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Effects of anti-miR-182 on Thrombospondin-1 expression in human colon cancer cells

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ABSTRACT

Background: MicroRNAs are small non-coding RNAs that regulate the expression of different genes, involved in cancer progression, angiogenesis and metastasis. Bio-informatic statistical analysis indicated that miR-182, over-expressed in colorectal cancer (CRC), has like predictive target Thrombospondin-1 (TSP-1), a protein inversely correlated with tumor vascularity and metastasis that results downregulated in different types of cancer including CRC. Early Growth Response 1 (Erg-1) and Specificity Protein 1 (Sp-1) are transcriptional factors that bind consensus sequence on TSP-1 gene promoter and are putative target of miR-96/182/183 cluster. MiR-182 could target SMAD4, which expression influences angiogenesis, increasing expression of TSP-1.

Aims: Considered that we investigated whether and how the silencing of miR-182 could restore TSP-1 expression levels in HT-29 and HCT-116 cells.

Materials and Methods: We determined the expression levels of TSP-1 and miR-182 in HT-29 and HCT-116 by qRT-PCR. Then, cells were transfected with synthetic oligonucleotides targeting miR-182 (anti-miR-182) and we evaluated TSP-1 mRNA, using qRT-PCR, and intracellular and secreted protein levels by Western blotting (WB) and ELISA. Next, we evaluated Erg-1 and Sp-1 total and fractionated protein levels by WB and we assessed their function by ChIP. Finally we evaluate intracellular SMAD4 levels by WB.

Results: We found that TSP-1 expression increased after transfection with anti-miR-182. Moreover, we observed that anti-miR-182 induced Egr-1 expression, nuclear traslocation and its binding on TSP-1 promoter in HCT-116. Instead Sp-1 was up-regulated both as total and as nuclear protein in HT-29 tranfescted and then recruited on the TSP-1 promoter consensus sequence. Finally, we found increased SMAD4 levels in transfected cells.

Conclusions: Our data suggest that anti-miR-182 could determine an up-regulation TSP-1 expression, modulating Egr-1 and Sp-1 function. Moreover, the restoration of SMAD4 expression induced by silencing of miR-182 could be another mechanism by which anti-miR-182 could restore the anti-angiogenic phenotype. Understanding the molecular mechanism by which miRNAs regulate gene expression could be used to restore TSP-1 expression to contrast angiogenic and invasive events in colon cancer.

Introduction

miRNAs: Function and mechanism of action

MicroRNAs (miRNAs) are a group of non-coding regulatory RNAs, 20-22 nucleotides in length, which have been shown to regulate several cellular processes such as proliferation, differentiation, apoptosis, cell metabolism and angiogenesis [1].

MiRNAs are transcribed by the RNA Pol-II as pri-miRNAs and then processed into mature miRNAs by the sequential action of Drosha and Dicer ribonucleases [1]. Upon maturation, miRNAs are incorporated into the RNA-induced silencing complex (RISC). The key components of the RISC are the Argonaute (Ago) proteins. All Argonaute proteins share two main, highly conserved, structural features: the PAZ domain and the PIWI domain. The PAZ domain binds dsRNA while the PIWI domain has extensive homology to the RNAse H catalytic domain [2] (Fig.1). In humans, only Ago2 (slicer) has endonucleolitic activity [3]. miRNAs are able to coordinate the expression of entire sets of genes, thus finely tuning the mammalian transcriptome [1]. MiRNAs can regulate gene expression by recognizing specific binding sites in the 3' untranslated region (UTR) of target mRNA molecules mainly through their seed sequence (nt.2-8). Additional complementary regions are often found in the non-seed sequence (nt.9-22) [1]. MiRNAs binding leads the degradation of the target mRNAs, to the inhibition of their translation, or both [1; 4]. In animals, it has been recently proposed that downregulation of target genes mostly occurs through mRNA destabilization, with only a very modest effect on translational efficiency [5].

To date over 1000 of miRNAs have been identified in humans. Interestingly, about 30% of miRNAs in both humans and mouse are organized in cluster [6; 7; 8]. They are transcribed as a single polycistronic unit that is rapidly processed in order to obtain the single mature miRNAs. Evolutionary, this organization facilitates functional overlap and cooperativity among miRNAs. This would allow several miRNAs to coordinately regulate multiple nodes belonging to the same pathway or different pathways in a timely fashion [7], although this hypothesis has not been formally tested yet. In the last few years miRNAs have been shown to have a great impact in both normal cellular processes and cancer. They are highly conserved across vertebrates and have a fundamental role in controlling developmental pathways. Genes controlling developmental processes such as cell proliferation, cell death and differentiation are commonly associated with cancer, and many microRNAs are implicated in tumour development and progression [9]. In fact, a significant number of miRNAs is located in tumour susceptibility loci in

mice [10]. miRNA ''signatures'' of human cancer have been found of great help for tumor classification and clinical outcome predictions [11; 12].

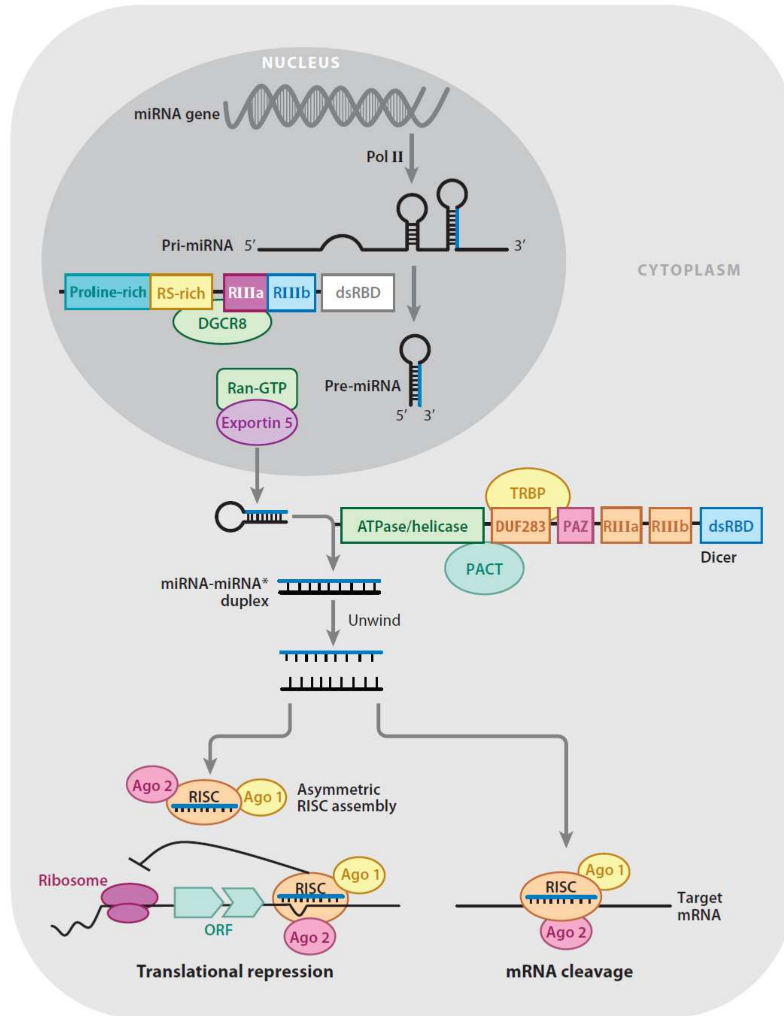

Figure 1: **microRNA biogenesis**. The production of miRNAs from pri-miRNA is a complex and coordinated process operated by different groups of enzymes and associated proteins in the nucleus or cytoplasm.

miRNAs and cancer

It is estimated that more than 60% of all protein coding genes are the potential targets of miRNA [13; 14]. Naturally the reported roles of miRNA are implicated in almost all aspects of cellular functions, including cell differentiation, cell death, cell cycle, developmental timing, inflammation, metabolism and stemness [11; 15; 16; 17; 18].

As expected from their involvement in normal physiological functions, dysregulation of miRNA expression has been shown to be involved in the pathogenesis of a wide variety of pathological conditions, such as heart disease, neurodegenerative disease and cancer. The dysregulation of miRNA is implicated in almost all aspects of cancer characteristics, including cell cycle, apoptosis, invasion ⁄metastasis, angiogenesis and hypoxia-resistance. The first evidence that miRNA is involved in the pathogenesis of cancer was obtained from the study of chronic lymphocytic leukemia (CLL), in which miR-15a and miR-16-1 were identified on a region of the genome that was frequently lost in CLL patients [19]. These miRNA target anti-apoptotic protein BCL2 and their downregulation promotes cancer. Hence, it was proposed that these miRNA have a 'tumor-suppressive' role in the pathogenesis of CLL [20]. Since then, a number of reports have demonstrated the involvement of miRNA in cancers. miRNAs have been classified as oncogene or tumor suppressor according to their ability to influence cancer related pathways [21] although their roles are sometimes dependent on cellular context (Fig.2). As suggested by their name, oncogenic miRNAs promote phenotypes associated with cancers, including cell proliferation, invasion and resistance to apoptosis. OncomiRs are in many cases upregulated in cancers and their elevated expression is indispensable for sustained growth of cancer cells [22]. Therefore, inhibition of these miRNAs by anti-miRNAs can be a new class of molecular targeted therapy. In contrast, tumor-suppressive miRNAs are miRNAs that have anti-tumor functions.

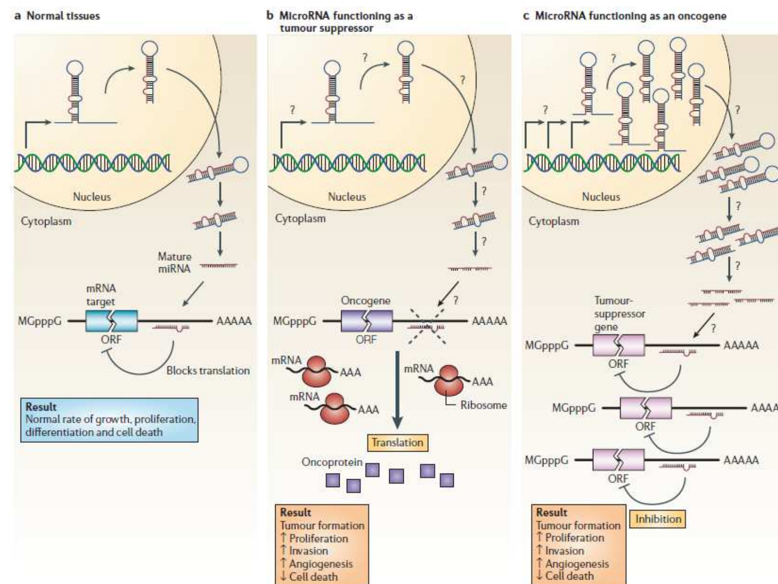

Figure 2: **MicroRNAs can function as tumour suppressors and oncogenes. A.** In normal tissues, miRNAs normally modulate cellular growth, proliferation, differentiation and cell death. **B.** The reduction or deletion of a miRNA that functions as a tumour suppressor leads to tumour formation. **C.** The amplification or overexpression of a miRNA that has an oncogenic role would also result in tumour formation

Because oncogenic and tumor-suppressive miRNAs confer cancer-promoting or cancer-suppressing characteristics to cancers, these miRNAs are regarded as potential targets for novel cancer therapies. In addition, aberrantly expressed miRNA can be used for the diagnosis of cancers. Considering that the functions of a substantial proportion of miRNAs are not known, systematic exploration of cancer-associated miRNAs might be beneficial to detect such clinically relevant miRNA.

MiRNA expression can be altered by several mechanisms in human cancers including (Fig.3):

- *Chromosomal abnormalities* [23]: MiRNAs often reside in particular genomic regions that are prone to alterations in cancer. These regions could include either a minimal region of loss of heterozygosity, which can harbor a tumor suppressor gene; a minimal region of amplification, which might contain oncogenes or fragile sites.
- *Epigenetic changes* [24]: An extensive analysis of genomic sequences of miRNA genes showed that approximately half of these genes are associated with CpG islands, suggesting that miRNAs can represent candidate targets of the DNA methylation machinery mutations and polymorphisms (SNPs).

- *Abnormalities in miRNA-processing genes and proteins* : Mutations and polymorphisms located in mature miRNAs, pre-miRNAs, or more likely in adjacent genomics regions can also change miRNAs expression by affecting their processing. The protein machinery that is involved in the biogenesis of miRNAs is complex. On consequence, alterations of these proteins should have dramatic effects on miRNAs expression.

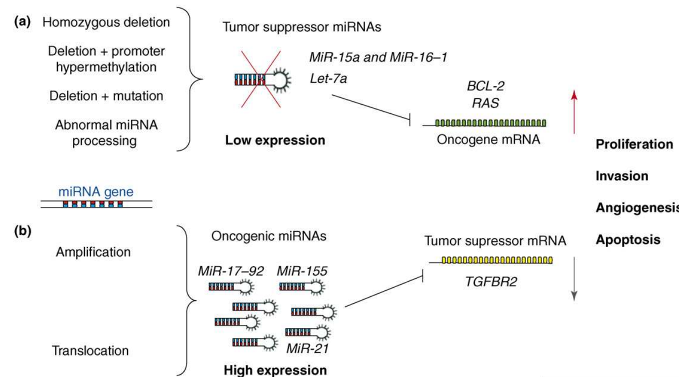

Figure 3: **MiRNA expression can be altered by several mechanisms in human cancers**.
a.The loss of function of a miRNA **b.** The amplification or overexpression of a miRNA

Involvement of microRNA in colorectal cancer pathogenesis

Overexpression and silencing or switching off of specific miRNAs, have been described in the carcinogenesis of CRC. Upregulation of mature miRNA may occur owing to transcriptional activation or amplification of the miRNA encoding gene, whereas silencing or reduced expression may result from deletion of a particular chromosomal region, epigenetic silencing, or defects in their biogenesis [25]. Many proteins involved in key signaling pathways of CRC, such as members of the Wnt/β-catenin and phosphatidylinositol-3-kinase (PI-3-K) pathways, KRAS, p53, extracellular matrix regulators as well as epithelial-mesenchymal transition (EMT) transcription factors [26] are altered and seem to be affected by miRNA regulation in CRC. Analyses of these miRNAs in functional studies are crucial to better understanding CRC pathogenesis and with an aim to eventually identify novel therapeutic targets. On the other hand, expression profiles of hundreds of miRNAs have been shown to have at least the same potential for identification of biomarkers as profiling of their mRNA or protein counterparts. This allows predicting prognosis and therapy response as well as distinguishing certain disease entities, including CRC. *miR-135a* and *miR-135b* decrease translation

of the APC transcript *in vitro*. Of note, *miR-135a* and *miR-135b* were also found to be upregulated *in vivo* in colorectal adenomas and carcinomas and correlated with low APC levels [27]. These observations suggest that alteration in the *mir-135* family can be one of the early events in CRC's molecular pathogenesis. KRAS oncogene has been reported to be a direct target of the let-7 miRNA family [28]. Another miRNA associated with KRAS regulation in CRC is *miR-143* [29]. miR-21 is the miRNA most frequently upregulated in CRC [30; 31; 32]. It seems that suppression of PTEN controlled by miR-21 is associated with augmentation of PI-3-K signaling and progression of CRC. The conserved miR-34a-c family was found to be direct transcriptional targets of p53. EMT is the conversion of an epithelial cell into a mesenchymal cell. Morphologically, EMT is characterized by a decrease of E-cadherin, loss of cell adhesion and increased cell motility leading to promotion of metastatic behavior of cancer cells (including CRC) [33]. The transcriptional repressor zinc-finger E-box binding homeobox 1 (ZEB1) is a crucial inducer of EMT in various human tumors, and it recently was shown to promote invasion and metastasis of tumor cells. The functional links to EMT comes from members of the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429). ZEB1 directly suppresses transcription of miRNA-200 family members miR-141 and miR-200c, which strongly activate epithelial differentiation in pancreatic, colorectal and breast cancer cells [34] (Fig.4).

A number of studies based on expression profiling has proven there are significant changes of miRNA expression levels in CRC tissue in comparison to colorectal epithelium and these have identified groups of miRNAs enabling prognostic stratification of CRC patients and prediction of their responses to selected chemotherapeutic regimens and radiotherapy. miRNAs' occurrence has been repeatedly observed also in serum and plasma, and miRNAs as novel minimally invasive biomarkers have indicated reasonable sensitivity for CRC detection and compare favorably with the fecal occult blood test.

