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Investigation of peroxisome proliferator-activated receptor-gamma gene Pro12Ala polymorphism and its effect on peroxisome proliferator-activated receptor-gamma mRNA expression in human cancer cell lines

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Abstract
The Peroxisome proliferator-activated receptor-gamma (PPARγ) is a member of the nuclear receptor family of transcription factors. In addition to its role in lipid and glucose metabolisms, PPARγ has possible roles in tumor suppression. One of the most common variations in PPARγ is the Pro12Ala polymorphism. It is the change of proline amino acid to alanine amino acid by the conversion of cytosin (C) to guanine (G) at 34th nucleotide of the 12th codon PPARγ gene. Several recent studies have shown that Pro12Ala polymorphism may be associated with many diseases, such as obesity, diabetes, and some types of cancer. Moreover, Pro12Ala polimorphism has been shown to affect PPARγ mRNA expression in the context of obesity. However, there have been no studies investigating the relationship Pro12Ala polymorphism and PPARγ mRNA expression in cancer. In this study, Pro12Ala polymorphism in PPARγ gene was investigated in cancer and normal cell lines via using restriction fragment length polymorphism and Sanger Sequencing. Depending on the Pro12Ala status, the effect of the polymorphism on PPARγ mRNA expression was investigated via using Real Time Polymerase Chain Reaction. In conclusion; amongst 13 cell lines that were screened, heterozygous Pro12Ala polymorphism was detected only in AGS and Caki-1 cancer cell lines. PPARγ mRNA expression in these cell lines was found to be lower than PPARγ mRNA expression in cell lines without Pro12Ala polymorphism such as MCF10A, SK-BR-3, MDA-MB-468. Taken together, Pro12Ala polymorphism may modulate the mRNA expression level of PPARγ. The role of this modulation in cancer should further be investigated.

Keywords: Peroxisome proliferator-activated receptor-gamma, Pro12Ala Polymorphism, Cancer, Cell line, Peroxisome proliferator-activated receptor-gamma mRNA expression

Introduction

Peroxisome proliferator-activated receptor-gamma (PPARγ) is a member of nuclear receptor family of ligand-dependent transcription factors. It is highly implicated in adipogenic regulation. Due to its important role in adipogenic differentiation and lipid storage, it is mainly expressed in adipose cells [1]. In addition to adipocytes, it is expressed in a variety of tissues such as liver, muscle, mammary gland where it performs several different functions extending from regulation of glucose and lipid metabolism to inflammation control [2].

The human PPARγ gene is located on the chromosome 3p25 (OMIM: 601487). It encodes for four different transcripts, namely PPARγ1, PPARγ3, PPARγ4 and PPARγ2 [3]. Two alternatively spliced protein isoforms, PPARγ1 and PPARγ2 are expressed from these transcripts [4].

Several sequence variants of PPARγ have been identified. One of the most common of these variants is a C to G transition at 34th nucleotide of the 12th codon PPARγ gene leading to a proline to alanine exchange at amino acid position 12 (Pro12Ala)(rs1801282) [5]. This variant is located on the ligand-independent activation domain of PPARγ. Therefore, it is thought that the proline to alanine exchange in this domain may cause a conformational change in the protein resulting in reduced DNA binding affinity and consequently reduced transcriptional activity [6].

In addition to several diseases including obesity and diabetes, PPARγ is also implicated in the pathology of cancer. PPARγ is thought to play possible anti-carcinogenic roles in a number of different cell types depending on its anti-proliferation, pro-differentiation and pro-apoptotic properties [7]. There are several studies on the association of Pro12Ala polymorphism with different cancer types

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such as breast cancer and gastric cancer. However, there is still some controversy among different studies, i.e. colorectal cancer, on whether there is any association or which allele is protective over the other in terms of cancer risk [8]. Although there seems to be differential effect of Pro12 and Ala12 alleles of PPARγ gene on the risk of different cancer types, this effect highly depends on the ethnicity of the populations in the studies. The exact molecular mechanism of these allele specific effects is yet to be elucidated.

In a relatively recent study, a correlation between Pro12Ala polymorphism and PPARγ mRNA expression was demonstrated in obesity patients. According to this study, in obesity patients Pro12 allele is associated with high expression of PPARγ in comparison with patients with Ala12 allele [9]. These data led us to think whether PPARγ Pro12Ala polymorphism and its possible effect on PPARγ mRNA expression polymorphism may have a role in carcinogenesis. To the best of our knowledge, there are no studies on the relationship between Pro12Ala polymorphism and PPARγ mRNA expression in cancer cell lines.

