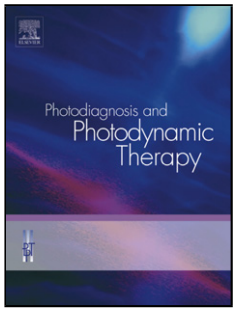

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PII: S1572-1000(16)30026-6
DOI: http://dx.doi.org/doi:10.1016/j.pdpdt.2016.03.006
Reference: PDPDT 755

To appear in: *Photodiagnosis and Photodynamic Therapy*

Received date: 5-1-2016
Revised date: 6-3-2016
Accepted date: 21-3-2016

Please cite this article as: Silva Luciana C, Ferreira-Strixino Juliana, Fontana Letícia C, Rocha Gonsalves António Md'A, Serra Arménio C, Pineiro Marta, Canevari Renata A.Molecular Analysis of Apoptosis Pathway after Photodynamic Therapy in Breast Cancer: animal Model study.*Photodiagnosis and Photodynamic Therapy* http://dx.doi.org/10.1016/j.pdpdt.2016.03.006

Molecular Analysis of Apoptosis Pathway after Photodynamic Therapy in Breast Cancer: animal Model study

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Acknowledgement: The Foundation for Research Support of the State of São Paulo (FAPESP) for providing the materials for the development of research (process number 2009/18440-1) and CNPq (process number 003/2012).

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Key words: apoptosis; breast cancer; photodynamic therapy, chlorin

Highlights

Pro-apoptotic *BAK1, CARD6, CASP8, CIDEA, CIDEB, DAPK1, TNF, TNFRSF10B, FASLG, LOC687813,* and *TP73* genes showed increased expression, after PDT.

CD40 anti-apoptotic gene showed decreased expression in the group who underwent PDT (G3) in relation to G2.

These genes are involved more directly with cellular apoptosis induced by PDT using the Chlorin photosensitizer.

Abstract

Background: Molecular investigation of breast tumors has permitted better understanding about interaction of genes and pathways involved in tumor progression.

Objective: The aim of this study was to evaluate the association between genes belonging to the pathway of apoptosis with tumor response to photodynamic therapy.

Study Design/Materials and Methods: The mammary tumors were induced in twenty-four *Spraguey-Dawley* female rats by oral gavage of 7,12-dimethylbenz(a) anthracene (8 mg/Kg body weight). Animals were divided into three groups: G1 (normal tissue), G2 (tumors without treatment), G3 (animals euthanized 48 h after treatment). The photosensitizer used was a chlorin, 5,15-bis-(2-bromo-5-hydroxyphenyl) chlorin) in the dose of 8mg/kg for each animal. Light source of diode laser at a wavelength of 660 nm, fluence rate of 100 mW/cm, and light dose of 100 J/cm was delivery to lesions for treatment. A sample from each animal was investigated by quantitative real time PCR using *Rat Apoptosis RT^2 Profiler™ PCR Array* platform.

Results: Pro-apoptotic *BAK1, CARD6, CASP8, CIDEA, CIDEB, DAPK1, TNF, TNFRSF10B, FASLG, LOC687813*, and *TP73* genes showed increased expression, and *CD40* anti-apoptotic gene showed decreased expression in the group who underwent PDT (G3) in relation to G2.

Conclusion: The results indicated that these genes are involved more directly with cellular apoptosis induced by PDT using the Chlorin photosensitizer.

INTRODUCTION

Breast cancer is an heterogeneous disease, where numerous genetic changes result in imbalance of cell proliferation, alteration of the mechanism of apoptosis, alteration in the expression of several genes, generation of genetic instability, among other factors [1]. Genetic heterogeneity contributes to the differences in clinical behavior and to the response to treatment in which tumors belonging to the same histological type may differ in sensitivity to chemotherapy, radiotherapy, and other treatments, contributing to the high mortality associated with these tumors [2].

For better cancer treatment, Photodynamic therapy (PDT) is an emerging technology that has been used as a therapeutic method to treat various solid tumors, tumors in early stages, and a palliative method in advanced cancers [3, 4]. It has been used in combination with other therapies such as chemotherapy, surgical removal, radiotherapy, or other therapies such as anti-VEGF therapy (vascular endothelial growth factor), with several studies reporting promising results [5, 4, 6]. This therapy combines the use of photosensitizers and an exogenous source of visible light with specific wavelength [7].

Photosensitizers (PS), such as chlorins, are excited from the singlet electron ground state to a higher energy singlet state upon illumination with light sources of the appropriate wavelength, typically between 600–800 nm. Chlorins are a second generation of photosensitizers, this PS has absorption bands in the red and near-infrared regions allowing better tissue light penetration. Studies showed the distribution of this PS in organelles like endoplasmic reticulum and lysosomes, with a large localization in mitochondria and nuclei. The PDT treatment with Chlorins has demonstrated that it could promote the apoptosis in human cancer cells [7, 8].

This event causes different reactions on tissue, leading to the generation of various cytotoxic reactive oxygen species (ROS). Beside that event, others are activated, such as, influences in proliferation decline, induced cell cycle arrest, apoptosis or necrotic cell death of affected cells. The cytotoxicity of the PDT agent is highly related to its localization within the cell [(8)]. The main advantage of this therapy is the ability to treat selectively lesions without many side effects as chemotherapy and radiotherapy. It does not lead to impaired organ function and integrity, as can often occur after surgical removal [(9)].

