Chapter 4

The Role of Cyclooxygenase-2, Epidermal Growth Factor Receptor and Aromatase in Malignant Mesothelioma

Rossella Galati

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1. Introduction

Malignant mesothelioma (MM) is a rare malignant disease originating from neoplastic mesothelial cells which compose the serous membranes of pleura, peritoneum, pericardium, or testis. Mesothelioma responds little to chemo and radiotherapy and is associated with a poor prognosis. In Western Europe, the incidence is increasing and is expected to peak in the year 2020 (Peto et al., 1999; Pelucchi et al., 2004) while in Japan and Australia, the peak is expected for 2025 and 2015 respectively. Thus in order to improve the clinical outcome in the pharmacological treatment of this refractory tumour, drugs directed against novel and/or characterized tumour-specific cellular targets are needed. Malignant pleural mesothelioma (MPM) originates from the pleural layers. Pleura is not just a limiting protective layer for lung, but a dynamic cellular structure regulating serial responses to injury, infection, and disease. Mesothelial cells are biologically active because they can sense and respond to signals within their microenvironment. The development of MM is associated in most patients with a history of asbestos exposure (Mossman et al., 1996). In addition, some investigations have implicated SV40 virus in the pathogenesis of a subset of mesotheliomas (Carbone et al., 2003). Exposure to asbestos typically occurs during mining and milling of the fibers or during industrial application of asbestos in textiles, insulation, shipbuilding, brake lining mechanics, and other areas. Non occupational exposure is usually related to asbestos fibers inadvertently released into the environment and transported by asbestos-contaminated clothing or other materials. After asbestos inhalation, fibers deposited in the lungs typically remain in close contact with lung epithelial cells. Since this fiber-cell interaction could potentially initiate or inhibit cellular functions, asbestos acts as a carcinogen by initiating the carcinogenic process. Carcinogens are known to modulate the transcription factors, anti-apoptotic proteins, proapoptotic proteins, protein kinases, cell cycle proteins, cell adhesion molecules, cyclooxygenase-2, and growth

factor signaling pathways. Research has demonstrated that asbestos exposure generates reactive oxygen species and activates macrophages and other cell types to produce these compounds as well as cytokines and growth factors (Kamp & Weitzman, 1999). Furthermore, the deposition of insoluble amphibole fibers results in a chronic inflammatory state and increased rates of MM in exposed individuals (Mossman & Churg, 1998). This article reviews recent studies regarding some MM molecular targets involved in inflammation for not only prevention but also for therapy of this deadly cancer.

2. MM molecular targets involved in inflammation

The existence of inflammation has been associated with up-regulation of the inducible cyclooxygenases-2 (COX-2), leading to an increase in its product prostaglandin-E2 (PGE-2) (Vane et al., 1994), and is associated with an increased risk of cancer (Ambs et al., 1999). Considerable evidence indicates that COX-2–derived PGE2 can activate epidermal growth factor receptor (EGFR) signaling and thereby stimulate cell proliferation. The mechanism(s) by which this occurs seem to be complex and context specific. Regardless of the precise mechanism for doing so, exposure to COX-2–derived PGE2 can initiate a positive feedback loop whereby activation of EGFR results in enhanced expression of COX-2 and increased synthesis of prostaglandins (Lippman et al., 2005). Although there is a crosstalk between EGFR and COX-2 in carcinogenesis it is important to stress that EGFR and its downstream effectors can be activated independently of COX-2/PGE2. For example, in MM, asbestos fibers activate the EGFR resulting in activation of extracellular signal regulated kinase downstream (Shukla et al., 2011). Similarly, COX-2/PGE2 and its downstream effectors can be regulated independently of EGFR signaling. For example, PGE2 is able to rapidly stimulate Erk phosphorylation in a subset of non–small cell lung cancer (NSCLC) cell lines via intracellular activation of kinase cascades independently of the proteolytic release of EGFR ligands via Src. (Gutkind, 1998; Krysan et al., 2005). These findings have provided the underpinnings for developing agents targeting EGFR or COX-2. A recent study with COX-2 and EGFR inhibitors in MM has shown that the differences in the susceptibility to drugs could be due to the differences in the signalling pathways affected, in addition to the responses that may depend on cell type. In particular it was demonstrated in the Ist-Mes-2 MM cell line a synergistic effect on the inhibition of cell growth between the active small molecule inhibitor of EGFR, gefitinib and rofecoxib, a drug that specifically targets COX-2. Interestingly, the other two cell lines sensitive to treatment with single drugs Ist-Mes-1 and MPP89, did not display this synergistic effect. Only in Ist-Mes-2, the cell line where p-AKT was not detectable, did the combination of rofecoxib and gefitinib result in a synergistic effect. This study suggests that identifying the mechanisms that underlie these differences in sensitivity of cell lines of MM single agents and their combinations, can help us to explore new proteins involved in drug resistance. (Stoppoloni et al., 2010). Lately a new therapeutic target, the Aromatase (CYP19A1), has been identified in MM. This new discovery has highlighted the possibility that there may be in MM as well as in breast cancer a relationship between inflammation, COX-2, EGFR and Aromatase (Fig.1)(Chumsri et al. 2011. These key molecules and pathways that connect chronic inflammation with inflammation associated

