Antioxidant Enzymes as New Biomarkers for Prediction of Tumor Progression in Breast Cancer

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1. Introduction

Today breast cancer represents the most frequent of all cancer pathologies in the world, with more than one million newly diagnosed cases and about 370000 cancer-related deaths in women each year, even with all the significant progress in its diagnosis and treatment (Ott et al., 2011). The development of breast tumor is a multistep process. It is generally thought that the initiation of breast cancer occurs after accumulation of genetic alterations that result in either activation of oncogenes and/or inactivation of tumor suppressor genes, leading to an abnormal cellular proliferation and promoting the development of tumor in mammary gland. A number of risk factors such as reproductive and hormonal factors, alcohol consumption, cigarette smoking, dietary factors and chronic inflammation have been identified for breast cancer, whose mechanisms by which they increase risk of the disease are not always clear (Mitrunen and Hirvonen, 2003). It has been proposed that the production of reactive oxygen species (ROS) leading to an oxidative stress is the linking factor between these carcinogens. The oxidative stress is defined as an imbalance between production of ROS, and their elimination by antioxidant defense system (redox imbalance). This imbalance leads to damage of important biomolecules and cells, with potential impact on the whole organism. ROS are products of a normal cellular metabolism and play vital roles in the stimulation of signalling pathways in cells in response to changes in intra- and extracellular environmental conditions. During endogenous metabolic reactions, aerobic cells produce ROS such as superoxide anion ($O_2 \bullet -$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH\bullet$), and organic peroxides as normal products of the biological reduction of molecular oxygen. Most ROS are generated in cells by the mitochondrial respiratory chain. Under hypoxic conditions following reoxygenation, the electron transfer at the level of complex III of mitochondrial respiratory chain to molecular oxygen occurs directly, leading to a high level of ROS. Beside oxidative phosphorylation, low levels of ROS are continuously formed by oxidase activities in peroxisomes, and by the cytochrome P450 system in endoplasmic reticulum. ROS as $OH\bullet$ is produced *in vivo* in the presence of reduced transition metals (ions of Fe, Cu) mainly *via* the Fenton reaction in contact with H_2O_2. Under normal metabolic conditions, these ROS are eliminated rapidly in normal cells by the antioxidant defense system. However, ROS may be overproduced when normal cells are exposed to a sustained environmental stress, including ionizing radiation and many

xenobiotics (Matés et al., 2008). In addition, during inflammation, neutrophils and macrophages are recruited to the site of damage, which leads to a "respiratory burst" due to an increased uptake of oxygen and, thus, an increased release and accumulation of ROS at the site of damage *via* a plasma membrane bound nicotinamide adenine dinucleotide phosphate, reduced form (NADPH)-oxidase (Reuter et al., 2010). An overproduction of ROS over a long time, associated with an exceeded antioxidant defense system, causes significant oxidative damages by reacting with macromolecules, including proteins, lipids and nucleic acids. These oxidative damages may be deleterious for cell structure and functions, and induce somatic mutations and neoplastic transformation. ROS can generate other reactive species (e.g., reactive aldehydes—malondialdehyde and 4-hydroxynonenal) by inducing excessive lipid peroxidation, which increases the membrane permeability. Oxidation of sensitive amino acids (cysteine, methionine, proline, phenylalanine, tryptophane and tyrosine) or reaction with reactive aldehydes from lipid peroxidation causes denaturation of proteins, leading to disturbance of cell signalling and metabolic pathways (Goetz and Luch, 2008). An excess of ROS causes several types of DNA damage, including depurination and depyrimidination, single and double-stranded DNA breaks, base and sugar modifications and DNA-protein crosslinks (Goetz and Luch, 2008). Permanent modification of genetic material resulting from the oxidative damage is one of the vital steps involved in mutagenesis that leads to carcinogenesis (initiation and progression to the development of cancer). Stimulation of DNA damage can either arrest or induce transcription, signal transduction pathways, replication errors and genomic instability, all of which are associated with carcinogenesis. The most frequent DNA mutations caused during oxidative stress, initiated by ionizing radiation and other environmental carcinogens are 8-dihydro-2 deoxyguanosine or 8-oxoguanosine (8-OHdG). Indeed, this oxidized DNA product is relatively easy to generate during oxidative stress and is mutagenic and carcinogenic. Thus, it is considered as a useful marker of oxidative stress and a potential biomarker of carcinogenesis. In addition to extensive studies devoted to the role of oxidative nuclear DNA damage in neoplasia, there exist several evidence about the involvement of the mitochondrial oxidative DNA damage in the carcinogenesis process. Mutations and altered expression in mitochondrial genes encoding for five complexes involved in the respiratory chain have been identified in various human cancers, including breast cancer (Rohan et al., 2010).

To protect against redox imbalance and to prevent oxidative damages, cells have developed a wide variety of enzymatic and nonenzymatic antioxidant defenses. Primary defense system prevents oxidative damage by scavenging ROS directly and includes superoxide dismutase (SOD), glutathione peroxidase/glutathione reductase (GPX/GR), catalase (CAT) and peroxiredoxins/thioredoxin reductase system (PRX/TRX). SOD destroys the highly reactive superoxide anion by converting into the less reactive hydrogen peroxide, which can be destroyed by CAT, GPX or PRX. The secondary defense system is composed of nonenzymatic and low weight molecules scavenging mainly OH• or chelating reduced metal transition to prevent Fenton reaction (ceruloplasmin for Cu, and transferrin and ferritin for Fe). This antioxidant defense includes tripeptide as glutathione or small proteins as metallothioneins, which are involved in the scavenging of OH•. In the case of glutathione (GSH), the oxidized form is rapidly regenerated by the NADPH-dependent glutathione reductase. The oxidized metallothioneins are rapidly degraded in cell. Dietary vitamins A (β-caroten), C (ascorbic acid) and E (α tocopherol), also have antioxidative properties against

