

## Evaluation of microphthalmia associated transcription factor (MITF) expression in peripheral blood of a population with malign melanoma and control population and cell lines\*

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### SUMMARY

**Background:** The incidence of malign melanoma tumours has increased more rapidly than any other type of cancer; this has intensified the searching for tools that facilitate early detection of melanoma. Microphthalmia associated transcription factor (MITF) is currently known as being a master melanocyte regulator. The article analyses MITF gene expression in peripheral blood of individuals suffering from melanoma, compared to people without any type of cancer and some cell lines.

**Materials and methods:** Thirty one samples of peripheral blood were used: 19 from patients having melanoma and 12 from healthy people. Then RNA was extracted from these samples. MITF and housekeeping genes ( $\beta$ 2M and GAPDH) expression levels were then quantified by real-time PCR. Five cell lines were also used to determine the MITF expression.

**Results:** MITF gene expression could be observed in all individuals, though no statistical significant differences were found among expression levels in the groups studied ( $p=0.09$ ). Even so, MITF expression in the group of patients suffering from melanoma was much more variable than that observed in the group of cancer-free people. Expression was detected in the cell line AGS (gastric adenocarcinoma), not yet described.

**Conclusions:** MITF gene expression levels were detected in the peripheral blood from both people suffering from melanoma and people without any type of cancer. However, variability in the number of molecules in MITF gene expression was observed in people with melanoma, this suggests the presence of tumour cells in circulation.

**Keywords:** Melanoma; MITF; qPCR; Circulation tumor cells; Cancer.

*Evaluación de la expresión del factor de transcripción asociado con microftalmia (MITF) en sangre periférica de individuos con y sin melanoma maligno y en líneas celulares*

### RESUMEN

**Introducción:** La incidencia de melanoma maligno se ha incrementado más rápido que cualquier otro tipo de cáncer, intensificando así la búsqueda de herramientas que faciliten la identificación temprana del melanoma. El factor de transcripción asociado con microftalmia (MITF) es conocido como el regulador maestro de melanocitos. En el presente estudio se analiza la expresión del gen MITF en sangre periférica de un grupo de individuos con melanoma, comparándola con un grupo de personas sin cáncer y en algunas líneas celulares.

**Materiales y métodos:** Se extrajo ARN de 31 muestras de sangre periférica: 19 de pacientes con melanoma y 12 de personas sin ningún tipo de cáncer. Se cuantificaron niveles de expresión tanto para el gen MITF como para los genes de expresión constitutiva ( $\beta$ 2M y GAPDH) mediante PCR tiempo real. Asimismo se evaluó la expresión de los mismos genes en cinco líneas celulares.

**Resultados:** En todos los individuos se observó expresión del gen MITF, aunque no hubo diferencias estadísticamente significativas entre los niveles de expresión en los grupos de estudio ( $p=0.09$ ). Sin embargo, la expresión de MITF en el grupo de pacientes con melanoma fue más variable que la observada en el grupo de personas sin cáncer. Asimismo, en la línea celular de adenocarcinoma gástrico se detectó expresión del gen MITF, no descrita hasta el momento.

**Conclusiones:** Se encontraron niveles de expresión del gen MITF en sangre periférica tanto de personas con melanoma como en personas sin cáncer. Sin embargo, la variabilidad en los niveles de expresión del gen MITF observados en personas

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con melanoma, sugiere la posible presencia de células tumorales en circulación.

**Palabras clave:** *Melanoma; MITF; qPCR; Células tumorales en circulación; Cáncer.*

The malign melanoma is considered in some countries (United States and Australia) as a problem of public health, and it is also classified as epidemic, because in the last years its incidence has increased<sup>1</sup>. It is considered among the most aggressive types of cancer and, in Colombia, causes approximately 40% of deaths for skin cancer<sup>2</sup>, reason why it becomes important to implement tests of early detection, prediction and pursuit based on molecular markers, besides, to guide and facilitate the development of new treatment options. At the moment the attention is focused in the detection of tumoral cells in circulation in peripheral blood, as promissory tool for the identification of either people with early stage of the illness and/or with clinical results potentially poor.