oncogenic transformation will be described. We emphasize how the increased understanding of the role of COX-2, EGFR and CYP19A1 in MM may provide novel preventive, diagnostic and therapeutic strategies for MM.

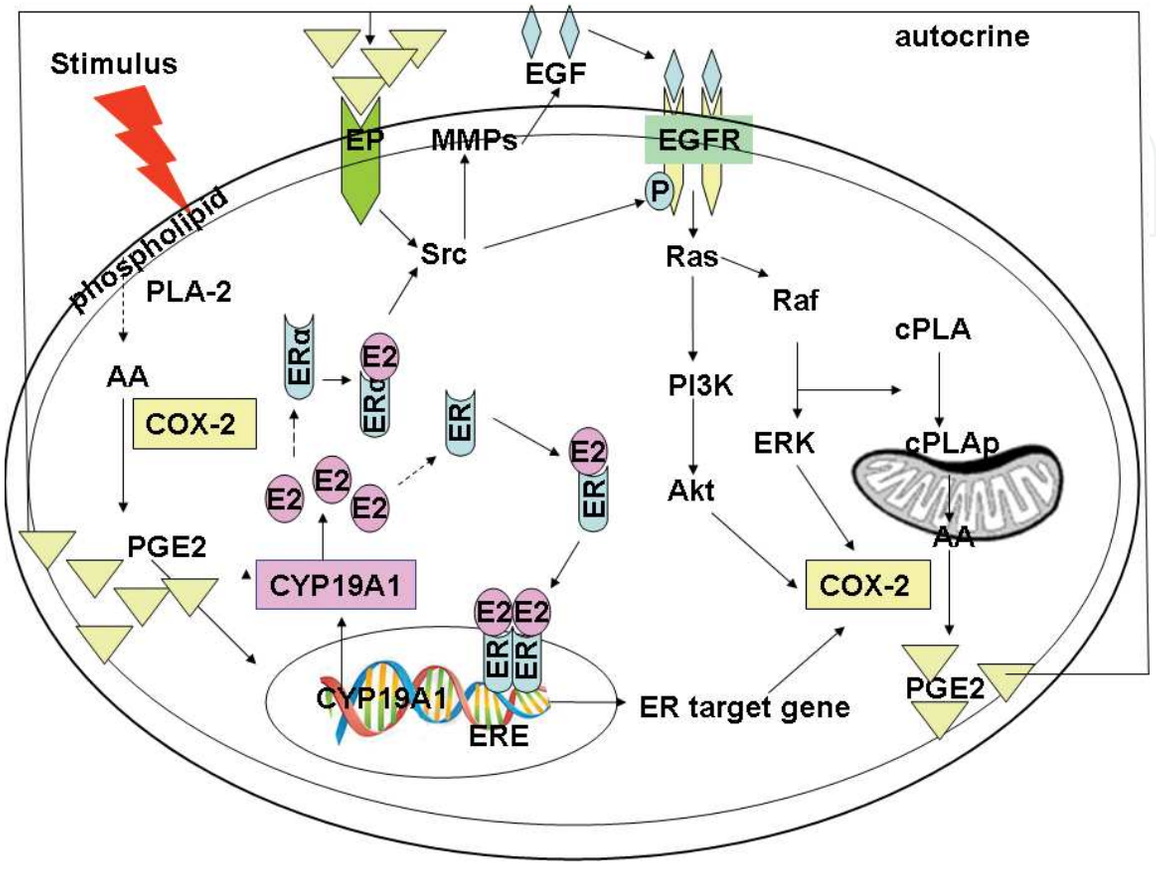

Figure 1. Posible relationship among inflammation, COX-2, EGFR and Aromatase.