The apoptotic mechanism plays an important role in tumor cell death caused by PDT, and it is responsible for rapid tumor elimination [(10)]. The induction of apoptosis has also been observed in many tumors at early times following PDT at doses leading to tumor eradication [(19, 11)]. It is known that multiple pathways are involved during PDT-mediated cell death [(12)], however there are reports that apoptosis PDT is related to the activation of two distinct: the intrinsic pathway, which is related to mitochondrial signals, and the extrinsic pathway activated by death ligands [(13)].

Molecular biology techniques have been involved in the research of the expression genes analysis and their interactions between different biochemical pathways that may be involved in cancer. The RT^2 Profiler PCR Array is the most reliable and accurate tool to analyze the expression of a focused panel of genes, and it offers a complete solution to examine gene expression profiles for any pathway of interest. Currently, little is known regarding gene expression analysis in mammary tumors induced in animal models after treatment by PDT. 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 [(14, 15, 16)]. Thus, it is important to determine which genes belonging to specific pathways are related to response to therapy. Therefore, the aim of

the present study was to evaluate the gene expression pattern of the apoptosis pathway after photodynamic treatment using a RT^2 profiler™ PCR array technique to find molecular markers which may be related to the response of the PDT in mammary tumors.

MATERIALS AND METHODS

This study follows the regulations governing research with animals 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).

Animals: In this study, were used twenty six young female rats, virgin, of *Sprague-Dawley* lineage, with mean age of 45-50 days and weighing between 230 and 380g, obtained from the Multidisciplinary Center of Biological Research, UNICAMP. These animals were submitted to conditions: room temperature with 50% humidity and 12h cycle of light and dark. The animals were weighted and anesthetized with intramuscular injection of sterile solution of Ketamine hydrochloride 5% (Syntec®) associated with muscle relaxant, analgesic, and sedative xylazine 2% (Syntec®) at a dosage of 0.2 ml/g. The animals were divided into three groups: G1 (rats that not received chemical induction and without any mammary tumor), G2 (control group consisting of rats with mammary untreated tumors) and G3 (rats with mammary tumors treated by PDT).

Chemical induction: Twenty-four animals had mammary tumors induced by 7,12-dimethylbenz (a) anthracene (DMBA) at a dose of 80 mg/kg dissolved in soybean oil and administered by gavage, with a single application. Chemical induction model was chosen to

simulate the process of carcinogenesis that occurs in humans, in order to evaluate the possible molecular alterations induced by PDT.

Photosensitizer: The photosensitizer used was a chlorin, 5,15-bis-(2-bromo-5-hydroxyphenyl) chlorin) synthetized by the Group of Coimbra University. The photosensitizer was diluted with the slowly adiction of 2ml/mg of ethanol 95%, 3 ml/mg of polyethylene glycol and 5 ml/mg of water, under agitation and was administrated intraperitoneally at a calculated dose of 8 mg/kg for each animal (approximately 0,8 ml per animal).

Light source: For the application of PDT, the light source used was a diode laser at a wavelength of 660 nm (QuantumTech® - Sao Carlos, Brazil), coupled to a cylindrical light diffuser (optical fiber model RD10 - Medlight), with 1 cm diameter. A low fluence rate of 100 mW/cm and light dose of 100 J/cm were used. The irradiation during 20 minutes, of tumor was interstitial, in all extention of the tumor and realized after 24 h administration of the photosensitizer.

Experimental procedure of photodynamic therapy: The animals were divided in two groups: a control group (G2) composed of 10 sick rats with breast tumor without any treatment and one group composed of 14 rats euthanized 48 h after they underwent PDT (G3). After the irradiation, the animals were put in a covering box with aluminum, for protection of light.

Extraction of RNA, reverse transcription and RT^2 Profiler PCR array: The 5 samples (G1), 10 tumor samples (G2), and 14 tumor samples (G3) were collected and stored at -80 °C for gene expression analysis. Before that, total RNA samples were isolated using the *Qiazol* reagent and protocol *RNeasy Lipid Tissue* (Qiagen). For RNA extraction a sample volume at about 100 mg (Qiagen) was used. The qualitative and quantitative characterization of the RNA extracted samples were performed using ultraviolet absorption spectroscopy (Nanodrop, ND-1000, Labtrade). In addition, its quality was visualized on agarose gel electrophoresis. After DNA digestion (RNase - Free DNase Set, Qiagen), RNA purification of the samples by RNeasy MinElute Cleanup Kit (Qiagen) and cDNA synthesis by the system preamp RT^2 First Strand kit (SuperArray Biosciences, USA) were performed. 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 Apoptosis RT^2 Profiler™ PCR Array (SuperArray, SA Bioscience, USA), consisting of 84 apoptosis-related genes, five housekeeping genes (*RPLP1*, *HPRT1*, *RPL13A*, *LDHA*, and *ACTB*), a control genomic DNA (GDC), three controls of reverse transcription (RTC), and three positive controls of PCR (PPC). In this study, housekeeping genes utilized were *RPL13A*, *LDHA*, and *ACTB* by their lower variation in threshold values (CT). The values of controls in the plate were used for calculations to determine the expression of each target gene present in the plate.

Equal aliquots of cDNA and RT^2 SYBR Green qPCR Master Mix (25 µl) were added to each well of the same PCR Array plate containing the predisposed gene-specific primer sets. The conditions of the PCR program consisted of cycling two steps, with an initial cycle at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

Upon completion of the qRT-PCR, the baseline and CT values were automatically set for each plate, and the same thresholds for all PCR arrays plates were kept. The data with the thresholds values were exported and analyzed by RT² Profiler PCR Array Data Analysis template v 3.3 (SuperArray Biosciences, USA).