Smith *et al.*<sup>3</sup> were the first ones in suggesting that melanoma cells could be detected in peripheral blood when identifying specific molecules of RNA of tumoral cells in circulation (tyrosine kinase RNA), but absent in non malign normal cells. Based on this original report, several groups of researches have used this and other methodologies to detect the expression of specific genes from melanocytes in circulation (tyrosine kinase, Dct, Mart-1)<sup>4,5</sup>. Nevertheless, in other studies is argued that these genes are not sufficiently good candidates for the analysis, mainly in advanced stages of the illness, because their molecules of RNA are not detected in a constant way in peripheral blood in individuals with melanoma<sup>6</sup>.

Regarding to microphthalmia associated transcription factor (MITF), it has been demonstrated that it plays a crucial role in the cell differentiation and proliferation, as well as in the post-natal (after life) viability of melanocytes because it regulates the expression of genes like p21, INK4a, CDK2, BCL2, TYR, DCT, among others, when it is activated mainly through the pathways MAPK, cAMP / PKA / CREB and Wnt-catenin<sup>7,8</sup>. There has been described nine different promoters in the gene MITF-, each one codifies a unique isoform with end 5', although they have in common the exons 2 to 9 which codify for the functional domain of the transcription factors. There is some information about the presence of some of the isoforms in the way of specific-tissue as

it is the case of the isoform MC, expressed specifically in mast cells and the M isoform expressed in melanocytes<sup>9</sup>. Also, immunohistochemistry techniques in tumoral tissue have shown that the MITF gene expression, in contrast to other specific markers of pigmented cells, remains in many, but in all the tumors of melanocytic origin, establishing it as a sufficiently sensitive marker to be kept in mind as possible tool to complement those which are used in the present time for the diagnosis of the melanoma and relieving their importance in the development of the illness<sup>10,11</sup>. For that reason, in the present analytic study of cases and controls, the MITF gene expression was evaluated in peripheral blood from individuals with malign melanoma (stage III and IV), and it was compared with the expression in peripheral blood from healthy people by means of real-time PCR (qPCR) and in different cell lines.

## MATERIALS AND METHODS

**Cell culture.** Five cell lines were used to evaluate the MITF gene expression. The cell line A-375 (malign melanoma) was used as a positive control; it was obtained directly from ATCC (American Type Culture Collection) while cell lines AGS and MCF-7 (gastric adenocarcinoma and breast cancer, respectively) were donated for the Instituto Nacional de Cancerología (INC.), Bogotá, Colombia. The cell lines were kept in cultivation according to the supplier specifications. In general, the means of cultivation DMEM was supplied with 10% fetal bovine serum (SFB) and they were incubated at 37°C with 5% CO<sub>2</sub>. From skin biopsies primary cultures of melanocytes (CPM) and fibroblasts (CPF) were generated, which were undergone the action of 5% Dispase II solution (Molecular Roche Biochemicals).

The disintegrated fragments of epidermis (melanocytes) were placed in a Petri sterile box with 2 ml of specific growth medium of melanocytes (Cell Application) and the dermis fragments (fibroblasts) were placed in a Petri sterile box with 2 ml of DMEM enriched with 10% SFB. The obtained cells were incubated at 37°C and 5% CO<sub>2</sub>, until they reached a 70% confluence; once it happened, they were treated with trypsin EDTA (0.25% for 60 seg) to remove the cells and using TRIZOL to prepare the cell extracts to isolate total RNA.

**Selection of cases and controls.** Keeping in mind the incidence of the illness in the population of the present study and previous reports<sup>5</sup>, representative sample size was calculated to be count on the study using the program sample size 1.1: 19 patients with malign melanoma diagnosed both clinically and histologically according to the AJCC (American Joint Committee on Cancer), 12 with metastasis to lymphatic regional nodules (stage III) and 7 with distant metastasis (stage IV); there were recruited to participate in this study (13 men, 6 women among 13 and 86 years of age). All patients were diagnosed, treated and supervised in the Units of Dermatology and Breast and Soft Tissue of the Instituto Nacional de Cancerología (INC), Bogotá, Colombia. There were excluded of the study the patients that had received systemic anti-neoplastic therapy (chemo or radiotherapy). The control group included 12 people (eight men and four women) without any sort of cancer who shared similar characteristics of gender, age, occupation and socioeconomic condition, in order to carry out a paired analysis and to control possible variables of confusion exposition to ultraviolet radiation. All participant individuals signed informed consent which were approved by the committees of ethics of the Universidad del Rosario and INC, according to the declaration of Helsinki.

