# *ESR2* gene variants (rs1256049, rs4986938, and rs1256030) and their association with breast cancer risk

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## Abstract

**Background:** Variants of the estrogen receptor β (*ESR2*) gene have been associated with different types of cancer. However, these associations have been inconsistent. We genotyped the *ESR2* variants (rs1256049, rs4986938, and rs1256030) in breast cancer (BC) patients and in healthy women.

**Results:** The variants rs1256049 and rs4986938 in the *ESR2* gene were not associated with risk susceptibility in BC patients. However, the rs1256030 variant had an association as a risk factor for BC patients when compared with controls and BC patients for the *TT* genotype [odds ratio (OR) 1.86, 95% confidence intervals (CI) 1.05–3.28, $p = 0.042$]. In addition, differences were observed in patients and controls carrying the *TT* genotype under 50 years of age (OR 1.85, 95% CI 1.05–3.27, $p = 0.043$). Thus, evident differences showed the rs1256030 variant in patients with *TT, TC,* and *TC+TT* genotypes with: 1) Stage IV (OR 1.60, 95% CI 1.06–2.54, $p = 0.033$), and 2) Luminal A (OR 1.60, 95% CI 0.47–0.21, $p = 0.041$), as well as in BC carriers of the *TT* genotype with indices of cellular proliferative (Ki-67) elevated (> 20%) and overweight (OR 1.67, 95% CI 0.85–3.28, $p = 0.041$), respectively. In BC HER2 with lymph node metastasis, the *TT* genotype was a protective factor (OR 0.38, 95% CI 0.18–0.78, $p = 0.005$). The identification

40   of haplotypes included two common *GAT* as risk factors (OR 3.1, 95% CI 1.31–7.72, $p = 0.011$)
41   and *GGC* as a protective factor (OR 0.7, 95% CI 0.60–0.97, $p = 0.034$). The combination of
42   alleles *GGGATC* was a risk factor (OR 2.5, 95% CI 1.28–5.0, $p = 0.008$).
43   **Conclusion:** The variant rs1256030 (*TT*) of the *ESR2* gene and haplotype *GAT* were associated
44   with susceptibility to BC as risk factors in this sample from the Mexican Add your abstract here.

## Introduction

46   Breast cancer (BC) is the most common type of cancer among women in the world (Sung et al.,
47   2020). In Mexico, BC is one of the major causes of mortality, and it is increasingly detected in
48   young women (Sung et al., 2020; Shoemaker et al., 2018). The gradual accumulation of
49   epigenetic and genetic events that occur in ductal and lobular normal breast cells can transform
50   into malignant tumor cells (Sung et al., 2020; Shoemaker et al., 2018; Gallegos et al., 2017).
51   Estrogens are important hormones that regulate the development of the mammary glands via an
52   estrogen receptor (ER), and they are often significantly overexpressed in BC (Gallicchio, 2006;
53   Maguire et al., 2005; Yu et al., 2011; Karlsson et al., 2016; Ghali et al., 2018). ER [known as
54   alpha (ERα) and beta (ERβ)] is a nuclear receptor formed by a protein family that functions as
55   transcriptional regulators activated by steroid hormones (Paterni et al., 2014; Karlsson et al.,
56   2016). ERα and ERβ have antagonistic functions regarding cell proliferation, while ERα
57   promotes and ERβ inhibits cell proliferation (Maguire et al., 2005; Treeck, 2009; Yu et al., 2011;
58   Paterni et al., 2014; Karlsson et al., 2016). It has been described that the estrogen receptor β gene
59   (*ESR2*) has GC-rich regions that are target sites for estrogen-responsive elements and that
60   variants of the *ESR2* gene have been associated with BC (Zhao, Dahlman-Wright & Gustafsson,
61   2010). ERs are often significantly overexpressed in many cancers, such as mammary
62   adenocarcinoma, and specifically the lack of expression of the *ESR2* gene is closely related to the
63   occurrence and progression of cholangiocarcinoma (Haldosén, Zhao & Dahlman-Wright, 2014;
64   Mahdavipour et al., 2017; Zhang et al., 2018; Carrillo et al., 2019). Approximately 75% of
65   primary BC patients express ER and more than half of these cancerous cells express
66   progesterone receptors. In postmenopausal women with BC, estrogen has been associated with
67   an increased risk (Carrillo et al., 2019). The *ESR2* gene is located on chromosome 14q22-24 and
68   has eight exons; the promotor and first intron contain regulatory sequences of other introns
69   (Haldosén, Zhao & Dahlman-Wright, 2014; Mahdavipour et al., 2017; Zhang et al., 2018).
70   Several variants have been identified, such as the rs1256049 (+1082*G/A*); it is characterized by a
71   change of *G* by *A* in exon 5 and its function remains unknown. The rs4986938 (+1730 *G/A*)
72   variant, located in the 3' untranslated sequence of exon 8, is characterized by a silent transition *G*
73   > *A*, their *A* allele correlates with a lower expression of the *ESR2* gene, and the rs1256030
74   variant is characterized by a transversion of *C* by *T* located in the intron 2 (Haldosén, Zhao &
75   Dahlman-Wright, 2014; Mahdavipour et al., 2017; Zhang et al., 2018). Studies of associations
76   between polymorphisms in *ESR2* and BC risk have been inconclusive. Some of them have
77   observed associations (Gold et al., 2004; Tsezou, 2008; Haldosén, Zhao & Dahlman-Wright,
78   2014), while others have not (Försti et al., 2003; Al-Eitan et al., 2019). However, in the Mexican
79   population, the association of *ESR2* variants (rs1256049, rs4986938, and rs1256030) in BC

80    remains unknown. Thus, the aim of this investigation was to determine the frequency and
81    association of *ESR2* variants (rs1256049, rs4986938, and rs1256030) in Mexican women with
82    BC risk.