Arachidonic acid (AA) metabolic pathway can be activated by inflammation (Stimulus). AA is released from membrane phospholipids by a phospholipase named phospholipase A2 (PLA-2) enzyme and converted to bioactive PGE-2 by COX-2. PGE_2 is an important regulator of *CYP19A1* gene expression and stimulates CYP19A1 activity to increase localized estrogen 17-beta-estradiol (E2). The E2 binds to the classical estrogen receptor (ER) to promote its dimerization and translocation to the nucleus where it modulates the expression of estrogen target genes (COX-2). The interaction of E_2 with ER-alpha also activates signaling cascades that promote cell proliferation, such as the activation of of c-Src tyrosine kinase (Src). Src can also be activated by binding of PGE2 to its receptor (EP). Src activation induces the EGFR phosphorilation and stimulates the matrix metalloproteinase cascade which culminates in the liberation of epidermal growth factor (EGF). Free EGF ligand binds to EGFR family receptors that activates extracellular-signal-regulate- kinase (ERK). Cytosolic phospholipase A_2 (cPLA) is a substrate for ERK and phosphorylation of cPLA (cPLAp) promotes its association with intracellular membranes such as those of the endoplasmic reticulum and mitochondria and releases lysophospholipids and AA from these membranes. COX-2 catalyses the conversion of AA into PGE-2.

2.1. Cyclooxygenases

COXs, also known as prostaglandin-endoperoxide synthases, are key regulatory enzymes in the biosynthesis of prostanoids, a class of hormones including prostaglandins, prostacyclins, and thromboxanes responsible for multiple inflammatory mitogenic, and angiogenic activities in various tissue and organ systems. Increasing interest on COXs is due to the many evidences showing the involvement of these enzymes not only in physiologic but even in pathophysiologic processes such as development and progression of cancer. Two COX isoforms have been identified as COX-1 and COX-2. COX-1 is expressed constitutively in several cell types of normal mammalian tissues, where it is involved in the maintenance of tissue homeostasis. In contrast, COX-2 is an inducible enzyme responsible for PGE2 production at sites of inflammation (Harris, 2007). The mechanism through which COX-2 exherts its tumorigenic action can be directly mediated by the enzyme or due to effects of its products. COX-2 is an oxygenase and its intermediates are highly reactive. It is possible that these compounds may cause free radical damage, for example, against DNA molecule (Cardillo et al., 2005) . There is considerable evidence that prostaglandins, participate both in normal growth responses and in aberrant growth, including carcinogenesis (Greenhough et al., 2009). PGE2 exerts its autocrine/paracrine effects on target cells by binding to four types of membrane-bound, G protein-coupled receptors termed as EP1, EP2, EP3, and EP4 (E-series prostanoid receptors) (Narumiya et al. 1999). These receptors are often coexpressed in the same cell type and use different, and in some cases, opposing intracellular signalling pathways (Breyer et al. 2001).Following ligand binding, the EP receptors activate different signal transduction pathways. EP1 raises intracellular Ca2_, whereas EP3 reduces or increases cyclic -adenosin monophosphate (cAMP) by activating inhibitory G (Gi) or stimulatory G (Gs) proteins depending on the particular splice variant expressed by the cell (Kotani, M et al. 1995). The EP2 and EP4 receptors increase intracellular cAMP by activating adenylate cyclase via Gs proteins. However, differences in the strength of Gs coupling, activation of other signal transduction pathways, agonist-induced desensitization, and agonist-induced internalization result in a differential response of the target cell to a ligand-induced activation of the EP2 or EP4 receptors (Akaogi et al. 2006).It was shown that PGE2 stimulation of both EP2 and EP4 receptors involves transactivation of the epidermal growth factor receptor (EGFR) signaling pathway to promote tumorigenesis (Buchanan et al.; Pai et al 2003; Sale set al. 2005). PGE-2 promotes tumor growth with subsequent enhancement of cellular proliferation, promotion and angiogenesis, stimulation of invasion/mobility, suppression of immune responses and inhibitiuon of programmed cell death by inducing expression of the Bcl-2 protooncogene (which can suppress apoptosis) (Cardillo et al.,2005) (Fig.2).

