# MOLECULAR PROFILING OF ANGIOGENESIS PATHWAYS AFTER PHOTODYNAMIC THERAPY IN BREAST CARCINOMA

Silva, L.C.C.[1], Strungis, N.[1], Batista, S.K.S.[1], Fontana, L.C.[1], Gonsalves, A. M. d'A. R.[2], Ferreira-Strixino J.[1], Canevari, R.A[1]

[1]IP&D–LEVB, Laboratório de Biologia Molecular do Câncer, Universidade do Vale do Paraíba – UNIVAP, São José dos Campos, SP, Brazil.
[2]Chymiotechnon, Departamento de Química, Universidade de Coimbra, 3049-535, Coimbra, Portugal.
e-mail: rcanevari@univap.br

***Abstract:****Photodynamic therapy (PDT) selectively destroys photosensitized tissue leading to various cellular and molecular responses. The establishment of new vessels occurs in a series of subsequential steps: PDT induces damage to the endothelium, which can lead to increased vascular permeability and, under intensive PDT conditions, even to platelet aggregation, vasoconstriction, and blood flow stasis. Eventually, ischemia, hypoxia, and inflammation can occur, resulting in angiogenesis. Molecular investigation related to this signaling pathway could provide a better understanding of the action of this therapy in breast tumors. After inducing mammary tumors in twenty female Sprague-Dawley rats with DMBA and performing PDT using two different photosensitizers (Chlorine and PDZ), a sample from each animal was analyzed by quantitative real time PCR using Rat Angiogenesis RT2 Profiler™ PCR Array platform. The rats were divided in four groups, including a normal group and control group (without PDT treatment). Comparisons of the molecular analysis were made between treated groups and control group, concerning genes involved in the angiogenic process and their relation to inhibition and promotion of this signaling pathway. The results indicate that multiple genes are related and involved in proangiogenic and antiangiogenic biochemical processes after PDT, thus providing a better understanding about genes involved in neovascularization and possibly evaluate novel strategies to improve PDT and photosensitizers responsiveness coupled with antiangiogenic therapy.*
***Keywords:*** *Breast cancer, Angiogenesis, Gene expression, Photodynamic therapy.*

## INTRODUCTION

Breast cancer is the most frequent carcinoma and the second most common cause of mortality in women [1]. This tumor is a heterogeneous disease with several molecular subtypes, that contributes tothe differencesinclinical presentation and treatment failure in some patients [2].

Photodynamic therapy (PDT) is an alternative a therapeutic modality in oncology treatment that is being used successfully in the treatment of many types of tumor, including skin cancer, surperficial esophageal cancer, lung cancer, and gastric cancer[3].The main advantage of PDT is the ability to selectively treat lesions without as many side effects as chemotherapy and radiotherapy because it does not lead to impaired organ function and integrity, as can often occur after surgical removal [4].

The neovascularization (angiogenesis) in tumor cells may occur during any period of neoplastic transformation, but usually coincides with increasing metabolic pressures, oncogene activation, or mutation of genes that encode tumor suppression proteins [5]. Likewise, oxidative stress caused by PDT results in a variable number of molecular and physiological responses may lead to angiogenesis [6]. This stress may increase tumor growth or evenlead to tumor recurrence by inducingthe production of pro-angiogenicmarkers by activemacrophages,such as VEGF, cyclooxygenase-2 (COX-2), matrix metalloproteinases (MMPs) and other cytokines [7].

The study of gene expression related to thissignaling pathway could provide a better understanding of the action mechanism of this therapy and, consequently, optimize it for clinical treatment of breast tumors.To date, metabolic pathways influenced by PDT have not been fully elucidated and published studies of gene expression analysis in mammary tumors induced in animal models after PDT are rare. Therefore, the aim of the present study was to evaluate the expression of genes that belongtothe angiogenesis pathway to find molecular markers that may be related to the effectiveness of PDT in mammary tumors.

## MATERIAL AND METHODS

This study follows the regulations related to animal research and the ethical principles of animal experimentation, published by the Brazilian Committee for Animal Experimentation (COBEA). It was approved by the Ethics Committee on Animal Use (CEUA) of the Universidade do Vale do Paraiba (registration number A003/CEAU/2012).