## Materials & Methods

84    Blood samples from 472 healthy blood-donating women and 400 patients with clinically and
85    histologically confirmed BC were included in the study. All samples for the study group were
86    obtained after the patients and controls provided written informed consent, as approved by the
87    ethical local committee (1305 and 785) registered under number R-2016-1305-4 of Centro de
88    Investigación Biomédica de Occidente (CIBO), Instituto Mexicano del Seguro Social (IMSS).
89    All procedures performed in studies involving human participants were in accordance with the
90    Helsinki Declaration. Clinical and demographical data were obtained using written
91    questionnaires. New samples were included in the study, as well as stored samples from previous
92    studies, in both cases a code was assigned to maintain the confidentiality of the study
93    participants.
94    DNA was extracted from peripheral blood lymphocytes using standard protocols (Miller, Dykes
95    & Polesky, 1988).
96    The PCR amplification of the rs1256049, rs4986938, and rs1256030 *ESR2* variants was achieved
97    followed by restriction enzyme analysis according to Table 1 and Figure 1.
98    Although these polymorphisms have been extensively studied in other parts of the world, in few
99    studies their association with BC has been reflected and given that these polymorphisms have
100   been little studied in the Mexican population, we consider it important to explore them.
101   However, the selected polymorphisms such as rs1256049 has very low MAF values in Mexican
102   population, for the above the size of the analyzed sample may be one of the limitations of the
103   study.
104   The sample size was calculated with a power of 80% and the alpha type error of 0.001 was 168,
105   342 and 194 cases for polymorphisms rs1256049, rs1256030, and rs4986938 of the *ERS2* gene,
106   respectively, using the online genetic power calculator program
107   https://zzz.bwh.harvard.edu&gpc/ (Purcell, Cherny & Sham, 2003)
108   Allele frequencies of each variant were obtained by direct counting. The Hardy-Weinberg
109   equilibrium (EHW) was tested by the chi-square goodness-of-fit test to compare the observed
110   genotype frequencies with the expected frequencies among control subjects. Odds ratios and
111   95% confidence intervals (CI) were also calculated. A two-tailed $p < 0.05$ was considered
112   statistically significant. The association analysis was determined by odds ratio, and binary
113   logistic regression was performed using PASW Statistic Base 18 software, 2009 (Chicago, IL,
114   USA). Pair-wise linkage disequilibrium (D′) and haplotype frequency were analyzed by the
115   SHEsis Online Version program (Shi & He, 2005).

## Results

117   Table 2 shows the epidemiological data from the BC patients and control individuals. The
118   observed average age in BC patients was 51.45 years, ranging from 23 to 84 years of age, and

119 both tobacco and alcohol consumption were statistically different in BC patients and controls ($p$
120 < 0.0001).
121 The genotype *AA* frequency of the rs1256049*G/A* and rs4986938*G/A* variants did not show
122 significant differences between patients and controls. However, in the rs1256030*C/T*
123 polymorphism, the genotype *TT* (OR 1.86, 95% CI 1.05–3.28, $p$ = 0.042), allele *T* (OR 1.37,
124 95% CI 1.09–1.73, $p$ = 0.007), and the recessive model (OR 1.40, 95% CI 1.05–1.87, $p$ = 0.027)
125 were observed as BC risk factors, respectively. The dominant model (OR 0.66, 95% CI 0.49–
126 0.89, $p$ = 0.007) and allele *C* (OR 0.72, 95% CI 0.57–0.91, $p$ = 0.007) show a protective factor,
127 respectively. The genotype distribution of the rs1256049, rs4986938, and rs1256030 variants in
128 the *ESR2* gene were in the Hardy-Weinberg equilibrium in the control group (Table 3).
129 Comparison of the variants (rs1256049, rs4986938, and rs1256030) in woman Mexican controls
130 with some woman control populations is show in the Table 3a.
131 The comparative analysis of the association between BC patients and controls, stratified by
132 demographic data (age < 50 years old, non-smoking, and non-drinking), shows statistically
133 significant differences with the rs1256030 variant (Table 4), while the rs1256049 and rs4986938
134 variants did not show significant differences between BC patients and controls (data not shown).
135 The association of clinical characteristics with the *ESR2* (rs4986938, rs1256030) variant in the
136 BC group is shown in Table 5. In BC patients who were overweight, the genotype *GG* of
137 polymorphism rs4986938 was a protector factor (OR 0.21, 95% CI 0.05–0.85, $p$ = 0.043),
138 whereas the *GA* genotype was a risk factor (OR 1.76, 95% CI 0.56–0.24, $p$ = 0.027) in Luminal
139 A patients. The genotypes *TC, TT,* and *TCTT* of the rs1256030 variant were a risk factor in BC
140 patients stratified by stage IV (*TC*, OR 1.6, 95% CI 1.06–2.54, $p$ = 0.033), Luminal A (*TC* and
141 *TCTT*, OR 1.6, 95% IC 0.47–0.21, $p$ = 0.040), and lymph node metastatic (*TT*, OR 2.67, 95% IC
142 1.29–5.53, $p$ = 0.010). Overweight BC patients had elevated Ki-67 with the *TT* genotype (OR
143 1.67, 95% CI 0.85–3.28, $p$ = 0.041) and Luminal A with ≤ 4 gestations (*TCTT*, OR 2.64, 95% CI
144 1.13–6.14, $p$ = 0.036). However, the *TT* genotype in the HER2 histological type with lymph node
145 positivity was a protective factor.
146 The rs1256049 and rs1256030 variants were found to be a strong linkage disequilibrium (D' =
147 1.0 and r´ = 1.0) in the control group (Figure 2).
148 The haplotype and alleles combination frequency comparisons among the study groups were
149 statistically significantly different in the *GAT* (OR 3.1, 95% CI 1.31–7.72, $p$ = 0.011), *GGC* (OR
150 0.7, 95% CI 0.60–0.97, $p$ = 0.034), and alleles combination *GGGATC* (OR 2.5, 95% CI 1.28–
151 5.0, $p$ = 0.008), respectively (Tables 6 and 7). In addition, the comparison between alcohol
152 consumption with *GGGGTC* in BC patients and controls was statistically significantly different
153 (OR 2.6, 95% CI 1.19–5.7, $p$ = 0.022) (data not shown). The association of the alleles
154 combination *GGGGCC* and *GGGGTC* with clinical characteristics of BC patients, stratified by
155 molecular classification, are shown in Table 8.