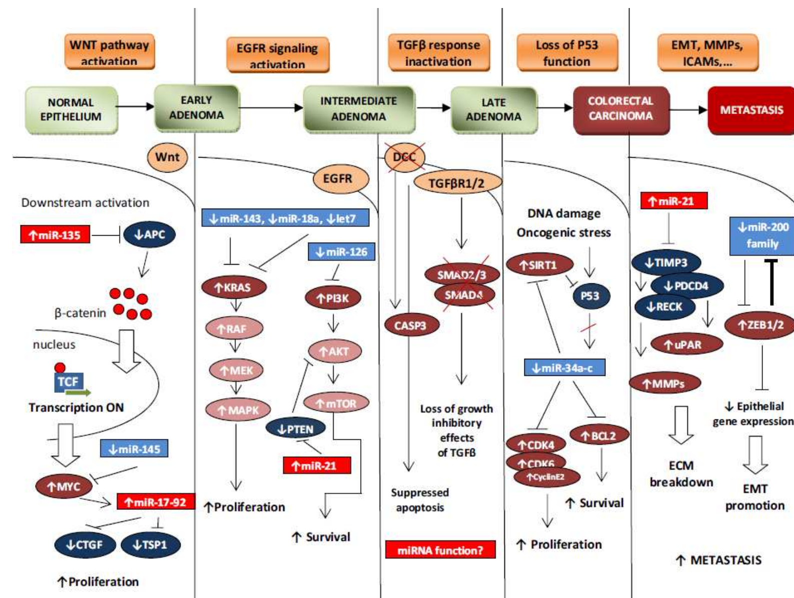

Figure 4: **MicroRNAs' involvement in Vogelstein's model of colorectal cancer pathogenesis**

miRNAs regulation of angiogenesis in CRC

Angiogenesis is defined as the growth of endothelial sprouts out of pre-existing post-capillary venules. During oncogenesis, tumor cells acquire characteristics that provide them with a growth advantage over normal cells. These characteristics, often referred to as the hallmarks of cancer, are shared by most, if not all, types of cancer and include infinite and self-sufficient growth, evasion of death signals, enhanced tissue invasion and metastasis, and sustained angiogenesis [35]. To induce angiogenesis, tumor cells have to undergo an angiogenic switch. More recent findings indicate that specific miRNAs might also be responsible for regulation of endothelial gene expression during tumor angiogenesis [36; 37]. Tumour neovascularization is partly driven by hypoxia, which stimulates tumour cell production of angiogenic factors such as vascular endothelial growth factor-A (VEGFA). An important regulator involved in the cellular response upon hypoxia is TP53 . Mutations in TP53 are common in CRC [38] and associated with increased tumour angiogenesis [39]. MiR-107 was identified as a downstream target of TP53 and proved capable of inhibiting the translation of hypoxia-inducible factor-1 beta (HIF1B)[40]. Upon hypoxic signalling in a CRC cell line, overexpression of miR-107 resulted in decreased VEGF expression. The oncogene c-Myc (MYC) is an important regulator of tumour angiogenesis.

MYC is often coactivated with RAS, of which HRAS and KRAS are known to up-regulate VEGF. In a CRC model, the combination of mutations in KRAS and TP53 yielded indolent, poorly vascularized tumours [41]. A robust tumour vasculature and progressive neoplastic growth only developed after overexpression of the MYC oncogene [41]. MYC-induced up-regulation of the miR-17-92 cluster is directly responsible for activating angiogenesis by down-regulation of the antiangiogenic Thrombospondin-1 (TSP-1) [41] (Fig.5). Other miRNAs have been found differentially expressed during the development of CC, among of them, the miR-96, miR-182 and miR-183 clusters are up-regulated [42]. In particular, miR-182 seems to control genes involved in melanoma cancer cells migration, invasion and, on consequence, influenced their metastatic potential [43], moreover miR-182 is involved in cellular DNA damage response [44].

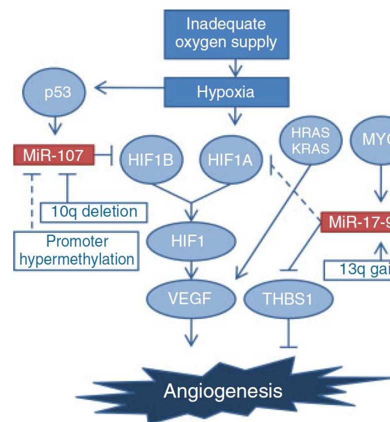

Figure 5: **Regulation of angiogenesis in CRC**

Thrombospondin-1 (TSP-1)

TSP-1 is a multifunctional extracellular matrix protein and one of a fivemember family of extracellular calcium-binding proteins [45]; it is a platelet- and cell-derived homotrimeric glycoprotein, secreted in a wide range of tissues where it is bound to the extracellular matrix [46] (Fig.6). Numerous in vitro and in vivo experiments have been carried out to identify multiple mechanisms by which TSP-1 inhibits angiogenesis. Three copies of the thrombospondin type 1 repeat (TSR), found in the middle of TSP-1 [47], are essential to inhibit tumor angiogenesis and growth. Through TSRs, TSP-1 binds to its receptor, CD36, to β1 integrins and to transforming growth factor-β (TGF-β).

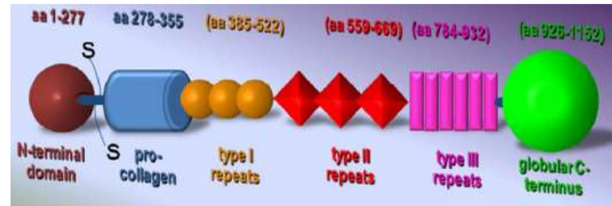

Figure 6: **Graphical representation of the structure of the TSP-1 molecule.** The thrombospondin monomer is a 150,000 MW glycopeptides. Regions containing amino acid sequence homologous to procollagen, properdin and EGF are shown. Heparin, calcium ion and cell binding domains are indicated.

Several evidences indicate that the anti-angiogenic activity of TSP-1 is mostly mediated by the inhibition of endothelial cell migration and the induction of apoptosis via interaction of TSP-1 with CD36 [48; 49]. In fact, CD36 expression has been shown to correlate with decreased stromal vascularization and a better prognosis in colon cancer [50], and in vitro, CD36 mediates TSP-1 inhibition of endothelial cell migration and tube formation [49; 51]. The interaction of TSRs and β1 integrins inhibits the migration of endothelial cells in a CD36- independent manner[52]. TSP-1 also indirectly influences angiogenesis through the activation of TGF-β, in fact TSP-1 is the only member of the thrombospondin family that can activate TGF-β [53] (Fig.7). Even if the precise mechanism underlying the activation of TGF-β by TSP-1 is not fully understood, seems that the amino acid sequence RFK between the first and second TSRs of TSP-1 is essential [54]. TSP1 is one of the genes that has been found to be aberrantly methylated in some colorectal cancers as well as in neuroblastomas and gastric cancers [55; 56; 57]. In consequence, the aberrant methylation of TSP1 promote tumor formation through inhibiting the TGF-β signaling pathway activation, showing a novel epigenetic mechanism for inhibiting the TGF-β signaling pathway in colorectal cancer [58].

TSP-1 has also been shown to inhibit angiogenesis by directly interacting with vascular endothelial growth factor (VEGF), and inhibiting matrix metalloproteinase-9 activation [45]. In addition, TSP-1 may inhibit angiogenesis by decreasing the level of circulating endothelial cell progenitors [59].

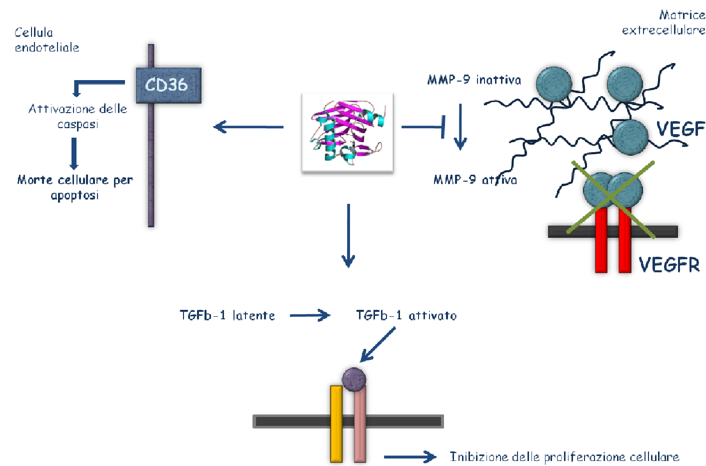

Figure 7: **Schematic representation of the functions of TSP-1 in tumors**.

TSP-1 levels expression is inversely correlated with tumor vascularity, in fact microvessel density is significantly higher in TSP-1-negative colorectal tumors and correlated with prognosis: it has been shown that patients with TSP-1-negative tumors had a significantly worse prognosis than did those with TSP-1-positive tumors. Moreover TSP-1 may be useful for predicting recurrence in patients with colon cancer because the frequency of hepatic recurrence was significantly higher whose colorectal tumors are VEGF-positive and TSP-1-negative [60] than in patients with TSP-1-positive colon cancer [61]. The fact that TSP-1 is a potent endogenous inhibitor of angiogenesis and is often down-regulated in tumor tissue lays the basis to explore therapeutic applications of TSP-1. These efforts fall into two basic approaches, the identification of strategies to up-regulate endogenous TSP-1, and the delivery of recombinant TSRs or synthetic peptides that contain sequences from the TSRs [62].

Transcriptional regulation of *tsp-1* gene

Is known that TSP-1 promoter region is important for basal transcriptional activity [63], and in the 5'-flanking region between -234 and +750 are localized several putative binding sites for Egr-1 and Sp-1 [63]. These transcriptional factors can compete to bind the same GC-rich sites. Huang et al. demonstrated that Egr-1 expression augments the Sp-1 activation of non-overlapping Sp-1 + Egr-1 sites, but inhibits Sp-1 activity when the sites are overlapping by competing with Sp-1 for the binding site. In addition, Sp-1 is

a strong inducer of Egr-1 suggesting a mechanism in which Sp-1 facilitates the inhibition of its own transactivating potential by induction of Egr-1. This ''facilitated inhibition'' of Sp1 transactivation activity by Egr-1 could be a common mechanism for the regulation of a wide range of growth-related genes [64].

A recent work shows that the 5-Fluoruracil (5-FU) induces the activation of Egr-1 via p38 mitogen-activated protein kinase (MAPK) pathway. This event determines the Egr-1 binding on TSP-1 gene promoter and, of consequence, the enhanced TSP-1 expression in human colon carcinoma KM12C cells[65], whereas Sp-1 plays a role in the EGF-induced activation of the TSP-1 gene [66].

In addiction TSP-1 expression seems to be regulated also by microRNAs . In fact, a down-regulation of *c-myc* increases the stability of TSP-1 mRNA through the decreased expression of *miR-17-92* cluster after treatment with 5-FU [67].

The role of SMAD4 in CRC

Smad4 was originally described as tumor suppressor gene deleted in pancreatic cancer (DPC4), and is located at chromosome 18q21.1 [68]. *Smad 4* gene mutation plays a significant role in colon carcinogenesis. Loss of *Smad* activation and/or expression occurs in approximately 10% of CRCs. This subset has a poor prognosis because of its association with advanced disease and the presence of lymph node metastases at diagnosis [69]. Loss of *Smad4* function occurs at later stages of malignancy, playing a role in the acquisition of advanced phenotypes. The frequency of mutational events of *Smad4* gene increases with the progression of carcinogenesis, being 0% in adenomas, 10% in intramucosal carcinomas, 7% in invasive carcinomas without distant metastasis, 35% in primary invasive carcinomas with distant metastasis, and 31% in carcinomas metastasized to the liver and distant lymph nodes, or disseminated [70]. In SMAD4 null (MC38 and SW620) cell lines, TGF-β induced invasion, migration, tumorigenicity and potentiality for metastasis, while incubation with LYLY2109761 (a potent TGF-β receptor kinase inhibitor) reversed these effects, suggesting that loss of SMAD4 might underlie the functional shift of TGF-β from a tumor suppressor to a tumor promoter. It has been shown that the restoration of SMAD4 to human pancreatic carcinoma cells blocked their ability to grow progressively as tumors in vivo. Unexpectedly, SMAD4 re-expression did not restore sensitivity to growth inhibition by TGF-β. Rather, Smad4 decreased the expression of VEGF and increased levels of the angiogenesis inhibitor TSP-1

causing the cells to switch from potently angiogenic to antiangiogenic in vitro and in vivo [71]. The SMAD4 effects on VEGF and TSP-1 expression may contribute to reduced tumor growth through diminished vascular supply (Fig.8).

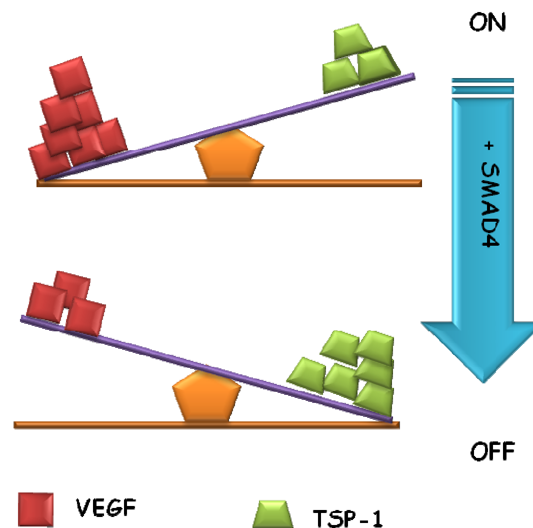

Figure 8: **Smad4 mediates an angiogenic switch**. Basal expression levels of the prominent angiogenesis regulator VEGF are reduced, whereas levels of the angiogenesis inhibitor are induced in a Smad4-dependent manner.

miRNAs and anticancer therapy

New technologies that utilize synthetic miRNAs or artificial target sites to exploit or inhibit endogenous miRNA regulation are emerging. Therapeutic strategies based on modulation of miRNA expression hold great promise owing to the ability of these small RNAs to regulate cellular behavior. The most promising therapeutic techniques tested to date are miRNA mimics and anti-miRNA oligonucleotides (AMOs). Because the loss of a miRNA inhibitory effect contributes to oncogene activation, it could be possible to inhibit the expression of dysregulated oncogenes by using synthetic miRNA mimics. On contrary, anti-miRNAs are modificated oligonucleotides designed to knock down miRNAs target. The 2’-*O*-methyl (2’-OMe), 2’-*O*-methoxyethyl (2’-MOE) are exemples of modificated oligonucleotides to target mi-RNAs. These modifications, frequently a methyl group, contributes

a limited amount of nuclease resistance and improves binding affinity to RNA compared with unmodified sequences. Recently, at the light of the success of siRNA as a potent and specific inhibitor of gene expression the scientists introduced the "locked nucleic acid (LNA)", an oligonucleotide that contains conformationally locked nucleotide monomers with a methylene bridge connecting the 2'-oxygen and 4'-carbon atoms of the ribose ring. Incorporation of LNA monomers into oligonucleotides and siRNA constructs has been shown to increase the nuclease resistance significantly, stabilize the duplex structure and improve mismatch discrimination[72]. A number of groups have shown that vectors expressing miRNA target sites can be used to saturate an endogenous miRNA, preventing the downregulation of its natural target. This technology, which has been defined with different names utilizes different gene delivery systems, including plasmids and vectors based on adenoviruses, retroviruses, and lentiviruses (Fig.9).

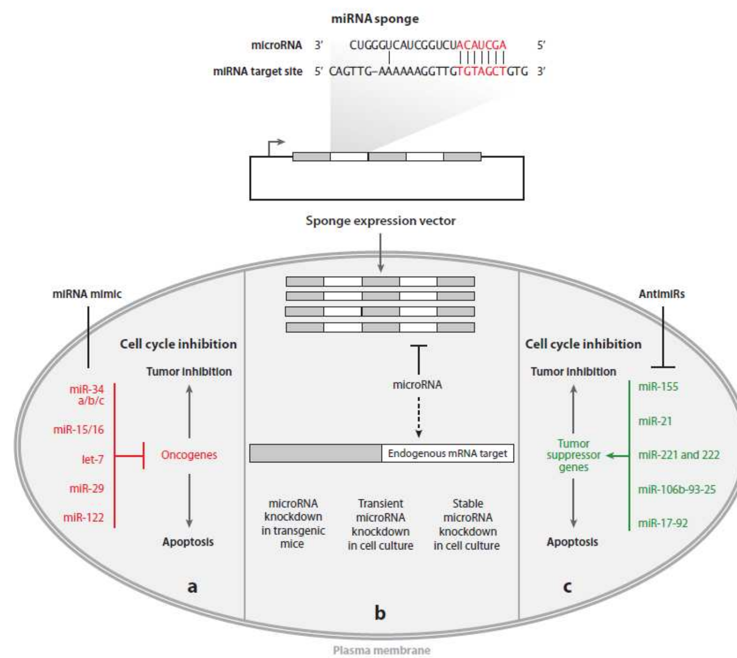

Figure 9: **miRNAs as therapeutics.** (*a*) The miRNA mimic technology. (*b*) Vectors expressing miRNA target sites (*c*) Knockdown of oncomiRs through anti-microRNA oligonucleotides

Aims of the thesis

TSP-1 expression in the tumoral microenvironment is correlated with the suppression of tumor growth and the inhibition of the angiogenic process, on consequence TSP-1 down-regulation in CRC is associated with the angiogenic-switch in favor of the formation of new vessels. As molecular basis about events that are responsible for the decreased expression of TSP-1 are almost clear, excluding the role played by the state of methylation of the promoter as confirmed by literature data, we hypothesized an regulatory role by miRNAs. MiR-182, belonging to the cluster miR-96/miR-182/miR-183, is highly expressed in tumoral colon samples respect normal speciments [42] and considered that bioinformatic analysis reveals that TSP-1 mRNA is a predictive target of miR-182, in this study we focused our attention on the effects of the silencing of miR-182 on TSP-1 expression regulation in HCT-116 and HT-29 colorectal cancer cells.

