

## SNP GENOTYPING BY TAQMAN ALLELE DISCRIMINATION TECHNIQUE

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**Abstract :** Breast cancer is the most frequent neoplasm in women worldwide and the principal cause of deaths by cancer, the majority being by metastatic disease. About half of breast tumors are hormone dependent, and in post-menopause women the preferred first line treatment uses third generation aromatase inhibitors. Aromatase is encoded by CYP19 gene on 15q21.1, and there is strong evidence that mutations in this gene affect its expression, with direct consequences on cancer phenotype and response to treatment. Several single nucleotide polymorphisms have been studied on CYP19A1 transcription variant, notably rs727479, rs10046, rs4646 and rs700518. We implemented a Taqman-based allele discrimination assay for the rapid investigation of the 4 SNPs in CYP19A1. We genotyped 22 metastatic breast cancer patients by the technique described.

### INTRODUCTION

Breast cancer is one of the leading causes of cancer morbidity and mortality worldwide (Bray et al, 2004). It is the second most common cancer in the world and, by far, the most frequent cancer among women with an estimated 1,67 million new cancer cases diagnosed in 2012 (25% of all cancers) (Ferlay et al, 2013). In Romania, the incidence of breast cancer in women was 66,2/100000/year, with a mortality rate of 21,6/100000/year (Steliarova-Foucher et al, 2015), the vast majority of deaths by breast cancer being by metastatic disease. The treatment options in metastatic breast cancer are mainly chemotherapy, targeted biological agents for HER-2/neu-positive tumours, and hormone therapy for hormone-dependent tumours (Cardoso et al, 2009). Up to 60% of breast tumours are positive for Estrogen Receptors alpha and beta, therefore being sensitive to estrogen binding. Estrogens induce target cells proliferation, such as breast epithelial cells and estrogen-dependent malignant cells (Weigel et al, 2001). For estrogen receptor positive breast cancer in postmenopausal women, the preferred first-line treatment is based on the administration of third generation Aromatase Inhibitors (Goldhirsch et al, 2009). Inhibitors that are in current clinical use include anastrozole, exemestane, and letrozole. Because estrogens play an important role in carcinogenesis and progression of breast cancer, genes encoding for enzymes involved in estrogen biosynthesis and metabolism are plausible candidates as breast cancer susceptibility genes.

Aromatase, also called estrogen synthetase or estrogen synthase, is an enzyme responsible for a key step in the biosynthesis of estrogens. It is a member of the cytochrome P450 superfamily (EC 1.14.14.1), which are monooxygenases that catalyze many reactions involved in steroidogenesis. In particular, aromatase is responsible for the aromatization of androgens into estrogens (Simpson et al, 2002). Aromatase is a member of the cytochrome P450 superfamily of enzymes. The cytochrome P450 proteins are monooxygenases which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. This protein localizes to the endoplasmic reticulum and catalyses the last steps of estrogen biosynthesis. Mutations in this gene can result in either increased or decreased aromatase activity; the associated phenotypes suggest that estrogen functions both as a sex steroid hormone and in growth or differentiation (Chen et al, 2004). In humans, the gene CYP19, located on chromosome 15q21.1, encodes the aromatase enzyme (OMIM 108330). CYP19 gene has nine coding exons and a number of alternative non-coding first exons that regulate tissue specific expression (Czajka-Oraniec et al, 2010). The gene expresses two transcript variants, the most studied being CYP19A1 (Entrez Gene ID 108330). Germline mutations in CYP1A1 could have either inhibition or gene expression stimulation effects and association of polymorphisms in the CYP19 with breast cancer risk has previously been studied (Raskin et al, 2009).