156 **Discussion**
157 The incidence of BC in Mexico has increased in the last 10 years and, as in other parts of the
158 world, is currently one of the principal causes of death in women (Chávarri et al., 2012; Gallegos

159 et al., 2017; Carrillo et al., 2019). In fact, it has been observed that it occurs at an average age of
160 50 years (Gallegos et al., 2017; Carrillo et al., 2019); the data has been consistent in this study
161 with the average patient age of 51.45 ± 11.86 years. New diagnostic techniques and changes in
162 health politics have contributed to better knowledge about BC, as well as to improving the
163 quality of life of BC patients in our country (Gallegos et al., 2017). However, as BC is a
164 multifactorial disease, it is necessary to implement new studies and strategies for the detection of
165 the disease in its early stages. In this study, we observed the differences in tobacco and alcohol
166 consumption between BC patients and controls. The relationship between these two factors and
167 cancer development is well established (Hydes et al., 2019). It is known that estrogen stimulates
168 mitotic activity in ductal and lobular breast cells, contributing to an increase in cancer risk
169 (Maguire et al., 2005; Mahdavipour et al., 2017). The *ESR1* and *ESR2* genes function as ligand-
170 activated transcription factors that are stimulated by hormones, translocate to the nucleus, bind to
171 their DNA response elements, and form transcriptional complexes with coactivators, which
172 increases the response of these receptors to regulate the transcription. ERα increases and ERβ
173 decreases the proliferation of cells (Harsløf et al., 2010). Many relevant studies in BC have been
174 associated with different variants of the *ESR2* gene, including the rs1256049, rs4986938, and
175 rs1256030 variants (Forsti et al., 2003; Maguire et al., 2005; Tsezou, 2008; Yu et al., 2012;
176 Karlsson et al., 2016; Haldosén, Zhao & Dahlman-Wright, 2014; Mahdavipour et al., 2017;
177 Ghali et al., 2018). The rs4986938 and rs1256049 are the most extensively studied variants of the
178 *ESR2* gene (Yu et al., 2011; Haldosén, Zhao & Dahlman-Wright, 2014), but they have
179 demonstrated contradictory results; some have been associated with susceptibility development
180 to BC (Dai et al., 2014), whereas others have not (Wang et al., 2013; Ghali et al., 2018).
181 Moreover, little is known about the association of the rs1256049, rs4986938, and rs1256030
182 variants in Mexican BC patients. In our study group, the frequency of genotypes, alleles, and
183 recessive models of both the rs1256049 and rs4986938 variants were not significantly different
184 between the BC patients and controls ($p > 0.05$). This data is in accordance with a study of a
185 Swedish population with a total of 723 BC cases and 480 controls, which did not find a
186 statistically significant association for any of the single polymorphisms shown for the rs1256049
187 variant (Maguire et al., 2015). However, one of the reasons for not observing an association
188 between the rs125049 polymorphism and breast cancer could be the low frequency of the
189 polymorphic allele and probably the sample size analyzed may be insufficient to observe such
190 association.
191 In our study group, the frequency of rs1256049 and rs4986938 did not represent a risk factor for
192 BC in the Mexican population. These data were consistent with meta-analysis studies that
193 examined different populations and cancer types (Dai et al. 2014, Li et al., 2019). However,
194 when comparing the rs1256049 and rs4986938 genotypes *GG* and *GA* of our women Mexican
195 control group with the control groups of other populations, differences were observed with the
196 Asians and Caucasian populations (Greendale et al., 2006). Similarities observed were with
197 respect to the distribution of the *AA* genotype of the rs1256049 polymorphism in the Caucasian
198 population. Regarding the rs1256030 polymorphism, it showed differences above all with the *CC*

199  genotype of the African, Caucasian and Asian population compared to the Mexican population.
200  Which points to the genetic heterogeneity of this polymorphism in other populations, as well as
201  demonstrating the importance of the sample size used in the different population studies.
202  Another study that included 436 postmenopausal women with BC from a Han Chinese
203  population found no statistically significant association for the rs4986938 variant (Yu et al.,
204  2011). A meta-analysis study that included both polymorphisms was not associated with BC risk
205  in any model (Breast and Prostate Cancer Cohort Consortium et al., 2008). In addition, we found
206  statistically significant differences with the genotype *TT*, allele *T,* and the recessive model
207  *CC+TC* of the rs1256030 variant ($p < 0.05$) between BC and control group, observing it as factor
208  of susceptibility risk to the development in BC. This variant has been studied in cognitive
209  development in menopause women (Karlsson et al., 2016), hepatic cirrhosis (Yang et al., 2015),
210  ovarian cancer (Lurie et al., 2009), and BC (Gold et al., 2004; Goulart, Zee & Rexrode, 2009;
211  Al-Eitan, 2019). The data are in accordance with a study that demonstrated the association of a
212  haplotype that includes the variant rs1256030 with a susceptibility development risk to BC (Gold
213  et al., 2004). How the rs1256030 variant had recognition sites with a different regulatory
214  sequence may have influenced gene expression or function of *ESR2* (Goulart, Zee & Rexrode,
215  2009); it is possible that the variant might affect their regulation and contribute to tumorigenicity
216  in breast tissue. Furthermore, an intronic mutation may affect splicing sites and lead to a
217  different final protein. In this study, we observed the genotype combinations *TC*, *TT,* and *TCTT*
218  of the rs1256030 variant as a risk factor for susceptibility to BC development in women who are
219  < 50 years old, do not drink alcohol, or smoke. Although there are no existing studies that
220  support these findings, these confounding factors show that this stratification is important for
221  contributing to differences in the *ESR2* variants' associations with BC risk. Molecular
222  mechanisms in the regulation of ERβ expression are relatively unclear, probably due to the
223  position of rs1256030 in the promotor region of the *ESR2* gene as a recognition site for
224  transcription binding in the regulation of the gene (Goulart, Zee & Rexrode, 2009; Lurie et al.,
225  2009).
226  We observed that the rs1256049 and rs1256030 variants were risk factors for susceptibility to the
227  development of BC, stratified by the different clinicopathological parameters, such as Luminal
228  A, stage IV, and positive lymph nodes. Although the expression of ERβ in BC has been analyzed
229  in different clinical materials, the regulation mechanisms of ERβ in the development of cancer
230  are still not understood. The expression of ERα and related genes has emerged as one of the
231  major determinants of molecular classification of invasive BC. The correlation of ERβ
232  expression with clinical pathological parameters of BC has been investigated in different studies
233  (Marotti et al., 2010; Rezende et al., 2017). Marotti et al. (Marotti et al., 2010), studied tissue
234  microarrays of paraffin blocks of 3093 BC and found a relationship between the expression of
235  ERβ and the molecular class of invasive BC. Overall, 68% of breast carcinomas were ERβ+. The
236  expression of ERβ was significantly associated with the expression of ERα ($p < 0.0001$) and PR
237  ($p < 0.0001$). ERβ expression was significantly related to molecular category ($p < 0.0001$) and
238  was more common in Luminal A (72% of cases) and B (68% of cases). Rezende et al. (Rezende