Statistical Analysis: Statistical analysis of expression gene data was performed with the nonpaired Student's t-test and considered statistically significant with p-values <0.05. 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

The criterion used in the study was the appearance of tumors in animals. All animals underwent a daily viability inspection and weekly physical examination (palpation) for tumor development observation. The tumor appeared in average after 6 weeks from the induction. Treated tumors showed different sizes, with different behaviors, some with exaggerated growth and others with slowly growth. When there was more than one tumor in the same animal, we chose one tumor to evaluate, giving preference to smaller tumors (2,2 cm^3 ± 4,3), making the treatment easier and more efficient. Thus, the averages found in the tumor measurements between G2 (control group) and G3 (treated with PDT) compared to the volume of the tumor area corresponding to the day of the biopsy were: 26.85 (± 20,0) cm^3 (G2) and 1.84 cm^3 (± 0,8) (G3). The tumor volume was measured by your height, length and width. The measurements were performed with the aid of a caliper.

Several analyses and comparisons were performed between groups. The *AVEN, BCL2L2, HRK, CARD10, CARD6, CASP14, CASP2, CFLAR, CIDEA, DAPK1, DFFA, FADD,*

GADD45A, LHX4, NOL3, RIPK2, TNF, TNFRSF1A, LOC687813, TRAF2, TP63, and *TP73* genes decreased expression, and the *BCL2A1D, NAIP2, BIRC5, CASP7, TNFRSF1B,* and *CD40LG* genes increased expression for G2 compared to G1 (data not shown). Of the 22 genes (the most with known function) detected with decreased expression in the tumor samples (G2), 16 are pro-apoptotic genes (*HRK, CARD10, CARD6, CASP2, CIDEA, DAPK1, DFFA, FADD, GADD45A, LHX4, RIPK2, TNF, TNFRSF1A, LOC687813, TP63,* and *TP73).* Of the 6 genes with over expression in the tumor samples (G2), 4 are anti-apoptotic (*NAIP2, BIRC5, CASP14,* and *CD40LG*). Of these, *CARD6, CIDEA, DAPK1, TNF, LOC687813,* and *TP73* pro-apoptotic genes showed decreased expression, and *NAIP2* and *CD40LG* anti-apoptotic genes showed over expression between tumors submitted to PDT (G3) compared to the non-treated tumors (G2) (Supplementary Table 1).

Of the 84 analyzed genes belonging to the apoptosis pathway, 21 showed significant changes in gene expression between tumors submitted to PDT (G3) compared to the non-treated tumors (G2). The increased expression for *BAK1, BCL2, NAIP2, CARD6, CASP8, CIDEA, CIDEB, DAPK1, IL10, LHX4, PRLR, TNF, TNFRSF10B, CD40LG, FASLG, LOC687813, TRAF2,* and *TP73* and a decreased expression for *CASP6, FADD,* and *CD40* genes were detected comparing G3 with G2. Table 1 shows the expression of 21 genes that show significant alteration and fold change values with respective p value.

Of the 18 genes with over expression in G3, 11 are pro-apoptotic genes (*BAK1, CARD6, CASP8, CIDEA, CIDEB, DAPK1, TNF, TNFRSF10B, FASLG, LOC687813,* and *TP73*), and of the 3 genes with under expression in G3, one gene (*CD40*) is anti-apoptotic. The controls used in PCR array platforms, control of reverse transcription (RTC) showed no contamination in the RNA samples, indicating the absence of inhibition of the reaction.

DISCUSSION

The induction of apoptosis by most physiological stimuli or toxic agents, proceeds through a series of signaling pathways, and PDT has been found to unregulated numerous signaling pathways, where some of these signals act as mediators or promoters of apoptosis in PDT-treated cells [5]. There are many studies in the literature that indicate the association between the apoptotic event pos PDT through histological analysis [12, 15, 17], but this process is still unclear. However, it is known in the literature that changes in RNA expression of apoptosis genes precede histological changes [18].

Rats as an experimental model are the most widely used for studies of mammary carcinogenesis, because they are standardized and comparable to the spontaneous disease in women. Furthermore, in rats, the tumors are subcutaneous, facilitating the access to the tumor lesion and its manipulation. Sprague–Dawley (SD) rats used in this study, are among the most sensitive to carcinogen-induced mammary cancers [19]. Based on these arguments, in this study Rat Apoptosis PCR Array was used to evaluate the relative expression of apoptosis-related genes to provide the identification of the pathway affected by PDT treatment in mammary tumors of samples obtained *in vivo* using photosensitizer derived from a chlorin.

During PDT-induced apoptosis, expression changes occurs of some genes and their proteins of apoptosis-related signaling pathway, such as BCL-2 family, which are known as critical regulators of the apoptotic pathway and along the intrinsic mitochondrial apoptosis pathway [20]. The BCL-2 family of proteins has expanded significantly and includes both pro- (for example, *BAK*, *BAX*, and *BOK*) as well as anti-apoptotic molecules [21]. This family is activated in response to different stress stimuli and can contribute to cell death triggered by members of the tumor necrosis factor family member such as *FAS*, *TNF*, or *TRAIL* [22, 23]. In this study, we found a significant

