobular	-	-	+	-	-	+
40	Lobular	+	-	+	-	+	+

Table 4. *ESR1* and *PGR* Methylation Promoter Status by Sample and Histological Type n/N (1)

	UM-*ESR1*	M-*ESR1*	UM-*PGR*	M-*PGR*
Ductal	18/20 (0.90)	5/20 (0.25)	18/20 (0.90)	5/20 (0.25)
Lobular	16/20 (0.80)	5/20 (0.25)	20/20 (1.00)	2/20 (0.10)
Total	34/40 (0.85)	10/40 (0.25)	38/40 (0.95)	7/40 (0.17)

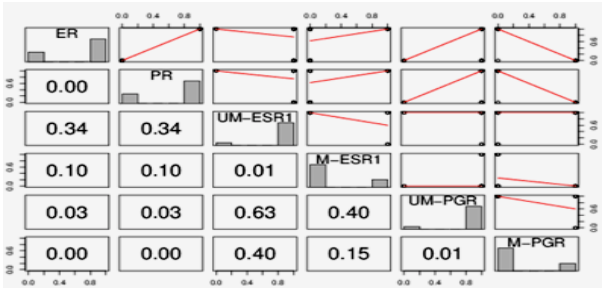

Figure 1. Ductal Breast Cancer. Correlation matrix of estrogen receptors (ER), progesterone receptors (PR), unmethylated *ESR1* promoter (UM-*ESR1*), methylated *ESR1* promoter (M-*ESR1*), unmethylated *PGR* promoter (UM- *PGR*), methylated *PGR* promoter (M-*PGR*). For every plot in the diagonally upper half, there is a corresponding p value in the lower half

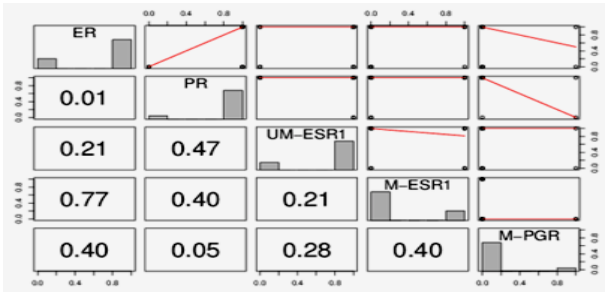

Figure 2. Lobular Breast Cancer. Correlation matrix of estrogen receptors (ER), progesterone receptors (PR), unmethylated *ESR1* promoter (UM-*ESR1*), methylated *ESR1* promoter (M-*ESR1*), methylated *PGR* promoter (M-*PGR*). For every plot in the diagonally upper half, there is a corresponding p value in the lower half

Correlation of promoter methylation status of ESR1 and PGR with the presence of ER and PR in lobular breast carcinoma

A positive correlation was observed between: ER and PR. A negative correlation was observed between: PR and M-*PGR* promoter. UM-*PGR* was not included in the analysis because the sample showed homogeneity. Correlation matrix is shown in Figure 2.

Discussion

Breast cancer is a heterogeneous and plastic disease with many subjacent genetic and epigenetic abnormalities. Additionally to the information supplied by the histopathological diagnosis of breast cancer, the molecular classification offers information of importance for evaluating multiple aspects as the groups differ in evolution, survival and response to treatment, by which is essential to determine the molecular type; this classification is based in the expression of ER, PR and HER2, including four molecular sub-groups: luminal A and B, HER2 over-expression and basal-like.

In spite of these achievements, there exists a consensus

with regard to the necessity of new biomarkers for the early detection of breast cancer; nowadays exists much interest for developing epigenetic biomarkers focused in methylation patterns of genes; with this in mind, the aim of this work was directed to analyze the methylation status of the promoters of the *ESR1* and *PGR* genes, as well as to determine their correlation with the expression of ER and PR determined by immunohistochemistry. The results showed that the promoters of *ESR1* and *PGR* are mostly in unmethylated state, which can be translated as gene expression; this is concordant with the fact that the majority of the cases of breast cancer are positive for ER and PR. The results are also concordant with the report of Gaudet et al. (2009), which describe for invasive breast cancer that DNA methylation of CpG islands in *ESR1* and *PGR* promoters is common but generally weak. The multiple correlation analysis of the 20 ductal cases showed significative positive correlations between ER and PR, which means that the methylation analysis mirrors the existing correlation between ER and PR by immunohistochemical analysis. Another positive correlation was that exhibited by the ER and PR with the UM-*PGR* promoter, which is a priori the expected because an unmethylated promoter is translated as gene expression. On the other hand, negative correlations were found between the M-*PGR* promoter, ER and PR, which also is the expected because the promoter methylation means gene silencing, although this correlation was not observed with the *ESR1* promoter. Other negative correlations observed were between M-*ESR1* and UM-*ESR1* promoter, as well as between the M-*PGR* and UM-*PGR*, which is biologically plausible and confirms the trend of the promoters towards any or other methylation status. In the lobular breast cancer type, a negative correlation was observed between PR and the UM-*PGR* promoter; in this histological type, there were few cases of non methylated promoter which impeded the study of some correlations. It is of note that M-*ESR1* and M-*PGR* promoters in both ductal and lobular cancer samples, were discordant in some individual samples, which merits further study.