Non-pathogenic single nucleotide polymorphisms (SNPs) of CYP19A1 showed to modify aromatase activity and to influence inhibitory medication in post-menopausal women (Miron et al, 2012). Most of the studies correlated CYP19A1 SNPs with lower or higher breast cancer risk (Sobczuk et al, 2009), or in prognostic of response to aromatase inhibitors treatment (Colomer et al, 2008). The furthergoing study of the presence, distribution and prevalence of CYP19A1 SNPs should bring insights in tumoral cell development, and could also allow individualization of personalized therapies based on host genetics. Therefore, developing, adapting and implementing robust and cost-effectiveness tools for SNP genotyping is a continuous challenge of cancer molecular biology.

In this study we describe a simple and efficient tool developed and implemented in order to quickly genotype the CYP19A1 SNPs rs727479, rs10046, rs4646 and rs700518, by Taqman-based allele discrimination assay.

## PATIENTS AND METHODS

Samples were obtained from post-menopausal metastatic breast cancer female patients. All patients agreed to participate by written informed consent. Genomic DNA was extracted in duplicate from 300 µl peripheral blood, using an adapted protocol of the Wizard<sup>®</sup> Genomic DNA Purification Kit, Promega<sup>™</sup> (Negura et al, 2011). DNA extractions were performed the same day with blood collection, or blood samples were kept at 4°C for a maximum of 48 hours. Extracted DNA was resuspended in TE 1x buffer. DNA was stored at 4°C for immediate evaluation, or at -20°C in the biobank. DNA quantity and quality was estimated by spectrophotometry, by measuring absorbance at 260 and 280 nm, on appropriate dilutions, using a Biowave DNA Spectrophotometer, Biochrome<sup>™</sup>.

Primers and probed (Life Technologies<sup>™</sup>) were adapted to flank polymorphic regions of CYP19A1. Sequences are detailed in the Results section. PrimerExpress<sup>®</sup> software (Applied Biosystems<sup>™</sup>) was used to verify specificity, T<sub>m</sub> and cross-annealing of oligonucleotides. PCR reactions were optimized for 25 µl total volume, containing 20 ng genomic DNA, 0.4 mM each dNTP, 0.8 µM of each primer, 0.25 µM of each probe, and one unit of AmpliTaq<sup>®</sup> Polymerase with appropriate Buffer, Life Technologies<sup>™</sup>. For optimizing PCR reactions without Taqman probes, the PCR program comprised an initial denaturation step at 94°C for 5 min followed by 30 cycles of 94°C for 20 s, 54°C for 20 s and 72°C for 30 s, and a final extension of 7 min at 72°C. 10 µl PCR product were mixed with 2 µl loading dye and deposited in a 1% agarose gel stained with 0.5 µg/ml ethidium bromide. Once the reactions optimized and specific, the Taqman PCR assay including the Taqman probes was performed using a program with an initial denaturation step at 94°C for 5 min, followed by 50 cycles of 94°C for 15 s denaturation and 60°C for 90s annealing/extension, in a two-step PCR. The run were performed in a 7500 Fast DX Real-Time System, Life Technologies, and the fluorescence results were interpreted and converted to genotypes with the 7500 software.

## RESULTS

The genotyping of CYP10A1 SNPs were realized by allelic discrimination in a Taqman system. Briefly, each region of interest containing the SNP was amplified in a Real-Time PCR system. Each reaction contained two **sequence-specific** primers for amplifying the polymorphism of interest (forward and reverse), as well as two **allele-specific** TaqMan probes for detecting the alleles for the specific polymorphism of interest. Each allele-specific TaqMan probe has a non-fluorescent quencher (NFQ) at its 3' end. The quencher molecule quenches the fluorescence emitted by the fluorophore. As long as the fluorophore and the quencher are in proximity, quenching inhibits any fluorescence signals. Also, each allele-specific TaqMan probe has a reporter dye at its 5' end (VIC dye is linked to the 5' end of the Allele 1 probe, and FAM dye is linked to the 5' end of the Allele 2 probe), which allows the allele-specific fixation of each probe. During the extension PCR step, the 5'-exonuclease activity of the Taq polymerase will degrade the annealed probe, liberating the associate fluorescence. In homozygous patients, one only fluorescence will be liberated, depending on the annealed probe. In heterozygous samples, each probe is fixed on each chromosome, and both fluorescences will appear. Each sample will be then distributed, based on the emitted fluorescence, in a allelic discrimination plot, within one of the 3 genotypes. Background noise is corrected and sample genotype is established.