increase of pro-apoptotic *BAK1*, *TNF,* and *TNFRSF10B* gene expression and of the anti-apoptotic *BCL2* gene expression in G3 in relation to the control (G2). The usefulness of Bcl-2 expression as a predictor of PDT response is controversial [(5)]. Granville et al. (1999) subsequently confirmed the ability of overexpressed protein Bcl-2 to suppress apoptosis in HL60 cells treated with BPD-MA-PDT [(24)]. These authors also reported that overexpressed Bcl-2 does not block the PDT-induced release of cytochrome c from mitochondria but rather inhibits subsequent steps in the caspase activation pathway [(25)]. However, Kawaguchi et al. (2000) showed no correlation between expression of Bcl-2 or p53 and local recurrence after PDT in a series of biopsies of squamous cell carcinomas of the bronchus previous to treatment with PII-PDT [(26)]. The levels of Bcl-2 have also been measured in biopsies of esophageal tumors treated with PII-PDT, but again no apparent correlation was found [(27)]. On the contrary, a screening of biopsies from patients with esophageal cancer treated with PDT suggested that Bcl-2 expression is associated with favorable response to PDT [(28)]. This finding can be explained by experimental studies showing that PDT leading to apoptosis by decreasing the Bcl-2/Bax ratio [(28, 29)]. It has proposed that elevated Bax may promote apoptosis even in the presence of Bcl-2 [(30)]. *BAK* gene is a member structurally similar to *BAX* gene, present in mouse liver mitochondria instead of *BCL-2* or *BAX*, showing the importance of this member in apoptotic process [(29)]. Bak protein should potentially work as tumor suppressors [(23)], and based on our results, may be a factor that induces apoptosis after therapy.

TNF gene encodes a multifunctional proinflammatory cytokine that belongs to the tumor necrosis factor (TNF) superfamily. This cytokine is involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation [(31,32)]. This gene participates of the

extrinsic pathway of apoptotic mechanism, characterized by a number of external signals to the cells and can be activated by the interaction of receptors involved in the process and their ligands, DNA repair defects, influence of radiation, and cytotoxic drugs [13]. *TNFRSF10B* is also a pro-apoptotic gene and is a death receptor that induces apoptosis by binding to the TRAIL. Through the domains in its structure, *TNFRSF10B* can recruit adaptor proteins and activate caspase-8, which in turn is responsible for activation of other members to cause release of cytochrome c from mitochondria by promoting the apoptotic mechanism [33,34,35].

CARD6, CIDEA, CIDEB, and *TP73* pro-apoptotic genes had increased expression in the group who underwent PDT (G3) in relation to G2. *CARD6* is a member of the death domain (DD)-fold superfamily, a homotypic protein-protein interaction module present in proteins that play pivotal roles in programmed cell death, NF-kB activation, and inflammation. Most proteins containing this motif, called caspase recruitment domain (CARD), are involved in pathways regulating apoptosis leading to activation of caspase family proteases as well as induction of NF-kB family transcription factor [36,37].

In this study, the up-regulation of pro-apoptotic gene *CIDEA* and *CIDEB* in G3 compared to G2 suggests that the expression of these genes is positively correlated with cellular apoptosis induced by PDT. *CIDEA* and *CIDEB* belong to the same family as CIDE and act as important regulators for various aspects of metabolism [38]. However, the molecular basis by which CIDE proteins regulate target gene expression remains unclear. VALOUSKOVÁ et al. (2008) hypothesize that *CIDEA* is sequestered in mitochondria, while transfer of this potentially dangerous protein from mitochondria into nucleus intensifies or even initiates apoptosis [39]. The function of *CIDEA* seems to be conserved between mouse and human. Recently, *CIDEA* was found to be localized to endoplasmic reticulum (ER) and lipid droplet, which are characterized as dynamic

organelles that are in close contact with multiple subcellular structures including endoplasmic reticulum, endosomes, mitochondria, and plasma membrane [38]. This evidence is very important because it may explain the effect of PDT in the tissue based on the subcellular localization of the photosensitizer, as demonstrated in literature. The results obtained in our study are in agreement with studies in the literature and indicate that *CIDEA* and *CIDEB* genes are critical regulators of apoptosis induced by PDT. In addition, the significant increase of gene expression in *TP73* gene elucidated the importance of this gene in effects of PDT. This gene is a tumor suppressor that has been found to be over-expressed in a variety of tumors and mediates apoptotic responses to genotoxic stress [40]. The increased expression of this gene in tumors treated by PDT (G3) indicates that it may be involved in apoptosis after PDT.

DAPK1, FASLG, and *LOC687813* pro-apoptotic genes also had increased expression in our study in G3 compared to G2, which suggests that they are also correlated with cellular apoptosis induced by PDT. The pro-apoptotic gene *DAPK1* (death associated protein kinase 1) is a serine/threonine (Ser/Thr) kinase and was originally identified by a positive mediator of gamma-interferon that mediates a range of processes, including induced cell death by various stimuli such as interferon-c, Fas and transforming growth factor-b [41,42], and autophagy [43]. *DAPK* gene expression and apoptotic activity is increased in response to transforming growth factor-beta (TGF-beta) and to stimuli that activate *p53,* such as DNA damaging agents [43,44]. *DAPK* also has the ability to counter tumor necrosis factor (TNF)-mediated apoptosis [43,45]. *DAPK1* loss expression correlates with recurrence and metastasis incidence [42]. *FASLG* gene encoded a protein that belongs to ligand for *FAS*. *FAS* gene interacts with this receptor *FASLG,* and this mechanism invokes a signal pathway involved in apoptosis regulation in several different cell types [46]. *FAS* gene has different types of signaling pathways proposed in

cell lines. The *FAS* type I pathway is characterized by binding of procaspase-8 to the death-inducing signaling complex (DISC) in response to *FAS* ligation. The internalized *FAS/FADD*/procaspase-8 complexes then further elicit downstream protease cascade activation and contribute to apoptosis execution. In type 2, caspase-8 activation occurs, resulting in apoptosis through mitochondria damage and subsequent cytochrome release into the cytoplasm [(46)]. The pro-apoptotic gene *LOC687813* (similar to TNF receptor-associated factor 1) encodes a protein involved in the regulation of apoptosis and signal transduction [(47)], and this process can be related to the events promoting by PDT. These results were expected because PDT promotes apoptosis in tumor tissues.