**Gathering and processing indictment of blood samples.** They were gathered in vacutainer tubes with EDTA samples of blood (5 ml) from patients with melanoma and healthy people and processed maximum from 2 to 3 hours after the gathering. Leucocytes and other mononuclear cells were separated using a gradient of Ficoll Histopaque-1077 (Sima Aldrich) and centrifuged to 2000 rpm for 20 min. Total RNA was isolated from mononuclear cells using TRIZOL (Invitrogen™, Life Technologies), technique on guanidinium thiocyanate-phenol-chloroform extraction method concentration, purity and integrity of the total RNA was determined by spectrofotometry and visualized by denaturing agarose gel electrophoresis at 1% for the later synthesis of the DNAc.

**RT-PCR and cDNA synthesis.** Total RNA was undergone to enzymatic digestion with DNase I amplification grade (Invitrogen™, Life Technologies), to eliminate traces of genomic DNA. For each sample included in the study, two reactions of RT-PCR (Reverse Transcription Polymerase Chain Reaction) were carried

out with the Kit SuperScript First-Strand Synthesis System (Invitrogen™, Life Technologies). cDNA was synthesized according to supplier's instructions. 1 µg of total RNA was mixed shortly with 0.4 mM of dNTPs, 20 ng oligo (dT) and DEPC water. The solution was undergone to a process of denaturation, heating at 65°C for 5 min. Then it was completed to a final volume of 25 µl adding buffer RT 1X, 4 mM MgCl<sub>2</sub>, 8 mM DTT, 40 RnaseOUT™ and 50 µ SuperScript™ II. The reaction mixture was incubated at 42°C for 50 min and the polymerase activity was blocked at 70°C for 15 min. 2u of RNase H were added to the mixture, which was incubated at 37°C by 20 min to eliminate traces of RNA.

**Real-time PCR (qPCR).** By means of real-time PCR, with specific primers of each gene some DNA regions were amplified to evaluate the GAPDH, β2M and MITF genes expression, using SYBR Green I™ fluorescent dye that binds to double stranded DNA in the detector of fluorescence Opticon II (MJ Research). 4 µl of cDNA were turned into a final volume of 20 µl, using the mixture of reaction of Kit Platinum® SYBR® Green qPCR SuperMix UDG 2X (Invitrogen™, Life Technologies). The final concentration of the components in the mixture for real-time PCR amplification was: Master Mix 1X, (1.5U Platinum® Taq DNA Polimerase, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM MgCl<sub>2</sub>, 200 µM c/u dNTPs, 1U UDG) and 0.5 µM for each primer (Table 1)<sup>12</sup> in distilled sterile H<sub>2</sub>O. The PCR conditions used were the same ones for the three genes: 50°C for 3 min, 94°C for 15 min, 40 cycles at 95°C for 15 seg, 58°C for 30 seg, Plate Read (Reading of Fluorescence) and 72°C for 30 seg, then 72°C for 5 min and lastly the dissociation curve was obtained among 70°C and 90°C. The amplified products of the genes β2M and GAPDH, were purified with Kit QIAquick® PCR Purification (Quiagen), quantified by spectrofotometry (TECAN® Genios) and used directly to carry out dilutions and to generate standard curves in the real-time PCR equipment. The PCR product of MITF gene was purified and cloned in the plasmid vector TOPO 2.1 (Invitrogen™, Life Technologies) in order to generate a more stable and specific product to be used in standard curves.