In conclusion, the results here shown suggest that the methylation of *ESR1* and *PGR* promoters is an infrequent event in the most frequent histological types of breast cancer; they also suggest that the methylation analysis of the *ESR1* and *PGR* promoters could represent an alternative or complement to the immunochemical expression analysis, although further studies are needed.

The DNA methylation analysis is highly sensible and specific, which is appealing for the development of methylation-based biomarkers; however, studies regarding quantitative methylation analysis, pyrosequencing, determination in serum DNA and many other issues are needed before they can be useful with diverse aim in breast cancer.

References

Allred DC (2010). Issues and updates: evaluating estrogen receptor-alpha, progesterone receptor, and HER2 in breast cancer. *Mod Pathol*, **23**, 52-59.

Baylin SB, Jones PA (2011). A decade of exploring the cancer epigenome-biological and translational implications. *Nat Rev Cancer*, **11**, 726-34.

Cancer Genome Atlas Network (2012). Comprehensive molecular portraits of human breast tumours. *Nature Oct,* **4**, 49061-70.

Castoria G, Migliaccio A, Giovannelli P, Auricchio F (2010). Cell proliferation regulated by estradiol receptor: therapeutic implications. *Steroids*, **75**, 524-7.

Esteller M (2008). Epigenetics in cancer. *N Engl J Med*, **358**, 1148-59.

Feinberg AP, Tycko B (2004). The history of cancer epigenetics. *Nat Rev Cancer*, **4**, 143-53.

Gaudet MM, Campan M, Figueroa JD, et al (2009). DNA hypermethylation of *ESR1* and *PGR* in breast cancer: pathologic and epidemiologic associations. *Cancer Epidemiol Biomarkers Prev*, **18**, 3036-43.

Goldhirsch A, Winer EP, Coates AS, et al (2013). Personalizing the treatment of women with early breast cancer: highlights of the St gallen international expert consensus on the primary therapy of early breast cancer 2013. *Ann Oncol*, **24**, 2206-23.

Hammond ME, Hayes DF, Dowsett M, et al (2010). American society of clinical oncology/college of american pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. *J Clin Oncol*, **28**, 2784-95.

Heichman KA, Warren JD (2012). DNA methylation biomarkers and their utility for solid cancer diagnostics. *Clin Chem Lab Me*, **50**, 1707-21.

Herman JG, Graff JR, Myohanen S, et al (1996) Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA*, **93**, 9821-6.

Knutson TP, Lange CA (2013). Tracking progesterone receptor-mediated actions in breast cancer. *Pharmacol Ther*, [Epub ahead of print].

Lapidus RG, Nass SJ, Butash KA, et al (1998). Mapping of ER gene CpG island methylation-specific polymerase chain reaction. *Cancer Res*, **58**, 2515-9.

Mc Cormack O, Harrison M, Kerin MJ, McCann A (2007). Role of the progesterone receptor (PR) and the PR isoforms in breast cancer. *Crit Rev Oncog,* **13**, 283-301.

Parrella P (2010). Epigenetic signatures in breast cancer: clinical perspective. *Breast Care*, **5**, 66-73.

Purdie CA, Quinlan P, Jordan LB, et al (2014). Progesterone receptor expression is an independent prognostic variable in early breast cancer: a population-based study. *Br J Cancer*, **110**, 565-72.

Radisky DC, Bissell MJ (2007). NF-kappaB links oestrogen receptor signalling and EMT. *Nat Cell Biol*, **9**, 361-3.

Ramezani F, Salami S, Omrani MD, Maleki D (2012). CpG island methylation profile of estrogen receptor alpha in Iranian females with triple negative or non-triple negative breast cancer: new marker of poor prognosis. *Asian Pac J Cancer Prev*, **13,** 451-7.

Renoir JM, Marsaud V, Lazennec G (2013). Estrogen receptor signaling as a target for novel breast cancer therapeutics. *Biochem Pharmacol*, **85**, 449-65.

Sasaki M, Dharia A, Oh BR, et al (2001). Progesterone receptor B gene inactivation and CpG hypermethylation in human uterine endometrial cancer. *Cancer Res*, **61**, 97-102.

Sturgeon SR, Balasubramanian R, Schairer C, et al (2012). Detection of promoter methylation of tumor suppressor genes in serum DNA of breast cancer cases and benign breast disease controls. *Epigenetics*, **7**, 1258-67.

Wessa P (2011) Free statistics software, office for research development and education, version 1.23-r7, available in www.wessa.net/.).