In this study, we aimed to investigate the presence of Pro12Ala polymorphism in PPARγ gene in cancer and normal cells lines and to determine the effect of this polymorphism on the PPARγ mRNA expression in cancer cell lines.

Material and Methods

Cell Culture

In our study; HUVEC (ATCC® CRL-1730 ™), MCF10A (ATCC® CRL-10317 ™), Caki-I (ATCC® HTB-46 ™), Jurkat (ATCC® TIB¬152 ™), SK-BR-3 (ATCC)) ® HTB-30 ™), HeLa (ATCC® CRM-CCL-2 ™), MCF7 (ATCC® HTB-22 ™), Ishikawa (Aydin University, Istanbul, Turkey), THP-1 (ATCC® TIB-202 ™), Caco-2 (ATCC® HTB-37 ™), AGS (ATCC® CRL1739 ™), MDA-MB-468 (ATCC® HTB-132 ™) and MDA-MB-453 (ATCC® HTB-131 ™) cell lines were used. Ingredients of growth media of each cell line are listed in Table 1. Cells were incubated at 37 ° C, under 5% CO_2 and 1 atm pressure.

Table 1. Table of ingredients of complete growth medium for each cell line

Cell Lines	Growth Medium	Added Ingredients
HUVEC	DMEM F12-Ham	%10 FBS %1 Antibiotics
MCF10A	%95 DMEM F12-Ham, EGF 20µl, Hydrocortizon 0,5µl, Insulin 274µl (per 100 ml)	%5 Horse Serum %1 Antibiotics
Caki-1	DMEM F12	%10 FBS %1 Antibiotics
Jurkat	RPMI-1640 Medium	%10 FBS %1 Antibiotics
SK-BR-3	DMEM F12	%10 FBS %1 Antibiotics
HeLa	DMEM F12	%10 FBS %1 Antibiotics
MCF7	DMEM F12	%10 FBS %1 Antibiotics
Ishikawa	RPMI-1640 Medium	%10 FBS %1 Antibiotics
THP-1	RPMI-1640 Medium	%10 FBS %1 Antibiotics
Caco-2	DMEM F12	%10 FBS %1 Antibiotics
AGS	DMEM F12	%10 FBS %1 Antibiotics
MDA-MB-468	DMEM F12	%10 FBS %1 Antibiotics
MDA-MB-453	DMEM F12	%10 FBS %1 Antibiotics
MDA-MB-468	DMEM F12	%10 FBS %1 Antibiotics
MDA-MB-453	DMEM F12	%10 FBS %1 Antibiotics

DMEM F-12-Ham (Dulbecco's Modified Eagle Medium / Nutrient Mixture F-12 Ham), RPMI 1640 (Roswell Park Memorial Institute 1640), EGF (Epidermal Growth Factor), Hydrocortisone, Insulin, PBS (1x Phosphate Buffered Saline) were used in the study . FBS (Fetal Bovine Serum), Horse Serum, Penicilin-Streptomycin(Antibiotics),Trypsin-EDTA (Ethylenediaminetetraacetic Acid) andDMSO (Dimethyl Sulfoxide) were commercially available.

All the experiments were performed with 80% confluency. As they reached 80% confluency, growth medium was discarded and cells were washed with PBS three times. After washing, cells were incubated with 0.25% Trypsin-EDTA (1mL/25 cm2 flask) to detach adherent cells from the flask surface. After the cells were detached, Trypsin-EDTA was inactivated by adding FBS. Cells were collected in a centrifuge tube and centrifuged at 1000 rpm for 5 minutes. Supernatant containing trypsin-EDTA-FBS was removed from the cells. The pellet was washed 3 times with PBS at 1000 rpm for 5 min. The supernatant was discarded. The pellet was stored at -20 ° C for DNA and RNA isolation.

DNA isolation

DNA isolation from each cell line was performed with Quick-DNA Miniprep Plus Kit (Zymo, Cat No: D4068) according to the manufacturer's instructions. Quality of DNA samples was assessed via using NanoDrop.

Polimerase Chain Reaction (PCR)

DNA samples were amplified with PPARγ (NG_011749.1)specific primers (Table 2) according to PCR conditions indicated in Table 3. Product size is 270bp.