OH•. Beside the antioxidant defense, cells have developed reparative mechanisms comprising enzymes involved in the elimination of oxidative damages. Oxidized proteins are specifically recognized by the 20S proteasome, which degrades them in an ATP and ubiquitin independent manner. Phospholipid hydroperoxides formed during lipid peroxidation are converted into the corresponding alcohol by the Ursini's glutathione peroxidase. Moreover, reactive aldehydes from the lipid peroxidation process may be inactivated in the cell by alpha-, pi-, mu-, and theta-class glutathione transferases (GST), which are able to transfer glutathione to the aldehyde group. Finally, the oxidative DNA damage as 8-OHdG in nuclear DNA is eliminated by a specific DNA repair enzyme as 8-OHdG glycosylase (Matés et al., 2008).
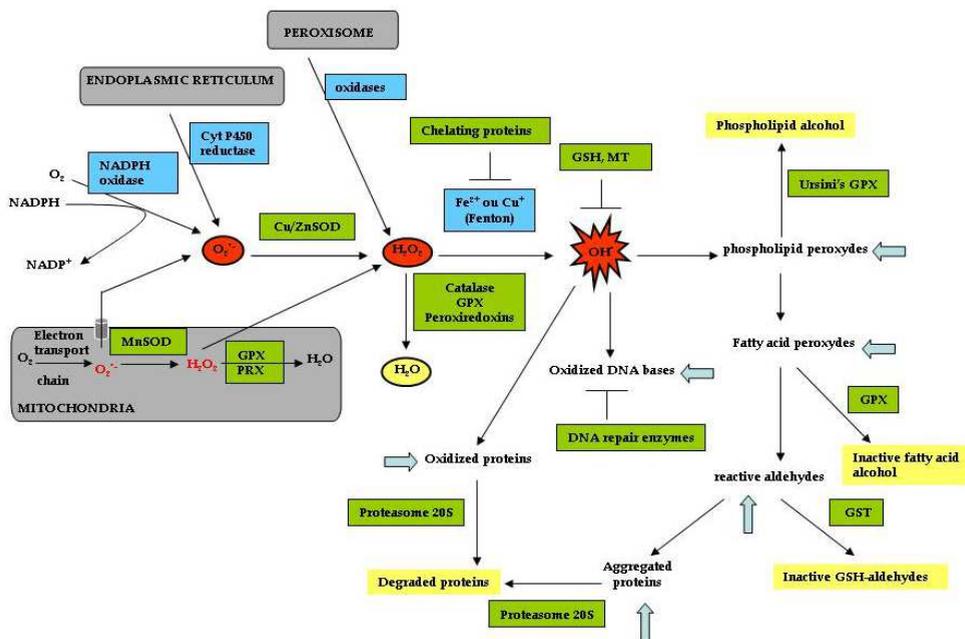

Fig. 1. Intracellular generation of ROS (in red) and main antioxidant defense system (in green). The oxidative damages are indicated by the large blue arrows. Antioxidant defense is constituted of enzymes and nonenzymatic molecules scavenging directly ROS. Antioxidant defense includes repair enzymes involved in the elimination of the oxidative damages (DNA repair enzymes, GPX, GST and proteasome 20S). For more details, see the text above.

During the initiation stage, low and chronic levels of ROS may produce DNA damages by introducing gene mutations and structural alterations into the DNA. In the promotion stage, ROS can contribute to abnormal gene expression, blockage of cell-to-cell communication, and modification of second-messenger systems, thus resulting in an increase in cell proliferation or a decrease in apoptosis of the initiated cell population. Finally, oxidative stress may also participate in the progression stage of the cancer process by adding further

DNA alterations to the initiated cell population. Indeed, carcinoma cells are frequently under persistent oxidative stress, what contribute to render them more aggressive. ROS are constitutively generated in carcinoma cells by altered metabolic pathways, particularly the respiratory chain in mitochondria, by an increase in some enzymatic activity as NADPH oxidase in plasma membrane, as well as by an imbalance in the expression of antioxidant enzymes.

2. Reactive oxygen species (ROS) in breast carcinogenesis and metastatic process

2.1 Breast cancer risk factors generating ROS

Many risk factors of breast cancer are sources of ROS, including caloric intake as fatty acid-enriched diet, cigarette smoking, endogenous and exogenous estrogens, and alcohol. A high caloric intake enriched with fatty acid, is able to modify mitochondrial function. The mitochondria use oxidative phosphorylation to convert dietary calories into usable energy, and they generate ROS as a toxic by-product at the respiratory chain level. These consequences can be exacerbated by polymorphisms in mitochondrial DNA which reduce the efficiency of mitochondrial functioning. Particularly, the A10398G polymorphism, which results in the substitution of threonine for alanine within the NADH dehydrogenase subunit of Complex I in the respiratory chain, has been associated with increased risk of breast cancer (Canter et al., 2005; Setiawan et al., 2008). This polymorphism may lead to impaired respiratory function and so to increased ROS production. The most important carcinogens in tobacco smoke are polycyclic aromatic hydrocarbons and are metabolized by the cytochrome P450 system to generate semiquinone radicals which undergo redox cycling to produce ROS. It is well known that a long-time exposure to estrogens increases the risk for breast cancer. Exposure of breast epithelium to endogenous estrogens is determined by several variables including timing of menarche, age at first full term pregnancy, number of pregnancies, and age at menopause. The serum levels of these endogenous estrogens, such as estrone, estradiol, estriol, have been considered as important risk factors for breast cancer. In addition, exposure of breast epithelium to exogenous estrogens from oral contraception, hormone replacement therapy, or xeno-estrogens (plasticizers, dyes, pollutants, pesticides and food preservatives) represents a potential risks for breast cancer. Indeed, beside their role in the cell proliferation mediated by a nuclear receptor, estrogens have been well described to generate ROS. Estrogen metabolism is mediated by cytochrome P450 reductase, mainly the cytochrome P450 1B1 reductase in human breast tissue, which generates reactive electrophilic estrogen *o*-quinones *via* the formation of hydroxylated estrogens (Mitrunen and Hirvonen, 2003; Gago-Dominguez et al., 2007). These compounds undergo redox cycling with the semiquinone radical, which may react with molecular oxygen to generate $O_2\bullet-$. Women consuming alcohol have been hypothesized to exhibit elevated estrogen levels. Apart from this, alcohol metabolism produces ROS. Alcohol as ethanol is converted to acetate by a simple, two step reaction involving the combined activities of alcohol dehydrogenase, that produces acetaldehyde, and the molybdenum xanthine oxidoreductase and/or aldehyde oxidase, that produce acetate from acetaldehyde. Both xanthine oxidoreductase and aldehyde oxidase can generate ROS from molecular oxygen (Wright et al., 1999; Seitz and Becker, 2007; Kabat et al., 2010).

Chronic inflammation is frequently seen in breast carcinomas and produces notable amounts of ROS, enough to cause additional genetic instability. The sources of inflammation

in breast cancer include exposure to allergens, radiation and toxic chemicals, consumption of alcohol or tobacco use. In addition, inflammatory cells also produce soluble mediators, such as metabolites of arachidonic acid, cytokines, and chemokines, which act by further recruiting inflammatory cells to the site of damage and producing more ROS. The main source of ROS, particularly $O_2 \bullet -$, is produced by the NADPH oxidase found in the plasma membrane of inflammatory cells. Inflammatory cells may also increase DNA damage by activating procarcinogens to become DNA damaging species; for example, neutrophils can activate aromatic estrogens and polycyclic aromatic hydrocarbons from cigarette smoking by ROS-dependent mechanisms. On the other hand, both neutrophils and macrophages have themselves been shown to release large quantities of $O_2 \bullet -$, H_2O_2 and $OH \bullet$ after activation of their redox metabolism (Reuter et al., 2010).