We designed and verify a primers/probes system for genotyping each SNP (Table 1).

The sequences of the regions of interest are presented below. Each amplicon is shown in the genomic context. Primers target sequences are underlined. Probes target sequences are double-underlined. SNP site is bold-red coloured.

Table 1. Sequence of primers and probes used for the Taqman allele discrimination assays. For the Taqman probes, the Allele 1 VIC dye-labeled probe corresponds to the first nucleotide inside the square brackets. The Allele 2 FAM dye-labeled probe corresponds to the second nucleotide inside the square brackets. Both nucleotides are red-coloured.

CYP19A1 SNP	Primers		Probe
rs727479	F	5' – GCTCAAGATGGGGTGGAGTAAAG – 3'	5' – TTCTTCTCCCTTTCCTTTGTTTCC[G/T] CCATGCCCCCTCTTTGTCTGTATT – 3'
	R	5' – CCACTATCACCACATTTCCAAGAA – 3'	
rs10046	F	5' – CCATCCTCGTTACACTTCTGAG – 3'	5' – TCTGGTGTGAACAGGAGCAGATGAC[G/A] AATAGCACCTAGCTTGGTGACAACC – 3'
	R	5' – CTTACCGACTATTTCTCCCTC – 3'	
rs4646	F	5' – CCTTGCACCCAGATGAGAC – 3'	5' – CTACTGATGAGAAATGCTCCAGAGT[T/G] GGTACTGACCAGCCTTCTCTAGTGT – 3'
	R	5' – CCTCAAACCTTTGGCCTCTG – 3'	
rs700518	F	5' – CTACTGCATGGGAATTGGAC – 3'	5' – GCCTGCACTACTACAACCGGGT[A/G] TATGGAGAATTCATGCGAGTCT – 3'
	R	5' – GAACAGACTCACTTGTGATA – 3'	

rs727479 – (CYP19A1 NM\_000499.3:c.145+418G>T, NG\_007982.1:g.101249G>T)

Amplicon – 229 bp. Probes anneal on reverse strand. Allele 1 is G (VIC probe). Allele 2 is T (FAM probe).

5' ATGAAAGCACTTAAAAAATATCTTAGATAAACCCCTCCACTCTGTGCTGTGCTTTCGTAAGTAGACTTGG  
CACAAGTACCCTTTAAAAACAAGCTCAAGATGGGGTGGAGTAAAGTAAAAATTCCAGGGTTTTCTGGTTAACT  
TAAAAAAAATAGATTGCAATTTGCTCTGAAGGTGAAAAATGCTCTGGAACATCTTCTCAGCTCTTTCTCTC  
TCCCTTTCCTTTGTTCCGCCATGCCCCCTCTTTGTCTGTATTATCCCTTCTCTTTATTCCACTCATCATT  
TCTCTTATTCTTGGGAATGTGGTGATAGTGGTCCCATTTTTCTCCACTGTCTGGATCCCATTGACCCTTA  
TGGGAGCCTGGTAGTGGGAGAAAAATGGGGACCCTCCAGTGCTCTTGGAAACATCATACCCCGGGAGAAT  
CTTCAACAAGATCT 3'

rs10046 – (CYP19A1 NM\_000499.3:c.151C>T, NG\_007982.1:g.132810C>T)

Amplicon – 414 bp. Probes anneal on forward strand. Allele 1 is C (VIC probe). Allele 2 is T (FAM probe).