239    et al., 2017), observed that rs4986938 sporadically modulates the severity of BC. Zhang et al.
240    (Zhang et al., 2012), correlated the ERβ with BC stage IV and node positive.
241    In addition, we observed the protective effect of the *TT* genotype of the rs1256030 variant in BC
242    HER2 with a positive lymph node. Observations that have been documented by other studies
243    have observed an inverse correlation in both ERβ expression in BC HER2 with positive lymph
244    nodes (Oueslati et al., 2017) and polymorphism in the *ESR2* gene (Chattopadhyay et al., 2014).
245    A plausible explanation would be that the rs1256030 variant located upstream in the promoter
246    region has sequence recognition to several transcription factor binding, and the polymorphism
247    might be modified by these binding sites and alter the transcription of *ESR1* expression,
248    consequently modulating *ESR2* target gene expression (Ghali et al., 2018).
249    The *ESR2* variants were in linkage disequilibrium, and haplotypes were constructed. Two
250    common haplotypes (rs1256049 and rs1256030), comprising approximately 99% of all
251    haplotypes, were found. The *GGC* haplotype was more frequent in BC patients (61%) and
252    controls (67%) and was associated as a protective factor for susceptibility to BC development.
253    However, the haplotype *GGT* and alleles combination *GGGATT* were associated as risk factors
254    for susceptibility to the development of BC. Although the analyzed number of cases for carrying
255    different variations with this allele was low these can be limitans of this studied, ever more
256    considering the allele frequencies, the MAF value in the study cohort of the rs1256049 variant is
257    very low, the distribution of alleles within the cohort may be considered insufficient to determine
258    the genotype-phenotype correlation. This limitation may be the main reason why a significant
259    association was not found for this variant.
260    However, these are a reflection of the common genotypes observed in the Mexican population,
261    no studies exist on these haplotypes analyzed here.
262    In this study, we also observed, as a protective factor, the association between alleles
263    combination *GGGGCC* in Luminal A with a partial response, HER2 with gastric (nausea,
264    diarrhea, vomiting, stomatitis, and mucositis), hematological (neutropenia, anemia, and
265    thrombocytopenia) toxicities during chemotherapy treatment, as well as the reduced risk of
266    *GGGGTC* alleles combination in BC triple negative in stage I–II. Investigations of the
267    expression of ERβ showed that the different molecular subtypes of BC, including Luminal A,
268    Luminal B, HER2, and triple negative, present different profiles of estrogen expression
269    (Mahdavipour et al., 2017). These depend on the moment of therapeutic treatment, before the
270    operation, or after chemotherapy and radiotherapy, which could be a predictive factor for the
271    prognosis of patients with BC (Haldosén, Zhao & Dahlman-Wright, 2014; Yu et al., 2016;
272    Mahdavipour et al., 2017; Carrillo et al. 2019). Moreover, the combined effects of the three
273    polymorphisms in the *ESR2* gene might confer a significant genetic predisposition to a complex
274    disease. Thus, a possible explanation for the protective role of polymorphisms in the *ESR2* gene
275    could be that the function of ERβ in BC cells displayed different roles depending on the presence
276    of ERα and other ERβ isoforms. When ERα is present, it has anti-proliferative function, but
277    exerts proliferative effects in the absence of ERα. These variations and the different variants in
278    the *ESR2* gene, as well as the epigenetic factor, could contribute to the variability of results in

279    clinical studies in BC (Haldosén, Zhao & Dahlman-Wright, 2014; Yu et al., 2016; Mahdavipour
280    et al., 2017; Carrillo et al. 2019).
281    Although one of the limitations of this study was the size of the sample analyzed by the MAF
282    very low frequency of two of the polymorphisms analyzed. One of the strengths of this study
283    was the strong linkage disequilibrium found between polymorphisms rs1256049 and rs1256030
284    of the ESR2 gene in control samples. It should be noted that the number of studies available
285    where the LD of these polymorphisms is analyzed is limited.

## Conclusions

287    In conclusion, our results showed that the rs1256030 variant had an association risk factor for BC,
288    comparing controls and BC patients in the genotype *TT*, allele *T,* and recessive model (*CC+TC*
289    genotype). In addition, differences were also observed in patients and controls with regard to
290    carriers of the *TT* genotype < 50 years old, and their non-smoking and drinking status. Thus, the
291    differences are evident in patients with *TT*, *TC,* and *TCTT* genotype with 1) Stage IV, 2) Luminal
292    A, and 3) positive lymph node; in addition, in BC with Ki-67 elevated and overweight with
293    genotype *TT* and Luminal A with a low number of gestations. The presence of the *TT* genotype is
294    a protective factor in HER2 patients with positive lymph nodes. The identification of haplotypes
295    included two common factors: the *GAT* as a risk factor and the *GGC* as a protective factor. The
296    alleles combination *GGGATC* as a risk factor and *GGGGCC* (Luminal A with partial
297    chemotherapy response and HER2 with toxicity gastric), and *GGGGTC* (triple negative with stage
298    I–II) as protective factors, respectively, confirming that these factors significantly contribute to
299    BC susceptibility in the analyzed sample from the Mexican population.
300    Further studies are required to confirm these observations, as well as the number of analyzed
301    variations in this study is limited to reflect more extensional results on the alleles combination in
302    a given population other polymorphisms of this gene need to be analyzed in the BC from Mexican
303    population.

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PCR conditions of rs1256049, rs4986938, rs1256030 polymorphisms in the *ESR2* gene

1    **Table 1. PCR conditions of rs1256049, rs4986938, rs1256030 polymorphisms in the *ESR2* gene**

| | rs1256049 (+1082*G/A*, exon 5) | rs4986938 (+1730 *G/A*, 3'UTR, exon 8) | rs1256030 *T/C* (Intron 2) |
|---|---|---|---|
| **\*PCR Primers: sense** <br> **antisense** | 5'-TTGCGCAGCTTAACTTCAAA-3' <br> 5'-ACCTGTCCAGAACAAGATCT-3' | 5'-CAATGCATATCCTGCCTGTG-3' <br> 5'-GGTTTAGGGGTGGGGTAGACTG-3' | 5'-CAATGCATATCCTGCCTGTG-3' <br> 5'-TCCCGGAAATCTGATACAGC-3' |
| **PCR product size** | 321 bp | 442 bp | 251 bp |
| **PCR conditions** | Buffer enzyme 1X, 7.5 pmol of each primer, 0.2 mM of dNTPs, 2.0 mM of MgCl2, 2.5 u of Taq polymerase, 100nM of genomic DNA | Buffer enzyme 1X, 7.5 pmol of each primer, 0.2 mM of dNTPs, 2.0 mM of MgCl2, 2.5 u of Taq polymerase, 100nM of genomic DNA | Buffer enzyme 1X, 7.5 pmol of each primer, 0.2 mM of dNTPs, 2.5 mM of MgCl2, 2.5 u of Taq polymerase, 100nM of genomic DNA |
| **Annealing PCR temperature** | 60°C | 55°C | 57°C |
| **Recognition enzyme restriction** | RsaI, 37°C, (5'…GT↓AC…3') | AluI, 37°C, (5'…AG↓CT…3') | AluI, 37°C, (5'…AG↓CT…3') |
| **\*\*Allele identified:** | | | |
| (Wild type) | *G*: 321 | *G*: 442 | *C*: 227+ 24 |
| (Polymorphic type) | *A*: 211+110 | *A:* 336+106 | *T*: 147+80+24 |