**Statistical analysis.** The expression levels among the groups were compared using analysis of relative quantification with the method published for Pfaffl, M<sup>13</sup>. Also, analyses of absolute quantification, to determine

**Table 1**  
**Oligo sequences used as primers**

Gene	Primer <sup>a</sup>	Sequence	S/AS	Product size
GAPDH	GAPD-5'	GAAATCCCATCACCATCTTCCAGG	S	121 Pb
	GAPD-3'	TGAGCCCCAGCCTTCTCCAT	AS	
β2M	B2M-5'	AATTCACCCCCACTGAAAAA	S	124 Pb
	B2M-3'	GCGGCATCTTCAAACCTC	AS	
MITF	MITF-5'	GCGCAAAGAAGCTTGAAGAAC	S	124 Pb
Consensus	MITF-3'	CGTGGATGGAATAAGGGAAA	AS	

a. The primers were designed using the program Oligo 5, according to the recommendations reported by Bustin SA<sup>12</sup>

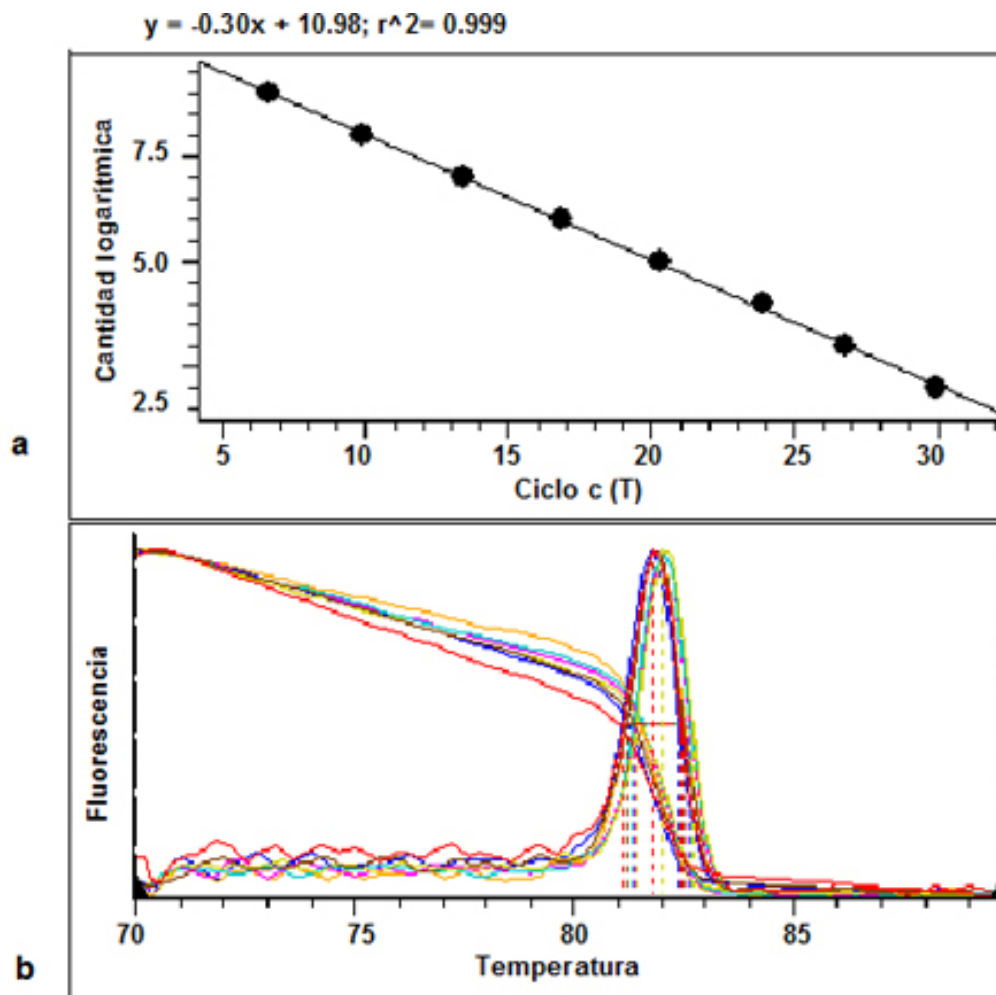
the number of molecules, made possible to the research team established a normal distribution of the data, applying the test Shapiro-Wilks. Significant differences among the studied groups for the levels of expression of number of molecules of MITF, GAPDH and β2M genes, were evaluated using the non parametric tests Kruskal-Wallis and the test of ranges with sign of Wilcoxon. For every test the significance level was 95% (p=0.05) in the statistical program SPSS (Version 12).