2.2 Causes and consequences of persistent oxidative stress in breast carcinoma cells

The oxidative stress exerted by ROS is involved in the breast cancer etiology by the accumulation of oxidative damages resulting to the oxidation of biological molecules, particularly the DNA leading to genomic instability. These events lead to the transformation of normal cells to carcinoma cells. However, the role of ROS may not be limited to early mutagenic events and cell transformation. A number of somatic mutations have been identified in breast cancer (Callahan et al., 1992). These include transversion mutations of p53, the breast cancer susceptibility genes BRCA1 and BRCA2. These key genes have been linked to breast cancer progression and the mutations found in them can be produced by ROS (Elledge et al., 1993; Hussain et al., 1994; Merlo et al., 1994). Although the direct link between ROS modification of DNA and mutation of these genes induced by breast cancer risk factors remains to be established, they should be considered important candidates for the induced carcinogenesis because mutations in these genes could be responsible for tumor initiation as well as tumor progression.

Some altered metabolic pathways have been described in breast carcinoma cells. The majority of the somatic alterations in mitochondrial DNA identified actually may simply represent the consequences of genomic instability and oxidative DNA damage during the multistep carcinogenic process. In humans, several studies have shown a relatively high frequency of mtDNA mutations in breast tumor tissues (range 20%–93%) (Rohan et al., 2010). Among them, we have cited above the A10398G (T114A) polymorphism in the NADH dehydrogenase subunit gene of the mitochondrial genome (Pezzotti et al., 2009). In consequence, a persistent dysfunction of mitochondrial respiratory chain is a source of ROS production in breast carcinoma cells. Actually, no nuclear mutations of mitochondrial proteins, particularly the succinate dehydrogenase proteins, have been associated with breast carcinoma cells, in contrast to some other solid tumors. It has been reported recently that NADPH oxidase contributes to persistent generation of ROS in breast carcinoma cells. This enzyme is overexpressed in breast tumors and carcinoma cell lines when compared to normal breast tissues and cells. Its activity is regulated by binding a partner as the small GTPase Rac1, which is itself downstream of the Ras oncoprotein (Brown and Bicknell, 2001). Carcinoma cell oxidative stress can also be induced by thymidine phosphorylase, an enzyme that is overexpressed in the majority of breast carcinomas. Thymidine phosphorylase catabolizes thymidine to thymine and 2-deoxy-D-ribose-1-phosphate; the latter is a very powerful reducing sugar that rapidly glycates proteins, generating ROS within the carcinoma cell. Thymidine phosphorylase activity has been shown to induce carcinoma cell oxidative stress *in vitro*. The frequent upregulation of thymidine phosphorylase in human

breast tumours suggests that this may be an important cause of oxidative stress in breast cancer (Brown et al., 2000). *In vivo*, an inadequate tumor vascular network can lead to a persistent oxidative stress in breast carcinoma cells. A breast tumor rapidly outgrows its blood supply, leading to glucose deprivation and hypoxia. Glucose deprivation rapidly induces cellular oxidative stress within the breast carcinoma cells because of the depletion of the intracellular antioxidant pyruvate which prevents in part the decomposition of endogenous ROS. Breast carcinomas usually support their growth by stimulating blood vessel development (angiogenesis). Blood flow within these new vessels is often chaotic, causing periods of hypoxia followed by reperfusion. It is well known that the reperfusion of tumor cause the generation of ROS, as observed after myocardial infarction or cerebral ischaemia. This ROS production during reperfusion may therefore be a cause of oxidative stress within breast carcinomas. One additional cause of persistent oxidative stress in carcinoma cells is the fact that breast tumors are frequently infiltrated by large numbers of macrophages (Leek et al., 2002). These may contribute to carcinoma cell oxidative stress, as tumor-associated macrophages have been shown to produce ROS by secreting tumor necrosis factor-alpha. This cytokine is known to induce cellular oxidative stress (Matés et al., 2008). Persistent generation of ROS in breast carcinoma cells is also associated with an alteration in the expression of antioxidant enzymes, those we describe below (see part 3).

Markers of the persistent oxidative stress have been detected in samples from *in vivo* breast carcinomas. For example, 8-OHdG has been reported to be increased 8- to 17-fold in breast primary tumors compared to non-malignant breast tissues (Malins et al. 1996; Matsui et al., 2000b). In addition, lipid peroxidation as evidenced by malondialdehyde (MDA) was enhanced in breast cancer tissues compared to non-malignant Tissues (Tas et al., 2005).

The persistent production of ROS cause additional genomic instability, as well as stimulate the expansion of initiated cell clones through modulating genes and activating signalling pathways (promotion stage of carcinogenesis) related to apoptosis or proliferation. In addition, it creates selection pressure for characteristics such as accelerated growth, invasion and metastasis. ROS are able to play a role as second messengers by activating small G protein, kinases and/or inhibiting phosphatases resulting in stimulation of signalling pathways. Ras oncoprotein can be activated by ROS via oxidative modification of its cysteine 118 residue which leads to the inhibition of GDP-GTP exchange. The effects of ROS occur through targeting a cysteine residues of the active sites of kinases and phosphatases, leading to activation and inactivation, respectively (Klaunig and Kamendulis, 2004). As shown in Figure 2, ROS can stimulate the proliferation of breast tumor cells by activating directly the extracellular signal-regulated kinase in Mitogen-activated protein kinase signalling pathway, as well as c-Src oncoprotein. ROS can stimulate the survival and resistance to apoptosis by activating phosphoinositide 3 kinase/Akt signalling pathway. In addition, these signalling pathways are regulated by the PTEN lipid phosphatase, which is reversely inactivated by ROS (Valko et al., 2006). In addition, ROS are known to induce considerable increase in intracellular calcium, which further may activate proto-oncogenes, such as c-fos, c-jun and c-myc or activate protein kinase C, which enhance tumor cell proliferation (Klaunig and Kamendulis, 2004). Moreover, the oncogenic protein c-Src, which is a nonreceptor tyrosine kinase has been reported to be activated by ROS and is often overexpressed in breast cancers. This oncoprotein binds to cell membranes by myristilation and initiates MAPK and PI3K signalling pathways (Leonard et al., 2004).