5' AGCCATTTGGCTTTGGGCCCCGTGGCTGTGCAGGAAAGTACATCGCCATGGTGATGATGAAAGCCATCCT  
CGTTACACTTCTGAGACGATTTCCACGTGAAGACATTGCAAGGACAGTGTGTTGAGAGCATAACAGAAGATAC  
ACGACTTGTCTTGCACCCAGATGAGACTAAAAACATGCTGGAAATGATCTTTACCCCAAGAAACTCAGAC  
AGGTGTCTGGAACTAGAGAAGGCTGGTCAGTACCCTCTGGAGCATTCTCATCAGTAGTTTCACATAC  
AAATCATCCATCCTTGCCAATAGTGTCATCCTCACAGTGAACACTCAGTGGCCCATGGCATTTTATAGGCAT  
ACCTCCTATGGGTTGTCACCAAGCTAGGTGCTATTCTGTCATCTGCTCTGTTACACCAGAGAACCAGGCTA  
CAAGAGAAAAAGCAGAGGCCAAGAGTTTGAAGGAGAAATAGTCTGGTGAAGAAACCGTATCCATAAAGAC  
CCGATTCCACCAATGTGCTTTGAGAAGGATA 3'

rs4646 – (CYP19A1 NM\_000499.3:c.1673T>G, NG\_007982.1:g.132952T>G)

Amplicon – 309 bp. Probes anneal on forward strand. Allele 1 is T (VIC probe). Allele 2 is G (FAM probe).

5' TTCCACGTGAAGACATTGCAAGGACAGTGTGTTGAGAGCATAACAGAAGATACACGACTTGTCTTGCACC  
CAGATGAGACTAAAAACATGCTGGAAATGATCTTTACCCCAAGAAACTCAGACAGGTGTCTGGAACACTAG  
AGAAGGCTGGTCAGTACCAGCTCTGGAGCATTCTCATCAGTAGTTCACATACAAATCATCCATCCTTGCCA  
ATAGTGTCATCCTCACAGTGAACACTCAGTGGCCCATGGCATTTTATAGGCATACCTCCTATGGGTTGTCAC  
CAAGTAGGTGCTATTTGTCATCTGCTCTGTTACACCAGAGAACCAGGCTACAAGAGAAAAAGCAGAGG  
CCAAGAGTTTGAAGGAGAAAAATAGTCTGGTGAAGAAACCGTATCCATAAAGACCCGATTCCACCAAAATGTGCT  
TTGAGAAGGATAGGCCCTTCAATAACAAAATGTATGTCTGGTTCCC 3'

rs700518 – (CYP19A1 NM\_000499.3:c.240A>G, NG\_007982.1:g.106684A>G)

Amplicon – 156 bp. Probes anneal on reverse strand. Allele 1 is A (VIC probe). Allele 2 is G (FAM probe).

5' AATTTTGGTGTAACATAAGATGTTGCTTATGCTCTGACACCTGTCTAGGTCCTGGCTACTGTCATGGGAAT  
TGGACCCCTCATCTCCACGGCAGATTCTGTGGATGGGGATCGGCAGTGCCTGCAACTACTACAACCGGG  
TATATGGAGAATTCATGCGAGTCTGGATCTCTGGAGAGGAAACACTCATTATCAGCAAGTGAGTCTGTTC  
TAATCGAAGACATACTTTTA 3'

We firstly optimized each PCR reaction, prior to the addition of the Taqman probes, in order to harmonize the reaction conditions to all polymorphisms. In figure 1, one can see specific unique bands corresponding to PCR products of rs727479 (Fig. 1a), rs10046 (Fig. 1b), rs4646 (Fig. 1c) and rs700518 (Fig. 1d). Figure 1e correspond to a multiplex-PCR assay we performed with all 8 primers, in order to verify cross-annealing and primer dimers absence.