## RESULTS

The cases group was constituted for 19 malign melanoma patients, whose main characteristic was the clinical stage of the tumor (III and IV). In the real-time PCR tests carried out for GAPDH and β2M genes, it was detected amplification of those genes in all the studied patients. However, seven cases were excluded because in the exploratory analysis with a box and whisker plot (not shown data), atypical value and ends atypical were observed, indicating that integrity and quality of the blood sample was low and it could also generate erroneous results in the final analysis. As a 1:1 equivalence was made by sex, age, stratum and occupation, the statistical paired analyses were carried out with 12 patients with malign melanoma and 12 people without any cancer type. The quantity of RNA obtained from this group of cases varied between 10 and 15 μg for sample, using for test 1 μg. Also, the absorbance at 280 nm was calculated and its relationship with the absorbance 260 nm (260/280) provided data superior to 1.7, indicating the good quality and purity of the extracted material.

**Standard curves and analysis of efficiency.** Quantification methods (absolute and relative) require equality or at least similarity in the PCR efficiencies of every gene with a low level of variation (preferably between 90% and 100% of efficiency), on purpose to obtain real and reliable data for analysis of quantification<sup>14</sup>. The purified and quantified fragments of the GAPDH and β2M genes, as well as of the MITF fragment gene, were cloned in the TOPO plasmid and for the standard curves. The detection limit for all the curves it was between 1x10<sup>9</sup> and 100 molecules that correspond to the minor and bigger C(t) (Cycle threshold) detected, respectively (Graphic 1a). The efficiency in the amplification of the three studied genes were between 95% and 100% and it was analyzed using the formula  $E = [10^{(-1/S)}]$  where S is the value of the slope of the curve. Also, the melting curve analysis in the range of temperature between 70°C and 90°C allowed to determine the primers specificity, because it was only observed a peak or melting point for each gene (Graphic 1b).

**Quantification of the MITF gene in cell lines.** In the cell lines (A-375, AGS, MCF-7) and in the melanocytes primary cultures (CPM,) and fibroblasts primary cultures (CPF), product of amplification was obtained when specific primers of GAPDH and β2M genes were used. At least two independent experiments were made for each cell line in culture, obtaining very similar data, which assured the reproducibility of the real-time PCR tests. The data among culture and genes, showed important similarity in their C(t)s, which allowed the research team to establish initially GAPDH and β2M as genes that are expressed in a relatively constant way in the cell cultures analyzed (data not show).

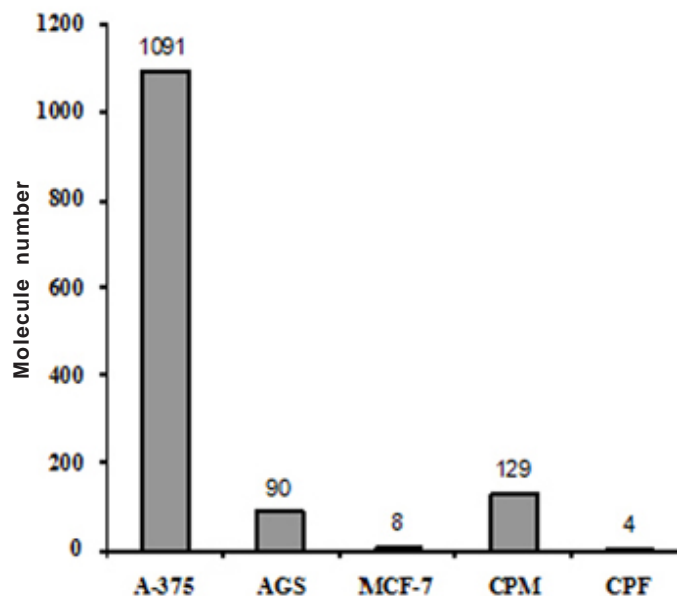


**Graphic 1. Standard curve of GAPDH gene. a.** Curve generated by the software Opticon Monitor 2.0 (MJ Research). There were made eight serial dilutions establishing an identical detection limit for the three analyzed genes:  $1 \times 10^9$  molecules (max) and 100 molecules (min). The slope data for GAPDH,  $\beta 2M$  and MITF were -0.30, -0.31 and -0.29 respectively (no curves shown for MITF and  $\beta 2M$ ). **b.** Dissociation Curve (curve of Melting), specific for the MITF gene, made with each sample included in the standard curve.