Because is known that miRNAs have a pleitropic effects, then we focused our attention on Egr-1 and Sp-1, predictive targets of miR-96/182/183 cluster, to establish if the effects of anti-miR-182 on TSP-1 expression are mediated by these transcriptional factors. Moreover, miR-182 could target SMAD4 that regulates VEGF and TSP-1 expression in a reciprocal manner in pancreatic cancer cells. On consequence, we evaluated if the effects of anti-miR-182 on TSP-1 could implicate SMAD4 signaling in HCT-116 cells, in addiction to transcriptional influence.

MicroRNA-based drugs represent a promising new class of therapeutic agents in degrees of abnormal or hyperactive silence genes responsible for many diseases. Newer chemotherapeutics agents, the so-called ‘targeted’ or ‘biological’ therapies have improved survival in patients with metastatic CRC, however, had a relatively small effect on survival outcomes [73; 74; 75]. For this reason new therapeutic approaches, as microRNAs, could be used to to contrast angiogenic spread and metastatic events.

Materials and methods

Cell cultures

To determine whether miR-182 regulates TSP-1 expression we used three human colon cancer cell lines: HT-29 (ATCC, Catalog No. HTB-38), Caco-2 (ECACC, Catalog No. 86010202), HCT-116 (ECACC, Catalog No. 91091005).HT-29 and HCT-116 were routinely grown in Gibco Mc-Coy's (Invitrogen, Carlsbad, CA). Caco-2 cell line were grown in Gibco DMEM:F-12 (L-Glutamine (+) , 15mM HEPES (+) NEAA (+), Invitrogen, Carlsbad, CA).
All media were supplemented with 10% Foetal Bovine Serum (FBS), 100 U/mL Penicillin and 50mg/mL Streptomycin.

Anti-miRNAs transfections

Transfections were performed with anti-miR-182 (Ambion, Austin, TX). HT-29 and HCT-116 cells were seeded at $5x10^5$ in 60mm culture dish. After 24 h (40–50% confluence), the cells were transfected with anti-miR-182 (100 nM) using siPort Neo Fx transfection agent (Ambion, Austin, TX), according to manufacturer's instructions. The mixture was transfected into all colon cancer cell lines for 24h in conditioned medium. Non-specific anti-miR (Ambion, Austin, TX) was used as negative control. The success of transfection was confirmed by quantitative Real-Time PCR.

Quantitative Real-Time PCR to determine the expression of miRNAs and TSP-1

Total cellular RNA and miRNAs were isolated using the miRNeasy Mini Kit (Qiagen Inc, Valencia, CA) and quantified through RNA 6000 Nano Assay (Agilent Techologies, Palo Alto, CA, USA) and using 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) we evaluated the integrity. For TSP-1 mRNA detection, 2 μg of total RNA were reverse transcribed using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA), according to manufacturer's instructions and 5 μl of the RT products were used to amplify TSP-1 mRNA sequence using the Hs00962914_m1 TSP-1 TaqMan gene expression assay (Applied Biosystems, Foster City, CA). Ten nanograms of total RNA were reverse transcribed using Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The obtained cDNA was amplified using Taqman hsa-miR-182 MicroRNA assay (Applied Biosystems, Foster City, CA). To normalize quantitative Real-Time PCR reactions, parallel reactions were run on each sample for RNU6B snRNA or Cyclophilin A. Changes in the target mRNA content relative to RNU6B or Cyclophilin were determined using the comparative Ct method to calculate

changes in Ct, and ultimately fold and percent change. An average Ct value for each RNA was obtained for replicate reactions.

TSP-1 detection by ELISA

A total of $5x10^5$ HT-29 and HCT-116 cells were transfected with anti-miR-182, as described before. After treatment, conditioned medium was collected to measure secreted TSP-1 expression levels using HumanQuantikine ELISA Kits (R&D Systems, Minneapolis, MN) following manufacturer's instructions. All points were done in triplicate and the experiments were repeated three times. All TSP-1 concentrations were within the range of curve standard. Linear regression analysis was performed to create the curve standard. The range of curve standards was 7.81, 15.6, 31.3, 62.5, 125, 250, 500 pg/mL; all TSP-1 concentrations in samples were within the range of curve standard. Linear regression analysis was performed to create the standard curve.

Western blotting (WB)

The cells, after transfection with anti-miR-182, were lysed to obtain total proteins using Complete Lysis-M Reagent (Roche, Mannheim, Germany) or cytoplasmic and nuclear protein fractions using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL). The expression of proteins was analyzed in 120 µg of total protein lysates and 80–50 µg of cytoplasmic and nuclear protein fractions. The following antibodies (Abs) were used for WB: anti-TSP-1 (3F357) mouse monoclonal IgG_1 (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA), anti-SMAD4 mouse monoclonal IgG_1 (1:500; 100 µg/ml, Gene Tex, Inc.), anti-Egr-1(S-25) mouse monoclonal Ab (1:500 Santa Cruz Biotechnology, Santa Cruz, CA), anti-SP-1 (C-20 X) rabbit polyclonal Ab (1:1000 Santa Cruz Biotechnology, Santa Cruz, CA), anti-GAPDH (6C5) mouse monoclonal Ab (1:1000 Santa Cruz Biotechnology, Santa Cruz, CA), anti-C23 (MS-3) mouse monoclonal Ab (1:1000 Santa Cruz Biotechnology, Santa Cruz, CA). The proteins were separated on an 8% polyacrylamide gel under denaturing conditions, and the specific signal was detected with ECL-WB substrate (Pierce Biotechnology Inc., Rockford, IL).

Chromatin immunoprecipitation (ChIP)

ChIP was performed using the Chromatin Immunoprecipitation Assay kit (Upstate, Temecula, CA), according to manufacturer's instructions. HT-29 and HCT-116 cells were transfected with 100 nM anti-mir-182 for 48 h, or left untreated. Next, the cells were cross-linked with 1% formaldehyde and chromatin was collected and sonicated. Soluble chromatin was

immunoprecipitated with the following Abs: 5 µg anti-Egr-1 (S-25) mouse monoclonal Ab (Santa Cruz Biotechnology) or 5 µg anti-SP-1 (C-20 X) rabbit polyclonal Ab (Santa Cruz Biotechnology). DNA–protein immune complexes were eluted, reverse cross-linked and DNA was extracted with phenol/chloroform and precipitated. The presence of the TSP-1 promoter domain containing consensus sequence for Egr-1 in immunoprecipitated DNA was identified by qRT-PCR using SYBR Green with the following primers: TSP-1 (region -482 to +162) forward 5'-AACGAATGGCTCTCTTGGTG-3', reverse 5'-GGGCGACTTACCTGTGTGTA-3'. The same primers amplify the promoter region containing Sp-1 consensus sequences. The PCR conditions for the TSP-1 promoter region were: 40 cycles at 1min, 30 sec at 95°C, 30 sec at 60°C, 1min at 72°C. In control samples, the primaries Abs were replaced with non-immune rabbit IgG. To normalize qRT-PCR reactions, chromatin inputs were used as control and were run on each sample. An average CT value for each samples was obtained for triplicate reactions.

Statistical analysis

The correlations were studied by Student's t-test. P-values of <0.05 were considered statistically significant.

Results

TSP-1 is down-regulated and miR-182 was over-expressed in HT-29 and HCT-116 colon cancer cell lines

Preliminary evaluations are performed to assess miR-182 and TSP-1 mRNA expression levels in HCT-116, HT29 using Caco-2 as control cells by qRT-PCR. We decided to use Caco-2 because it has been shown that these cells spontaneously differentiate in culture and form a polarized monolayer similar to that of the small intestine [76]. We observed that miR-182 was over-expressed by 3-fold in HT-29 and by 2.1-fold in HCT-116 cells respect to Caco-2 cell line (Fig.10A). Then, we evaluated TSP-1 mRNA expression levels in the same cells. Data showed that TSP-1 mRNA levels are down-regulated by 80% and by 95% in HT-29 and HCT-116 cells respectively, respect to Caco-2 cells (Fig.10B). These first results confirm the inverse correlation of expression levels of TSP-1 and miR-182 in the in vitro model that we proposed as in literature reported [61 42].

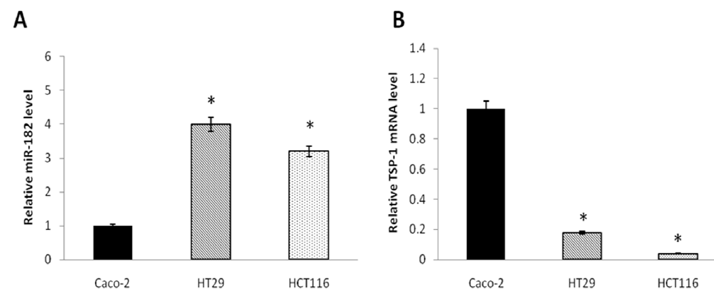

Figure 10: **miR-182 and TSP-1 expression in colon cancer cell lines**. A: miR-182 is up-regulated in HT29 and HCT-116 cancer cell lines. RUN6B was used as endogenous control. The graphs represent respectively the fold abundance of miR-182 relative to Caco-2 colon cancer cell B: TSP-1 mRNA is down-regulated in HT29 and HCT-116 cancer cell lines. To normalized TSP-1 quantitative real-time PCR reactions, parallel reactions was run on each sample for Cyclophilin A (PPIA). The graphs represent respectively the reduction of TSP-1 mRNA relative to Caco-2 colon cancer cell line

Effects of anti-miR-182 on TSP-1 expression

Hypothesizing the possible role of miR-182 in TSP-1 regulation and in the light of their inverse correlation, we investigated the effects of a synthetic oligonucleotide designed to target the mature forms of miR-182 on TSP-1 expression in colorectal cancer cells (anti-miR-182). Following miR-182 silencing, we observed that TSP-1 mRNA increased by 2.7-fold and 3.1-fold in HT-29 and HCT-116 respectively relative to untransfected and negative control cells (Fig. 11A). We also evaluated intracellular and secreted TSP-1 protein levels. Western blotting results, obtained on total cellular lysates,

indicated that intracellular TSP-1 protein levels increased in transfected HT-29 and in HCT-116 cells (Fig. 11B), while, by ELISA, we analyzed the amount of secreted TSP-1 that was elevated by 2.2-fold and by 1.9-fold in HT-29 and HCT-116 respectively (Fig. 11C).

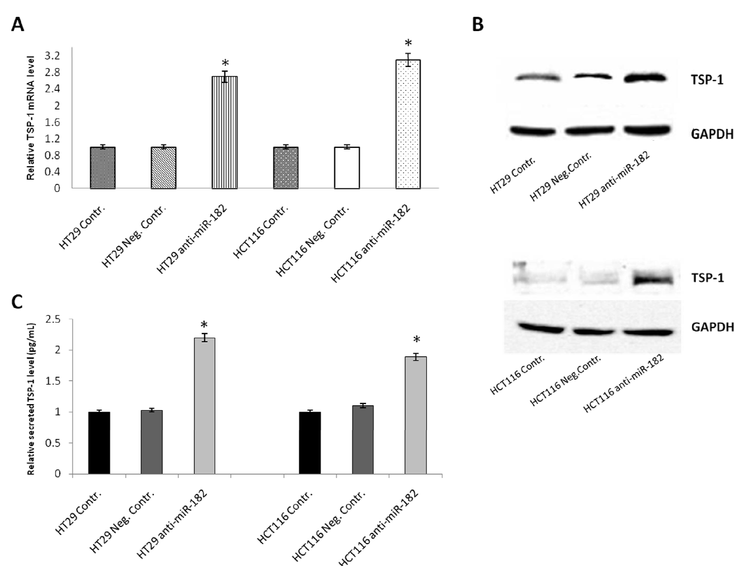

Figure 11: **Anti-miR-182 influences TSP-1 expression**. A. The expression of TSP-1 mRNA was studied after HT-29 and HCT-116 transfection with anti-miR-182 or with negative control anti-miR molecules. The graph indicates that TSP-1 mRNA levels increase in transfected cells relative to negative control. B. The abundance of TSP-1 total protein was determined by WB in 120 μg of total proteins. The pictures indicates the increase of TSP-1 protein levels relative to untrasfected cells C: The concentration of secreted TSP-1 was measured by ELISA in cells transfected with anti-miR-182 or with negative control anti-miR.

Silencing of miR-182 increases Egr-1 and Sp-1 protein levels in HCT-116 and HT-29 respectively

Since Egr-1 and Sp-1 are predictive target of miR-96/182/183 cluster [77], we investigated the molecular mechanism by which anti-miR-182 could determine up-regulated TSP-1 expression in colon cancer cells, as reported previously. For this reason, we evaluated if anti-miR-182 influenced Egr-1 and Sp-1 expression in the in vitro model that we decided to study. Western blotting analysis revealed that Egr-1 total protein levels were up-regulated after transfection with anti-miR-182 in HCT-116 cell lines and that Sp-1 was slightly increased in HT-29 transfected with anti-miR-182 respect to untransfected cells (Fig.12). These results may be indicate that miR-182 regulates different transcription factors involved in activation of TSP-1

expression in different cell types confirming that the actions of miRNAs is citotype-specific.

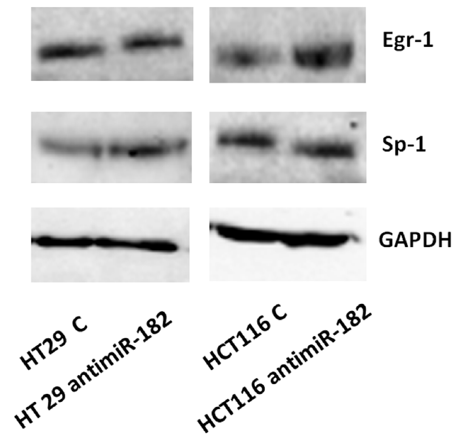

Figure 12: **Effects of anti-miR-182 on Egr-1 and Sp-1 on protein levels.**

Anti-miR-182 modulates Egr-1 and Sp-1 nuclear translocation in colon cancer cells

At the light of these data, we studied Egr-1 expression on fractionated lysates after transfection with anti-miR-182 in colon cancer cells. We found both cytosolic and nuclear accumulation of Egr-1 in transfected HCT-116 cells respect to untransfected. Moreover, we observed that anti-miR-182 affects nuclear accumulation of Egr-1 in HT-29. In fact, western blotting data evidenced a reduction of cytosolic fraction and an increase of nuclear translocation of Egr-1 contemporaneously in HT29 transfected with anti-miR-182. Probably miR-182 has a regulatory role in this event that here we did not investigate (Fig.13). Furthermore, western blotting analysis on fractionated protein extracts revealed that nuclear abundance of Sp-1 increased in HT-29 cells transfected with anti-mir-182 respect to untransfected and negative control, whereas anti-miR-182 seems not affect Sp-1 nuclear accumulation in HCT-116 cell line (Fig.13).