ROS are able to modulate the regulation of gene expression, by oxidizing directly a cysteine residues, that this leads to a change in the native conformation promoting an activation of

certain redox sensitive transcription factors. These latter include particularly hypoxia inducible Factor (HIF), Nuclear Factor-kappa B (NF-κB) and Activator protein-1 (AP-1) . ROS generated during hypoxia is responsible for stabilizing HIF. This latter is a heterodimer consisting of a constitutively stable subunit HIF-β and a redox sensitive subunit HIFα, whose the major isoform is HIF-1α. This transcription factor is involved in the tumor growth by activating angiogenesis (Brown and Bicknell, 2001). NF-κB is constitutively activated in many breast tumor cells, because the persistent production of ROS accelerates the degradation of IκB, the cytosolic inhibitory subunit of NF-κB (Nakshatri et al., 1997). This transcription factor is known to regulate target genes involved in proliferation, survival and migration of tumor cells. AP-1 is a heterodimeric transcription factor composed of the c-Jun and c-Fos proteins, which regulates a number of genes involved in the progression of cell cycle, such as cyclins D (Shen and Tergaonkar, 2009).

In addition to regulating tumor growth and survival, ROS also control mechanisms which are associated with the formation of breast tumor metastases. The cellular processes linked to this function are a decrease in the cell adhesion to the basal lamina and an increase in the migratory and invasive potential which favour breast cancer cells to enter the blood vessels. The loss of adhesion of normal cells is always accompanied with a particular type of apoptosis, widely known as anoikis, which is essential for prevention of the dissemination of cells to inappropriate sites. Resistance to anoikis is thus emerging as a hallmark of metastatic cancer cells, mainly because anchorage-independent growth of tumor cells is a classic feature of human malignancies (Storz, 2005).

In this context, increasing blood vessel growth due to hypoxia and reoxygenation increases the risk of blood-borne metastasis. Persistent production of ROS may augment breast tumor invasion and metastasis by increasing the rates of cell migration. As described earlier, the small GTPase Rac1 can activate the NADPH-oxidase in breast tumor cells, causing superoxide production. These ROS have been shown to mediate the role of Rac1 in actin cytoskeleton reorganization which is an important step in the loss of cell-cell adhesion and cell-matrix adhesion to laminin and fibronectin. The resistance to anoikis of cancer cells including breast cancer cells, which is an important step during the metastatic process, has been associated to the oxidation of c-Src by ROS, leading to a sustained activation of pro-survival signals through the ligand-independent phosphorylation of EGFR (Valko et al., 2006). ROS within breast tumors may also facilitate invasion and metastasis by activating MMPs and inhibiting antiproteases, directly or through activation of transcription factors. MMP-2 and -9 are gelatinases, which play a major role in breast cancer invasion and metastasis. High levels of MMP-2 and -9 correlate with poor prognosis in breast cancer patients and active MMP-2 and -9 are detected more frequently in malignant than in benign breast tumors (Jinga et al., 2006). MMP-2 and -9 expressions are regulated by AP-1 and NF-κB, respectively, which can be activated by ROS. In addition, ROS can modulate MMP-2 and -9 activities, by reacting with thiol groups in the protease catalytic domain. Like all MMP, they are secreted in a latent zymogen form in which the cleavage of the pro-domain is ROS dependent (Nelson et al., 2004; Kattan et al., 2008). Due to the expression of cell surface protein ICAM-1 (Intercellular Adhesion protein-1) and CD54 in consequence of the ROS-dependent activation of NF-κB, the trans-endothelial migration of breast tumor cells is favourable for development of metastasis. Protease inhibitors, such as α1-proteinase inhibitor and plasminogen activator inhibitor, may be inactivated by oxidation of methionine residues at their active sites. This facilitates the activity of various proteases,

increasing invasion and the likelihood of metastasis. For example, plasminogen activator is believed to play a role in metastasis in breast cancer. The other major regulator of metastasis is the hypoxic microenvironment. The degree of hypoxia correlates positively with metastasis. Hypoxia stimulates the epithelial-mesenchymal transition (EMT), which is characterized by loss of epithelial cell adhesion with repression of E-cadherin, and increased cell mobility. EMT is largely described as a process leading to breast tumor cell dissemination. The ROS-dependent activation of HIF under hypoxia can lead to subsequent activation of the transcription factor Twist, resulting in EMT. In addition, ROS produced during hypoxia can inhibit the activity of glycogen synthase kinase 3β (GSK3β). This results in the up-regulation of the EMT-inducing transcription factor Snail. Together, Twist and Snail are involved in the up-regulation of genes, leading to the breast tumor cell dissemination (Micalizzi et Farabaugh, 2010). From these, it is speculated that ROS are likely to be important regulators of metastasis (Cannito et al., 2010).
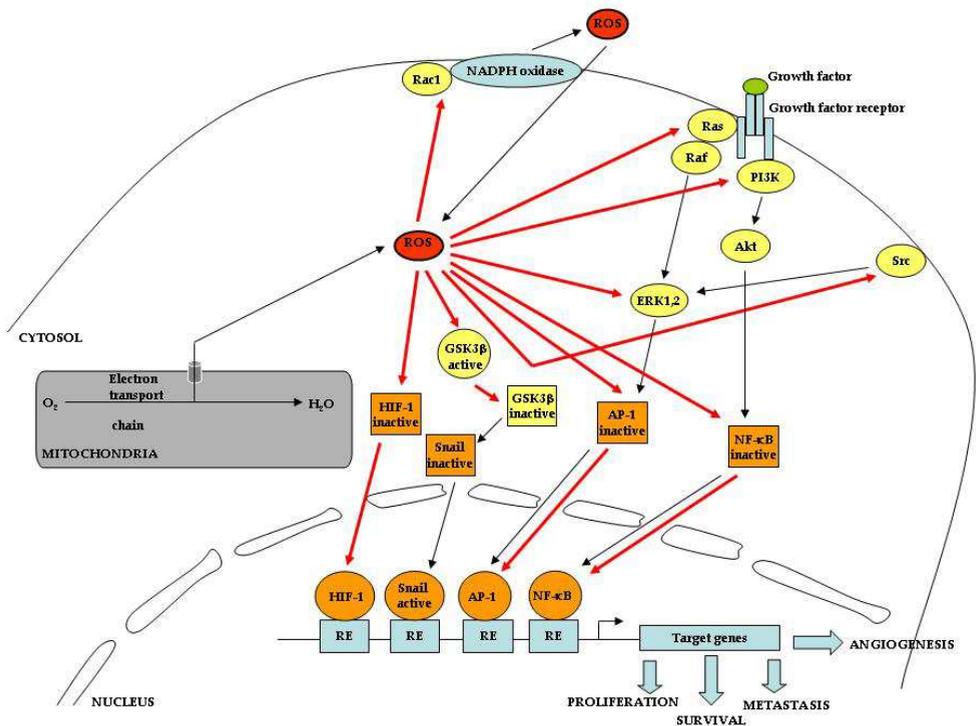

Fig. 2. Interaction between ROS generated from mitochondria and NADPH oxidase activity and signalling pathways leading to proliferation, survival and metastasis of breast tumor cells. Red arrows indicate action of ROS on their targets. RE for response element of transcription factors (in orange) in promoter region of target genes. Small G proteins, kinases and phosphatases in signalling pathways are represented in yellow.