2    \*The primers previously described (Mahdavipour et al. 2017); \*\* the genotypes were identified in 6%
3    polyacrylamide gels (29:1), followed by silver nitrate staining.

Demographic data for the study groups

1 **Table 2. Demographic data for the study groups**

| | BC patients(n=400) | | Controls(n=472) | | *p*-value |
|---|---|---|---|---|---|
| **Age** (years) | | | | | |
| Mean (SD) | 51.45 | (11.86) | 50.99 | (12.18) | 0.09* |
| < 50 years [(n), %] | (215) | 54.0 | (256) | 54.0 | |
| ≥ 50 years [(n), %] | (185) | 46.0 | (216) | 46.0 | 0.939** |
| **Tobacco consumption** | | | | | |
| No [(n), %] | (292) | 73.0 | (423) | 90.0 | |
| Yes [(n), %] | (108) | 27.0 | (49) | 10.0 | <0.0001** |
| **Alcohol consumption** | | | | | |
| No [(n), %] | (288) | 72.0 | (430) | 91.0 | |
| Yes [(n), %] | (112) | 28.0 | (42) | 9.0 | <0.0001** |

2 SD (standard deviation); * Student's T-test; **Chi-square test.

Genotype and allelic distribution of the rs1256049*G/A*, rs4986938*G/A*, rs1256030*T/C* variants of *ESR2* in BC patients and controls. Frequency comparison of the rs1256049, rs4986938 and rs1256030 *ESR2* gene polymorphisms in wom

**Table 3. Genotype and allelic distribution of the rs1256049*G/A*, rs4986938*G/A*, rs1256030*T/C* variants of *ESR2* in BC patients and controls**

| Variant | | BC | | Controls* | | OR | 95%(CI) | *p*- value |
|---|---|---|---|---|---|---|---|---|
| **rs1256049** | *Genotype* | (n=400) | % | (n=324) | % | | | |
| | GG | (391) | 98 | (322) | 99 | 1 | | |
| | GA | (9) | 2 | (2) | 1 | 3.70 | (0.79-17.2) | 0.123 |
| | AA | (0) | 0 | (0) | 0 | | | |
| Dominant | GG | (391) | 98 | (322) | 99 | 0.27 | (0.05-1.25) | 0.123 |
| | GA+AA | (9) | 2 | (2) | 1 | | | |
| Recessive | AA | (0) | 0 | (0) | 0 | 1.23 | (0.07-19.8) | 1.0 |
| | GG+GA | (400) | 100 | (324) | 100 | | | |
| | *Allele* (2n800) | | | (2n=648) | | | | |
| | G | (791) | 0.988 | (646) | 0.996 | 0.272 | (0.05-1.26) | 0.124 |
| | A | (9) | 0.012 | (2) | 0.004 | 3.67 | (0.79-17.6) | 0.124 |
| **rs4986938** | *Genotype* | (n=400) | % | (n=447) | % | | | |
| | GG | (300) | 75 | (347) | 78 | 1 | | |
| | GA | (96) | 24 | (93) | 21 | 1.20 | (0.86-1.66) | 0.301 |
| | AA | (4) | 1 | (7) | 1 | 0.63 | (0.18-2.18) | 0.553 |
| Dominant | GG | (300) | 75 | (347) | 78 | 0.86 | (0.62-1.18) | 0.373 |
| | GA+AA | (100) | 25 | (100) | 22 | | | |
| Recessive | AA | (4) | 1 | (7) | 1 | 1.57 | (0.45-5.42) | 0.553 |
| | GG+GA | (396) | 99 | (440) | 99 | | | |
| | *Allele* (2n800) | | | (2n=894) | | | | |
| | G | (696) | 0.870 | (787) | 0.880 | 0.909 | (0.68-1.21) | 0.577 |
| | A | (104) | 0.130 | (107) | 0.120 | 1.099 | (0.82-1.46) | 0.577 |
| **rs1256030** | *Genotype* | (n=400) | % | (n=346) | % | | | |
| | CC | (199) | 50 | (201) | 58 | 1 | | |
| | TC | (162) | 40 | (126) | 37 | 1.18 | (0.88-1.59) | 0.285 |
| | TT | (39) | 10 | (19) | 5 | 1.86 | (1.05-3.28) | 0.042 |
| Dominant | CC | (199) | 50.5 | (201) | 58 | 0.66 | (0.49-0.89) | 0.0.007 |
| | CT+TT | (201) | 49.5 | (145) | 42 | | | |
| Recessive | TT | (39) | 10 | (19) | 5 | 1.40 | (1.05-1.87) | 0.027 |
| | CC+TC | (361) | 90 | (327) | 95 | | | |
| | *Allele* (2n800) | | | (2n=692) | | | | |
| | C | (560) | 0.700 | (528) | 0.763 | 0.724 | (0.57-0.91) | 0.007 |
| | T | (240) | 0.300 | (164) | 0.237 | 1.379 | (1.09-1.73) | 0.007 |

OR (odds ratio), CI (confidence intervals, *p*-value (significant < 0.05). * Hardy-Weinberg equilibrium in controls for rs1256049 (chi-square test = 0.0031, *p* = 0.9555), rs4986938 ((chi-square test = 0.071; *p* = 0.7887), and rs1256030 (chi-square test = 0.016; *p* = 0.8974). From sample total of controls (n=472) only were obtained the genotypes for rs1256049*G/A* (n=324), rs4986938*G/A* (n=447), rs1256030*T/C* (n=346) of *ESR2* variants.