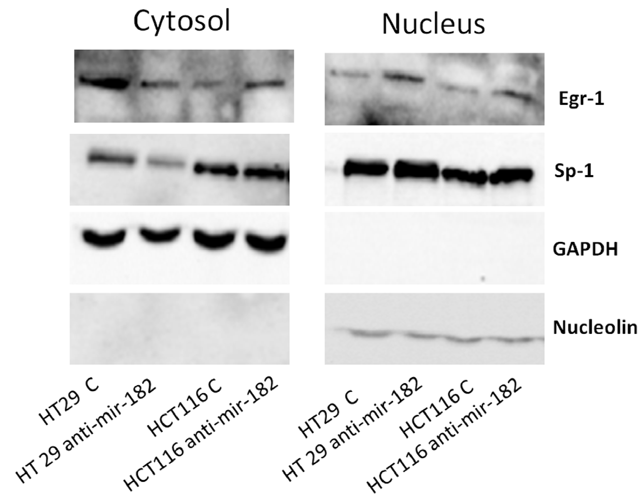

Figure 13: **Anti-mir-182 modulates Egr-1 and Sp-1 nuclear accumulation.** HT-29 and HCT-116 cells were transfected with anti-miR-182 or left untreated. Protein loading was controlled by re-probing WB filters for the expression of a nuclear marker nucleolin or cytosolic protein GAPDH.

Egr-1 and Sp-1 binding to TSP-1 promoter is influenced by anti-miR-182 in HT-29 and HCT-116 cells

Next, we assessed whether these factors bind specific consensus sequences into TSP-1 promoter (from -482 to +162) after transfection with anti-miR-182. Using ChIP assay, we found that the Egr-1 binding to TSP-1 promoter significantly increased by 21.3-fold in HCT-116 cells transfected with anti-miR-182 compared to untransfected cells. Although our data showed that anti-miR-182 induces the nuclear accumulation of Egr-1 in HT-29 cells, as before describe, we did not observe the Egr-1 recruitment on the consensus sequence into TSP-1 promoter in this cell line (Fig.14A). Since Sp-1 binds to two GC boxes lying between -267 and -71 at the 5'-flanking region of the TSP-1 gene [66], we assessed the recruitment of Sp-1 on TSP-1 sequence promoter after tranfection with anti-miR-182. Data indicated that Sp-1 bound by 4.4-fold TSP-1 sequence promoter in transfected HT-29 respect to control cells, on the contrary Sp-1 did not show significant binding TSP-1 consensus region both in transfected with anti-miR-182 and untranfected HCT-116 cells (Fig.14B).

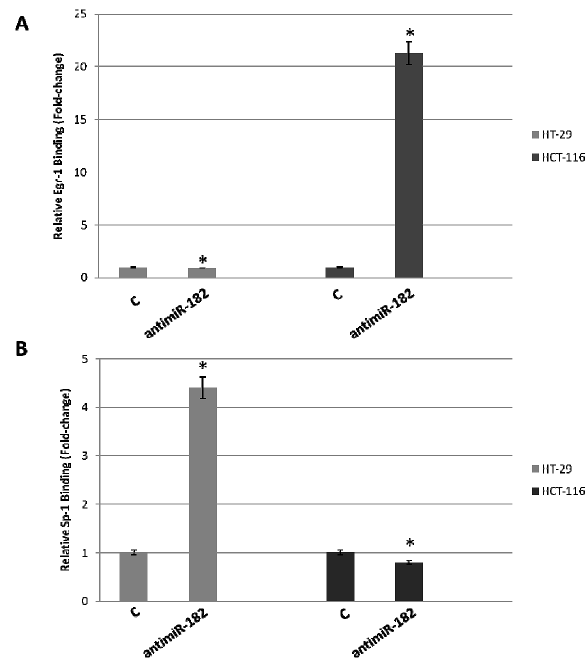

Figure 14: **Egr-1 and Sp-1 binding to TSP-1 promoter is modulated by miR-182 in HT-29 and HCT-116 cells.** A: Increased Egr-1 binding on TSP-1 promoter sequence in tranfected HCT-116 cells; Egr-1 is not recruited after tranfection with anti-miR-182 in HT-29. B: Increased Sp-1 binding on its consensus motif in HT-29 induced by anti-miR-182; Sp-1 is not loaded in HCT-116 cells.

Effects of anti-miR-182 on SMAD4 expression

It has been shown that the restoration of SMAD4 to human pancreatic carcinoma cells determines up regulation of mRNA of TSP-1. To test the possibility that miR-182 could target SMAD4 and control, on consequence, angiogenic activity influencing TSP-1 expression in colon cancer cells, we evaluated SMAD4 intracellular protein levels by WB.

The colon cancer cell line HT-29 has a known nonsense mutation in the SMAD4 gene and exhibited the loss of an entire 18q arm in the aCGH data. Thus, the HT-29 cell line was classified as SMAD4 loss. HCT-116 had no known coding mutations or significant genomic aberrations at the SMAD4 locus on Chromosome 18. This cell line was assumed to possess intact copies of the SMAD4 gene [78].

We found that SMAD4 increased in HCT-116 after transfection with anti-miR-182 respect to negative control and untransfected cells (Fig.15).

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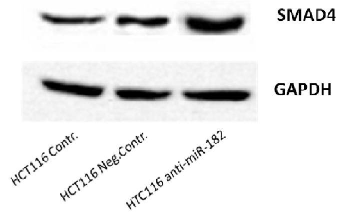

Figure 15: **The increased SMAD4 levels induced by anti-miR-182 determined**. The intracellular abundance SMAD4 was assessed by WB of 120 mg of total proteins using specific Abs. The picture shows the increased SMAD4 levels normalized respect to GAPDH. The pictures indicates the increase of SMAD4 protein levels relative to untrasfected and negative control cells

Discussion and Conclusions

TSP-1 has been shown to contrast angiogenesis by inhibiting endothelial cell migration [79], inducing endothelial cell apoptosis [80], antagonizing VEGF-mediated survival signaling [81], inhibiting VEGF mobilization by MMP9 [82], and directly binding VEGF [83]. In addition, TSP-1 may inhibit angiogenesis by decreasing the level of circulating endothelial cell progenitors. Down-regulation of TSP-1 in tumor cells is a frequent step toward the acquisition of an angiogenic phenotype.

Oncogenes such as Myc [84] and Src [85] inhibit TSP-1 expression, whereas oncosuppressor genes such as WT1 [86], PTEN [87], and Smad4/DPC4 [71]enhance TSP-1 expression.

Considering that several miRNA have been found to regulate the process of cancer metastasis independent of primary tumorigenesis [88], in this study, we described the effects of anti-miR-182 in the expression of TSP-1 in colon cancer cell lines. First, we assessed the expression levels of miR-182 and TSP-1 in HT-29 and HCT-116 cells. Data indicated an inverse correlation of expression that induced us to investigate whether contrasting the action of miR-182 could regulate TSP-1. For this purpose we silencing miR-182 using a synthetic oligonucleotide, anti-miR-182, that it targets the mature forms of miR-182 and thereby contrasts their expression and function in HT-29 and HCT-116. We found that the transfection with anti-miR-182 increased TSP-1 mRNA, intracellular e secreted protein. Moreover, supposing that anti-miR-182 could be affect TSP-1 expression by affects transcriptional factors expression that bind *TSP-1* gene promoter, we focused our attention on Egr-1 and Sp-1. Both are Cys_2-His_2–type zinc-finger transcription factors and binds to GC-rich, cis-acting promoter elements, in fact overlapping Sp-1 and Egr-1 sites are frequent [64]. In addition, Egr-1 and Sp-1 DNA binding capacities are regulated by phosphorylation.

Our results, obtained after transfection, indicated that anti-miR-182 could influence nuclear accumulation both Egr-1 and Sp-1 in HT-29, whereas anti-miR-182 could affects nuclear translocation of Egr-1 only in HCT-116 cells. Subsequent ChIP analyses revealed an increased Sp-1 binding on proximal promoter region of TSP-1 gene as a consequence of the its increased nuclear accumulation in HT-29, on contrary we did not observe Egr-1 recruitment despite of its major nuclear amount. Moreover we found an increased Egr-1 loading on TSP-1 promoter in HCT-116 transfected with anti-miR-182, and we did not observe the recruitment of Sp-1, which not shows any variation between cytosolic and nuclear levels.

Sp-1 increases the amount of Egr-1, which then becomes a potent competitor of Sp1 for binding to consensus sequence [64]. Since that Egr-1

inhibits the trans-activation of Sp-1 on overlapping Sp-1/Egr-1 and that Sp-1 activity could be augment by Egr-1 at non-overlapping sites in the Egr1-gene promoter, we hypothesized a possible regulatory mechanism in which anti-miR-182 increased the nuclear amount both Egr-1 and Sp-1 in HT-29 cells, in this context probably there are not a competition between Egr-1 and Sp-1 to load consensus sequence because Sp-1 binds non over-lapping sites. Reduced levels of miR-182 make Sp-1 is the major transcriptional factor implicated in the TSP-1 expression without involvement of Egr-1 in HT-29 cells. On contrary, in HCT-116 cell line the molecular mechanism by which anti-miR-182 could indirectly modulate TSP-1 is based on the physical displacement of Sp-1 on DNA by Egr-1.

In human pancreatic cancer cells SMAD4 re-expression reduces tumor growth through diminished vascular supply decreasing the expression of VEGF and increased levels of the angiogenesis inhibitor TSP-1 causing the cells to switch from potently angiogenic to antiangiogenic in vitro and in vivo [71]. Here, we found that SMAD4 protein levels increased after transfection with anti-miR-182 in SMAD4-wild type colon cancer cells that we studied.

In this work we showed that anti-miR-182 mediates suppression of angiogenic activity through the induction of the angiogenesis inhibitor TSP-1 by two mechanism: regulating transcriptional events on *tsp-1* promoter and inducing SMAD4.

The fact that TSP-1 is a potent endogenous inhibitor of angiogenesis prompted several groups to explore therapeutic applications of TSP-1, endeavoring to identify strategies to increase endogenous TSP-1 and the delivery of recombinant TSP-1 repeats (TSRs) or synthetic peptides that contain sequences from TSRs. Future investigation are expected to reveal the molecular basis for the differing effects of TSP-1 on tumorigenesis in different tumor types, and to describe the molecular pathways for the regulation of TSP-1 by multiple tumor suppressor and oncogenes. In particular we focused our attention on miRNAs, small regulatory noncoding RNAs which regulate mRNA function and which play a crucial role in cancer, where they may act as oncogenes or tumor suppressor genes.

In this work we showed the potential regulatory role of anti-miR-182 on TSP-1 expression in colon cancer. In the light of these results that remark the involvement of miRNAs in cancer pathogenesis, miRNAs could become new targets for the development of novel therapeutic strategies to restore physiological expression levels to modulate indirectly their target expression.

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LAST THREE YEARS PHD CURRICULUM VITAE

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PROFESSIONAL CAREER

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- Academic year 2009/2010 and 2010/2011. **Biotechnologies applied at the diagnosis and therapy of the solid tumors** (Prof. A. Russo) – Teaching assistant
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- October 2008 - **Biomedicine Master's Degree (MD)** 110/110 summa cum laude – University of Palermo, Italy
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SCIENTIFIC ACTIVITIES

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- **January 2009 – October 2009:** Graduate student, Molecular Genetic and Oncology Unit - University of Palermo. Research in the following projects: "***Expression of angiogenic regulators, VEGF and leptin, is regulated by the EGF/PI-3K/STAT3 pathway in colorectal cancer cells***", "***miR-20b Modulates VEGF Expression by Targeting HIF-1a and STAT3 in MCF-7 Breast Cancer Cells***",. Tutor: Prof. Antonio Russo.

MEETINGS

- XXVII CONFERENZA NAZIONALE DI CITOMETRIA (GIC) – FERRARA, 14-16 OTTOBRE 2009
- 11° CONGRESSO NAZIONALE DEL GOIM "ONCOLOGIA 2009: LE TERAPIE CHE PROLUNGANO LA VITA E LA RENDONO MIGLIORE"- CATANIA 15-17 GIUGNO 2009
- CONGRESSO NAZIONALE DELLA SOCIETA' ITALIANA DI SENOLOGIA CLINICA – PALERMO, 7-8 MAGGIO 2009
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BOOKS, PAPERS AND ABSTRACTS PUBLISHED DURING THE PHD COURSE

Papers

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6. N. Margarese, M. Perez, L. La Paglia, L.R. Corsini, D. Fanale, M. Terrasi, **V. Amodeo**, L. Insalaco, S. Cimino, G.B. Damiani, L. Napoli, L. Bruno, V. Calo` , V. Bazan, A. Russo **Clinical significance of intronic variants of *BRCA* genes of sicilian patients with hereditary breast/ovarian cancers** Cancer Treatment Reviews 36S3 (2010) S95-S119

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22. L.R. Corsini , D. Fanale , L. Insalaco , L. La Paglia , M. Perez , M. Federico , C.E. Symonds , V. Bazan , E. Surmacz , A. Russo **The Proximal Leptin Gene Promoter is Regulated by Ppar-γ Agonist in MCF-7 and MDA-MB-231 Breast Cancer Cells** M. Terrasi , A. D'Andrea, **V.Amodeo** , ONCOLOGY 2009;77(SUPPL 1):13162

Appendix

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Analysis of molecular mechanisms and anti-tumoural effects of zoledronic acid in breast cancer cells

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Abstract

Zoledronic acid (ZOL) is the most potent nitrogen-containing bisphosphonate (N-BPs) that strongly binds to bone mineral and acts as a powerful inhibitor of bone resorption, already clinically available for the treatment of patients with osteolytic metastases. Recent data also suggest that ZOL, used in breast cancer, may provide more than just supportive care modifying the course of the disease, though the possible molecular mechanism of action is still unclear.As breast cancer is one of the primary tumours with high propensity to metastasize to the bone, we investigated, for the first time, differential gene expression profile on MCF-7 breast cancer cells treated with low doses of ZOL (10 μM). Microarrays analysis was used to identify, describe and summarize evidence regarding the molecular basis of actions of ZOL and of their possible direct anti-tumour effects.We validated gene expression results of specific transcripts involved in major cellular process by Real Time and Western Blot analysis and we observed inhibition of proliferation and migration through MTT and Matrigel assay. We then focused on changes in the cytoskeletal components as FN1, actin, and anti angiogenic compounds as TGF-β1 and THBS1. The up-regulation of these products may have an important role in inhibiting proliferation, invasion and angiogenesis mediated by ZOL.

Keywords: ZOL • FN1 • TGF-β1 • THBS-1 • invasion • breast cancer

Introduction

Breast cancer is the most frequently diagnosed cancer in women around the world and bone is its most common associated site of metastasis [1]. ZOL is a potent N-BPs, inhibitor of bone resorption that reduces the risk of skeletal complications and prevents treatment-induced bone loss [2]. In oncology, its role in metastatic bone disease is well established [3], but there is increasing interest in its potential role in preventing and treating cancer-induced bone loss and its possible anti-tumour effects [4].

N-BP shave been shown to inhibit the mevalonate pathway involved in the synthesis of cholesterol, through inhibition of the enzyme farnesyl diphosphate synthase. This process leads to the decreased production of the isoprenoid lipids farnesyl diphosphate and geranyl geranyl diphosphate both enzymes required for the prenylation of small GTP-ases, such as Rho, Rac, cdc42 and Rab. Small GTP-ases signalling regulates key cellular processes including proliferation, cell motility, angiogenesis, survival and migration, all mechanisms implicated in the development and spreading of many types of cancer including breast cancer [5–8]. Bisphosphonates, ZOL in particular, induce also tumour cell apoptosis and stimulate γδ T cell cytotoxicity against tumour cells. *In vivo*, ZOL inhibits bone metastasis formation and reduces skeletal

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tumour burden in mouse models. This may reflect direct antitumour effects and indirect effects *via* inhibition of bone resorption. In addition, ZOL inhibits experimental angiogenesis *in vitro* and *in vivo* [9]. Data from *in vitro* and pilot studies suggest that ZOL reduces circulating levels of vascular endothelial growth factor (VEGF) in metastatic breast cancer patients [10, 11], suggesting these drugs could interfere with tumour-associated angiogenesis. Evidence *in vivo* already exists that ZOL treatment inhibits tumour-associated angiogenesis by inducing a profound reduction in macrophages infiltrating mammary or cervical carcinoma lesions, associated with decreased VEGF and matrix metalloprotease-9 (MMP-9) levels in the tumour microenvironment [12]. Interactions of cells with their surroundings can have profound influences on gene expression and cellular behaviour [13–15].