Table 2. Primer Sequences for PCR [10]

Primers	Sequence (5'→3')
Forward Primer	GCC AAT TCA AGC CCA GTC
Reverse Primer	GAT ATG TTT GCA GAC AGT GTATCA GTG AAG GAA TCG CTT TCC G

Table 3. PCR Conditions

Step	Temperature	Time	Cycle
Initial Denaturation	94°C	8 min	1
Denaturation	94°C	50 sec	35
Annealing	59°C	50 sec	35
Elongation	72°C	1 min	35
Final Elongation	72°C	5 min	1
Hold	4°C	∞	

RFLP (Restriction Fragment Length Polymorphism)

PCR products (expected size: 270bp) were cut by using BstU-I enzyme (incubation at 60°C for 15 minutes). Restriction enzyme cutting products was checked via running an agarose gel. Band from homozygous WT (Pro12/Pro12) is expected to be 270bp because of lack of BstU1 cut site. However, samples with homozygous Pro12Ala polymorphism (Ala12/Ala12) are expected to show two bands (227 bp and 43 bp). Samples from heterozygous Pro12Ala polymorphism (Pro12/Ala12) are expected to show three bands (270bp, 227 bp and 43 bp)

Sanger Sequencing
RFLP results were validated via Sanger sequencing by using ABI 3100 Sequencer. Primer Sequence is 5'-GCC AAT TCA AGC CCA GTC-3'. Data were analyzed via SnapGene Software.

RNA Isolation
RNA isolation from each cell line was performed with NucleoSpin® RNA Kit (Bioline, Cat No: BIO-65053) according to the manufacturer's instructions.

cDNA Synthesis
cDNA synthesis was performed with SensiFAST™ cDNA Synthesis Kit (Macherey-Nagel, Ref No: 740955.10) according to the manufacturer's instructions.

Quantitative Real-time PCR and Data Analysis
SYBR Green was used in quantitative Real-time PCR. Primers were indicated in Table 4 and Real-time PCR conditions are indicated in Table 5.

Table 4. Primer Sequences in Quantitative Real-Time PCR. *NM_001101.5, **NM_001354670.2

Primer	Sequence (5'→3')	Time
β -actin * Forward Primer	TGAAGTGTGACGTGGACATC	151bp
β -actin * Reverse Primer	GGAGGAGCAATGATCTTGAT	
PPARγ ** Forward Primer	AATCAAAGTGGAGCCTGCAT	169bp
PPARγ ** Reverse Primer	ACCCTTGCATCCTTCACAAG	

Table 5. Quantitative Real-Time PCR Conditions

Step	Temperature	Time	Cycle
Inital Denaturation	95°C	5 min	1
Denaturation	95°C	10 sec	45
Annealing	55°C	10 sec	45
Elongation	72°C	30 sec	45
Final Elongation	95°C	10 min	1

Normalization was performed against β-actin. ΔΔCt value was calculated by using delta cycle threshold (ΔCt). Fold changes in expression was calculated via using $2^{-\Delta\Delta Ct}$ formula. Error bars were calculated according to standard deviation value of biological replicates. Statistical analysis was performed via Student`s t-test. $p<0.05$ was considered statistically significant.

Results

In order to analyze presence/absence of PPARγ Pro12Ala polymorphism, RFLP method was used. The polymorphism creates a BstU-1 cut site on the DNA by the conversion of C to G. Therefore, PPARγ specific PCR followed by BstU-1 restriction enzyme cut were used for polymorphism analysis.

The target region of PPARγ in 13 different cell lines (HUVEC, MCF10A, AGS, Caki-1, MDA-MB-468 MDA-MB-453, SK-BR-3, MCF7, Jurkat, THP-1, Caco-2 Ishikawa, HeLa cell lines) were amplified via using specific primers. The target amplicon is known to be 270 bp long [11]. In order to check PCR reaction, PCR products were run in agarose gel (Figure 1)

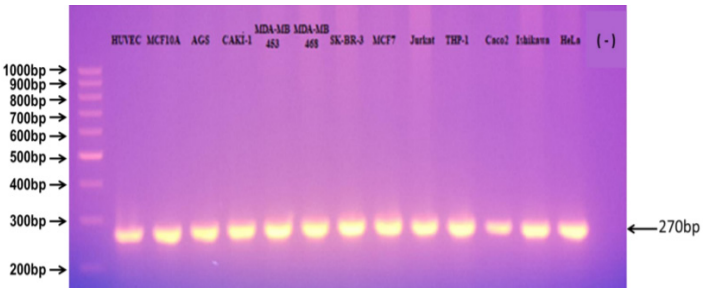

Figure 1. PCR results from HUVEC, MCF10A, AGS, Caki-1, MDA-MB-468 MDA-MB-453, SK-BR-3, MCF7, Jurkat, THP-1, Caco-2 Ishikawa, HeLa cell lines (Lane #2 to Lane #14). Last lane (#15) shows negative control as (-). First lane shows DNA ladder (GeneRuler 100 bp DNA Ladder) where lowest band corresponds to 200bp