Besides *BCL-2*, some anti-apoptotic genes also showed over-expression in G3 group in relation to G2: *PRLR*, *LHX4*, *TRAF2*, and *NAIP2*. The biological effects of *PRLR* receptor gene are mediated by interaction with this ligant *PRL* (prolactin) [(48)], which promotes the activation of several signaling pathways, including the Janus kinase-Signal transducer and activator of transcription (*JAK-STAT*), the Mitogen-activated protein kinases (*MAPK*), and the phosphoinositide 3 kinase (*PI3K*) [(48)]. Some studies found that altered expression of *PRLR* gene and its ligant *PRL* is implicated in breast and other types of cancers [(48)]. *TRAF2* encoded a protein that is a member of the TNF receptor associated factor (TRAF) protein family. This protein directly interacts with TNF receptors, and forms a heterodimeric complex with TRAF1 that interacts with the inhibitor-of-apoptosis proteins (IAPs), and functions as a mediator of the anti-apoptotic signals from TNF receptors. The interaction of this protein with TRADD, a TNF receptor associated apoptotic signal transducer, ensures the recruitment of IAPs for the direct inhibition of caspase activation [(49,50)]. *NAIP* gene (neuronal apoptosis inhibitory protein) is a member of the conserved inhibitor of apoptosis protein (*IAP*) family. NAIP is required in the mammalian innate immune response and has been implicated in some

diseases such as spinal muscular atrophy [51]. Over-expression of NAIP and other apoptosis inhibitory proteins has been demonstrated to prevent apoptosis induced by a variety of death signals [52]. The under-regulation of these anti-apoptotic genes in G3 compared to G2 suggests that they may not be involved in the apoptosis event that occurs after therapy.

PDT is responsible for inducing a large number of immune systems effects in the tumor tissue. There have been substantial advances in the understanding of this mechanism, with two possible aspects for the effect of PDT on the immune response against cancer: anti-tumor activity of PDT-induced inflammatory cells and generation of a long-term anti-tumor immune response. These effects can be elicited by phototoxic damage that is not necessarily lethal to all tumor cells and creates an inflammatory stimulus. The inflammatory signaling after PDT initiates a massive regulated invasion of neutrophils, mast cells, and monocytes/macrophages involved in this process [53]. Our results found altered expression of genes involved with immune and inflammatory process (over-expression in *IL10* and *CD40LG* genes, down-expression in *CD40* gene), when comparing G3 with G2. IL10 is a potent cytokine with a dual ability to immunosuppress or immunostimulant effects. They are produced predominantly by T cells, monocytes, macrophages, and endothelial cells. They have multiple functions including facilitating communication between immune cells, controlling genes, regulating transcription factors, and governing the inflammation, differentiation, proliferation, and secretion of antibodies [54]. The over-expression of IL10 gene indicates the relationship of this gene with the inflammatory response that occurs after PDT. *CD40LG* encodes a protein that is expressed on the surface of T cells and regulates B cell function by engaging CD40 on the B cell surface. *CD40* gene mediates antiapoptotic signaling, proliferation, and differentiation in B cells [55]. *CD40* is a member of the tumor necrosis factor receptor

super family (*TNFR*), and it is expressed on the surface of several immune and nonhematopoietic cells, such as B cells, macrophages, dendritic cells, fibroblasts, and endothelial cells in certain pathogenic conditions [56]. Recent experimental and clinical observations suggest that the *CD40* pathway can be exploited to treat malignancy and that *CD40* gene and its ligand *CD40LG* play important roles in mediating cellular and humeral immune response by regulating the functions of antigen presenting cells (APC) and T cells [56]. The down-regulation of the *CD40* gene in G3 suggests that it might be involved in apoptosis observed in the samples obtained after treatment. In addition, the *IL10*, *CD40LG* and *CD40* genes with differential expression observed in our study suggests that it can be associated with immune response to PDT.

Our study demonstrated that caspases also showed differential expression when compared the two groups. Caspases are proteins involved to the mechanisms of proliferation, differentiation, or migration and are directly related to the promotion mechanism of apoptosis [57]. The pro-apoptotic *CASP8* gene had over-expression and *CASP6* had decreased expression in the treated group when compared with control group. Considering that animals of this study were sacrificed 24 hours after PDT was performed and that this treatment exerts its effects in the tissue instantly after application, we assume that the differential expression observed for these two genes was due to the different role played by caspases in cells, because they can be defined as executioners (caspase -3, -6 and -7), or initiators caspases (caspase-8, -9 and -10) [58]. This could explain the increased expression observed to *CASP8* gene and the decreased expression to *CASP6* gene in the treated group.

In this study, five genes that had differential expression in the treated group when compared with control group are members of the tumor necrosis factor (*TNF*) receptor superfamily that belong to extrinsic pathway and are associated with death signaling

pathway, known as mediators of several apoptotic pathways. The pro-apoptotic *TNF*, *TNFRSF10B, FASLG,* and *LOC687813* genes had over-expression and the anti-apoptotic *CD40* and pro-apoptotic *FADD* had under expression. *FADD* gene also showed decreased expression in the treated group (G3) compared to the control group (G2). These genes belong to *TNF* receptor superfamily and are associated with receptors of apoptotic death signaling pathway. *FADD* associates adaptor proteins and triggers multiple cell signals, including the activation of caspases, mitogen-activated protein kinases (*MAPKs*), and nuclear factor-κB (*NF-κB*). *FADD* is thought to be essential for *TRAIL*-induced apoptosis [(59)]. The under-regulation of these anti-apoptotic genes and the over-expression observed in other genes belonging to this family in G3 compared to G2 suggests that this family could be related with the effect of the PDT and that *FADD* gene is not involved in the apoptosis event that occurs after therapy.