Angiogenesis and regulation of tumour environment is essential for cancer growth and progression, and therefore, anti-angiogenesis is one promising strategy to treat cancer [16]. Numerous anti-angiogenic factors have been described as transforming growth factor β-1 (TGF-β1) and relative TGF-β1/SMAD (small mother against decapentaplegic) signalling pathway plays an important role in cancer cells and leads to growth inhibition, differentiation and apoptosis [17]. The TGF-βs represents a family of multifunctional cytokines that modulate the growth and function of many cells, including those with malignant transformation. Their signalling pathways are frequently involved in suppressing the growth of human tumours [18]. Recent data suggest that activation of the TGF-β pathway leads to the induction of apoptosis closely followed by the induction of cytostasis, resulting in different carcinoma regression [19, 20]. An important natural activator of TGF-β1 is Thrombospondin 1 (THBS1), a trimeric glycoprotein strongly bound to the extracellular matrix (ECM) [21] and a potent natural inhibitor of angiogenesis [22]. Its ability to block migration of endothelial and cancer cells *in vitro* has been shown to be independent of the activation of TGF-β1 [23, 24]. THBS1 affects ECM structure and function both through direct interactions and indirect effects on other components that are secreted by the cell [25]. Consider that cell adhesion to ECM is crucial to several steps in tumour progression and metastasis, many studies have demonstrated that THBS1 mediates cellular adhesion of numerous cell types and several transformed cell lines [24, 26]. Inhibition of angiogenesis is also a consequence, in part, of re-organization of the actin cytoskeleton and disassembly of focal adhesions in endothelial cells and to inhibit cellular motility, cellular migration and invasion [27].The molecular and physical composition of the ECM can be affected by tumour cells themselves, as well as multiple stromal cell types. Alterations in the expression of ECM-related genes have been identified in gene expression signatures related to poor prognosis and metastases in breast cancers. Indeed, changes in the cytoskeletal components such as production and organization of fibronectin (FN1), actin and collagen have been implicated in eliciting the transition from dormancy to metastatic growth [3, 28–32].

Consequently, we studied the potential mechanisms by which ZOL may regulate global gene expression profile, cellular proliferation, invasion and angiogenesis in MCF-7 breast cancer cells, an ideal model of bone metastatizing cells [33], centering our discussion on FN1, actin, TGF-β1 and THBS1, proteins with a central role respectively on cytoskeletal re-organization, cellular motility, invasion and angiogenetic process.

Materials and methods

Cell culture

Human breast cancer cell lines, MCF-7, purchased from the American Type Culture Collection (Rockville, MD, USA) were grown in Dulbecco's modified Eagle's medium Gibco DMEM:F12 (Invitrogen, Carlsbad, CA, USA) containing 10% foetal bovine serum (FBS) and 1% Penicillin/Streptomycin (P/S) (Gibco). Cells were incubated at 37°C in a humidified atmosphere of 5% of CO_2. Eighty per cent confluent cultures were stimulated with either 10 μM of ZOL for 24, 48 and 72 hrs. ZOL was kindly provided by Novartis Pharma AG. The stock solution of ZOL was prepared at a concentration of 4 mg/ml in distilled water, and aliquots were stored at −20°C.

Cell growth assays

Seventy per cent confluent cultures were treated with 10, 50 and 100 μM of ZOL. Cell numbers before and after 1, 2 and 3 days of treatment were determined by counting the cells. All assays were done in triplicate and repeated at least twice.

Cell viability assay

Cell viability in human breast cancer cell lines, MCF-7, treated with 10, 50 and 100 μM of ZOL for 24 hrs, was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described in literature [34] with minor modifications. Briefly, MCF7 cells were seeded in flat-bottomed 96-well plates at a density of 10,000 cells/well. Twenty-four hrs later, growing cells were washed and treated for 24 hrs with the ZOL (10 and 100 μM). Cell viability was measured using MTT at a concentration of 0.5 mg/ml (20 μl/well). After 1 hr incubation at 37°C, cells were solubilised in DMF (Dimethyl formamide) solution (DMF/H_2O, 1:1, pH 4.7) containing 20%SDS for an additional incubation time of 16 hrs at 37°C to dissolve the blue formazan product. Optical density was measured at 570 nm using a 96-well plate reader (EL800 Biotek Instruments). All the experiments were run in sextuplicate and repeated twice.

Microarray analysis

Total cellular RNA was isolated from MCF-7 cells treated with ZOL (10 μM) for 24 hrs using the miRNeasy Mini Kit (Qiagen Inc, Valencia, CA, USA) and quantified through 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Five micrograms of total RNA was reverse transcribed using the high capacity cDNA archive kit (Applied Biosystems) according to vendor's instructions.

Then cDNAs were *in vitro* transcribed for 16 hrs at 37°C using the IVT Labelling Kit (Affymetrix) to produce biotinylated cRNA. Labelled cRNA was isolated using the RNeasy Mini Kit column (QIAGEN). Purified cRNA was fragmented to 200–300 mer using a fragmentation buffer. The quality of total RNA, cDNA synthesis, cRNA amplification and cRNA fragmentation was monitored by capillary electrophoresis (Bioanalizer 2100; Agilent Technologies). Fifteen micrograms of

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fragmented cRNA was hybridised for 16 hrs at 45°C with constant rotation, using a human oligonucleotide array U133 Plus 2.0 (Genechip; Affymetrix, Santa Clara, CA, USA). After hybridization, chips were processed using the Affymetrix Gene Chip Fluidic Station 450 (protocolEukGE-WS2v5_450). Staining was made with streptavidin-conjugated phycoerythrin (SAPE) (Molecular Probes), followed by amplification with abiotinylated anti-streptavidin antibody (Vector Laboratories), and by a second round of SAPE. Chips were scanned using a Gene Chip Scanner 3000 G7 (Affymetrix) enabled for High-Resolution Scanning. Images were extracted with the Gene-Chip Operating Software (Affymetrix GCOS v1.4). Quality control of microarray chips was performed with the AffyQCReport software [35]. A comparable quality between microarrays was demanded for all microarrays within each experiment.

Microarray statistical analysis

The background subtraction and normalization of probe set intensities was performed with the method of Robust Multi array Analysis described by Irizarry *et al.* [36]. To identify differentially expressed genes, gene expression intensity was compared using a moderated test and a Bayes smoothing approach developed for a low number of replicates [37]. To correct for the effect of multiple testing, the false discovery rate was estimated from *P*-values derived from the moderated *t*-test statistics [38]. The analysis was performed with the affylmGUI Graphical User Interface for the limma microarray package [39].

Matrigel invasion assay

The invasive potential of breast cancer cells was assessed *in vitro* in matrigel-coated invasion Chambers (BD BioCoat Matrigel Invasion Chamber; Becton Dickinson Biosciences, Franklin Lakes, NJ, USA) in accordance with the manufacturer's instructions. Cell invasion experiments were performed with a 24-well companion plate with cell culture inserts containing 8 um pore size filters. Untreated MCF-7 cells and drug-treated MCF-7 cells with ZOL 10 μM for 24 and 48 hrs (5×10^4/500 μl) were added to each insert (upper chamber), and the chemoattractant (FBS) was placed in each well of a 24-well companion plate (lower chamber). After 22 hrs incubation at 37°C in a 5% CO_2 incubator, the upper surface of the filter was wiped with a cotton-tipped applicator to remove non-invading cells. Cells that had migrated through the filter pores and attached on the under surface of the filter were fixed and stained by Diff-Quik staining kit (BD, Becton Dickinson Biosciences). The membranes were mounted on glass slides, and the cells from random microscopic fields (×40 magnification) were counted. Five fields per membrane were randomly selected and counted in each group. All experiments were run in duplicate, and the percentage of invasive cells was calculated as the percentage invasion through the matrigel membrane relative to the migration through the control membrane, as described in the manufacturer's instructions.

Real time-quantitative PCR (Q-PCR)

Total cellular RNA was isolated from MCF-7 cells treated with ZOL (10 μM) for 24hrs using the miRNeasy Mini Kit (Qiagen Inc) and quantified through 2100 Bioanalyzer (Agilent Technologies). Five micrograms of total RNA was reverse transcribed using the high capacity cDNA archive kit (Applied Biosystems), according to vendor's instructions. Five microlitre of the RT products was used to amplify FN1 (hs01549976_m1), ACTIN (hs99999903_m1), TGF-β1 (hs00998133) and Trombospondin 1 (THBS1) (hs00962914) sequences using the TaqMan gene expression assay (Applied Biosystems). To normalize quantitative real-time PCR reactions, parallel TaqMan human Cyclophilin (4326316E) control reagents assays (Applied Biosystems) were run on each sample. Changes in the target mRNA content relative to control mRNA were determined using a comparative Ct method to calculate changes in Ct, and ultimately fold and percent change. An average CT value for each RNA was obtained for triplicate reactions.

Western blotting (WB)

The cells were treated with 10 μM ZOL for 24, 48, 72, and also 96 hrs or left untreated, and then were lysed to obtain total proteins using complete Lysis-M reagent (Roche, Mannheim, Germany)Protein concentration was determined by the Bradford method and the expression of proteins was analyzed in 150 μg of total protein lysates. Proteins were separated on a gel with 8 and 10% polyacrylamide under denaturing conditions and transferred by electrophoresis to a nitrocellulose membrane. Nonspecific binding was blocked by soaking membranes in 1 × TBS, 5% powdered milk, and 0.05% Tween-20 for at least 60 min. at room temperature. Membranes were incubated with the following primary antibodies: p44/42 MAPK (Erk1/2) mouse mAb# 9107 (at 1:2000), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (197G2) Rabbit mAb #4377 (at 1:2000), Akt1 (2H10) Mouse mAb #2967 (at 1:100) and Phospho-Akt (Ser473) (193H12) Rabbit mAb #4058 (at 1:1000) were from Cell Signalling Technology, Fibronectin antibody, Rabbit polyclonal antibody to FN1 GTX112794 (at 1:1000), beta Actin [AC-15] antibody, mouse monoclonal GTX26276 (at 1:5000) and TGF beta [TB21] antibody, mouse monoclonal GTX21279 (at 1:1000) were from Gene Tex, Inc., Smad4 (MAB1132 at 1 μg/mL) and anti-Smad2/3 (#07-408 at 1:500) were from Millipore Corporation, THBS1 mouse monoclonal and GAPDH (6C5): sc-32233 were from Santa Cruz Biotechnology. After the membranes were washed three times with TBS plus 0.05% Tween-20 for 30 min., they were incubated with the following peroxidase (HRP)-conjugated secondary: anti-rabbit, anti-mouse and anti-goat antibody (2030; Santa Cruz Biotechnology) diluted to 1:1000, followed by three washes with TBS plus 0.05% Tween-20. Detection was performed with chemiluminescence detection reagents (ECL; Pierce Biotechnology Inc., Rockford, IL, USA).

Results

ZOL inhibit breast carcinoma cells proliferation

To identify the lower dose of ZOL sufficient to induce an anti-proliferative effect on MCF-7 cell proliferation, we tested different concentrations (10–100 μM) of ZOL for 24, 48 or 72 hrs.Cell count showed that cell growth was inhibited by ZOL *versus* control at all concentrations used (Fig. 1). In particular, tumour cell growth was reduced to about 40% at a ZOL concentration of 100 μM over a period of incubation of 24 hrs whereas the lower ZOL concentration (10 μM) was slightly less efficient (20%), but effective. Consider that inhibition

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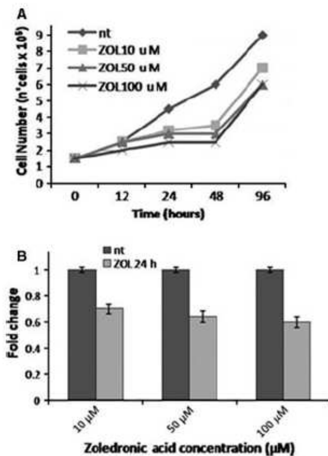

Fig. 1 A, B ZOL inhibits cell growth *in vitro*. About 10^5 and 10^4 cells were cultured in 6-well and 96-well tissue culture plates and exposed to ZOL at a concentration ranging from 10 to 100 µM for different times. Cellular viability was analyzed by cellular count (**A**) and MTT assay (**B**).

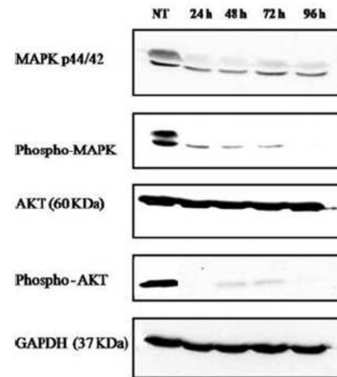

Fig. 2 Effects of ZOL addition on MAPK and Akt-dependent pathways on MCF-7 cells. Cells were treated with 10 µM ZOL for 24, 48, 72 and 96 hrs. Thereafter, both the expression and activity of MAPK p44/42 and AKT were evaluated. Determination of the expression and phosphorylation of MAPK p44/42 and AKT evaluated as described in 'Materials and Methods'. Expression of the house-keeping protein GAPDH, used as loading control.

rates of 10 and 100 µM of ZOL were not shown a significant difference, we can assert that the treatment at lower concentration for only 24 hrs is sufficient to induce an inhibition of proliferation, also confirmed by determination of number of metabolically active cells by MTT assay (Fig. 1 A and B). On the basis of these data, we have selected this concentration of ZOL for all the subsequent experiments.

To elucidate the mechanisms by which cell proliferation is suppressed, we have analysed the effects of ZOL on specific proliferative pathways. Time-course experiments were performed using WB to determine phosphorylation and thus activation of MAPK and AKT pathways. We found that phosphorilation of MAPK and AKT was decreased significantly after both 24 hrs and 48 hrs exposure to 10 µM ZOL (Fig. 2).

Thus, as expected and previously reported with higher doses [40], also low doses of ZOL induced decrease of both MAPK and Akt activity, by which ZOL inhibits the cell proliferation and the ability of tumour cells to expand once they colonize bone [41–44].

Gene expression profile of MCF-7 breast cancer cells treated with low doses of zoledronic acid

The main aim of this study was to investigate the molecular mechanisms by which low doses of ZOL exert their antitumour effects in breast cancer cells. Though ZOL have clearly demonstrated to inhibit proliferation and induce apoptosis in cancer cell lines by interfering with the mevalonate pathway [5–8], the type and pattern of downstream genes modulated by ZOL treatment are still unknown.

To investigate molecular basis of anti-tumoural effect of low doses of ZOL on breast cancer cells, we have evaluated the expression profiling of MCF-7 treated with 10 µM of ZOL for 24 hrs *versus* untreated, using a cDNA microarray platform Affymetrix. Of the 33,000 independent features on the microarrays, 126 were found to be differentially expressed after 24 hrs of treatment. In particular, 17 genes were downregulated (−1.57 to −2.88), and 109 genes were upregulated (+1.52 a +5.27). We considered for following analysis only the genes with fold change >2 as significative upregulation or downregulation and with statistical difference of expression of each gene was at least $P < 0.001$ (Fig. 3A).

We grouped genes related to biological process, molecular function categories and finally in cellular component categories, that have changed in a statistically significant manner (P-value ≤ 0.05) after treatment with ZOL (Fig. 3 B, C and D). The most significant changes in biological processes confirmed the involvement of ZOL in metabolic processes, in fact 38 genes are differentially regulated. Other changes were observed in the cellular localization (24 genes regulates), cell communication (20 genes regulated) and in cell proliferation pathways (eight genes) (Fig. 3B).

Analysis also showed a regulation of molecular function categories, as protein (37 genes) and ion binding (27 genes), and transporter activity (11 genes) affected by ZOL (Fig. 3C). Cellular

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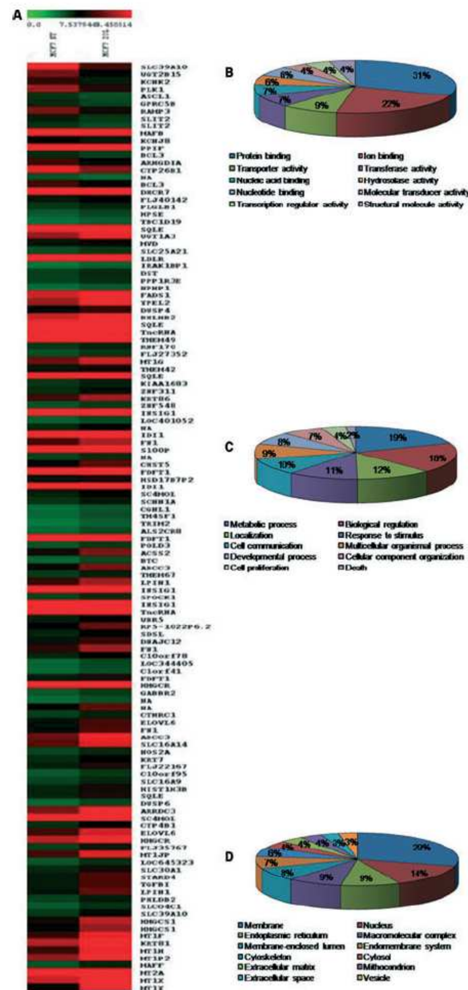

Fig. 3 (**A**) Treatment with ZOL globally affects gene expression profile in MCF-7 cells. (**B, C, D**) Corrected microarray signal values of genes involved in different biological process, clustered by specific functions (Biological process, Cellular function, Cellular component) of MCF-7 cells treated for 24 hours with 10 μM ZOL in comparison to control cells.