PCR products from Figure 1 were cut by BstU-1 restriction enzyme. According to this restriction enzyme cut, only AGS and Caki-1 cell lines carry heterozygous Pro12Ala polymorphism. The other cell lines are homozygous WT (Pro12/Pro12) (Figure 2). Although samples with heterozygous Pro12Ala polymorphism (Pro12/Ala12) are expected to show three bands (270bp, 227 bp and 43 bp), depending on technical difficulties, i.e. presence of primer dimer and small size of 43bp band, two bands (270bp, 227 bp) were shown in the figure.

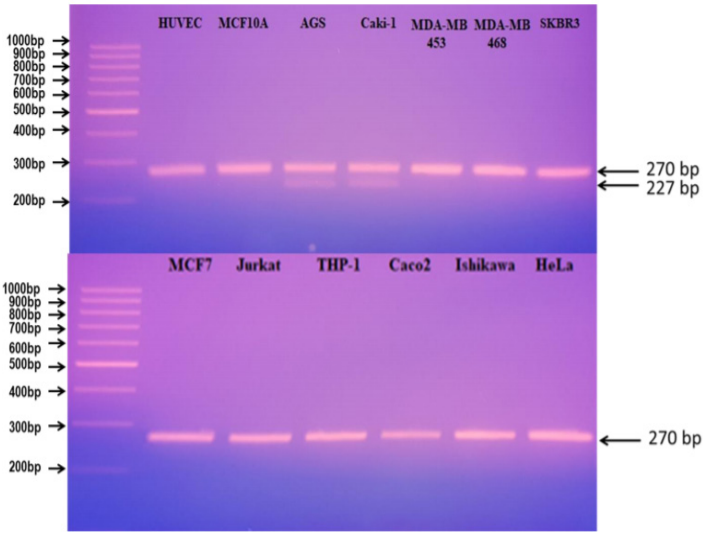

Figure 2. RFLP results from HUVEC, MCF10A, AGS, Caki-1, MDA-MB-468 MDA-MB-453, SK-BR-3, MCF7, Jurkat, THP-1, Caco-2 Ishikawa, HeLa cell lines. Two bands (270bp, 227 bp) represent samples with heterozygous Pro12Ala polymorphism (Pro12/Ala12), due to restriction enzyme cut. One band (270bp) represents samples with homozygous WT (Pro12/Pro12). First lanes show DNA ladder (GeneRuler 100 bp DNA Ladder) where lowest band corresponds to 200bp.

RFLP results from all 13 cell lines were validated with Sanger Sequencing. PCR products from Figure 1 were sequenced with specific primers. As shown in RFLP, only at AGS and Caki-1 cell lines were found to carry heterozygous Pro12Ala polymorphism. Figure 3 shows chromatograms of AGS and Caki-1 and MCF-10A (Pro12/Pro12) cell line.

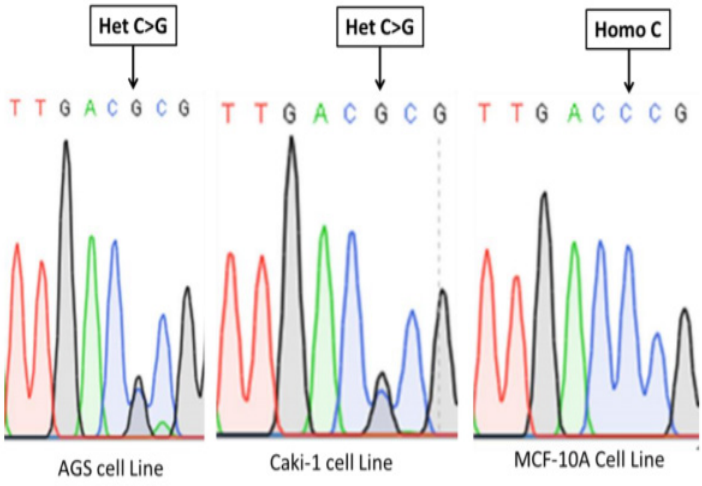

Figure 3. Chromatogram representations of Sanger sequencing results from AGS, Caki-1 and MCF10A cell lines. Het C>G represents heterozygous Pro12Ala polymorphism (Pro12/Ala12); Homo C represent homozygous WT (Pro12/Pro12)