ROS play a role in angiogenesis, which is required for tumor growth as well as metastasis. ROS within the tumor microenvironment may promote metastasis by increasing vascular

permeability, either by direct damages to endothelial cells or by the upregulation of inducible nitric oxide synthase (iNOS). By this mechanism, ROS may also increase the blood supply to breast tumor carcinoma by triggering vasodilatation, because the nitric oxide produced would activate cGMP within nearby smooth muscle cells, leading to vasodilatation. In addition, low concentrations of ROS ($O_2 \bullet -$ and H_2O_2) produced by breast tumor cells can stimulate endothelial migration and tube formation in an *in vitro* model of angiogenesis, through the activation of the secreted MMP-1, a collagenase that aids vessel growth within the tumor microenvironment. Finally, ROS has been shown to increase production of the angiogenic factor such as vascular endothelial growth factor (VEGF) by tumor cells. As described above, HIF may be increased by ROS, and this transcription factor is known to induce VEGF expression (Ushio-Fukai and Nakamura, 2008).

3. Antioxidant enzymes in breast carcinogenesis and metastatic process

3.1 Relationship between antioxidant enzymes and breast cancer risk factors

As described above, there are three main types of antioxidant defense enzymes: the superoxide dismutases (SOD), including MnSOD and cytosolic CuZnSOD, catalase (CAT), and the peroxidases (GPX1 and GPX4 and peroxiredoxins I to VI). All of them function to protect the cell from damage due to ROS. In addition, several other enzymes are implicated in oxidative damage repair (Figure 3). The reduction of oxidized glutathione (GSSG) produced by action of GPXs is catalyzed by glutathione reductase (GSR). The thioredoxins (TXN and TXN2) and thioredoxin reductases (TXNRD1 and TXNRD2) are also involved in antioxidant defense through the thioredoxin redox cycle which allows the reduction and so the regeneration of peroxiredoxins (Arner and Holmgren, 2006; Matés et al., 2008). Genetic polymorphisms in a number of the genes encoding these enzymes may be important in affecting levels of ROS and oxidative damages when the mammary epithelial cells are exposed to risk factors, and could also have an impact for risk to develop breast cancer.

Common variants in oxidative damage defense and repair genes, including MnSOD, GPX1, catalase, GST and catechol-O-methyl transferase (COMT), may be good candidates for both cancer susceptibility and prognosis. The case of MnSOD will be described in the part 4.

The antioxidant enzyme GPX is involved in the detoxifying hydrogen- and lipid peroxides depending on GSH and GSH redox cycle by GSR. Among GPX, it has been identified a genetic polymorphism in the GPX1 gene at codon 198, resulting in either a proline (Pro) or leucine (Leu) at the corresponding position of the encoded protein, which has drawn increasing attention in the etiology of several cancers, including breast cancer (Hu and Diamond, 2003; Hu et al., 2010). The selenium-dependent activity of GPX198Leu mutant enzyme is lower than for the GPX [198]Pro wild-type enzyme, and is associated weakly with higher breast cancer risk, depending on the population (Cox et al., 2004).

Among other primary antioxidant enzymes neutralizing ROS, CAT is the most potent enzyme and inducible by exposure to ROS, particularly hydrogen peroxide (H_2O_2). Located in the peroxisomes of all cells, CAT is a heme enzyme converting H_2O_2 into H_2O and O_2 to directly reduce the production of OH• and lipid hydroperoxides. A C/T substitution at nucleotide position 262 has been identified in the promoter region of the human CAT gene, resulting in reduced enzyme activity. The consequence of the low activity for this polymorphism has been associated with increased breast cancer risk, particularly among low consumers of fruits and vegetables (Ahn et al., 2005).

For other antioxidant enzymes such as GSR, GPX4, Cu/ZnSOD, TXN and TXNRD, no association with a risk of developing of breast cancer was found for any of the polymorphisms reported (Cebrian et al., 2006). In addition, no significant association was observed between common variants in these genes coding for antioxidant defense enzymes (Cu/ZnSOD, MnSOD, CAT, GPX1, GPX4, TXN, TXN2, TXNRD1 and TXNRD2) and susceptibility to breast cancer (Oestergaard et al., 2006).
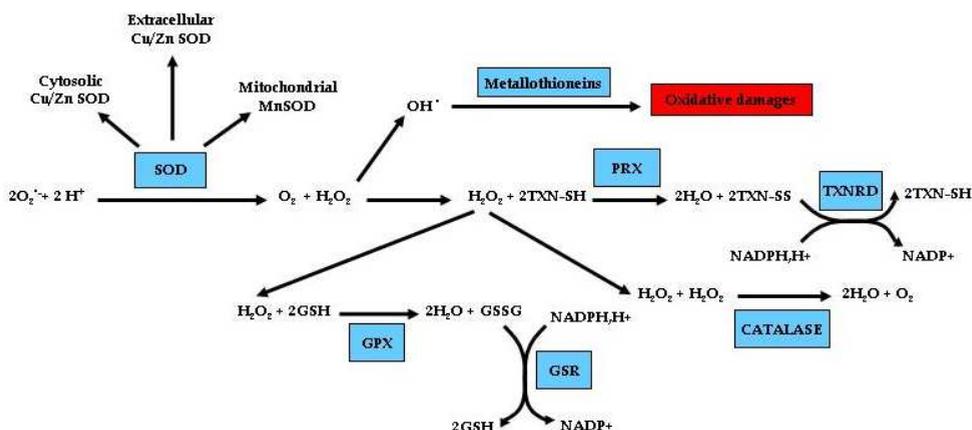
Fig. 3. The main antioxidant enzymes (in blue) involved in the direct elimination of ROS in cells and whose expression is altered in breast cancer cells. The systems involved in the reduction of the cofactor TXN or GSH are indicated with the corresponding enzyme.