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component categories that result differentially regulated by gene expression profile comprend membrane and cytoskeletal components (48 genes), nucleus (19 genes) and endoplasmic reticulum components (13 genes) (Fig. 3D).

Alterations in gene expression identified by microarray analysis show modification of possible early-response genes as the treatment with ZOL was carried out for only 24 hrs, and were further investigated by real-time quantitative reverse transcription-PCR.

Effects of ZOL on breast carcinoma cells invasion

In light of previous observation, we hypothesized that the inhibitory effect of ZOL on cellular growth and deregulation of cytoskeletal component observed by analysis of gene expression, could result in inhibition of tumour cell invasion. To address this question, alterations in gene expression, identified by microarray analysis, were further investigated by real-time quantitative reverse transcription-PCR and WB analysis to investigate whether alterations in gene expression were translated into corresponding changes in protein levels.

We found that treatment with ZOL induces transcription and protein expression of some matrix and cytoskeletal components, such as Fibronectin and actin, involved in cancer microenvironment. In particular, the up-regulation of gene coding for FN1 shown by microarray (fold change of 1.93) was confirmed by Real Time RT-PCR with a fold change of 2.3 compare with control (Fig. 4A) and mRNA expression of actin, analysed by Real time RT-PCR, showed a fold change of 1.5. Interestingly, a high protein expression is maintained even at longer treatment (at 96 hrs), and with the most activating effect in the protein products, indicating the potential consequences of ZOL treatment on the morphology and cell motility, considered the cellular roles of FN1 and actin as factors that can change the ECM (Fig. 4B).

Then, the effects of ZOL, on the *in vitro* invasion of MCF-7 were investigated by Matrigel assays. We observed that cells treated even with only 10 μM of the drug, resulted in a reduction in invasion in a time dependent manner, reaching 60–90% inhibition after 24 hrs (Fig. 5).

These results demonstrate that ZOL treatment has a strong inhibitory effect not only on MCF-7 cells growth but also on invasiveness and that possibly the alteration of FN1 and actin expression, could be involved in invasion of human breast cancer cell lines.

ZOL increases expression of anti angiogenetic factors in breast carcinoma cell lines

ZOL can inhibit angiogenesis of tumour cells and emerging evidence suggests that the use of this agent may impede the development of bone metastases [45, 46].THBS1, TGF-β1 and its signalling effectors regulate many aspects of tumour cell biology, such as growth arrest and cell motility, the latter of which is important for the metastatic dissemination of tumour cells from their primary location to lymph or blood vessels [47–49].

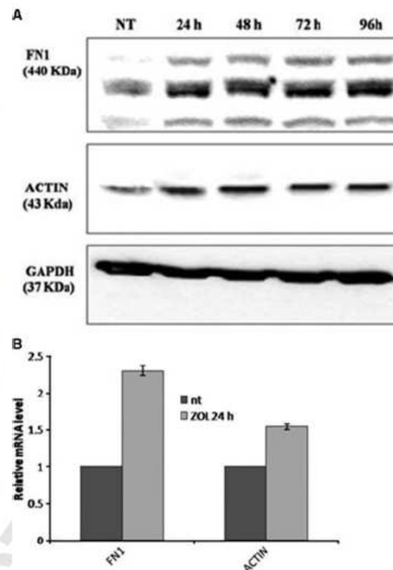

Fig. 4 Effect of ZOL on the mRNA expression and protein levels of FN1 and ACTIN. (**A**) Effect of ZOL 10 μM on the mRNA expression of FN1 and ACTIN, as quantified by real time PCR in MCF-7 cells. (**B**) Effect of ZOL on FN1 and ACTIN protein levels. MCF-7 cells were incubated with low concentration of ZOL for different times, and protein expression were examined by Western blot developing with the enhanced chemoluminescence reagent (ECL). Each membrane was also probed with GAPDH to confirm equal loading.

To investigate the effects on angiogenesis induced by low dose of ZOL, we observed specific mRNA expression and protein levels of TGF-β1and THBS1, to confirm overexpression observed by microarrays analysis, in particular, TGF-β1showed a fold change of +2.3 and THBS1 a fold change of +2.6 compare with untreated control. After only 24 hrs exposed to ZOL, both transcription and protein expression was significantly increased (Fig. 6), indicating possible implication of these two protein in anti-angiogenetic process mediated by low doses of ZOL.

Moreover, as classic TGF-β signalling involves the activation of Smad2/3 and Smad4, direct mediators that accumulate into the nucleus, we examined Smad expression using WB method. Smad complexes interact with transcription factors, co-activators and co-repressors where they participate in the regulation of different target gene expression [50].

Treated MCF-7 cells exhibited a substantial increase in Smad 2/3 at 24 hrs whereas Smad4 peaked at 24 hrs and began to decrease

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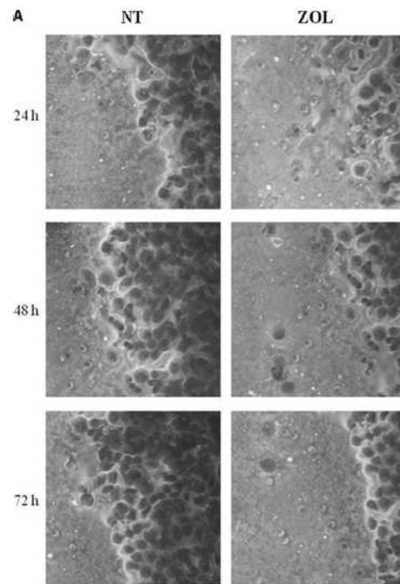


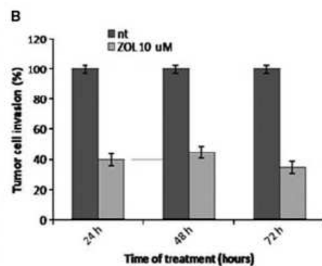
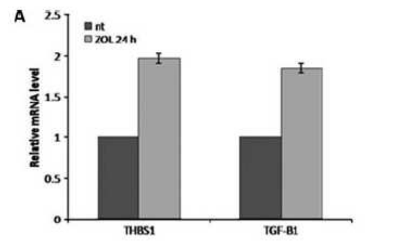


Fig. 5 ZOL decrease the invasive potential of human breast cancer cells. Effect of ZOL on the invasion of MCF-7 cells. Treated or not with ZOL 10 μM for 24 hrs, were plated onto Matrigel invasion chambers as described in 'Materials and Methods', and the cell invasion was evaluated. The invaded cells for each insert were stained and quantified. The results are expressed as a percentage of MCF-7 not treated cells (**B**). The experiments were performed at least three different times, and the results were always similar. Data are represented as percentage of control (100%).



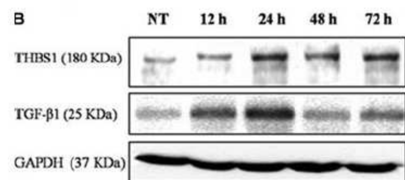


Fig. 6 Effect of ZOL on the transcript and protein levels of THBS1 and TGFβ1. A. mRNA expression of THBS1 and TGFβ1, as quantified by real time PCR in MCF-7 cells treated. (**B**) Effect of ZOL on THBS1 and TGFβ1 protein levels. MCF-7 cells were incubated with low concentration of ZOL for different times, and protein expression were examined by Western blot. The house-keeping protein GAPDH was used as loading control. The experiments were performed at least three different times, and the results were always similar.

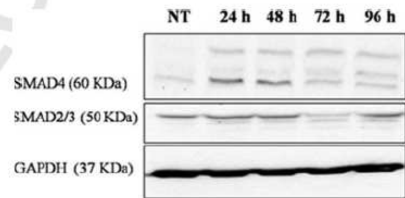


Fig. 7 Effects of ZOL on TGF-β1-dependent pathway and Smad protein expression. Cells were treated with 10 μM ZOL for 24, 48, 72 and 96 hrs. Determination of the expression SMAD2/3 and SMAD4 evaluated after blotting with specific antibodies, as described in 'Materials and Methods'. Expression of the house-keeping protein GAPDH, used as loading control. The experiments were performed at least three different times, and the results were always similar.

after 72 hrs (Fig. 7), indicating that MCF-7 cells possibly contain sufficient quantities of receptors and Smads to signal in response to TGF- β 1 (Fig. 7).

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Discussion

Preclinical studies have demonstrated that ZOL can inhibit proliferation, invasion, migration and angiogenesis of tumour cells. Emerging evidence also suggests that the use of this agent may impede the development of bone metastases in mouse models [45, 46]. The mechanism by which ZOL exerts its anti-cancer properties have already been investigated, and its direct effect on cancer cells, as well as the inhibitory effect on tumour angiogenesis, has been confirmed [51, 52]. Several studies have demonstrated that, *in vitro*, the binding of breast and prostate cancer cells to bone surfaces is inhibited by ZOL, that this treatment also has an inhibitory effect on cell proliferation and that a decrease of cellular migration was observed when prostate and breast cancer cell lines were cultured with ZOL [4, 53, 54]. This mechanism seems to be mediated by the effects on the cytoskeleton through Rho A [5].

The main aim of our study was to investigate the molecular mechanisms by which ZOL exerts its antitumour effects in breast cancer cells by Microarray analysis.

To identify the lower dose of ZOL sufficient to induce a moderate anti-proliferative effect on MCF-7 cell proliferation, we first performed cell proliferation assays, by cellular count and MTT. We tested different concentrations (10-100 μM) of ZOL for 24, 48 or 72 hrs, and we found that the highest inhibitive rate reached to nearly 50%. Considering that 10 μM of ZOL had shown a sufficient inhibitory effect, we have selected this concentration of ZOL for all the subsequent experiments.

Data obtained from observation of the activation of major cellular pathways are indicative of mechanisms by which this drug is able to block cellular proliferation. In particular, we confirm, also with low doses, the inhibition of the phosphorylation state of AKT and MAPK protein [40], responsible for key cellular pathways.

To deeply investigate the molecular mechanism by which ZOL acts as antitumour drug, we have performed a gene expression profiling of MCF-7 breast cancer cells treated with low doses of ZOL, and we have demonstrated that ZOL induce differential expression of 126 genes with a strongly up-regulation of different cytoskeletal and ECM component. Based on these results, we also hypothesized that low concentrations of ZOL may affect the processes of invasiveness in cancer cells by altering their ability to invade the tumour microenvironment and thus inhibit their metastatic potential.

As tumour cell invasion requires both cell migration and digestion of the basement membrane, we hypothesized that ZOL inhibited MCF-7 tumour cell invasion was mostly dependent on the cell surface activity driven by FN1 expression and on remodelling of cytosckeletal components. Several studies suggest that FN1 is related to tumour invasion and metastasis [55, 56] playing a key role in the tissue remodelling and cell migration events that occur during normal development; it has been thought to have an important role in both tumour invasion and metastasis. In particular, FN1 is a major constituent of the cell surface of many cultured cells, and it is either eliminated or reduced on the surface of oncogenically transformed cells [55]. Many reports have suggested that there is a correlation between the loss of cell surface FN1 and the ability of a cell to metastasize [44].

In our study, after treatment with10 μM of ZOL, FN1 and actin result up-regulated both by Real Time RT-PCR and WB, indicating their possible involvement in cytoskeletal re-organization induced by ZOL.

On the basis of these considerations, we have performed a Matrigel assay of MCF-7 breast cancer cells treated with ZOL at 10 μM for 24 hrs, and we have demonstrated that ZOL strongly inhibits invasion of these cells. These data agreed with some earlier research *in vitro* [18, 56]. However, the regulatory mechanism of FN1 expression of breast carcinoma is not clear. It is thought it could be regulated by a variety of growth factors such as TGF-β1 frequently involved in suppressing the growth of human tumours [18].

In fact our analysis confirmed that ZOL treatment have induced an up-regulation of transcription and of protein product of TGF-β1, letting us to speculate its involvement in transcriptional control of FN1. As classic TGF-β signalling involves the activation of Smad2/3 and Smad4, we also demonstrated that ZOL induce, at 24 hrs, an increase of Smad2/3 and Smad4 as direct mediators of TGF-β signalling in final activation of anti angiogenetic effects of ZOL. ZOL can also inhibit angiogenesis of tumour cells and emerging evidence suggests that the use of this agent may impede the development of bone metastases [45, 46].

We also found that low dose of ZOL, increased expression of THBS1, a factor involved in the angiogenesis process [55, 56], but also in the regulation of FN1 and actin. THBS1, TGF-β1 and its signalling effectors regulate many aspects of tumour cell biology, such as growth arrest and cell motility, the latter of which is important for the metastatic dissemination of tumour cells from their primary location to lymph or blood vessels [47, 48].

Finally, our results suggested that ZOL showed anti-proliferative and anti-invasive effects in MCF-7 cells and that these data may depend on the activator effect of ZOL in the expression of ECM, cytoskeletal component, and anti-angiogenc factors found in this study. On the basis of this preliminary results *in vitro*, it could be interesting to develop molecular therapeutic strategies based on the specific activation of the expression of particular component for inhibit tumoural growth and angiogenesis, or to evaluate in particular specific roles of FN1 and actin, blocking their expression, in inducing effect antiproliferative and anti-invasive of ZOL in human breast cancer cells.

This study strongly encourage the new experimental design for treatment of breast cancer based on administration of ZOL and to discover their target molecular in cancer cells for future more effective synergistic treatments.

In conclusion, in the present studies, we investigated the role of ZOL in the regulation of breast cancer cell invasion. Our results demonstrated that ZOL, *via* cytoskeletal remodelling, plays an inhibitory role in breast cancer cell invasion, possibly by specifically up-regulating the TGF-β1/Smad signalling pathway, and the downstream activity of FN1 and ACTIN.

On the basis of these results, future work has been hypothesized, it could be interesting to develop molecular therapeutic strategies based on the specific regulation of expression and/or function of cytoskeletal components.

Therefore, as subject of the next experiments, will be evaluated the activity of ectopic regulation of FN1 mRNA expression to study effective potential antiproliferative and anti-invasive of ZOL in human breast cancer cells, focusing more attention on the other factors or

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protein families that influence invasive potential of MCF-7 tumour cells in particular.

Finally, these data strongly encourage the design of clinical trials based on the concomitant administration of ZOL and ectopic additional expression of matrix proteins for efficacy testing.

Conflicts of interest

There are no conflicts of interest in relation to this work.

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Review

Expert Opinion

1. Introduction
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The role of microRNAs in cancer: diagnostic and prognostic biomarkers and targets of therapies

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Introduction: miRNAs are noncoding RNAs that target specific mRNA with subsequent regulation of particular genes, implicated in various biological processes. In cancer, miRNAs could show a different expression from normal tissues. miRNAs have a role as oncogenes when they target tumor suppressor genes and similarly they are tumor suppressors when they target oncogenes.
Areas covered: In this review, areas covered include the role of miRNAs in cancer diagnosis, prognosis and research for achievement of therapeutic strategies implicating miRNAs in oncology. As biogenesis of miRNAs is fundamental to understand their usefulness, this has also been discussed. Both miRNA expression profiles in cancer tissues and miRNA levels in peripheral blood were studied for improvement in the management of cancer patients.
Expert opinion: miRNAs have the potential for better understanding of tumor biology, but could also provide clinical advancement in management and therapy of various malignancies. The possibility of miRNA detection in peripheral blood would allow an eager expansion of their application in various clinical settings for cancer. The applicability of miRNA expression profiles still needs to be defined.

Keywords: biomarkers, cancer, miRNAs, therapy

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1. Introduction

MicroRNAs (miRNAs) are small, endogenous noncoding RNAs, 21 to 24 nucleotides (nt) long, which induce posttranscriptional gene silencing, recognizing their target mRNAs by base complementarity [1]. MiRNA genes are encoded in introns or exons of a protein-coding gene or in the intergenic regions, and it has been estimated that they regulate up to 30% of human genes [2]. miRNAs are key regulatory molecules involved in regulation of a wide variety of fundamental cellular processes, such as proliferation, death, differentiation, motility and invasiveness [3]. Aberrant expression of miRNAs has been observed in a diversity of pathological events. Importantly, deregulation or genetic changes of miRNAs have been critically implicated in the pathogenesis of most human cancer [4]. The knowledge of mechanisms underlying the role of miRNAs in cancer initiation and progression provided a tool to create different strategies for using miRNAs as potential targets for cancer treatment.