In the malign melanoma A-375 cell line and in the primary culture of melanocytes (CPM), it was possible to detect the MITF gene expression. MITF gene expression was also evidenced in the of gastric cancer (AGS) cell line, which was an unexpected fact, because these cells are from gastric epithelial origin; in those cells it was not expected to find MITF gene expression and there is any report type concerning to the evaluated gene expression. The gene expression levels in the primary culture of fibroblast (CPF) and in the breast cancer (MCF-7) cell line were very low, being located outside the detection limit. For that reason they were

considered negatives for the MITF gene expression (Graphic 2).

**MITF gene quantification in the cases and controls groups.** When analyses of absolute quantification were made, the calculated mean for the expression levels of GAPDH gene was between 10 and 12 million of molecules; it applies for the cases group as well as for the controls group. On the other hand, the mean in  $\beta 2M$  gene expression were between 41 and 43 millions of molecules for patients with malign melanoma and people without cancer (Graphic 3). As controls were selected keeping in mind similar characteristics of



**Graphic 2. Level of expression of the MITF gene in cell lines. The expression levels in MCF-7 and CPF were very low, therefore these cellular cultivations were considered negatives for the MITF gene expression.**

gender, age, occupation and socioeconomic condition to those of the patients, the analyses were made in a paired way, applying the Wilcoxon signed-rank non parametric test which didn't show significant differences in the levels of GAPDH expression ( $p=0.388$ ) and  $\beta 2M$  expression ( $p=0.347$ ) genes between the pairs of the study groups.

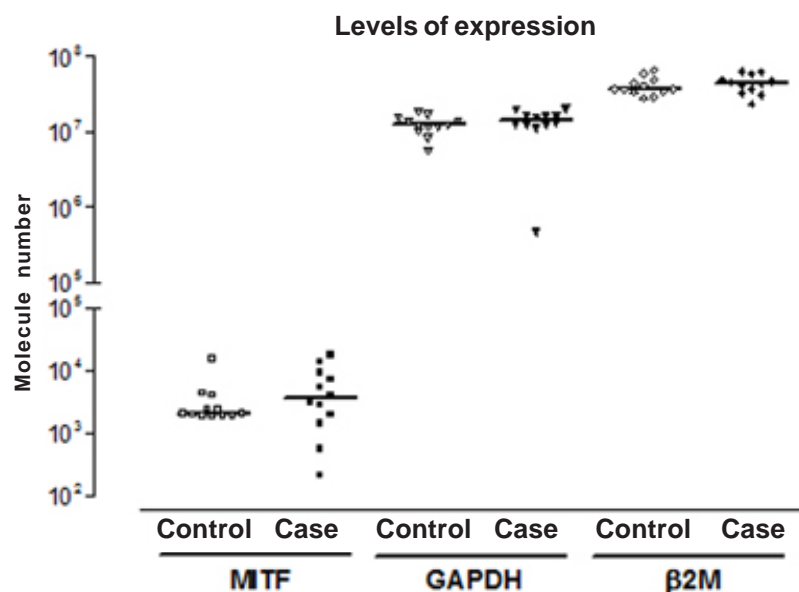
MITF gene expression was detected in every individual with malign melanoma and everyone without any kind of cancer through absolute quantification analysis. However it was observed bigger variability in the levels of expression in the cases group, fact that is confirmed when the dispersion of their samples was compared with the dispersion of the samples of the control group (Graphic 3). The expression levels for each one of the evaluated groups, were between 2,700 and 3,300 molecules. When the statistical tests were applied with raw data of C(t) (Wilcoxon  $p=0.38$ ) there were not significant differences ( $p>0.05$ ) in the levels of MITF gene expression between patient with melanoma and people without any cancer type. In the same way, there were not significant differences when comparing the number of molecules of MITF gene, between cases and controls groups ( $p>0.05$ ). However, there are reflected small differences in the fact that in 8 of the 12 analyzed couples, the cases showed bigger levels expression (Table 2a, Graphic 3). Relative quantification analysis carried out with

the method published by Pfaffl<sup>12</sup>, which keep in mind the amplification efficiency of the white gene (MITF) and the normalizer gene (GAPDH), allowed to evidence that on average, the difference in the level of MITF expression gene was 1.34 times higher in the cases group than the control group (Table 2a, b). This result confirm the small differences that were found in the absolute quantification analysis.