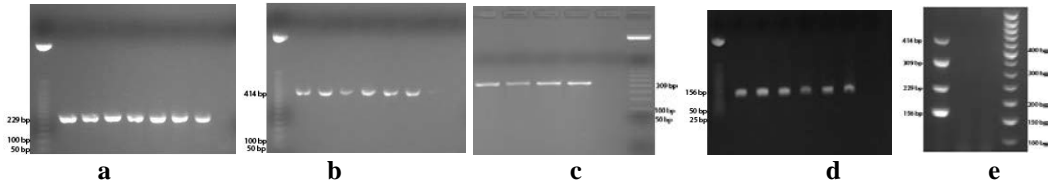


Figure 1. Electrophoresis of PCR products corresponding to amplifications in the regions of interest of rs727479 (1a), rs10046 (1b), rs4646 (1c) and rs700518 (1d). Multiplex-PCR of all regions is shown in 1e.

Subsequently, we performed the Taqman assay in a Real-Time PCR system. 22 metastatic breast cancer patients were genotyped by the technique described, in order to verify reproducibility and efficiency. The results are presented in Figure 2 to 5.

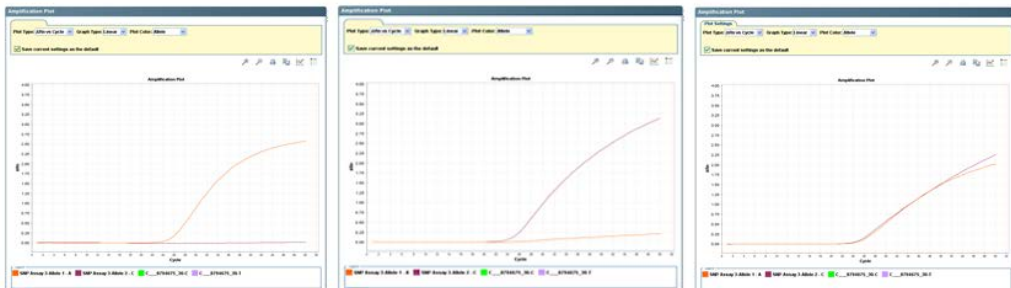


Figure 2. Genotyping of rs727479 revealing GG homozygous (left), TT homozygous (right) and heterozygous (middle)



Figure 3. Genotyping of rs10046 revealing CC homozygous (left), TT homozygous (right) and heterozygous (middle)

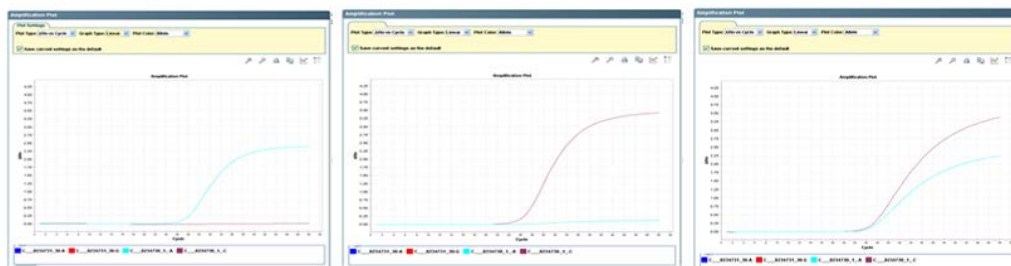


Figure 4. Genotyping of rs4646 revealing TT homozygous (left), GG homozygous (right) and heterozygous (middle)

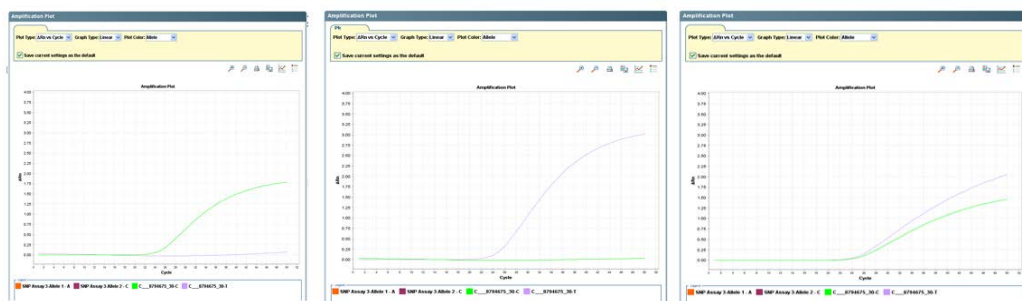


Figure 5. Genotyping of rs700518 revealing AA homozygous (left), GG homozygous (right) and heterozygous (middle)