1.1 Biogenesis of miRNAs

MiRNA biogenesis in the human cell is a multistep complex process, which begins in the nucleus where miRNA genes are transcribed by RNA polymerase

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Article highlights.

- miRNAs have a crucial role in cancer biology.
- OncomiRs and anti-oncomiRs represent their role as oncogenes or tumor suppressor genes, respectively.
- Aberrant miRNA expression could be exploited for cancer diagnosis.
- Differential expression pattern in tissue and body fluids helps to define prognosis in cancer patients.
- The application of biotechnologies to miRNAs is providing new therapeutic options.

This box summarizes key points contained in the article.

II into long primary miRNAs (pri-miRNAs), which have stem and terminal loop structure with flanking segments [2]. Pri-miRNAs are subsequently cleaved into smaller, stem-looped, hairpin-like miRNA precursor (pre-miRNA) of ~ 70 nt in length by RNase III-type enzyme Drosha that form a microprocessor complex with its binding partners DGCR8. Pre-miRNAs are exported from the nucleus into the cytoplasm by Exportin-5. In the cytoplasm, pre-miRNAs are then cleaved by RNase III-type enzyme Dicer and its binding partners, the transactivator RNA-binding protein (TRBP), to generate a 19- to 23-nt RNA duplex, which contains both the mature miRNA strand and its complementary strand. The mature miRNA strand is preferentially incorporated into a microRNA-induced silencing complex (miRISC), while the other strand of miRNA is degraded by the RISC. The miRNA strand guides the RISC to its mRNA target, containing complementary sequence to the mature miRNA and subsequently induces the cleavage or silencing of the target mRNA [5]. The complementarity between miRNA and its target mRNA is a crucial factor for the posttranscriptional regulatory mechanism induced by miRNAs.

miRNAs imperfectly complementary to the binding site in the 3' UTR (untranslated regions) of their mRNA target repress the protein expression by translation inhibition, whereas miRNAs perfectly complementary to mRNA target promote its degradation [2].

1.2 Aberrant expression of miRNAs in cancer

miRNAs are involved in the regulation of different biological processes such as development, differentiation, apoptosis and proliferation. Several studies confirmed the important role of deregulated expression of miRNAs in the pathogenesis of different human cancers and a spectrum of cancer-associated miRNAs have been found in various types of cancer cell lines and clinical tumor specimens [6,7]. Aberrant expression of miRNAs in cancer is correlated to different mechanisms, which include chromosomal abnormalities, genomic mutations, polymorphism, epigenetic changes and alteration in miRNA biogenesis [8], which can play a substantial role in carcinogenesis. Almost 50% of human miRNA genes are frequently located at fragile site and genomic regions involved in cancers [6] and high frequency of genomic alterations in miRNA loci was observed in human melanomas, ovarian and breast cancer using high-resolution array-based genomic hybridization [9,10].

The possibility to evaluate cell-free miRNAs in serum and other body fluids was reached in 2008. Circulating cell-free miRNAs are stable under various conditions. It was hypothesized that passive release occurs during tissue injury, for example, after myocardial infarction. The same mechanism could also be valid for cancer. In fact cell lysis due to rapid proliferation might contribute to release of miRNAs in the peripheral blood. Alternatively, miRNAs may be included in small particles, such as exosomes, which protect them against RNase activity. miRNAs are transported in exosomes and can exert gene silencing in recipient cells. Cells are able to actively secrete endogenous miRNAs [11]. Their role has not yet been clearly defined.

1.3 miRNAs as oncogenes and tumor suppressors

miRNAs are involved in crucial biological processes and their deregulated expression may lead to several pathological events [12]. Because many mRNA targets of miRNAs are involved in tumor initiation and progression, miRNAs can possess oncogenic or tumor-suppressive activities; therefore, their aberrant expression may be functionally involved in cancer [13].

miRNAs with oncogenic activities are termed *Oncomirs* [14]. This term indicates the miRNAs are constitutively overexpressed that promote tumor cell growth by inhibiting tumor suppressor genes or genes that control cell cycle progression, differentiation or apoptosis [15].

Mir-21 is a good example for oncogenic miRNA. Its target tumor suppressor gene is *PTEN* and several studies showed that miR-21 overexpression, correlating to *PTEN* downregulation, leads to proliferation and metastasis in glioblastoma, pancreatic, breast and hepatocellular cancers [16]. By contrast, tumor suppressor miRNAs *Anti-oncomiR* usually prevent tumor development by inhibiting oncogenes [17].

miRNA let-7 negatively regulates *K-RAS* and is considered as *Anti-oncomiR* [18]. let-7 downregulation with a consequently RAS overexpression is found in NSCLC than in normal lung [19]. In addition, a reduced expression of let-7 has been associated with shortened postoperative survival in various types of cancer [20].

miRNAs involved in the acquisition of invasive abilities are called *MetastamiRs* [21]. This miRNAs regulating positively or negatively epithelial-to-mesenchymal transition (EMT), loss of cellular adhesion, can play a pro- and anti-metastatic role [22].

Some of these miRNAs are miR-192/215, for which target is E-cadherin repressors ZEB-1 and ZEB-2 [23], miR-30 and miR-200 family that regulate the TGFβ pathway [24]. Based on these concepts, the inhibition of *OncomiRs* and *MetastamiRs* as well as restoration of *AntioncomiRs* could represent an important promise for the treatment of cancer.

2. Diagnostic role of miRNAs

Numerous studies suggest that miRNA expression signatures, intended as the expression of the differentially regulated miRNAs across solid cancers, could represent biomarkers in breast cancer diagnosis [5,25,26].

miRNAs can help to unreveal the tissue of origin for cancers of unknown primary origin. A classifier included 48 miRNAs evaluated in primary or metastatic tumor tissue to achieve this goal [27,28]. MiRNA expression patterns were analyzed in human serum to identify five types of human cancers including those developing in prostate, colon, ovarian, breast and lung. This analysis allowed to distinguish in serum of cancer patients, specific miRNA expression patterns for lung cancer and colorectal cancer (CRC), suggesting that circulating miRNAs could provide fingerprints of different malignancies [29].

Single miRNA levels were also evaluated to help diagnosis of cancer. Various studies found statistically significant difference between cancer patients and healthy donors.

miR-145 has been considered to have potential clinical applications as a novel biomarker for breast cancer diagnosis [30]. The polymorphisms, located within the sequence of miRNA precursor, were also proved to be associated with the risk of breast cancer [26].

A similar procedure measure seven candidate miRNAs to identify breast cancer patients instead of healthy controls. In particular, miR-195 and let-7a were expressed at a higher level in circulating blood of breast cancer patients than in controls. Interestingly, the levels of these two miRNAs decreased significantly after curative tumor resection [31].

A recent study demonstrated a stronger association of miR-21 with breast cancer and higher stage of neoplastic disease [32]. From 11 Chinese NSCLC patients, serum was drawn and compared with that obtained from 21 normal controls. Twenty-eight miRNAs were downregulated and 63 miRNAs were upregulated. miR-25 and miR-223 were independently validated in a wider sample, and they were found to be more expressed in lung cancer patients [29]. In prostate cancer patients, among a panel of six candidate miRNAs, miR-141 showed the greatest differential expression in plasma between two pools of 25 metastatic prostate cancer patients and 25 healthy controls, and this miRNA was confirmed to be expressed at a higher level in cancer patients on an individual level [33].

Furthermore, 120 primary CRC patients and 37 advanced adenoma patients, both taken before surgery, were compared with 59 age-matched healthy controls. MiR-29a and miR-92a were upregulated in CRC plasma compared with controls. These miRNA expression was higher in adenoma patients and significantly lower than that in true cancer patients and decrease after surgery [34].

MiR-92a and miR-17 also were found to have a higher expression in the plasma of CRC patients compared with controls, decreasing after tumor resection. Both miRNAs did not have higher expression in patients with gastric cancer or inflammatory bowel disease, confirming their specificity [35]. Another study showed that in 103 CRC patients, circulating miR-221 expression was higher than that in 37 controls although with a low specificity of 41% at the optimal cut-off level [36].

3. Prognostic role of miRNAs

The advent of high-throughput techniques has revealed that miRNA expression is deregulated in almost all human tumors with respect to the normal tissue counterpart, and increasing evidence supports a role for miRNAs as prognostic biomarkers of human cancers [37]. The roles of microRNAs in cancer biology and prognosis prediction in non-small cell lung cancer (NSCLC) have been widely studied [38,39].

Increased miR-34a expression was associated with fewer relapses in a small retrospective study of resected NSCLC patients and overexpression of let-7a was shown to be related to increased overall survival in NSCLC patients representing possible protective prognostic factors that could prevent the recurrence in surgically resected NSCLC [40]. Moreover, overexpression of two oncomiRs, miR-21 and miR-155, was shown to be related to decreased overall survival in NSCLC patients [7] [41].

In relation to different types of cancers, the same miRNAs may not have the same role in prognosis.

For example, in a recent work, the prognostic role of miR-34a has evaluated in tumor samples and cell lines of SCLC. The authors show that, unlike NSCLC, the expression of miR-34a is not prognostic in SCLC patient, and because it does not correlate with the malignant behavior of cancer, it is unlikely to be a therapeutic target [42]. In a recent work, the expression profiles of miR-335 and miR-126 were correlated with metastasis-free survival of breast cancer patients [43]. When primary tumors displayed, low expression of miR-335 and miR-126 patients had a shorter median time to metastatic relapse, compared with the group with high levels of miR-335 and miR-126 in primary tumor tissue. miR-335 and miR-126 regulate a set of metastasis-related genes playing a role as metastasis suppression; therefore, the evaluation of expression levels of these miRNAs could have a prognostic role in human breast cancer. Preliminary studies correlate the decreased miRNA biogenesis with tumor progression and could serve to identify several prognostic indicators in different malignancies. For example, in a subset of NSCLC, a reduced *DICER1* expression is found to correlate with survival of surgically treated patients, with a significant prognostic impact ($p = 0.001$) that appears to be independent of disease stage ($p = 0.001$) [44]. In ovarian cancer patients, high *DICER1* and *DROSHA* expression were associated with increased median survival (> 11 vs 2.66 years for other subgroups; $p < 0.001$) [45]. These studies suggest the important role of miRNA biogenesis in cancer, and supporting an overall antitumorigenic role of miRNAs, this impaired miRNA processing could enhance the tumorigenic activity and the invasiveness of cancer cells.

In CRC, a wide range of dysregulated miRNAs have been identified. This altered expression is correlated to epigenetic mechanisms, including hypermethylation of promoter regions [46].

Abnormal hypermethylation of promoters of miR-9, miR-34a, miR-34b, miR-34c, miR- 129 and miR-137 is associated with reduced expression in CRC tissues, suggesting a contribution to transcriptional downregulation of miRNAs [47]. In a recent work, two epigenetically modified miRNAs, miR-34b/c and miR-148a, were identified in fecal specimens and the results obtained suggest a possible role of these miRNAs in CRC prognosis and screening strategies [48].

4. Therapeutic potential of miRNAs

The association of the aberrant miRNA expression with cancer development and their role as tumor suppressors or oncogenes implicate that the achievement of novel miRNA-based therapeutic strategy for cancer will exploit a proper modulation of miRNA expression.

One specific miRNA can target the mRNA of various genes. This characteristic implies that by inhibiting one miRNA, we could obtain the suppression of numerous genes and possibly a whole pathway or even more pathways could be silenced.

In the acknowledgment of miRNAs' dual role in carcinogenesis, two possible approaches have been developed for using miRNAs as cancer therapy: antisense-mediated inhibition for oncogenic miRNAs and expression replacement for miRNA tumor suppressor with miRNA mimetics or viral vector-encoded miRNAs.

4.1 Targeting oncogenic miRNAs

4.1.1 Anti-miRNA Oligonucleotides

The base-pair interaction between miRNAs and their target mRNAs is essential for the function of miRNAs. Therefore, a logical approach of silencing oncogenic miRNAs is to use an inhibitory anti-miRNA oligonucleotides (AMOs), which sterically blocks the interaction between miRNA and its target mRNA by competition [49].

The therapeutic efficacy of AMOs depends on their ability to migrate through the body, resist to nuclease degradation and to obtain an adequate organ distribution. Chemical modifications can significantly improve the stability of AMOs and are necessary to increases the thermal stability upon hybridization with complementary single-strand RNA target molecules.

One example of AMO chemically modified is locked nucleic acid (LNA). LNA is an oligonucleotide that contains conformationally locked nucleotide monomers with a methylene bridge connecting the 2'-oxygen and 4'-carbon atoms of the ribose ring [50]. This modification increases the nuclease resistance [51], stabilize the duplex structure and improve mismatch discrimination [52]. LNA oligonucleotides show high stability and low toxicity in biological systems, efficient transfection into mammalian cells and potent antisense activity *in vivo* [53]. A recent work revealed that miR-31 acts as an oncogenic miRNA (*oncomir*) in NSCLC; therefore, LNA oligonucleotides could offer a therapeutic possibility to repress the miR-31 expression [54]. Currently, an LNA-based miR-122 inhibitor is in Phase II clinical trial for the treatment of hepatitis C [55]. Another chemically modified ribonucleotides is the 2'-O-methyl oligonucleotides, wherein 2'-hydroxyl on the ribose is replace with the 2'-O-methyl group. This modification improves the biostability and makes the oligonucleotides more resistant to degradation [56].

MiR-21 is overexpressed in several cancer types and plays a critical role in cell proliferation by *PTEN* downregulation [16]. Si *et al.*, using a xenograft carcinoma model, injected MCF-7 cells transiently transfected with 2'-O-methyl oligonucleotides complementary to miR-21. They found that tumors derived from MCF-7 cells transfected with anti-miR-21 were 50% smaller in size than control MCF-7 tumors [57].

However, the use of 2'-*O*-methyl antisense oligonucleotide has several limitations. A direct measurement of the depletion of miRNAs is difficult, because miRNAs bind to the miRNA target but not induce its degradation. Therefore, the only possible method to confirm the decrease in number of miRNAs is to measure the level of expression of a reporter gene containing the target sequence of the miRNA.

4.1.2 Small-molecule inhibitor

Small-molecule inhibitors against specific miRNAs have been investigated to inhibit the oncogenic miRNA. In a recent work conducted *in vitro*, it has been shown that azobenzene is an efficient inhibitor of biogenesis of miR-21. This specific inhibitor provides a tools for the investigation of miRNAs function and could represent a promising molecule to boost patient response to existing chemotherapies or stand-alone cancer drugs [58].

4.2 Restoring Suppressor miRNAs

Since the tumor suppressor expression can inhibit tumor growth, it has been proposed that restoring tumor-suppressive miRNAs may also have antitumor effect.

miRNA replacement therapy can be made by two different strategies: using viral vector-based gene restoration or by miRNA mimics.

In a recent work, the use of mimics in complex with a novel neutral lipid emulsion, for the tumor suppressors miR-34a and let-7, induces therapeutic benefit in mouse models of NSCLC [59]. A significant growth reduction of both murine and human NSCLC was observed when let-7 overexpression was induced from lentiviral vectors [60]. These results support the evidence that synthetic miRNA mimics or lentiviral vectors can be a promise for future target therapy for lung cancer. These findings suggest that a selection of miRNAs that are highly expressed and tolerated in normal tissues but lost in cancer cells can be a general strategy for restoring tumor suppressor miRNAs as therapy in human cancers.

Table 1. miRNAs with a possible prognostic and diagnostic role in breast, lung and colorectal cancers.

Tumor	Prognostic miRNAs	Diagnostic miRNAs
Breast cancer	miR-335 miR-126	miR-145 miR-195 let-7a
Non-small cell lung cancer (NSCLC)	miR-34a let-7a miR-21 miR-155	miR-25 miR-223
Colorectal cancer (CRC)	miR-34b/c miR-148a	miR-29a miR-92a miR-17 miR-221

5. Conclusions

This review summarizes the potential role of miRNAs as molecular markers for cancer prognosis, diagnosis and their potential role as possible therapeutic targets. Despite the efforts and advances made in developing miRNA-mediated therapy, there are still restrictions on the possibility of using miRNAs as potential targets for the development of new therapeutic approaches. First is to maintain target specificity. miRNA targeting is known to be sequence specific instead of gene specific and gene silencing requires only a partial complementary between miRNA and protein-coding transcripts. Therefore, the effect of a specific miRNA-mediated therapy could be evaluated by a proteome-wide scale to prevent unwanted gene alteration.