The extent of photodamage and cytotoxicity after PDT in vivo is multifactorial and can depend of different cell types, incubation and illumination conditions, and concentration of oxygen in tumor tissue [(60)]. Chemical and physical properties, subcellular location, and concentration of the photosensitizer may all influence the mode and extent of cell death and consequently significantly alter the treatment efficacy of PDT [(53, 60, 61)]. A previous study of our group [(62)], which evaluated the expression of genes involved in the apoptosis pathway by RT^{2}profiler™ PCR array technique and using the photosensitizer chlorine Photodithazine® (PDZ - Veta Grand, Russia), detected over-expressed in 6 genes (*CASP4, CASP12, CIDEA, GADD45A*, *PRLR,* and *FAS)* and decreased expression in 14 genes (*BAK1, MAPK8IP1, TNFRSF11B, TP53, TP63, BAD, BIK, NAIP2, BOK, DFFA, FADD, CD40, TRADD,* and *LOC687813*) between tumors treated by PDT with non-treated. When comparing our results with the previous study, only the genes *CIDEA* and *PRLR* with over expression and *CD40* gene with under expression

showed concordant results. In the present study, was used the photosensitizer chlorin similar to Foscan. The relative contribution of each photosensitizer depends on the nature of this compound and its localization within the tumor tissue, on the tumor type (vascularity and macrophage content), and on the time delay of irradiation after photosensitizer administration [63]. Therefore, we believe that the low concordance in relation to genes differentially expressed between the two studies is related to the type of photosensitizer used, because other conditions employed between the two studies were the same, such as incubation and illumination conditions.

The results of this study indicates that the pro-apoptotic *BAK1, CARD6, CASP8, CIDEA, CIDEB, DAPK1, TNF, TNFRSF10B, FASLG, LOC687813,* and *TP73* genes with increased expression and *CD40* anti-apoptotic gene with decreased expression, in the group who underwent PDT (G3) in relation to G2, are more directly involved with cellular apoptosis induced by PDT using the chlorin group photosensitizer. We conclude that discovery of these markers can contribute to a better understanding of the action of PDT in breast cancer and can assist in greater antitumor effectiveness of PDT in breast tumor.

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FIGURE CONTENTS

Figure 1. Chemical structure of the chlorin, 5,15-bis-(2-bromo-5-hydroxyphenyl) chlorin), synthesized by Coimbra University – Chemical Department – Portugal.

FIGURE CONTENTS

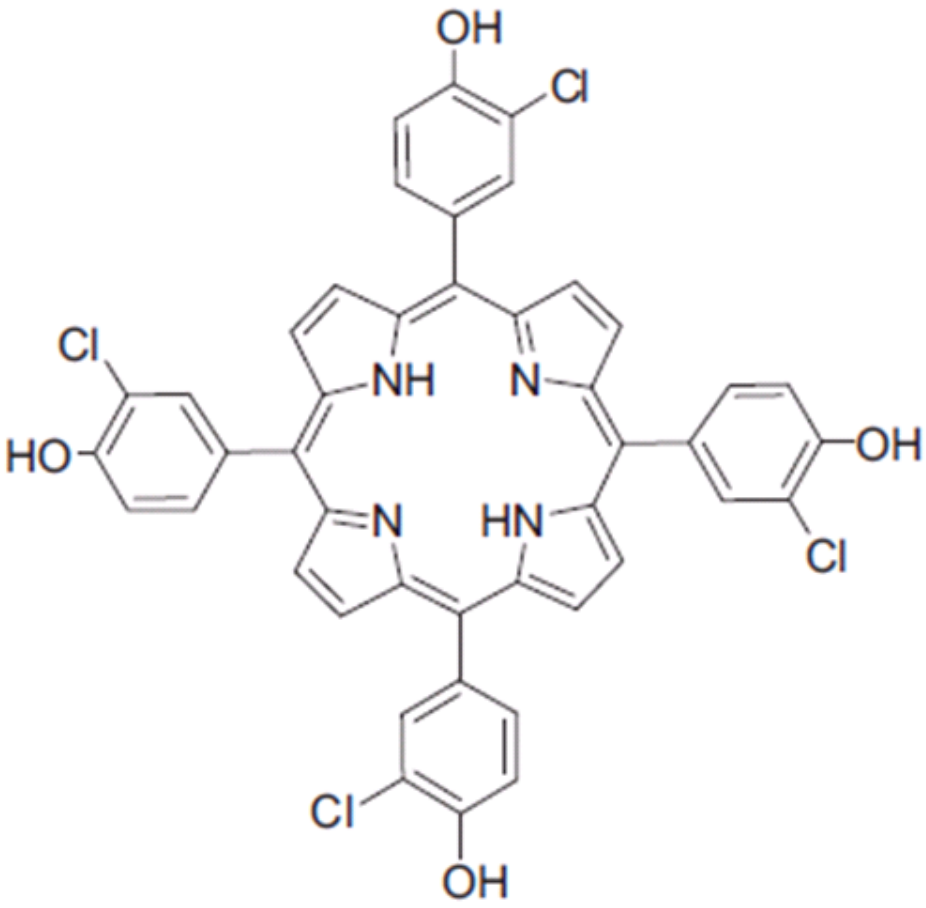

Figure 1

Table 1. Differential expression of the 84 genes of the apoptosis pathway (*platform Rat Apoptosis RT2 Profiler™ PCR Array*): comparison between G3 (mammary tumors treated by PDT) and G2 (mammary untreated tumors) groups and between G2 and G1 (without mammary tumor). The fold change, fold regulation and p-values values are listed in both comparisons.