## DISCUSSION

The detection of tumoral cells in peripheral blood of patient with cancer is a procedure that has been broadly used to identify specific tumor RNAm molecules in several types of malignancy, for example in prostate cancer, breast cancer and malign melanoma<sup>15</sup>. However, there are some difficulties to detect and quantify the RNAm, particularly, because of the great variability and lack of sensitivity of the used techniques, being RT-PCR one of the techniques with high level of sensitivity and specificity, reason why it is frequently used for genetic expression analysis. Also, the more used biomarker to detect tumoral cells in blood from patient with melanoma, the tyrosine kinase (key enzyme for the biosynthesis of melanin), shows considerable variations in its detection of which goes from 0%<sup>16</sup> to 100%<sup>17</sup>. It makes necessary the search of other markers with lesser variability. Differing from the studies mentioned in the present work, the quantitative PCR was used (qPCR) to detect the MITF gene expression levels in peripheral blood in patients with malign melanoma as indicator of the presence of tumoral cells in circulation.

The quantitative analysis of the MITF gene expression was made applying the methods of absolute and relative quantification, based on the generation of standard curves. These were created from a plasmid that contained a fragment the of 124pb corresponding to the consensus sequence of the gene (the melanocytes expresses other isoforms of MITF-m). Cell cultures analyses revealed MITF gene expression levels in the malign melanoma (A-375) cell line and in the primary culture of melanocytes (CPM). In the breast cancer (MCF) cell line -7 and the primary culture of fibroblasts (CPF), signs of belated amplification were detected, data which



**Graphic 3. Absolute quantification analysis.** The distribution of cases and controls for the GAPDH and  $\beta$ 2M genes show very similar levels of expression, indicating the utility of those as constituent expression genes, as well as the good data processing and quality of the analyzed samples. The distribution of the cases reflects the variability in the MITF gene expression compared with its expression the group of controls. The mean in the expression of the group of cases was 3,269 molecules (Ct: 24,64) while in the group of people without cancer was 2,730 (Ct: 24,91). The variability in the MITF gene expression can be corroborated by the fact that, even the statistics tests didn't reveal significant differences ( $p > 0.05$ ), their value are located very near the limit ( $p = 0.09$ ), suggesting that in future studies, it is necessary to enlarge the panel of markers and the sample size to be analyzed.

were practically not quantifiable and therefore considered negatives for the MITF gene expression. The reproducibility of data, obtained from two independent experiments by triplication of the cell lines (MCF-7 and CPF), could be explained by illegitimate gene transcription, reported in some investigations<sup>18</sup>, as the responsible one of generating very low expression levels of any gene in any cellular type. In the gastric cancer (AGS) cell line, an average of 90 molecules was detected after carrying out two independent experiments by triplication. The MITF gene expression in the gastric tissue has not been sufficiently informed. Therefore these data are may be the first report of the gene expression in this cell line and possibly, the first one that suggest the expression of one or more isoforms of the MITF gene in the gastric tissue.

It was found MITF gene expression in the 12 patients with malign melanoma (stages III-IV) as well as in 12 people without any cancer type. The relative quantification analysis showed that on average, the cases group expressed 1.34 times more the MITF gene than the group of people