### Animal models

Twenty-five young, virgin Sprague-Dawley female rats were used in this study, with mean age of 40 days and weighing between 230 and 380g, obtained from the

Multidiciplinary Center of Biological Research (UNICAMP).

Five animals were used as a normal group (G1 - normal tissue). Twenty animals had mammary tumors induced by a single dose (70 mg/kg) of 7,12-dimethylbenz(a)anthracene (DMBA) diluted in soybean oil given by gavage. These animals were submitted to special environmental conditions: room temperature with 50% humidity and a 12 hour cycle of exposure to light and darkness. The animals underwent a daily viability inspection and weekly physical examination for tumor development.

**Photodynamic treatment**

In order to compare the influence of PDT in gene expression, a control group (G2) was composed of 10 sick rats without any PDT treatment. For PDT experiments, two groups were analyzed: G3 (five animals treated with PDT and photosensitizer Photodithazine® - PDZ) and G4 (five animals treated with PDT and photosensitizer Chlorine). The photosensitizer PDZ (Veta Grand, Moscow, Russia) is a chlorine obtained from the cyanobacterium*Spirulinaplatensis*and the photosensitizer Chlorine is a properly halogenated Chlorine derived from Foscan, belonging to the group of Coimbra photosensitizers [5].

Photosensitizers were administrated intraperitoneally at a calculated dose of 8 mg/kg for each animal. The animals were anesthetized with xylazine 2% (Coopazine® - Coopers) and ketamine (Vetanarcol® - Konig) at a dosage of 0.2 ml/g. For the applicationof PDT, the light source used was a diode laser at a wavelength of 660 nm coupled to optical fiber diffuser, using a fluence rate of 100 mW/cm and light dose of 100 J/cm (QuantumTech®- ,Brazil).

**Expression gene analysis**

The evaluation of changes in gene expression was performed by quantitative real-time PCR (qRT-PCR) on the ABI Prism 7500 Sequence Detection Systems (Life Technologies, USA). PCR reaction was done using the RT$^2$ SYBR Green qPCR Master Mix and the platform Rat Angiogenesis RT$^2$ Profiler™ PCR Array (SuperArray, SA Bioscience, USA), consisting of 84 angiogenesis-related genes,

Equal aliquots of cDNA and RT$^2$ SYBR Green qPCR Master Mix were added to each well of the same PCR Array plate containing the predisposed gene-specific primer sets. The conditions of thePCRprogramconsisted ofcyclingtwo steps,withan initial cycleat95 °C for 10 min, followed by 40 cycles of15 sat95 °C and1 min at60 °C.

The data with the thresholds values were exported and analyzed by RT² Profiler PCR Array Data Analysis template v 3.3 (SA Biosciences, USA). Value of the fold-change greater than 2 was defined as increased expression, and the fold-change value of less than 0.5 as decreased gene expression.

**RESULTS**

Several analyses were performed between groups. The increased expression for *CXCL2*, *CXCL9*, *FGF1*, *IL1B*, *IL6*, *MDK*, *MMP19*, *MMP3*, *MMP9*, *PGF*, *PTGS1* and *TIMP1* genes, and a decreased expression for *ANPEP*, *BAI1*, *EGF*, *EPAS1*, *F2*, *FGF16*, *FGF2*, *FGF6*, *FIGF*, *ID1*, *ID3*, *IFNA1*, *LECT1*, *LEP*, *NRP2*, *PECAM1*, *SERPINB5*, *SPHK1*, *TEK*, *TGTB2*, *THBS4*, *TIMP3* and *TNF-alpha* genes were detected comparing G2 when G1.

In the comparison between G3 (tumors treated with PDZ) with G2 (tumors without PDZ treatment), 15 genes showed increased expression: *ANPEP*, *CDH5*, *CXCL2*, *CXCL9*, *ENG*, *EPAS1 (HIF-2alpha)*, *FGF2*, *FIGF (VEGF-D)*, *FLT1*, *LEP*, *NPR1*, *PDGFA*, *PLG*, *TIMP3* and *TNF-alpha*, where all are proangiogenic, and only the *CXCL9* gene showed decreased expression (Table 1, Figure 1).