Gene symbol	Gene name	Fold change G3/G2	p-value	Fold Regulation G3/G2	Fold change G2/G1	p-value	Fold Regulation G3/G2
Apaf1	Apoptotic peptidase activating factor 1	1.85	0.039856	1.85	1.58	0.003336	1.58
Api5	Apoptosis inhibitor 5	1.60	0.270018	1.60	0.62	0.002260	0.62
Aven	Apoptosis, caspase activation inhibitor	1.92	0.061507	1.92	0.46	0.000000	0.46
Bad	BCL2-associated agonist of cell death	1.18	0.085021	1.18	1.00	0.810166	1.00
Bag1	BCL2-associated athanogene	0.36	0.058793	-2.81	3.48	0.577531	3.48
Bak1	BCL2-antagonist/killer 1	4.12	0.002445	4.12	1.87	0.024466	1.87
Bax	Bcl2-associated X protein	1.02	0.260293	1.02	1.04	0.822358	1.04
Bcl10	B-cell CLL/lymphoma 10	0.41	0.574667	-2.41	1.09	0.401016	1.09
Bcl2	B-cell CLL/lymphoma 2	2.44	0.020143	2.44	0.60	0.000961	0.60
Bcl2a1d	B-cell leukemia/lymphoma 2 related protein A1d	0.56	0.174731	-1.77	4.80	0.005872	4.80
Bcl2l1	Bcl2-like 1	0.98	0.310126	-1.02	0.54	0.013697	0.54

Bcl2l11	BCL2-like 11 (apoptosis facilitator)	8.21	0.130457	8.21	0.87	0.525280	0.87
Bcl2l2	Bcl2-like 2	1.01	0.489127	1.01	0.22	0.000048	0.22
Bclaf1	BCL2-associated transcription factor 1	0.24	0.175377	-4.16	1.64	0.059168	1.64
Bid	BH3 interacting domain death agonist	1.58	0.077518	1.58	1.21	0.304979	1.21
Hrk	Harakiri, BCL2 interacting protein (contains BH3 domain)	10.21	0.227446	10.21	0.20	0.001528	0.20
Bik	BCL2-interacting killer (apoptosis-inducing)	0.67	0.697981	-1.49	0.87	0.388826	0.87
Naip2	NLR family, apoptosis inhibitory protein 2	2.91	0.02002	2,91	1.86	0.110201	1.86
Birc3	Baculoviral IAP repeat-containing 3	0.82	0.336060	-1.22	0.92	0.820966	0.92
Xiap	X-linked inhibitor of apoptosis	1.44	0.310673	1.44	0.55	0.000217	0.55
Birc5	Baculoviral IAP repeat-containing 5	0.33	0.058700	-3.06	2.98	0.005126	2.98
Bnip1	BCL2/adenovirus E1B interacting protein 1	0.52	0.804208	-1.94	0.57	0.007435	0.57
Bnip2	BCL2/adenovirus E1B interacting protein 2	1.30	0.068411	1.30	0.52	0.000166	0.52
Bnip3	BCL2/adenovirus E1B interacting protein 3	1.52	0.068429	1.52	1.22	0.332300	1.22
Bok	BCL2-related ovarian killer	0.63	0.986602	-1.59	0.55	0.024017	0.55
Card10	Caspase recruitment domain family, member 10	1.74	0.191990	1.74	0.31	0.001374	0.31
Card6	Caspase recruitment domain family, member 6	4.26	0.025471	4.26	0.35	0.000607	0.35
Casp1	Caspase 1	0.67	0.575102	-1.49	1.04	0.706870	1.04
Casp4	Caspase 4, apoptosis-related cysteine peptidase	0.12	0.697836	-8.39	1.06	0.840928	1.06

Casp12	Caspase 12	1.88	0.182076	1.88	0.57	0.012332	0.57
Casp14	Caspase 14	28.96	0.085678	28.96	0.00	0.000046	0.00
Casp2	Caspase 2	1.86	0.029626	1.86	0.37	0.001099	0.37
Casp3	Caspase 3	1.01	0.310811	1.01	1.67	0.005566	1.67
Casp6	Caspase 6	0.29	0.000265	-3.47	1.26	0.109335	1.26
Casp7	Caspase 7	0.78	0.777036	-1.28	2.15	0.001998	2.15
Casp8	Caspase 8	3.73	0.003524	3.73	0.72	0.050699	0.72
Casp8ap2	Caspase 8 associated protein 2	13.33	0.015377	13.33	1.86	0.110201	1.86
Casp9	Caspase 9, apoptosis-related cysteine peptidase	1.46	0.185916	1.46	0.69	0.072762	0.69
Cflar	CASP8 and FADD-like apoptosis regulator	0.21	0.908644	-4.82	0.38	0.000094	0.38
Cidea	Cell death-inducing DFFA-like effector a	5.02	0.047000	5.02	0.04	0.000012	0.04
Cideb	Cell death-inducing DFFA-like effector b	5.28	0.047495	5.28	0.49	0.971385	0.49
Cradd	CASP2 and RIPK1 domain containing adaptor with death domain	0.28	0.482576	-3.53	0.75	0.032636	0.75
Dad1	Defender against cell death 1	0.84	0.366710	-1.19	1.06	0.500024	1.06
Dapk1	Death associated protein kinase 1	8.10	0.031868	8.10	0.32	0.004282	0.32
Dffa	DNA fragmentation factor, alpha subunit	0.62	0.719950	-1.60	0.25	0.000000	0.25
Dffb	DNA fragmentation factor, beta polypeptide	3.03	0.062935	3.03	0.77	0.661642	0.77