In order to investigate whether presence of Pro12Ala polymorphism has an effect on PPARγ mRNA expression, RNA from cell lines with heterozygous Pro12Ala polymorphism(AGS and Caki-1) and cell lines carrying homozygous Pro12/Pro12 (SK-BR-3 and MDA-MB-468) were isolated and cDNA from these samples were synthesized. Real-time PCR was performed to assess expression differences with respect to SK-BR-3 as control. As shown in Figure 4, PPARγ mRNA expression is lower in cell lines with heterozygous Pro12Ala polymorphism (AGS and Caki-1) compared to WT cancer cell lines including SK-BR-3 and MDA-MB-468.

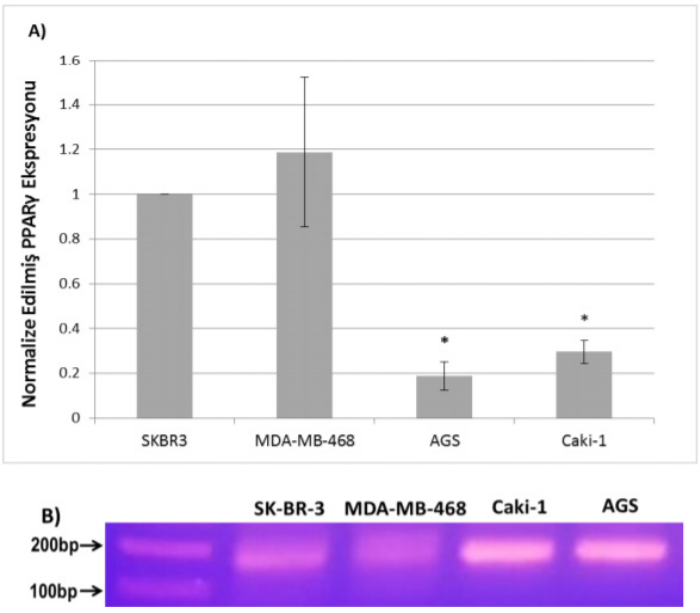

Figure 4. PPARγ expression in different cell lines. A)Normalized PPARγ mRNA expression in cell lines with different Pro12Ala polymorphism status. Normalization was done against β actin mRNA expression. SK-BR-3 cell line, rather than MCF-10A, was used as control in the data analysis in order to assess the effect of the polymorphism in cancer background. * denotes significance in expression level differences where $p<0.05$($p=0.02$ for Caki-1 vs SK-BR-3; $p=0.02$ for AGS vs SK-BR-3) B) PCR gel image of quantitative real-time PCR showing PPARγ expression levels. First lane indicates marker. Amplicon size is 169bp

Discussion

In this study, we aimed to investigate PPARγ Pro12Ala polymorphism in different cell lines and to understand whether this polymorphism has an effect on PPARγ mRNA expression. Pro12Ala polymorphism has been shown to be associated with many diseases such as obesity, diabetes, and some types of cancer [2]. However, to the best of our knowledge, there are no studies examining the relationship between cancer and Pro12Ala polymorphism, at the cellular level. The relationship between Pro12Ala polymorphism and PPARγ mRNA expression has been studied in obesity patients [9] but this relationship has never been investigated in cancer. Moreover, Pro12Ala polymorphism that we identified in AGS and Caki-1 cell lines could not be found in cancer cell line databases including GEMICCL database. Thus, our study has novel aspects that can contribute to the field.

There are several studies investigating the association between a polymorphism and cancer. Blood samples from patients and normal individuals in the population are commonly used to investigate this association. Even if any kind of association is found between the variant and disease, these types of studies fail to explain the underlying mechanism of these possible associations. Using cell lines will be more useful to see the effects of variants at the cellular level. In addition to providing ease of use in terms of technical difficulties and ethical considerations, using cell lines confers the opportunity of elucidating cell-specific effects by keeping the extracellular variables constant. These properties make cell lines a suitable working model. In the literature, polymorphism studies in cell line models are not very common, but SNPs are found in the genome of certain cell lines [12]. These studies are important for understanding the effects of different variants of genes on cellular phenotype such as mRNA and protein expression, protein localization as well as the function of the gene product. In addition, by screening cell lines for the variants, it is possible to get an idea about specific properties of cell lines in order to select correct cell lines in further studies.