**Table 1**. Differential expression of the 15 genes of the angiogenesis pathway, comparing G3 to G2.

| Gene | *Fold Change G3/G2* | *Pvalue G3(PDZ)/G2* |
|---|---|---|
| *ANPEP* | 3,26 | 0,002 |
| *CDH5* | 2,54 | 0,018 |
| *CXCL2* | 7,97 | 0,028 |
| *CXCL9* | 0,20 | 0,001 |
| *ENG* | 3,47 | 0,004 |
| *EPAS1* | 4,19 | 0,001 |
| *FGF2* | 2,27 | 0,018 |
| *FIGF* | 3,19 | 0,018 |
| *FLT1* | 2,06 | 0,192 |
| *LEP* | 6,75 | 0,046 |
| *NPR1* | 3,40 | 0,018 |
| *PDGFA* | 2,49 | 0,347 |
| *PLG* | 2,20 | 0,016 |
| *TIMP3* | 3,33 | 0,002 |
| *TNF* | 2,53 | 0,017 |

Fold-change greater than 2 and P values <0.05 are indicated in red; fold-change values less than 0.5 and P <0.05 are indicated in blue.

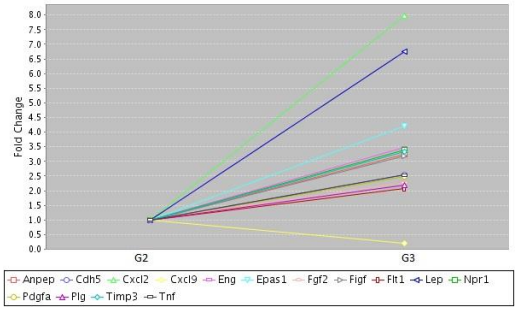


**Figure 1.** Differential expression of 15 genes of the angiogenesis pathway, comparing G3 to G2.

Subsequently, comparing G4 to G2, it was observed increased expression of the genes *CXCL1*, *LEP* and *MMP2*, and decreased expression of the *CTGF*, *CXCL9*, *IFN-gama*and *NPR1* genes. All genes with over expression are proangiogenic and those with decreased expression are proangiogenic, except for IFN-gama. (Table 2, Figure 2).

The CXCL9 gene present in all results, present in either underexpressed or overexpressed forms, plays sometimes dual roles in tumor biology: proangiogenic or antiangiogenic, depending on which receptor it is binded. [8]

**Table 2.** Differential expression of the 7 genes involved in the angiogenesis pathway, comparing G4 to G2.

| Gene | *Fold Change G3/G2* | *Pvalue G3(PDZ)/G2* |
|---|---|---|
| *CXCL1* | 2,40 | 0,026 |
| *LEP* | 5,02 | 0,043 |
| *MMP2* | 2,06 | 0,216 |
| *CTGF* | 0,31 | 0,006 |
| *CXCL9* | 0,18 | 0,000 |
| *IFN-gama* | 0,35 | 0,026 |
| *NPR1* | 0,46 | 0,018 |

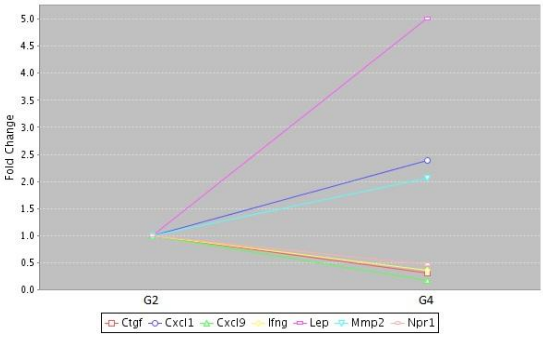


**Figure 2**. Differential expression of 7 genes involved in the angiogenesis pathway, comparing G4 to G2.

## DISCUSSION

PDT is a selective treatment modality that affects mainly the target tissue and is still under investigation. Many studies have used *in vitro* models, such as cell culture, but few of them were conducted *in vivo*, using animal models for research of mammary tumours [9, 10, 11]. In order to determine the mechanism and effectiveness of PDT, a comprehensive approach is necessary to understand the influence of this therapy on gene expression.