Figure 2. PGE2 in carcinogenesis

For several types of cancer the real risk factor seems to be chronic inflammation (Prescott & Fitzpatrick, 2000) that maintains high level of COX-2 and increase events that promote tumor formation. A tragic example of this mechanism is MM. Although molecular mechanisms of asbestos tumorigenicity have not been elucidated, research has shown that deposition of insoluble amphibole fibers results in a chronic inflammatory state (Mossman & Churg, 1998) and that this state generates reactive oxygen and nitrogen species, as well as cytokines and growth factors, through the activation of macrophages and other cell types (Kamp & Weitzman, 1999).

As expected, the prolonged inflammation causes the increase of COX-2 level, that is actually recognized as an important MM prognostic factor (Edwards et al., 2002; Mineo et al., 2010). A study clearly demonstrated that COX-2 expression is a strong prognostic factor in human mesothelioma, which contributes independently to the other clinical and histopathologic factors in determining a short survival (Edwards et al., 2002). Although the regulation of mRNA stability appears to be the most important regulatory step for COX-2 expression, several studies have reported that other mechanisms, such as transcriptional control or hypermethylation (Dixon et al., 2000), also are involved in the regulation of COX-2 expression. In cancer cells, it was demonstrated previously that altered post-transcriptional regulation of COX-2 is mediated by increased cytoplasmic mRNA binding of the mRNA stability factor HuR (Dixon et al., 2001). In MM, the cytoplasmic expression of HuR was correlated significantly with high COX-2 expression and with poor survival (Stoppoloni et al.,2008). Finally, COX-2 has been proposed to exert its influence on mesangial cell proliferation *in vitro* by a novel mechanism involving the tumor suppressor p53 and the cell cycle inhibitors p21 and p27 (Zahner et al., 2002). Interestingly, several studies have investigated the potential prognostic value of p53, p21 and p27 in malignant mesotheliomas, thus reinforcing the evidence of a primary role of COX-2 in the pathogenesis and progression of MM (Bongiovanni et al., 2001; Baldi et al., 2002). Due to the lack of a reliable treatment capable of achieving long-term control in mesothelioma patients, these enzymes are becoming more and more appealing as potential therapeutic targets (Veltman et al., 2010; Stoppoloni et al., 2010; O'Kane et al., 2010)

2.2. Epidermal growth factor receptor

The epidermal growth factor receptor (EGFR) is the cell-surface receptor for members of the epidermal growth factor family (EGF-family) of extracellular protein ligands (Herbst, 2004). Upon activation by its growth factor ligands, EGFR undergoes a transition from an inactive monomeric form to an active homodimer. In addition, EGFR may pair with another member of the ErbB receptor family, such as ErbB2/Her2/neu, to create an activated heterodimer. EGFR dimerization stimulates its intrinsic intracellular protein-tyrosine kinase activity. As a result, autophosphorylation of several tyrosine residues in the C-terminal domain of EGFR occurs (P-EGFR). This autophosphorylation leads to the activation of downstream signalling cascades including the RAS/extracellular signal regulated kinase (ERK) pathway, the phosphatidylinositol 3-kinase/AKT (PI3K/AKT) pathway and the Janus kinase/Signal transducer and activator of transcription (JAK/ STAT) pathway (Fig.3).