Glutathione-associated metabolism is a major mechanism for cellular protection against breast cancer risk factors that generate ROS. GSTs catalyse the conjugation of glutathione to cytotoxic products (aldehydes, lipid hydroperoxides) of lipid peroxidation induced by ROS and thus protect normal mammary epithelial cells (Rundle et al., 2000). These cytosolic and dimeric enzymes are induced under conditions of oxidative stress, and can be differentiated into five classes in mammalian cells termed alpha, mu (M), pi (P), theta (T) and zeta (Di Pietro et al., 2010). Allelic variation has been found in genes encoding for these GST, and the absence of specific isoenzymes affects the tolerance of cells to chemical challenges and may result in increased somatic mutation rate and thereby higher susceptibility to malignancies. In estrogen metabolism, GSTs play a role in the catalysis of glutathione conjugation of catechol estrogen quinones, the reactive intermediates of estrogen metabolism which are able to form DNA adducts. Only GSTM1 and 3 and GSTP1 are expressed in breast tissue and involved in the estrogen metabolism. Several recent studies have examined the GSTM1 and 3 and GSTP1 genotype in relation to individual breast cancer risk. The homozygous deletion (null genotype) of the GSTM1 gene leads to the total absence of the respective enzyme activity. An association has been observed between the GSTM1 null genotype and increased breast cancer risk in some populations, such as postmenopausal Caucasian, French and African-American women, and premenopausal Korean women. It can be noted that the genetic polymorphisms of *GSTM1* have also been found to influence the risk-enhancing effect of alcohol in breast cancer (Zheng et al., 2003). Concerning GSTM3, a variant allele has been reported which differs from the wild-type allele by a three base pair

deletion in intron 6. This mutation in the noncoding region of the gene generates a binding site for the YY transcription factor and leading to an expression of the variant allele at different levels with less efficiencies in the metabolism of catechol estrogen quinones. However, only one study has been shown an association between variant allele of GSTM3 and breast cancer risk for women consuming alcohol (Mitrunen et al., 2001). The GSTP1 is the major GST expressed consistently in both normal and breast tumor tissue. Two variant alleles have been detected in addition to the wild-type allele. In both of the variant alleles, a point mutation at position 133 in nucleotide sequence results in a single amino acid change from isoleucine (Ile) to valine (Val) at codon 105. The Ile/Val[105] polymorphism has been demonstrated lower specific activity. One variant has another point mutation resulting in alanine (Ala) to valine (Val) change at codon 114, which does not modify the enzyme activity. Whereas no significant overall association has been seen between the Ala/Val[114] polymorphism and breast cancer risk, a significant increase in the risk was observed for former smokers for the homozygous Ile/Val[105] polymorphism (Millikan et al., 2000).

It is known a genetic polymorphisms in estrogen metabolizing enzymes which can be associated with risk of breast cancer. Estrogens play a crucial role in the development and evolution of human breast cancer, because they are converted by cytochrome P450 1B1 to 4-hydroxyestradiol (4-OHE2), a putative carcinogenic metabolite of estrogen. This catechol estrogen metabolite is oxidized further to produce a reactive quinone *via* semiquinone radical, which can generate ROS by undergoing redox cycling. It exists catechol-O-methyl transferase, a phase II enzyme under soluble or membrane-bound form that inactivates catechol estrogens by transfer of a methyl group. This enzyme is considered as an antioxidant enzyme by preventing the conversion of catechol estrogen metabolite to semiquinones and quinones and, therefore, blocks the generation of ROS. A single G to A base pair change in the COMT-L low activity allele containing genotypes (*HL* or *LL)* results in a valine to methionine amino acid change at codon 108/158 in the cytosolic/membrane–bound form of the protein. This change was associated with a decreased activity of the COMT compared with the wild-type COMT-H allele (Mitrunen and Hirvonen, 2003). It has been observed an increased risk for premenopausal women carrying the *COMT-L* allele-containing genotypes and decreased risk for postmenopausal women with these genotypes. Furthermore, the increase in the risk was confined to never alcohol users and ever smokers (Matsui et al. 2000a).

3.2 Relationship between antioxidant enzymes and persistent oxidative stress in breast carcinoma cells

The persistent oxidative stress in carcinoma cells is often associated with an alteration in the expression of antioxidant defense enzymes (Figure 3). According to the type of antioxidant enzyme has an altered expression, an accumulation of the corresponding ROS ($O_2\bullet-$ or H_2O_2) leads to the tumor cell proliferation or migration and invasion.

The case of MnSOD will be described in the part 4. Concerning catalase, it has been observed a relationship between ER status and the enzyme level in breast carcinoma cell lines. It has been reported that catalase expression is higher in ER-positive than in ER-negative breast cancer cells (Kattan et al. 2008). *In vivo*, a decrease in its activity has been found in tumor tissue of breast cancer patients as well as the stage of the tumors (Tas et al., 2005).

In contrast to catalase, an inverse correlation between ER expression and GPX-1 expression has been observed in breast cancer cell lines (Esworthy et al., 1995). However, the GPX level

in breast tumor tissues is relatively low in contrast to the normal counterpart tissue. The GPX-1 being the main mitochondrial and H_2O_2-detoxifying enzyme with peroxiredoxin III, its deficient expression may be associated with the deficient activity of the respiratory chain in aggressive breast cancer cells, explaining in part the high H_2O_2 release from mitochondria, a ROS involved in the metastatic process. In addition, peroxiredoxin III (PRX III) expression may decrease with the aggressiveness of breast cancer cells, which is often associated with a dysfunction in the mitochondrial activity. In addition to PRX III, the other peroxiredoxins, also called thioredoxin peroxidases, are a distinct expression between tumor and normal cells and tissues. These enzymes are characterized by one (PRX VI) or two (PRX I-V) cysteines as their active site and are reduced to the initial state by thioredoxin. In contrast to catalase, PRX are widely distributed subcellularly, with PRX I, II, III, V and VI in cytosol, PRX IV and VI in peroxisomes, endoplasmic reticulum and Golgi apparatus (PRX IV), and mitochondria (PRX III) (Rhee et al., 2005). In response to an increased production of ROS, it has been reported that only PRX III, IV, V and VI is overexpressed in breast cancer cells and tissues in relation to normal cells and tissues. Whereas PRX III, IV and VI overexpression is associated with progesterone and estrogen receptor expression, tumor cell proliferation and with a better prognosis, PRX V was related to the larger tumor size and positive lymph node status and also a shorter survival (Karihtala et al., 2003).

Thioredoxins (TXN), involved in the reduction of PRX, are also overexpressed in breast tumor cells. This overexpression is observed rather in the nuclei of invasive tumor cells or tissues, probably related to the role of these thiol-containing antioxidants in the activation of the transcription factor NF-κB, which is known to play a crucial role in the invasive processes by regulating target genes. In addition, TXN are also a key antioxidant proteins for DNA synthesis by directly serving as an electron donor to ribonucleotide reductase and this redox function is essential for breast cancer cell proliferation (Arner and Holmgren, 2006).