Angiogenesis signaling pathways influenced by PDT have not been fully elucidated.To our knowledge, this is first study of expression gene analysis in the angiogenesis pathway affected by PDT of breast samples obtained *in vivo*. Among the 15 genes obtained in the first comparison between G3 and G2, classic potent proangiogenic can be seen in genes with over expression, such as: *VEGF-D*, *HIF-2alpha*, *FGF2* and *TNF-alpha*. Whereas, when comparing G4 to G2, the three genes with over expression are proangiogenic, and of the four that showed underexpressed, three of them had antiangigenic properties.

These results suggest that changes in the expression of genes involved in the angiogenesis pathway played an important role in the angiogenesis induced by PDT and these angiogenesis-associated genes can contribute to a better understanding of the PDT action mechanism in breast cancer. In addition, these findings may yield a new insight into the development of strategies for PDT treatment of patients with breast cancer, such as usingantiangiogenic regulators coupled with photosensitizers to improve PDT efficacy.

## ACKNOWLEDGEMENTS

The Foundation for Research Support of the State of São Paulo (FAPESP) for providing the materials used in the development of this research (process number 2009/18440-1) and CNPq/PIBIC for granting the scholarship (process number 003/2012).

## REFERENCES

Oliveira CLC, Vieira CRS, Giannella-Neto A. Transdutor de pressão diferencial capacitivo para medição de fluxo ventilatório. Revista Brasileira de Engenharia Biomédica - Caderno de Engenharia Biomédica. 1982; 1(1):5-29.

[1] INCA, Institute, N.C.I., Cancer. Available from: http://www.inca.gov.br, 2014. [cited 2014 Jul 31]

[2] Groce, CM Molecular origins of cancer oncogenes and cancer. N. Engl. J. Med. 2008; 358(5):502-511.

[3] Shishkov N., Kuznetsova O., Termirbolat B. Photodynamic Therapy for Gynecological Diseases and Breast Cancer CancerBiol Med. 2012; 9(1): 9–17.

[4] Agostinis P. *et al.* Photodynamic Therapy of Cancer: An Update CA Cancer J Clin 2011; 61(4):250–281.

[5] Langley, RR, Kim, SJ, Fidler IJ The Use of Orthotopic Models to Validate Antivascular Therapies for CancerIn: Marmé, D., Fusenig, N., editors, Tumor Angiogenesis: Basic Mechanisms and Cancer Therapy.Springer, 2008, p. 481.

[6] Gomer, CJ, Ferrario, A., Luna, M., Rucker, N., Wong S. Photodynamic Therapy: combined modality approaches targeting the tumor microenvironment. Lasers surg. med. 2006; 38(5): 516-521.

[7] Ferrario, A., Tiehl, KFV, Rucker, N. Antiangiogenic treatment enhances photodynamic Therapy responsiveness in a mouse mammary carcinoma. Cancer Res. 2000; 60:4066-4069.

[8] Verbeke H. *et al* The role of CXC chemokines in the transition of chronic inflammation to esophageal and gastric cancer Biochim Biophys Acta. 2012; 1825(1):117-29.

[9] Gibson SL *et al* Efficacy of photodynamic therapy on original and recurrent rat mammary tumors. PhotochemPhotobiol 1995; 61(2):196-199.

[10] Bressenot A. *et al* Assessment of apoptosis by immunohistochemistry to active caspase-3, active caspase-7, or cleaved PARP in monolayer cells and spheroid and subcutaneous xenografts of human carcinoma. J Histochem. Cytochem. 2009; 57(4):289-300.

[11] Duanmu J. *et al* Effective treatment of chemoresistant breast cancer in vitro and in vivo by a factor VII-targeted photodynamic therapy. Br J Cancer. 2011; 104:1401-1409.