In our study, two different approaches were used to analyze the effect of PPARγ Pro12Ala polymorphism on mRNA expression. In the first approach, gene expression analysis was performed by taking the normal breast cell line MCF10A as control. The comparison between cancer cells and normal breast cell line MCF10A would be useful to obtain information about both Pro12Ala polymorphism and PPARγ mRNA expression between cancer and normal cells. In this analysis, three different breast cancer cell lines (MDA-MB-453, MDA-MB-468, MCF-10A, SK-BR-3) were compared with normal breast line MCF10A and PPARγ mRNA expression of these breast cancer cells were found to be lower compared to PPARγ mRNA expression in normal breast cell line. In Caki-1 and AGS cell lines where Pro12Ala polymorphism was detected, PPARγ mRNA expression showed a higher decrease compared to cell lines without Pro12Ala polymorphism (data not shown). This decrease can be attributed both to the expression difference between cancer and normal cells and to the effect of polymorphism. In order to exclude the effect of differences between cancer and normal cell lines and to see the effect of the polymorphism in cancer cell lines, the second analysis was performed by taking SK-BR-3 as control (Figure 4). In this analysis, while PPARγ mRNA expression did not change much

in the cell lines where Pro12Ala polymorphism was not detected, there seems to be a significant decrease PPARγ mRNA expression in Caki-1 and AGS cell lines with Pro12Ala polymorphism. Therefore, it was concluded that the decrease in PPARγ mRNA expression might be due to the Pro12Ala polymorphism. The next step would be to investigate the PPARγ expression levels in the absence of Pro12Ala polymorphism in cancer cell lines originating from the same tumor type as Caki-1 and AGS cell lines compared to Caki-1 and AGS, respectively ; so that one can exclude the possibility that this expression difference might be due to the difference between the cancer types.

As indicated above, PPARγ mRNA expression was found to be one of the lowest among other cancer cell lines in AGS. Although we speculated that this low expression might depend on Pro12Ala polymorphism, Sato et al. compared PPARγ mRNA expression and protein levels in different gastric cancer cell lines including AGS and found that both mRNA and protein levels of PPARγ were lowest in AGS cell lines [13]. AGS cell line in our study also has a very low PPARγ mRNA expression compared to other cancer cell lines. To examine whether this low expression is due to the Pro12Ala polymorphism or not, it is necessary to determine whether the other gastric cell lines carry the Pro12Ala polymorphism.

There are several studies showing the association between Pro12Ala polymorphism and breast cancer. Although some of the studies demonstrate controversial data from different populations, it is generally thought that Pro12Ala polymorphism may be protective against breast cancer [14-16]. In our study, five different breast cancer cell lines (MCF-7, MDA-MB-453, MDA-MB-468, SK-BR-3) and normal breast cell line (MCF-10A) were screened in terms of Pro12Ala polymorphism status. Pro12Ala polymorphism could not be detected in any of these cell lines. Therefore, no conclusions have been reached regarding the effect of Pro12Ala polymorphism in breast cancer. As indicated above, when the breast cancer cell lines were compared among each other in terms of PPARγ mRNA expression PPARγ, mRNA expression in cancer cell lines was found to decreased compared to normal breast cell line(data not shown). Elstner et al. found that PPARγ protein expression in breast adenocarcinoma cells from breast cancer patients is higher in comparison to PPARγ protein expression of normal epithelial cells. [17]. Although it seems controversial to our data, mRNA expression and protein expression may not always be comparable in cells depending on possible post-transcriptional and post-translational modifications. One of our future perspectives is to study PPARγ protein expression to further elucidate the role of Pro12Ala in cancer cells.

Conclusions

In conclusion, PPARγ gene Pro12Ala polymorphism was detected only in AGS and Caki-1 cell lines in 13 different cell lines. PPARγ mRNA expression was found to be low in those cell lines with Pro12Ala polymorphism compared to cancer cell lines without Pro12Ala polymorphism. Our data suggest that the presence of Pro12Ala polymorphism in cancer cells may reduce PPARγ mRNA expression which might contribute carcinogenesis at the cellular level.

Conflict of interest
The authors declare that there are no conflicts of interest.

Financial Disclosure
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Ethical approval
Before the study, permissions were obtained from local ethical committee.

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