12  **Table 3a. Frequency comparison of the rs1256049, rs4986938 and rs1256030 *ESR2* gene**
13  **polymorphisms in women Mexican control with other populations**

| Polymorphism | | | | | | Genotypes *p-value* | | |
|---|---|---|---|---|---|---|---|---|
| **rs1256049** | | | *GG* | *GA* | *AA* | *GG* | *GA* | *AA* |
| Race | Reference | Country | | | | | | |
| Mestizo | (this study) | Mexico | 322 | 2 | 0 | | | |
| | | | | | *Vs.* | | | |
| Caucasian | (Dai et al., 2014) | Germany | 5086 | 389 | 5 | 0.00001 | 0.00001 | 0.332 |
| Asian | (Dai et al., 2014) | Brazil | 230 | 208 | 29 | 0.00001 | 0.00001 | 0.00001 |
| Caucasian | (Dai et al., 2014) | USA | 6751 | 734 | 70 | 0.00001 | 0.00001 | 0.371 |
| Caucasian | (Dai et al., 2014) | Sweden | 356 | 41 | 1 | 0.00001 | 0.00001 | 1.0 |
| Caucasian | (Dai et al., 2014) | Finland | 198 | 39 | 1 | 0.00001 | 0.00001 | 0.577 |
| Asian | (Dai et al., 2014) | China | 537 | 541 | 131 | 0.00001 | 0.00001 | 0.00001 |
| **rs4986938** | | | *GG* | *GA* | *AA* | *GG* | *GA* | *AA* |
| Mestizo | (this study) | Mexico | 347 | 93 | 7 | | | |
| | | | | | *Vs.* | | | |
| African | (Li et al., 2019) | Tunisia | 94 | 120 | 69 | 0.00001 | 0.00001 | 0.00001 |
| Caucasian | (Li et al., 2019) | Brazil | 109 | 115 | 29 | 0.00001 | 0.00001 | 0.00001 |
| Caucasian | (Li et al., 2019) | Germany | 2169 | 2557 | 752 | 0.00001 | 0.00001 | 0.00001 |
| Asian | (Li et al., 2019) | Japan | 281 | 102 | 5 | 0.097 | 0.074 | 0.964 |
| Caucasian | (Li et al., 2019) | Japan | 236 | 171 | 51 | 0.00001 | 0.00001 | 0.00001 |
| Asian | (Li et al., 2019) | India | 9 | 81 | 159 | 0.00001 | 0.0008 | 0.00001 |
| Caucasian | (Li et al., 2019) | USA | 3229 | 3304 | 984 | 0.00001 | 0.00001 | 0.00001 |
| Caucasian | (Li et al., 2019) | USA | 470 | 612 | 190 | 0.00001 | 0.00001 | 0.00001 |
| Caucasian | (Li et al., 2019) | Sweden | 175 | 190 | 56 | 0.00001 | 0.00001 | 0.00001 |
| Caucasian | (Li et al., 2019) | Sweden | 105 | 103 | 30 | 0.00001 | 0.00001 | 0.00001 |
| Caucasian | (Lidaka et al. 2021) | Russia | 10 | 49 | 8 | 0.00001 | 0.00001 | 0.00001 |
| Caucasian | (Marini et al., 2012) | Italy | 31 | 45 | 13 | 0.00001 | 0.00001 | 0.00001 |
| **rs1256030** | | | *TT* | *TC* | *CC* | *TT* | *TC* | *CC* |
| Mestizo | (this study) | Mexico | 9 | 126 | 201 | | | |
| | | | | | *Vs.* | | | |
| African | (Greendale et al., 2006) | USA | 12 | 134 | 147 | 0.444 | 0.0443 | 0.0188 |
| Caucasian | (Greendale et al., 2006) | USA | 126 | 343 | 220 | 0.00001 | 0.0002 | 0.00001 |
| Asian | (Greendale et al., 2006) | USA-Chinese | 10 | 73 | 67 | 0.0654 | 0.0269 | 0.0026 |
| Asian | (Greendale et al., 2006) | USA-Japanese | 27 | 75 | 57 | 0.00001 | 0.0514 | 0.00001 |

Association of *ESR2* rs1256030 variant with age, non-drinking and non-smoking in the BC patients and controls

1   **Table 4. Association of *ESR2* rs1256030 variant with age, non-drinking and non-smoking**
2   **in the BC patients and controls**

| Variant | Genotype | Variable | OR | 95%(CI) | *p*-value |
|---------|----------|----------|-----|---------|-----------|
| **rs1256030** | *TT* | <50 years old | 1.85 | (1.05-3.27) | 0.043 |
| | *TT* | Non-smoking | 2.03 | (0.70-0.29) | 0.018 |
| | *TT* | Non-drinking | 2.06 | (0.72-0.29) | 0.019 |
| | *TCTT* | Non-smoking | 1.40 | (0.34-0.15) | 0.026 |
| | *TCTT* | Non-drinking | 1.39 | (0.39-0.15) | 0.036 |

3   OR (odds ratio), CI (confidence intervals, *p*-value (significant < 0.05).

Association of *ESR2* rs1256049, rs1256030 variants with clinical variables of BC patients.