Trx reductase (TRXRD) utilizes NADPH to reduce and activate TRX as well as other proteins. There are three different TRXD proteins in human cells with a distinct localization: TRXD1 are observed in extracellular space, nucleus, cytoplasm and in plasma membrane, while TRXD2 and TRXD3 are present in mitochondria. The TRX and TXRD contribute to maintain a reduced state in cell by maintaining protein thiols in the reduced form, like some active transcription factors which promote breast cancer cell proliferation and invasion (Arner and Holmgren, 2006). Recently, TRX and TXRD have been considered as a tumor growth promoting factors for estrogen-sensitive breast cancer cells (Cadenas et al., 2010). They were identified in a complex associated with the DNA-bound estrogen receptor alpha (ERα) to regulate the expression of estrogen-responsive genes in estrogen-sensitive breast cancer cells (Rao et al., 2009).

Metallothioneins (MTs) are another group of antioxidant proteins which protect cells to the OH• radical production by chelating the transition metal as copper and by scavenging this ROS directly. These MTs are a family of ubiquitous and low molecular weight cysteine-rich proteins encoded by 10 genes which play an important role in tumor cell proliferation (Eckschlager et al., 2009). However, it has been reported in many studies an association between high MT expression, particularly the MT2A isoform, and both poorer prognosis of patients and aggressive histopathological features of tumors (Jin et al., 2004). These observations can be associated with the fact that expression of the MT genes are activated by

hypoxia though the binding of metal response element of their promote region by the metal transcription factor. Thus this molecular mechanism contributes to the survival of hypoxic breast tumor cells which acquire invasive and metastatic abilities.

4. MnSOD in breast carcinogenesis and metastatic process

SODs were the first characterized antioxidant enzymes. Three different types of SOD are expressed in human cells (Figure 3). This part 4 will be focused on MnSOD, which is considered as one of the most important antioxidant enzymes in mammals, for the following reasons. Whereas cytosolic and extracellular Cu/ZnSOD were not essential for survival of mice in knockout studies, mice lacking MnSOD had severe metabolic acidosis, degeneration of neurons and cardiac myocytes and died prenatally of dilated cardiomyopathy (Lebovitz et al., 1996). In addition, the role of MnSOD in cancer has been largely studied even if is still rather ambiguous and is associated with profound alterations in the gene expression of the antioxidant enzyme by different molecular mechanisms (Miao and St Clair, 2009).

4.1 Relationship between MnSOD and breast cancer risk factors

It has been observed a relationship between low MnSOD activity and risk of breast cancer development. The low MnSOD activity depend on a main gene polymorphism identified as a single nucleotide substitution of C to T at the second nucleotide of codon 16 of the MnSOD gene changes which encoded amino acid substitution from alanine (GCT) to valine (GTT) at the position-9 of the mitochondrial targeting sequence of the mature protein. This alteration designated as the MnSOD Ala[16]Val polymorphism has been found to affect the transport of MnSOD into the mitochondria, thus altering its enzymatic activity. The human MnSOD Ala variant has been found to generate 30-40% more active MnSOD protein compared to the Val variant in mitochondria (Sutton et al., 2003). The MnSOD Val/Val genotype having a low MnSOD activity as consequence could be considered as deleterious for mammary epithelial cells exposed to environmental carcinogens such as alcohol, tobacco smoke or estrogens generating $O_2\bullet-$ during their metabolism (Bica et al., 2009). However, several epidemiologic studies have looked at the association between a MnSOD gene polymorphism and breast cancer risk. Different studies in diverse populations have resulted in identifying conflicting roles for the Ala/Val[16] polymorphism and cancer risks. In summary, breast cancer risk is slightly increased in women carrying the MnSOD Ala/Ala genotype compared to those carrying the Val/Val genotype, especially in premenopausal women. This risk is further increased in premenopausal women with low intakes of fruits, vegetables, and various dietary supplements (antioxidant vitamins and selenium). Some other epidemiologic studies reveal a relationship between this Ala/Ala genotype and smoking or alcohol consumption in diverse populations (Wang et al., 2009). Recently, an epidemiological study on a large population of patients has been focused on examining associations between combined gene polymorphisms in antioxidant enzymes and breast cancer risk. An increase in the risk of breast cancer has been observed in patients who carry both the MnSOD Ala/Ala genotype and the GPX-1 Leu/Leu genotype, while neither allele alone show any change in breast cancer risk (Cox et al., 2006).

Another polymorphism in the MnSOD gene has also be identified. MnSOD exists as a homotetramer, and Ile to Thr amino acid change at codon 58 has been shown to result in

lower MnSOD activity due to destabilization of the tetrameric structure of the enzyme. However, its frequency seems to be low to have any detectable effect on breast cancer risk (Mitrunen and Hirvonen, 2003).

4.2 Relationship between MnSOD and persistent oxidative stress in breast carcinoma cells

It is known that MnSOD plays a role in breast cancer, depending on its basal expression. *In vitro* studies show that a low MnSOD expression correlates with a high rate of tumor cell growth, whereas high MnSOD content is associated with the invasive and metastatic properties of tumor cells (Nelson et al., 2003). These latter display altered transcription of MnSOD gene, which is also associated with that of H_2O_2-detoxifying enzymes, as compared with the normal counterparts, leading to an imbalance in the redox state by an increase in the level of ROS. Concerning breast cancer cells, the estrogen-sensitive and nonmetastatic tumor MCF-7 cell line exhibits a low basal expression of MnSOD, leading to an accumulation of $O_2\bullet-$, which act as second messenger molecules promoting cell proliferation by activating the Ras-mediated signalling (Li et al., 1995). MnSOD-forced overexpression in these breast cancer cells after transfection of cDNA encoding the antioxidant enzyme reduces cell proliferation and regulates the activation of MMP-2, suggesting a potential modulation of their invasiveness, despite a slower growth rate (Zhang et al., 2002). In contrast, the estrogen-independent and metastatic breast cancer MDA-MB231 and SKBR3 cell lines exhibit a high basal MnSOD expression, which correlates with the invasive and metastatic properties of cells. Up-regulation of MnSOD in estrogen-independent and metastatic cancer cells is associated with an unexplained low expression of catalase, GPX and PRDX3. As a consequence, H_2O_2 from MnSOD activity is overproduced and plays a role in the invasive ability of estrogen-independent and metastatic breast cancer cells, by activating particularly matrix metalloprotease 9 (Kattan et al., 2008). *In vitro* studies are correlated with a clinical investigation which reports that the MnSOD level is positively correlated with the *in vivo* tumor grade in breast carcinomas, and, particularly with the invasive and metastatic phenotypes of advanced breast cancers (Tsanou et al., 2004). Taken together, these observations suggest that an elevated level of MnSOD may reflect tumor progression to a metastatic phenotype in breast cancer cells. The increase of MnSOD expression in breast cancer cells may represent a mechanism by which, by boosting the intracellular concentration of H_2O_2, they reduce their proliferation rate and increase their invasive capacity (Figure 4). In this case, it can be postulated that MnSOD up-regulation would be associated with a poor prognosis in advanced breast cancer. Moreover, the high MnSOD activity may play a role in angiogenesis though the release of H_2O_2, which is able to activate VEGF synthesis (Ushio-Fukai and Nakamura, 2008).