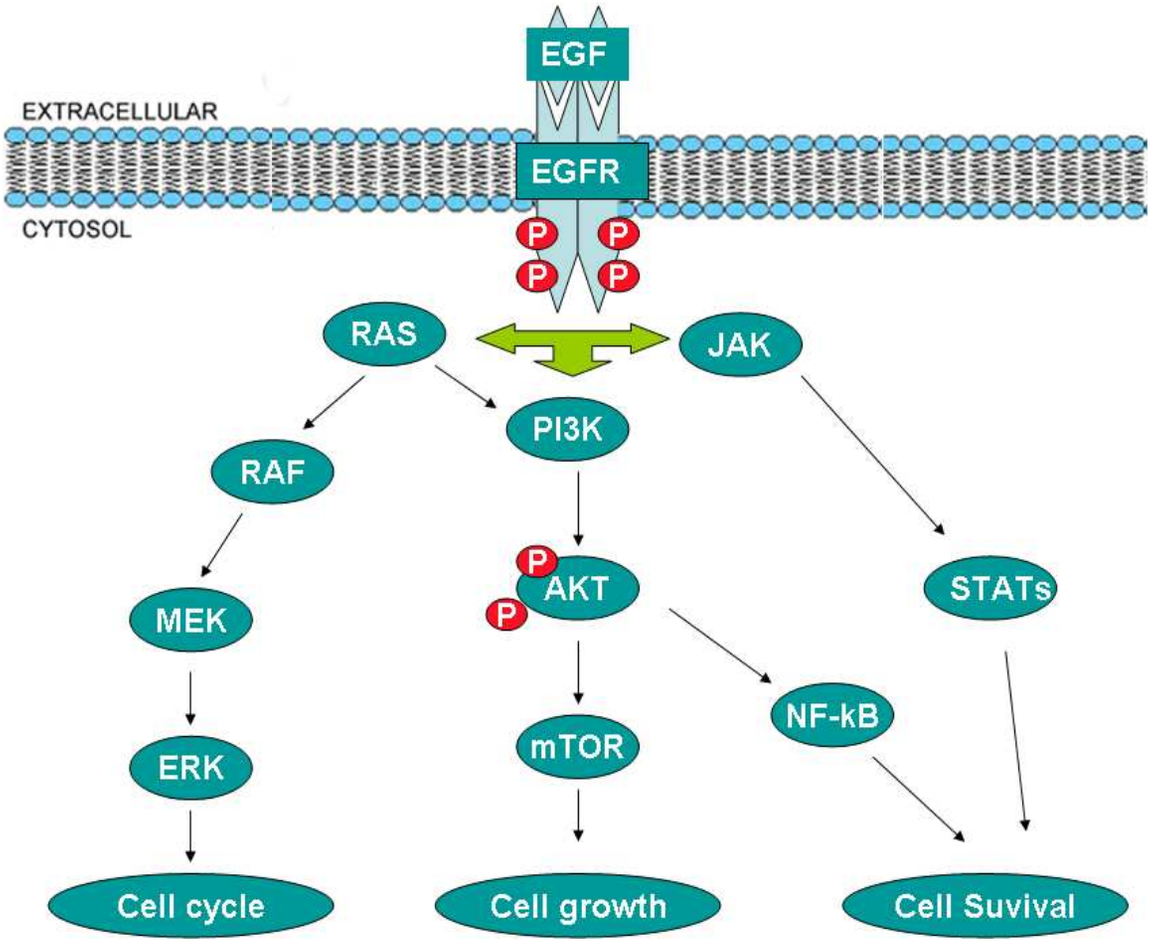

Figure 3. Activation of downstream signaling cascade by P-EGFR

These pathways act in a coordinated manner to promote cell survival (Oda et al., 2005). Such proteins modulate phenotypes such as cell migration, adhesion, and proliferation. EGFR is reportedly over-expressed in a wide variety of malignancies. Various studies suggest that receptor tyrosine kinase activation participates in the oncogenic progression of non neoplastic mesothelial progenitor cells to malignant mesothelioma. Asbestos fiber interact with the external domain of the EGFR to cause dimerization, activation and increased EGFR mRNA and protein levels in rat and human SV-40 immortalized mesothelial cells (Shukla et al., 2011). Up-regulated EGFR and resulting tyrosine phosphorylation leads to the Ras activation which phosphorylates directly and activates Raf (Rapidly Accelerated Fibrosarcoma). Raf is responsible for phosphorylation of the mitogen associated / extracellular regulated kinase-1 (MEK) which in turn phosphorylates extracellular regulated kinases (ERK) on specific residues of threonine and tyrosine (Ras-Raf-MEK-ERK mitogen activated protein kinase (MAPK) pathway). ERK activates a variety of substrates involved in cell cycle. The ERK family consists of at least seven isoforms, and little is known about their regulation and function. ERK1/2 phosphorylation by asbestos, is dependent on phosphorylation of the EGFR. Moreover, has been shown that ERK5, a redox-sensitive kinase known to mediate c-*jun* proto-oncogene expression is activated by asbestos. ERK1/2