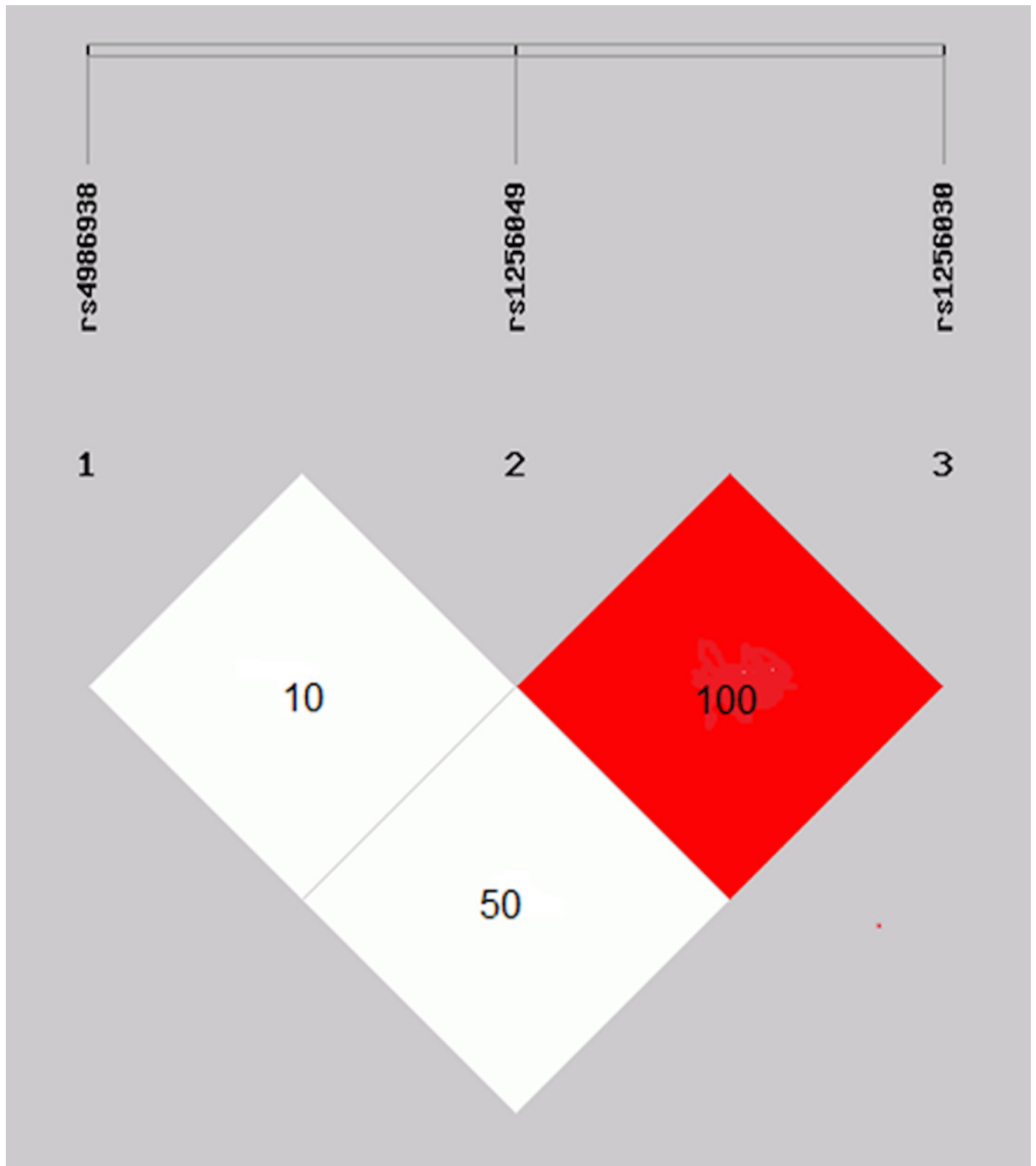
3  **Table 5. Association of *ESR2* rs1256049, rs1256030 variants with clinical variables of BC**
4  **patients.**

| Variant | Histological status | Genotype | Clinical variable* | OR | 95% (CI) | *p*-value |
|---------|--------------------|----------|--------------------|-----|----------|-----------|
| **rs4986938*G/A*** | | *GG* | **Overweigh (25-29.9 BMI) | 0.21 | (0.05-0.85) | 0.043 |
| | | *GA* | Luminal A | 1.76 | (0.56-0.24) | 0.027 |
| **rs1256030*T/C*** | | *TC* | **Stage IV | 1.6 | (1.06-2.54) | 0.033 |
| | | *TC* | Luminal A | 1.6 | (0.47-0.21) | 0.041 |
| | | *TT* | lymph node metastatic | 2.67 | (1.29-5.53) | 0.010 |
| | | *TCTT* | Luminal A | 1.6 | (0.47-0.21) | 0.040 |
| | **Ki-67 (≥20%)** | *TT* | **Overweigh | 1.67 | (0.85-3.28) | 0.041 |
| | **Luminal A** | *TCTT* | Gestations (≤ 4) | 2.64 | (1.13-6.14) | 0.036 |
| | **HER2** | *TT* | lymph node metastatic | 0.38 | (0.18-0.78) | 0.005 |

5  OR (odds ratio), CI (confidence intervals, *p*-value (significant < 0.05). **\*Non-significance clinical
6  variables included in the analysis: Age (<50, ≥ 50 years), tobacco and alcohol consumption,
7  menopause, cancer type (ductal, lobular), Chemotherapy (non-chemotherapy response, non-
8  chemotherapy response by recurrence. The non-response to chemotherapy treatment with
9  Anthracyclines (e. g. doxorubicin, epirubicin, liposomal doxorubicin), taxanes (docetaxel,
10  paclitaxel) and trastuzumab was evaluated according to the pathological Ryan's classification
11  described as follows: 1. Moderate response (single cells or small groups of cancerous cells), 2.
12  Minimum response (residual cancer surrounded by fibrosis), and 3. Poor response (minimal or
13  no tumor destruction, extensive residual cancer). Molecular classification (Luminal B, Triple
14  negative).

# Figure 1

Linkage disequilibrium of the rs1256049 and rs1256030 polymorphisms of *ESR2*

gene were found to be in strong linkage disequilibrium (D' = 1.0 and r´= 1.0). The rs4986938 variant was moderately LD of rs1256049 variant.

Haplotype frequency of the rs1256049, rs4986938, rs1256030 variants of *ESR2* in BC patients and controls

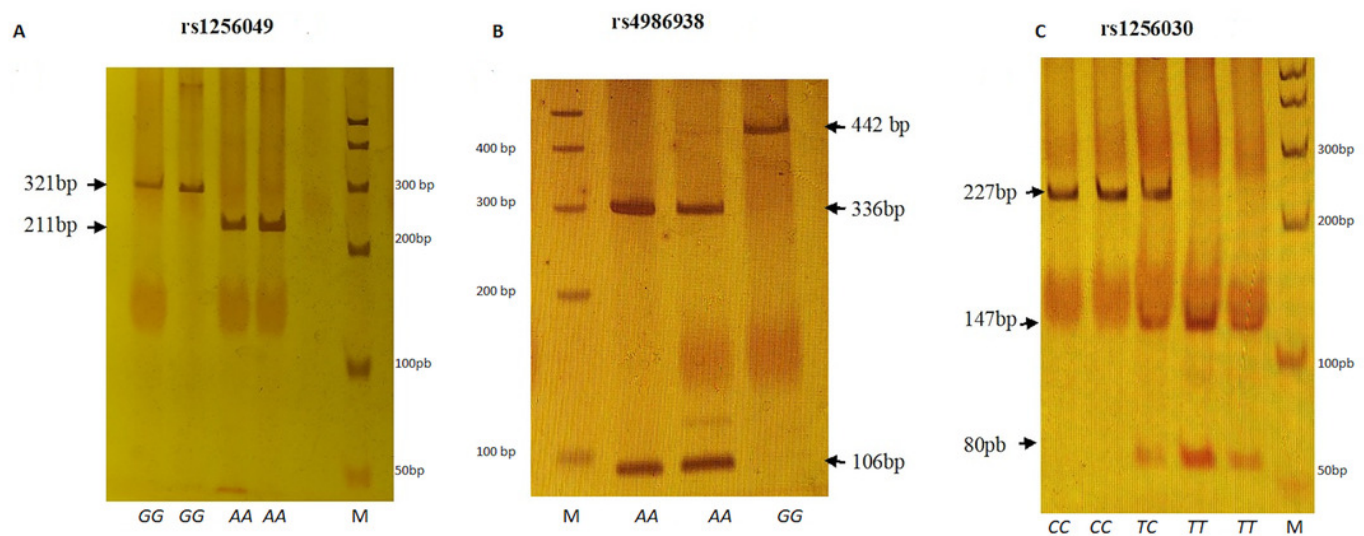
1 **Table 6. Haplotype frequency of the rs1256049, rs4986938, rs1256030 variants of *ESR2* in**