Fadd	Fas (TNFRSF6)-associated via death domain	0.18	0.002295	-5.41	0.49	0.001204	0.49
Faim	Fas apoptotic inhibitory molecule	1.82	0.064352	1.82	1.33	0.199534	1.33
Gadd45a	Growth arrest and DNA-damage-inducible, alpha	0.71	0.335227	-1.42	0.38	0.000027	0.38
Il10	Interleukin 10	8.84	0.035053	8.84	1.39	0.561666	1.39
Lhx4	LIM homeobox 4	14.43	0.031975	14.43	0.20	0.013858	0.20
Lta	Lymphotoxin alpha (TNF superfamily, member 1)	2.80	0.117734	2.80	2.31	0.271585	2.31
Ltbr	Lymphotoxin beta receptor (TNFR superfamily, member 3)	3.80	0.097939	3.80	0.82	0.842191	0.82
Mapk8ip1	Mitogen-activated protein kinase 8 interacting protein 1	0.24	0.175377	-4.16	1.64	0.059168	1.64
Mcl1	Myeloid cell leukemia sequence 1	0.14	0.531249	-6.91	0.52	0.000471	0.52
Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	0.11	0.717355	-8.85	0.63	0.137995	0.63
Nol3	Nucleolar protein 3 (apoptosis repressor with CARD domain)	0.81	0.444371	-1.23	0.10	0.000100	0.10
Polb	Polymerase (DNA directed), beta	3.51	0.070997	3.51	0.55	0.000676	0.55
Prdx2	Peroxiredoxin 2	0.22	0.612310	-4.56	1.43	0.023899	1.43
Prlr	Prolactin receptor	14.85	0.000363	14.85	2.44	0.187911	2.44
Prok2	Prokineticin 2	1.53	0.323105	1.53	2.83	0.190483	2.83
Pycard	PYD and CARD domain containing	0.50	0.962028	-2.00	0.57	0.002135	0.57

Ripk2	Receptor-interacting serine-threonine kinase 2	0.57	0.548507	-1.75	0.24	0.000020	0.24
Sphk2	Sphingosine kinase 2	0.96	0.549479	-1.05	0.60	0.011070	0.60
Tnf	Tumor necrosis factor (TNF superfamily, member 2)	52.44	0.028810	52.44	0.22	0.000000	0.22
Tnfrsf10b	Tumor necrosis factor receptor superfamily, member 10b	13.33	0.015377	13.33	1.86	0.110201	1.86
Tnfrsf11b	Tumor necrosis factor receptor superfamily, member 11b	1.78	0.062462	1.78	0.87	0.609560	0.87
Tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1a	1.02	0.355947	1.02	0.40	0.002889	0.40
Tnfrsf1b	Tumor necrosis factor receptor superfamily, member 1b	1.07	0.201261	1.07	2.05	0.033271	2.05
Cd40	CD40 molecule, TNF receptor superfamily member 5	0.30	0.000029	-3.36	1.08	0.712489	1.08
Fas	Fas (TNF receptor superfamily, member 6)	1.68	0.020206	1.68	0.54	0.011784	0.54
Tnfsf10	Tumor necrosis factor (ligand) superfamily, member 10	2.09	0.072343	2.09	2.23	0.209091	2.23
Tnfsf12	Tumor necrosis factor ligand superfamily member 12	3.31	0.061511	3.31	0.62	0.210946	0.62
Cd40lg	CD40 ligand	2.82	0.048448	2.82	4.02	0.013033	4.02
Faslg	Fas ligand (TNF superfamily, member 6)	4.51	0.024477	4.51	0.98	0.995476	0.98
Tp53	Tumor protein p53	0.81	0.455654	-1.24	1.22	0.226293	1.22
Tradd	TNFRSF1A-associated via death domain	1.77	0.048296	1.77	0.69	0.137976	0.69
LOC687813	Similar to Tnf receptor-associated factor 1	5.28	0.006031	5.28	0.40	0.002734	0.40
Traf2	Tnf receptor-associated factor 2	2.88	0.047990	2.88	0.47	0.001465	0.47
Traf3	Tnf receptor-associated factor 3	0.48	0.769499	-2.08	0.62	0.041331	0.62

Traf4	Tnf receptor associated factor 4	0.75	0.327684	-1.33	1.33	0.229377	1.33
Tp53bp2	Tumor protein p53 binding protein, 2	0.60	0.385919	-1.68	0.54	0.011927	0.54
Tp63	Tumor protein p63	0.90	0.413513	-1.11	0.22	0.000031	0.22
Tp73	Tumor protein p73	17.46	0.044285	17.46	0.13	0.000064	0.13
Rplp1	Ribosomal protein, large, P1	0.65	0.873373	-1.53	0.89	0.290728	0.89
Hprt1	Hypoxanthine phosphoribosyltransferase 1	8.04	0.077626	8.04	1.13	0.252257	1.13
Rpl13a	Ribosomal protein L13A	0.46	0.011885	-2.15	0.89	0.287768	0.89
Ldha	Lactate dehydrogenase A	0.93	0.512136	-1.08	0.95	0.433959	0.95
Actb	Actin, beta	2.31	0.001954	2.31	1.19	0.123667	1.19
RGDC	Rat Genomic DNA Contamination	1.82	0.064352	1.82	1.33	0.199534	1.33

pro-apoptotic gene: blue color; anti-apoptotic gene: red color; genes with both functions: green color