and ERK5 are all important in asbestos-induced proliferation and this may be the result of increases in the mRNA levels of AP-1 family members. The ERK5 pathway may be contributing selectively to the regulation of c-*jun*, whereas ERK1/2 pathways may regulate c-*fos*, *fra*-1 and c-*jun*. Has been linked ERK1/2-dependent *fra*-1 expression to mesothelial cell transformation by asbestos and the protracted expression of this gene may be a result of initial increases in c-*fos* and c-*jun* (Scapoli et al., 2004). The phosphoinositide 3-kinase (PI3K)/AKT pathway, plays a critical role for the cell cycle progression in human MM cells [Altomare et al.,2005). AKT, and the downstream mTOR are involved in cell growth and survival, and they are often found to be activated in MM (Carbone et al., 2012). It was reported previously that STAT1 and STAT3 are deregulated MM (Kothmaier et al., 2008).The JAK/STAT signalling pathway is the principal signalling mechanism for growth factors in mammals. JAK activation induces a variety of biological responses such as cell proliferation, diff erentiation and cell migration. In addition, MM cell lines are reported to express EGFR and transforming growth factor-α (TGF-α), suggesting an autocrine role for EGFR in MM (Cai et al., 2004; Jänne et al., 2002). EGFR immunopositivity has been indicated as a poor prognostic factor in many solid tumors in the past (Nicholson et al., 2001). The EGFR expression in MM has been previously reported, with controversial results, possibly due to the lack of standardized method for EGFR detection and quantification (Dazzi et al., Destro et al., 2006; 1990; Govindan et al., 2005; Ramael et al., 1991; Trupiano et al., 2004). Until now, the role of immunohistochemistry (IHC) EGFR positive staining in influencing prognosis of MM is not clear. Some authors did not find differences in survival when IHC EGFR positive or negative staining were compared (Destro et al., 2006; Okuda et al., 2008). This is because only few reports analyzed the effect of IHC EGFR positive status and cell subtype in MM patients. Recently EGFR overexpression is identified by IHC in 52% of epithelial MM and is demonstrated to be a factor negatively affecting prognosis (Rena et al., 2011). In view of these studies, EGFR was targeted for MM therapy, but despite the high expression of EGFR not all cells are sensitive to EGFR inhibitors (Garland et al., 2007). Many efforts are now directed to understand the lack of sensitivity of MM to EGFR inhibitors. In one such study, EGFR mutations were found in 31% (9 of 29) of malignant mesothelioma cases. Seven of these mutations were novel, and one was the L858R mutation described in NSCLC (Foster et al., 2009). Activating EGFR mutations in MM associated with optimal resectability and prolonged survival. Clinically these mutations may ultimately have utility in patient selection for surgery, systemic therapy, and selection for EGFR-TKI (tyrosine kinase inhibitor). The clinical course of MM patients with EGFR mutant tumors appear to share same 'relative' improved clinical outcome like mutant EGFR-NSCLC (Foster et al., 2010 . Study shows the ineffectiveness of the EGFR inhibitors due to coactivation of multiple receptor tyrosine kinase (EGFR, ERBB3, MET, and AXL) in individual mesothelioma cell lines (Ou et al., 2011) Thus, a combination therapy, could be a winning strategy in the treatment of mesothelioma.

2.3. Aromatase

A novel marker of MM recently identified is the CYP19A1 (Stoppoloni et al., 2011). CYP19A1 is the cytochrome P450 enzyme complex that converts C19 androgens to C18

estrogens. The human CYP19A1 gene, located in the 21.2 region on the long arm of chromosome 15 (15q21.2), spans a region that consists of a 30 kb coding region and a 93 kb regulatory region. Its regulatory region contains at least 10 distinct promoters regulated in a tissue- or signalling pathway-specific manner. Each promoter is regulated by a distinct set of regulatory sequences in DNA and transcription factors that bind to these specific sequences. These partially tissue-specific promoters are used in the gonads, bone, brain, vascular tissue, adipose tissue, skin, foetal liver, and placenta for estrogen biosynthesis necessary for human physiology (Bulun et al., 2004). Estrogens contribute to differentiation and maturation in normal lung (Patrone et al., 2003) and also stimulate growth and progression of lung tumors (Stabile et al., 2002; Pietras et al., 2005). Two major pathways, generally termed genomic and non-genomic, are known to mediate estradiol effects on cells. (Fig.4)