The understanding of the molecular mechanisms involved in the distinct basal expression of MnSOD between nonmetastatic and metastatic breast cancer cells may likely have important clinical implications in predicting tumor progression in breast cancer. Two molecular mechanisms are identified to be involved in the low basal MnSOD expression in nonmetastatic breast cancer cells. Epigenetic processes, such as methylation of CpG islands localized in the proximal gene promoter and a decrease in the histone modifications (methylations and acetylations) are involved in the transcriptional repression of MnSOD gene in some breast cancer cell lines (Hitchler et al., 2006). The other mechanism involves the occupancy of the proximal promoter of MnSOD gene by the both transcription factor Activator protein-2 alpha (AP-2α) and the Damaged DNA binding 2 (DDB2) protein. This

latter protein has been described originally for its role in the nucleotide excision repair of DNA lesions (Sugasawa, 2010). It has been described recently that DDB2 regulates negatively the basal MnSOD expression through its binding to a specific and characterized DNA sequence, which is associated with the loss of acetylated histones, and with the recruitment of the AP-2α (Minig et al., 2009). This latter is known to play a role as repressor of MnSOD gene by binding a response element localized in the GC-rich region in the proximal promoter (Zhu et al., 2001).
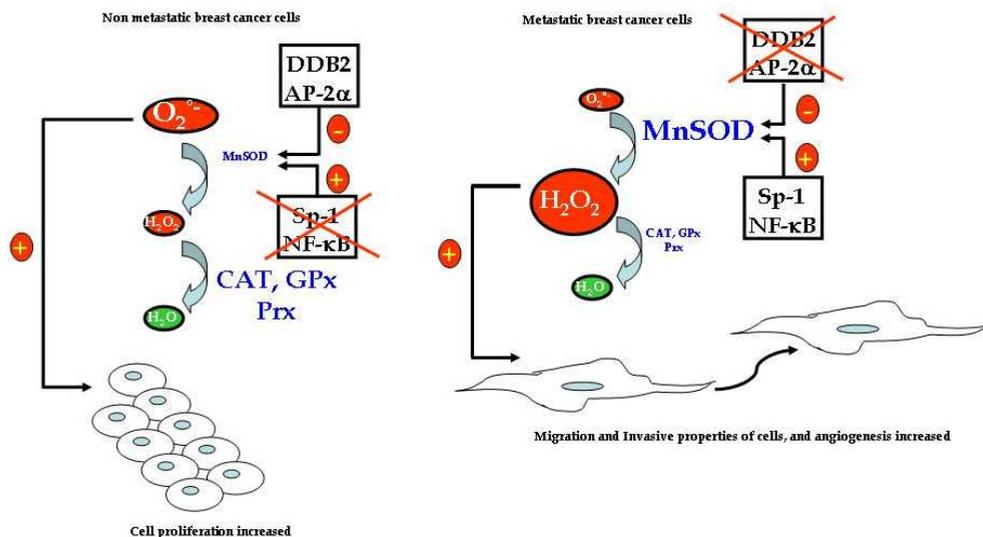
Fig. 4. Role of MnSOD in growth of nonmetastatic and metastatic breast cancer cells. Details are described in the text.

The molecular mechanism, by which the metastatic breast cancer cells exhibit a high basal MnSOD expression, involves essentially the transcription factors Specific-1 (Sp-1) and NF-κB. In absence of DDB2 expression as observed in metastatic breast cancer cells, Sp-1 can bind one of these response elements localized in the GC-rich region in the proximal promoter of MnSOD gene, and cooperates with NF-κB interacting with its binding site localized in intron 2 of the gene and characterized as enhancer element of the transcription (Ennen et al., 2011).

5. Conclusions

In summary, oxidative stress is an important risk factor for cancer development and for disease progression. It seems that polymorphisms in gene encoding the antioxidant enzymes contribute to the accumulation of ROS from the metabolism of risk factors and result in the transformation of normal mammary epithelial cells. Actually, relations between these gene polymorphisms and breast cancer risk are controversial as yet, because of the lack of large case-control studies which need to be drawn, and the difficulties related to the difference between ethnic groups, exposure to environmental factors and the life style. However, reducing ROS by the administration of antioxidants has been considered a good

alternative for cancer prevention, since high consumption of fruits and vegetables has been related to the reduction of breast cancer risk. In addition, antioxidant strategy represents the possibility to inhibit in theory some of the stimuli that contribute to cancer transformation and the expression of an aggressive phenotype, minimizing the acquisition of new mutations and the onset of molecular pathways that promote cancer growth, survival and spreading.

The observations showing an alteration in gene expression of antioxidant enzymes in breast carcinoma cells have been pertinent to better understand the role of ROS in the breast tumor progression toward metastatic phenotype. It appears that some antioxidant enzymes represent actually a good potential biomarkers for the breast tumor progression, particularly MnSOD. The overexpression of this latter enzyme, resulting to an altered expression of its gene, seems to be clearly defined and to depend on the malignant phenotype. Finally, it has been already observed that different intracellular antioxidant capacities may determine the ability of metastatic breast carcinoma cells to resist radiotherapy and anticancer drugs whose activity is dependent on the massive ROS production.

6. References

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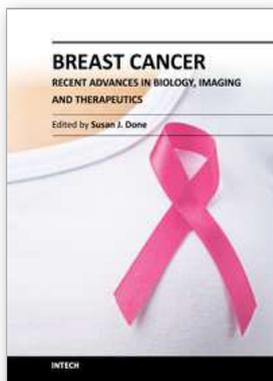
Breast Cancer - Recent Advances in Biology, Imaging and Therapeutics
Edited by Dr. Susan Done

In recent years it has become clear that breast cancer is not a single disease but rather that the term encompasses a number of molecularly distinct tumors arising from the epithelial cells of the breast. There is an urgent need to better understand these distinct subtypes and develop tailored diagnostic approaches and treatments appropriate to each. This book considers breast cancer from many novel and exciting perspectives. New insights into the basic biology of breast cancer are discussed together with high throughput approaches to molecular profiling. Innovative strategies for diagnosis and imaging are presented as well as emerging perspectives on breast cancer treatment. Each of the topics in this volume is addressed by respected experts in their fields and it is hoped that readers will be stimulated and challenged by the contents.

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