without cancer; in the absolute quantification analysis when comparing the obtained data (number of molecules) in a paired way (case-control), no significant statistical differences were found in the MITF gene expression level, between the evaluated groups ( $p > 0.05$ ). Erroneous estimates were discarded during the quantification process because there were not found significant differences in the genes of constituent expression GAPDH and  $\beta$ 2M ( $p > 0.05$ ), indicating that the procedures were carried out technically correctly. MITF is essential for the development and survivor of melanocytes and apparently, it also performs this important role in other cell types when codifies for MITF isoforms that have different N-term endings. One of them is MITF-A expressed in mast cells<sup>19</sup>, which are recognized as the principal effectors in allergic and inflammatory reactions, derived from hematopoietic stem cells. The movement of these cells along the blood and lymph vessels, as well as the identification of the MITF protein by immunohistochemical analyses in lymphocytes<sup>20</sup>, could explain the detection of the gene in people with malign melanoma as well as in people without any type of cancer and in the same way, it could justify why no significant differences between the studied groups were found. Although no significant differences were observed in the absolute quantification, small differences ( $p = 0.09$ ) were reflected by the wide variability in the levels of MITF gene expression (Graphic 3), as well as, the small increase in expression level of the observed cases when relative quantification analysis were made

**Table 2**  
**Relative expression analysis**

Match Case/Control	Ct MITF case/Ct control	Ct GAPDH case/Ct control	Match Case/Control	Ct MITF Ct case/ Ct control	Ct GAPDH Ct case/ Ct control	Relative expression Pfaffl <i>et al.</i> 2002
1 (Mel12/Cont7)	22.08 / 22.30	12.52 / 12.80	1	-0.22	-0.28	0.96
2 (Mel13/Cont8)	25.30 / 24.30	12.31 / 12.96	2	1.09	-0.65	0.31
3 (Mel14/Cont9)	23.11 / 24.22	12.55 / 12.47	3	-1.10	0.07	2.20
4 (Mel15/Cont15)	23.90 / 25.38	12.22 / 13.01	4	-1.49	-0.80	1.56
5 (Mel17/Cont12)	24.39 / 25.51	12.61 / 13.62	5	-1.12	-0.91	1.12
6 (Mel19/Cont10)	23.46 / 25.45	12.81 / 14.09	6	-2.00	-1.28	1.58
7 (Mel20/Cont21)	22.48 / 25.32	12.57 / 12.35	7	-2.84	0.23	7.81
8 (Mel21/Cont18)	24.51 / 25.10	12.94 / 12.62	8	-0.59	0.33	1.86
9 (Mel24/Cont22)	24.84 / 25.42	12.90 / 13.06	9	-0.58	-0.16	1.32
10 (Mel26/Cont17)	27.06 / 25.45	12.94 / 13.22	10	1.61	-0.28	0.28
11 (Mel27/Cont20)	28.73 / 25.34	17.65 / 12.77	11	3.39	4.88	3.02
12 (Mel28/Cont16)	25.72 / 25.13	13.86 / 13.04	12	0.62	0.83	1.17
<b>Promedio</b>	<b>24.64 / 24.91</b>	<b>13.16 / 12.99</b>	<b>Promedio</b>	<b>0.27</b>	<b>0.16</b>	<b>1.34</b>

**a. The figure shows the cases, each one matched up with their respective control according to the similar characteristics of gender, age, occupation and socio-economic condition to the patients, as well as respective C(t) of the MITF and GAPDH genes for each couple analyzed.**

(Figure 2b), suggesting that a higher genetic instability could exist in the cases group of cases circulating cells, which is confirmed by cytogenetic studies made by the research team; it could be explained by the presence of tumoral cells in peripheral blood of patient with melanoma.

The similarities in the MITF gene expression levels between the studied groups, suggests that this gene is broadly expressed, indicating that it can be a gene of constituent expression that can little contribute in the specific diagnosis of the melanoma, just as it is expressed by some authors<sup>9,10</sup>. Nevertheless, it must keep in mind that while the MITF gene expression in people without cancer was relatively constant, in the group of people with melanoma the observed expression was much more variable. Therefore, it is possible that enlarging the number of individuals to be analyzed and using a panel of biomarkers that include, for example, tyrosine kinase and the melanocytes specific isoform of the MITF gene (MITF-m), it could be obtained a higher specificity and sensitivity to develop and validate molecular diagnosis

**b. Relative quantification analysis according to the method of Pfaffl, carried out taking as normalizer the GAPDH gene. No shown data of the House Keeping Gene expression  $\beta 2M$ .**

tests, that allow to detect circulating tumoral cells in patient with melanoma.

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