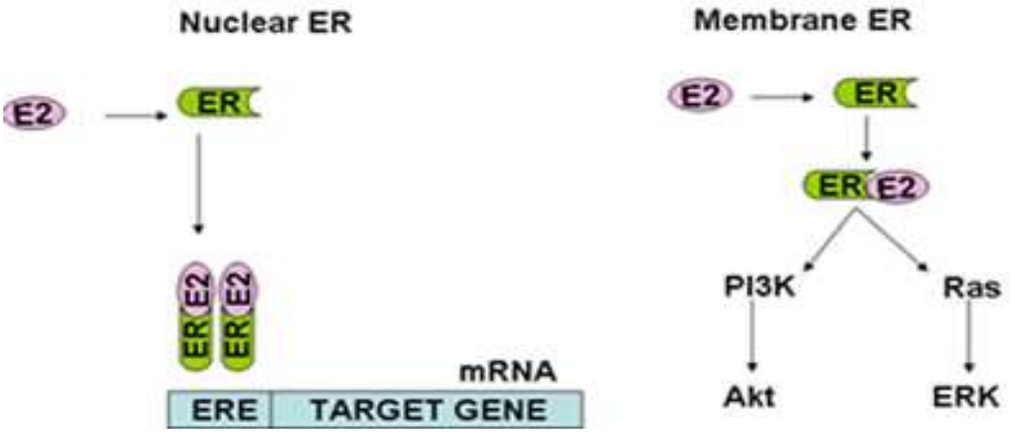

Figure 4. Estrogen Receptor fuction: Genomic (Nuclear ER) and Non Genomic (Membrane ER) action

Estradiol has traditionally been described to mediate its effects via intracellular receptors located in the cytoplasm or on the nuclear membrane and thus studies have investigated the effect of estradiol on transcription factors in the regulation of target genes . Estradiol also acts on the plasma membrane to initiate signaling pathways in the cytoplasm and regulate cellular functions, which is called the non-genomic pathway (Simoncini et al., 2004; Simoncini & Genazzani, 2003). PGE2 is thought to be an important regulator of CYP19A1 gene expression (Zhao et al., 1996). PGE2 increased CYP19A1 activity level in MM cell lines (Stoppoloni et al., 2011). Over the last decade many studies have been carried out to identify potential CYP19A1 stimulatory factors: IL-6 was the most potent factor detected that could stimulate CYP19A1 activity (Reed et al., 1992). The MM cell lines were capable of releasing a constitutively high amount of IL-6 (>1,100 pg.mL supernatant-1 of confluent cultures) (Orengo et al., 1999). This could explain the presence of CYP19A1 in MM cells. Furthermore, estrogen receptor (ER) were also detected in MM cell lines by western blot. The classic 67 kDa and a variant 46 kDa of ERα and 59 kDa of ERβ were expressed in MM cell lines. In support of these results there are recent literature data pointing to a role for estrogens in MM pathogenesis. Epidemiologic studies have identified female gender as a positive prognostic factor for MM (Pinton et al., 2009), although no experimental explanation of this

finding has been provided thus far. CYP19A1 was expressed in the majority of samples from patients with MM. Cytoplasmic expression of CYP19A1 significantly correlated with poor survival (Stoppoloni et al., 2011). The World Health Organization classifies MM into epithelial, sarcomatoid, and biphasic types, each of which can be subdivided further (Travis et al., 1999). This classification has implications for both diagnosis and prognosis. Prognosis is poor for all MMs, but sarcomatoid MMs have a particularly poor response rate to treatment (Neragi-Miandoab et al., 2008). A significant association between high expression of CYP19A1 and sarcomatoid MMs was found (Stoppoloni et al., 2011). These observations strongly suggest that CYP19A1 plays a role in tumour progression in MM. MM cell proliferation was significantly reduced by exemestane (aromatase inhibitor) treatment. Treatment of MM cells with exemestane led to significant reduction of tumor cell growth, perturbation of cell cycle, caspase activation, PARP cleavage, down-regulation of p-AKT and Bcl-xL. . Since Akt pathway as well as Bcl-xL are implicated in conferring resistance to conventional chemotherapy exemestane could open new treatment strategies to be associated with standard therapy for patients afflicted with MM (Stoppoloni et al., 2011).

3. Conclusion

COX-2, EGFR and CYP19A1 are investigational at the present time. The cross-talk between markers that have been described and their value as prognostic indicators will need to be validated in prospective studies in larger patient populations. Their role at the present time is to give us direction towards development of newer therapies in this very resistant tumor. The standard of care at the present time for malignant mesothelioma does not involve checking for these markers and making patient care decisions based on them. But we hope that in the near future this would become a reality with a better treatment approach and prognosis for these patients. Furthermore the possibility of using natural anti-inflammatory products in the chemoprevention of people at risk of MM can not exclude.

Author details

Rossella Galati
Regina Elena Cancer Institute, Rome, Italy

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