2 **BC patients and controls**

| *ESR2* gene | | | BC(n=800) | | Controls(n=472) | | | |
|---|---|---|---|---|---|---|---|---|
| rs1256049*G/A* | rs4986938*G/A* | rs1256030*T/C* | n | % | n | % | OR95%(CI) | *p*-value |
| A | G | C | 5 | 0.6 | 1 | 0.2 | 2.9 (0.34-25.4) | 0.53 |
| A | G | T | 2 | 0.3 | 1 | 0.2 | 1.1 (0.10-13.0) | 1.0 |
| G | A | C | 72 | 9.0 | 46 | 9.6 | 0.9 (0.62-1.35) | 0.73 |
| G | A | T | 30 | 4.0 | 6 | 1.0 | 3.1 (1.31-7.72) | **0.01** |
| G | G | C | 487 | 61.0 | 316 | 67.0 | 0.7 (0.60-0.97) | **0.03** |
| G | G | T | 203 | 25.0 | 102 | 22.0 | 1.2 (0.94-1.61) | 0.14 |
| A | A | C | 1 | 0.1 | 0 | 0.0 | | |
| A | A | T | 0 | 0.0 | 0 | 0.0 | | |

# Figure 2

Electrophoretic running on 6% polyacrylamide gel (29:1).

**A)** Rsa I digestion. Genotypes *GG* (321 bp), *AA* (211+110bp). **B)** Alu I digestion. Genotypes *GG* (442bp) and *AA* (336+106bp). **C)** Alu I digestion. Genotypes *CC* (227+ 24bp), *TC* (227+ 147+ 80+24bp) and *TT* (147+ 80+24bp). M represents 50bp DNA ladder.

Alleles combination distribution of *ESR2* variants rs1256049, rs4986938, rs1256030 of
in study groups

1 **Table 7. Alleles combination distribution of *ESR2* variants rs1256049, rs4986938,**
2 **rs1256030 of in study groups**

| *ESR2* gene | | | BC(n=400) | | Controls(n=234) | | | |
|---|---|---|---|---|---|---|---|---|
| rs1256049*G/A* | rs4986938*G/A* | rs1256030*T/C* | n | % | n | % | OR (95%CI) | *p*-value |
| *GG* | *GG* | *CC* | 152 | 38.0 | 105 | 45.0 | 0.7 (0.55-1.06) | 0.133 |
| *GG* | *GG* | *TC* | 110 | 27.5 | 66 | 28.0 | 0.9 (0.66-1.35) | 0.843 |
| *GG* | *GG* | *TT* | 32 | 8.0 | 12 | 5.0 | 1.5 (0.80-3.14) | 0.241 |
| *GA* | *GG* | *CC* | 4 | 1.0 | 1 | 0.5 | 2.3 (0.25-20.9) | 0.657 |
| *GA* | *GG* | *TC* | 1 | 0.25 | 1 | 0.5 | 0.57 (0.03-9.3) | 1.0 |
| *GA* | *GG* | *TT* | 1 | 0.25 | 0 | 0.0 | 1.1 (0.10-12.7) | 1.0 |
| *GA* | *GA* | *CC* | 1 | 0.25 | 0 | 0.0 | 1.1 (0.10-12.7) | 1.0 |
| *GA* | *GA* | *TC* | 2 | 0.50 | 0 | 0.0 | 1.7 (0.17-16.7) | 1.0 |
| *GG* | *GA* | *CC* | 43 | 10.75 | 32 | 15.0 | 0.74 (0.4-1.22) | 0.301 |
| *GG* | *GA* | *TC* | 45 | 11.25 | 11 | 5.0 | 2.5 (1.28-5.0) | **0.008** |
| *GG* | *GA* | *TT* | 5 | 1.25 | 3 | 1.0 | 0.96 (0.22-4.0) | 1.0 |
| *GG* | *AA* | *CC* | 2 | 0.50 | 3 | 0 | 1.7 (0.17-16.7) | 1.0 |
| *GG* | *AA* | *TC* | 2 | 0.50 | 0 | 0.0 | 1.7 (0.17-16.7) | 1.0 |

Association of alleles combination with clinical variables of BC patients group

1    **Table 8. Association of alleles combination with clinical variables of BC patients group**

| Haplogenotype | Histological status | Clinical variable* | OR | 95% (CI) | *p*-value |
|---|---|---|---|---|---|
| *GGGGCC* | Luminal A | Partial response | 0.25 | (0.07-0.07) | 0.011 |
| | HER2 | Gastrict toxicity | 0.21 | (0.06-0.74) | 0.016 |
| *GGGGTC* | Triple negative | Stage I-II | 0.3 | (0.10-0.87) | 0.044 |

2    OR (odds ratio), CI (confidence intervals, *p*-value (significant < 0.05). **\***Non-significance clinical
3    variables included in the analysis: Age (<50, ≥ 50 years), tobacco and alcohol consumption,
4    menopause, cancer type (ductal, lobular), Chemotherapy (non-chemotherapy response, non-
5    chemotherapy response by recurrence. The non-response to chemotherapy treatment with
6    Anthracyclines (e. g. doxorubicin, epirubicin, liposomal doxorubicin), taxanes (docetaxel,
7    paclitaxel) and trastuzumab was evaluated according to the pathological Ryan's classification
8    described as follows: 1. Moderate response (single cells or small groups of cancerous cells), 2.
9    Minimum response (residual cancer surrounded by fibrosis), and 3. Poor response (minimal or
10   no tumor destruction, extensive residual cancer). Molecular classification (Luminal B).