## DISCUSSION AND CONCLUSIONS

Single Nucleotide Polymorphisms occur once in every 300 nucleotides on average, which means there are roughly 10 million SNPs in the human genome. When SNPs occur within a gene or in a regulatory region near a gene, they may play a more direct role in disease by affecting the gene function. Some of these genetic differences have proven to be very important in the study of human health, being involved in the predisposition to the disease or in the response to medication and treatment. Given the huge amount of SNPs possibly involved in cancer diseases, developing robust and efficient techniques for quickly genotyping an important number of samples is the present challenge of molecular medicine. Here we describe a rapid, cost-effectiveness method of genotyping CYP19A1 SNPs by Taqman allele discrimination assay. By analyzing a consecutive series of 22 metastatic breast cancers, we verified the reproducibility of the method, therefore recommending it for large scales genotyping in the evaluation of the response to breast cancer treatment.

## REFERENCES

- Bray F, McCarron P, Parkin DM**, (2004): The changing global patterns of female breast cancer incidence and mortality. *Breast Cancer Res*, 6, 229-239.
- Cardoso F et al.**, (2009): Locally recurrent or metastatic breast cancer: ESMO Clinical Recommendations for diagnosis, treatment and follow-up. *Ann Oncol*, 20(4), 15-18.
- Chen S**, (2004): Aromatase and breast cancer. *Front.Biosci.* 3, d922–33.
- Colomer R, et al.**, (2008): A single-nucleotide polymorphism in the aromatase gene is associated with the efficacy of the aromatase inhibitor letrozole in advanced breast carcinoma. *Clin Cancer Res*, 14(3), 811-816.
- Czajka-Oraniec I, Simpson ER**, (2010): Aromatase research and its clinical significance. *Endokrynol Pol*, 61(1): 126–134.

- Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, Rosso S, Coebergh JWW, Comber H, et al.**, (2013): Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. *Eur J Cancer*, 49(6), 1374-1403.
- Miron L, Negură L, Peptanariu D, Marinca M**, (2012): Research on aromatase gene (CYP19A1) polymorphisms as a predictor of endocrine therapy effectiveness in breast cancer. *Revista medico-chirurgicala a societatii de medici si naturalisti Iasi*, 116(4), 997-1004.
- Negură L, Popoiu G, Matei M, Azoicăi D, Negură A**, (2011) Optimization and comparative evaluation of nucleic acids extraction protocols. *Analele Științifice ale Universității “ Alexandru Ioan Cuza” Iași (Serie nouă). Secțiunea IIa. Genetică și Biologie Moleculară*, Tom XII(4), 41-46.
- Raskin L et al.**, (2009): BRCA1 breast cancer risk is modified by CYP19 polymorphisms in Ashkenazi Jews. *Cancer Epidemiol Biomarkers Prev*, 18(5), 1617-1623.
- Simpson ER, Clyne C, Rubin G, Boon WC, Robertson K, Britt K, et al.**, (2002): Aromatase – a brief overview. *Annu.Rev.Physiol*, 64, 93–127.
- Sobczuk A et al.**, (2009): The CYP17 and CYP19 gene single nucleotide polymorphism in women with sporadic breast cancer. *Pol J Pathol*, 60(4), 163-167.
- Steliarova-Foucher E, O’Callaghan M, Ferlay J, Masuyer E, Rosso S, Forman D et al.**, (2015): The European Cancer Observatory: A new data resource. *Eur J Cancer*, in press
- Weigel NL, et al.**, (2001): Estrogen and progesterone action. In: DeGroot LJ, Jameson JL, Burger HG, eds. *Endocrinology*. Philadelphia: WB Saunders, 2053-2060.

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