The second limiting factor is to achieve high therapeutic efficiency due to the amplitude of target gene modulation and the number of cells that can be targeted. This is dependent on the difficulty of delivering therapeutic oligonucleotides in the tumor site and poor resistance to degradation by nuclease. However, in several tumors, especially in breast, lung and CRC, different miRNAs with a possible prognostic and diagnostic role have been identified (**Table 1**). Despite the encouraging results obtained, the introduction of this and other miRNAs in clinical practice is still too distant. Further investigations are needed to specifically evaluate these approaches in various human tumors.

6. Expert opinion

miRNAs appear key regulators of various biological processes. Increasingly, new implications in disease development and cancer are found for miRNAs. The more relevant results are their double function as oncogenic and tumor suppressors. In fact, those miRNAs targeting mRNA as transcript of oncogenes result tumor suppressor, while those targeting tumor suppressor genes transcripts have oncogenic activity. This finding has various consequences: i) miRNA expression could provide a complementary and specular counterpart of cancer-related gene expression level, albeit miRNAs are more stable than mRNA; ii) the variation of miRNA levels could help for the identification of cancer in earlier stages and prognostically define the progression toward advanced stages; iii) miRNAs could be delivered, be targeted or mimicked to regulate gene expression for attempting to block cancer development and progression.

To date, some databases of target genes for miRNAs are available. This association was established by relating complementary base pair sequence between mRNAs and miRNAs. Another limitation of miRNAs' clinical application relates to the difficulty in reaching the target organs or tissues. Currently, there are numerous studies trying to discover a better way to deliver miRNAs as therapeutic molecules.

A potential result of such research includes a better understanding of the biological processes implicated in cancer development and progression. This aim, derived from the clinical application of miRNAs, is represented by improvements in diagnostic, prognostic and therapeutic tools. Moreover, the development of new strategies for miRNA targeting will allow a more precise evaluation of the functions of cancer-related genes.

One future question is whether the variations of miRNA expression patterns are able, per se, to lead the neoplastic transformation or whether they are a consequence of differential expression of cancer-related genes for genetic changes. If we could find an answer for this question, we could experience an important advancement of knowledge for tumor biology, which poses the basis for an improvement in therapeutic strategies.

miRNAs are versatile for clinical employment because they are stable and can be studied both in tumor tissues and in peripheral blood. For this reason, miRNAs were proposed as screening tests for early diagnosis. The realization of this goal will help clinicians in this area to submit to more specific or invasive diagnostic detections. However, sensibility and specificity need to be defined for singular or groups of miRNAs before these molecules are considered for clinical indication.

In conclusion, it seems that most benefit is derived from the clinical application of miRNAs in regard to therapeutic function. However, it seems that actually the employment of miRNAs to define prognosis of cancer patients is becoming increasingly developed with positive results. Usage of diagnostic miRNAs in the diagnostic setting requires more consistent data.

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Declaration of interest

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REVIEW

Breast cancer genome-wide association studies: there is strength in numbers

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Breast cancer (BC) is a heterogeneous disease that exhibits familial aggregation. Family linkage studies have identified high-penetrance genes, *BRCA1*, *BRCA2*, *PTEN* and *TP53*, that are responsible for inherited BC syndromes. Moreover, a combination of family-based and population-based approaches indicated that genes involved in DNA repair, such as *CHEK2*, *ATM*, *BRIP* and *PALB2*, are associated with moderate risk. Therefore, all of these known genes account for only 25% of the familial aggregation cases. Recently, genome wide association studies (GWAS) in BC revealed single nucleotide polymorphisms (SNPs) in five novel genes associated to susceptibility: *TNRC9*, *FGFR2*, *MAP3K1*, *H19* and lymphocyte-specific protein 1 (*LSP1*). The most strongly associated SNP was in intron 2 of the *FGFR2* gene that is amplified and overexpressed in 5–10% of BC. rs3803662 of *TNRC9* gene has been shown to be the SNP with the strongest association with BC, in particular, this polymorphism seems to be correlated with bone metastases and estrogen receptor positivity. Relevant data indicate that SNP rs889312 in *MAP3K1* is correlated with BC susceptibility only in *BRCA2* mutation carriers, but is not associated with an increased risk in *BRCA1* carriers. Finally, different SNPs in *LSP1* and *H19* and in minor genes probably were associated with BC risk. New susceptibility allelic variants associated with BC risk were recently discovered including potential causative genes involved in regulation of cell cycle, apoptosis, metabolism and mitochondrial functions. In conclusion, the identification of disease susceptibility loci may lead to a better understanding of the biological mechanism for BC to improve prevention, early detection and treatment.
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Introduction

Breast cancer (BC) is the most common cancer and the second leading cause of cancer death among women (Parkin *et al*., 2005).

The family history is the main risk factor for BC, indicating that the genetic factors are very important in the development of disease (Antoniou and Easton, 2006).

In the 1990s, linkage studies in multiple case families have identified two major susceptibility genes in BC *BRCA1* and *BRCA2* (Miki *et al*., 1994; Wooster *et al*., 1995).

Germline mutations in *BRCA1* and *BRCA2* genes occur rarely in the general population but confer high risks of breast and ovarian cancer and a lower risk for other cancers (Antoniou *et al*., 2003; Thompson and Easton, 2004).

TP53 and *PTEN* mutations are also present in the population at low frequency and lead to very high BC risk associated with rare cancer syndrome, however, population-based studies have estimated that alterations in these genes account only the 15% of the familial risk of BC (Sidransky *et al*., 1992; FitzGerald *et al*., 1998; Peto *et al*., 1999; Dite *et al*., 2003).

Further, genetic linkage analyses failed to identify additional high-penetrance susceptibility genes and the identification of rare variants of genes involved in DNA repair, such as *CHEK2*, *ATM*, *BRIP* and *PALB2* in families lacking *BRCA* mutations (Meijers-Heijboer *et al*., 2002; Thompson *et al*., 2005; Rahman *et al*., 2007; Hollestelle *et al*., 2010), associated with a moderate risk of disease, can explain only a small portion of familial risk.

Therefore, all of these known genes account for only 25% of the familial aggregation cases (Thompson and Easton, 2004), suggesting that most of the familial risk of BC can plausibly involve a combination of multiple low-penetrance susceptibility alleles, each conferring a small effect on BC risk (Antoniou and Easton, 2006; Table 1).

According to this model defined 'polygenic', proposed to explain the genetic susceptibility to BC, a large number of low-risk variants occurs with high frequency in populations, therefore, it may have a multiplicative effect in determining the overall risk of disease (Pharoah *et al*., 2002; Figure 1). A significant part of polygenic contribute to low-penetrance susceptibility may rise by non-conservative missense mutations in evolutionarily conserved domains.

Table 1 Genetic loci implicated in breast cancer susceptibility

High penetrance, low frequency	*Low penetrance, low frequency*	*Low penetrance, high frequency*
BRCA1	CHEK2	FGFR2
BRCA2	ATM	TNRC9
p53	PALB2	LSP1
PTEN	BRIP1	MAP3K1
		SLC4A7
		COX11

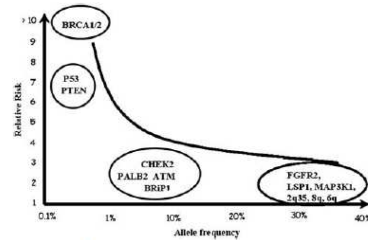

Figure 1 Allelic variants at low frequency tend to be associated with higher relative risk of BC (for example, BRCA1, BRCA2), high-frequency allelic variants are associated with lower RR (for example, FGFR2, LSP1, and so on) configuring an inverse correlation.

Genome-wide association studies (GWAS)

In recent years the research of low-penetrance allelic variants was conducted mainly through GWAS. These studies use a large number of common genetic single nucleotide polymorphisms (SNPs) to identify associations with disease that rely upon patterns of linkage disequilibrium (LD) in the human genome (Hirshfield *et al*., 2010). The power of GWAS is to evaluate the association of genetic variants at different loci on different chromosomes (LD) in large series of cases versus controls, analyzing a panel of hundred thousand SNPs simultaneously, to identify new alleles of susceptibility to BC (Orr and Chanock, 2008). In the human genome has been estimated that there are seven million of common SNPs that have a minor allele frequency (m.a.f.), $>5\%$ and because recombination occurs in different hot-spots, the nascent polymorphisms are often strongly correlated.

These studies therefore provide a powerful tool to identify novel markers for susceptibility and prognosis of disease (Peto, 2002; Houlston and Peto, 2004; Easton and Eeles, 2008). In the GWA studies the accumulation of a large number of data is crucial. Houlston and Peto, (2004) have estimated the number of cases required to identify low-penetrance alleles conferring a relative risk of two both in an unselected population and in families with first-degree relatives affected. In an unselected population the identification of a susceptibility allele with a frequency of 5% requires over 800 cases. In the same population, the identification of a susceptibility allele with a frequency of 1% requires over 3700 unselected cases, whereas about 700 would be enough if three affected families are selected. Therefore, the power of association studies can be significantly increased using selected cases with a family history of cancer because less cases are required to demonstrate the association with disease (Houlston and Peto, 2004).

The potential of the association studies of cases with a family history to identify low-penetrance alleles conferring a relative risk of 2 has been demonstrated by the mutation *CHEK2* 1100delC in patients with BC. This variant carried by 1% of the population confers an increased risk of 1.7-fold. The frequency was not significantly increased in unselected cases (1.4%), but it was strongly increased in familial cases without *BRCA1* and *BRCA2* mutations (5.1%; Meijers-Heijboer *et al*., 2002).

In the past years several novel risk alleles for BC were identified by four recent GWA studies: Breast Cancer Association Consortium, Cancer Genetic Markers of Susceptibility, DeCode Islanda, Memorial Sloan–Kettering Cancer Center (Easton *et al*., 2007; Hunter *et al*., 2007; Gold *et al*., 2008; Stacey *et al*., 2008).

In each of them the association study was shared into three phases: the first phase identifies the common SNPs in cases and controls, the second phase evaluates how many of the above SNPs are common to a greater number of cases and controls and, finally, the third phase aims to identify new alleles of susceptibility of BC.

Easton *et al*., in their study, identified five independent loci associated with increased susceptibility to BC ($P<10^{-7}$). This multistage study involved in the first stage 390 BC cases with a strong family history and 364 controls, and 3990 cases and 3916 controls in the second stage.

To define the risk associated with the 30 most significant SNPs, a third stage of the study was conducted involving 21 860 cases and 22 578 controls from 22 additional studies in the Breast Cancer Association Consortium.

These combined analyses allowed to observe that the SNPs showing a stronger statistical evidence of association with an increased familial risk were: rs2981582 lies in intron 2 of *FGFR2*, rs12443621 and rs8051542 within *TNRC9*, rs889312 lies in a region that contain *MAP3K1* gene, rs3817198 lies in intron 10 of lymphocyte-specific protein 1 (*LSP1*) and rs2107425 within the *H19* gene.

Unlike other BC susceptibility genes previously identified that are involved in DNA repair and sex hormone synthesis, in this work three of the five loci reported contain genes involved in regulation of cell growth and cell signaling (Easton *et al*., 2007).

Starting from this study of Easton *et al*., in attempt to identify further loci associated with BC risk, Ahmed *et al*. have genotyped in a third stage further 814 SNPs, involving 3878 cases and 3928 controls from three studies of the Cancer Genetic Markers of Susceptibility.

These analyses allowed to identify three additional SNPs (rs4973768, rs4132417 and rs6504950) that have been evaluated in a fourth stage from 27 studies in the Breast Cancer Association Consortium.

rs4132417 showed no evidence of association in the fourth stage, it was probably a false positive. rs4973768 showed strong association with age that was higher for ER-positive than ER-negative disease, moreover there was no evidence of association with a positive family history of BC.

Similarly, rs6504950 showed statistical evidence of association with ER-positive disease and no association with the family history, and unlike of rs4973768 no association with the age.

Moreover, in this study additional association analyses showed that another SNP rs1357245 is located in the same LD block as rs4973768 in the 3p24 region (Ahmed *et al.*, 2009). Genotyping the 28 SNPs, known to be present in this region, in 2301 cases and 2256 controls, it presents a further SNP rs2307032 that was correlated with both SNPs.

Hunter *et al.*, in a recent work, have identified alleles in *FGFR2* associated with risk of sporadic postmenopausal BC.

In this study, the National Cancer Institute Cancer Genetic Markers of Susceptibility identified four SNPs, two (rs1219648 and rs2420946) in intron 2 of *FGFR2* and two (rs11200014 and rs28981579) in *FGFR2* gene, using GWAS of BC by genotyping 528 173 SNPs in 1145 postmenopausal women of European ancestry with invasive BC and 1142 controls.

These polymorphic variants showed a strong association with the risk of disease (Hunter *et al.*, 2007).

A similar experimental design was conducted by Gold *et al.* in 249 Ashkenazi Jewish women, containing multiple cases of BC but lacking *BRCA1* or *BRCA2* mutations, presented at the Memorial Sloan–Kettering Cancer Center.

This study confirmed the BC association with the *FGFR2* locus, identified by Easton and Hunter studies, but showed an association with the *RNF146* and *ECHDC1* region at 6q22.33 not seen in the previous works (Gold *et al.*, 2008).

ECHDC1 gene encodes for a trifunctional protein involved in mitochondrial fatty acid oxidation (Hashimoto *et al.*, 1996) and *RNF146* encodes for a protein, called actylidin, that functions as a ubiquitin protein ligase (E3; Mani and Gelmann, 2005), that could have a role in breast tumorigenesis.

To identify new risk variants associated with BC susceptibility, Stacey *et al.* have carried out a GWA study genotyping 1600 Icelandic individuals with BC and 11 563 controls.

Two SNPs showed statistically significant association with BC: the A allele of rs13387042 on chromosome 2q35 and the T allele of rs3803662 on 16q12.

The 25% of individuals of European descent are homozygous for allele A of rs13387042 and have an estimated 1.44-fold greater risk than noncarriers and about 7% are homozygous for allele T of rs3803662 and have a 1.64-fold greater risk.

These risk alleles were not associated with histopathological subtype, stage and grade of tumors, but confer preferential risk for estrogen receptor (ER)-positive BC.

In the LD block where lies rs13387042 there are no known genes, but there are proximally and distally *TNP1*, *IGFBP5*, *IGFBP2* and *TNS1* genes (Stacey *et al.*, 2007).

rs3803662 is near the 5′ end of *TNRC9*, whose increased expression is highly predictive of metastasis to bone of BC (Smid *et al.*, 2006).

Comparing the results obtained from four major studies of GWA, it has been highlighted a correlation of allele frequency of some SNPs located on the genes: *FGFR2* encoding a receptor tyrosine kinase, *TNRC9* encoding a high-mobility group chromatin-associated protein, *MAP3K1*, which encodes the signaling protein mitogen-activated protein kinase 1 (MAPK1), *LSP1* encoding *LSP1* and *H19* an untranslated messenger RNA involved in regulation of the insulin growth factor gene 2 (Table 2).

These new discovered susceptibility genes are differentially expressed between the five distinct molecular subtypes of BC, based on differential gene expression profiles: luminal A, luminal B, basal like, ErbB2 + and normal like (Sorlie *et al.*, 2003). These distinct molecular subtypes of BC are associated with different clinical outcomes (Sorlie *et al.*, 2001).

If the probability to develop a given subtype of BC is genetically determined, we would expect to find that the

Table 2 Comparative analysis of the SNPs identified in the four studies (BCAC, CGEMS, MSKCC and DeCode Islanda)

Gene	*Location*	*BCAC*	*CGEMS*	*MSKCC*	*DeCode Islanda*
FGFR2	Chr 10q	rs2981582 (in intron2)	rs1219648 rs2420946 (in intron2) rs11200014 and rs28981579	rs2981582	No
TNRC9	Chr 16q	rs12443621 rs8051542	rs8049226 (within 200 kb of TNRC9)	rs3803662 and rs3112625	rs3803662 (near TNRC9)
MAP3K1	Chr 5q	rs889312	rs726501	No	No
LSP1	Chr 11p	rs3817198 rs498337	rs7120258	No	No
H19	Chr 11p	rs2107425	rs7120258 rs7578974	No	No

Abbreviation: BCAC, Breast Cancer Association Consortium; CGEMS, Cancer Genetic Markers of Susceptibility; MSKCC, Memorial Sloan–Kettering Cancer Center; SNPs, single nucleotide polymorphisms.

newly discovered susceptibility genes (Easton *et al.*, 2007) are differentially expressed in the various tumor subtypes.

Recently a significantly differential mRNA expression of *TNRC9*, *FGFR2*, *MAP3K1*, *H19* and *LSP1* from 112 breast tumor samples, representing all five subtypes, has been identified by analysis of variance (Nordgard *et al.*, 2007).

These data show the necessity to conduct stratified SNP disease association studies and to select patients by their molecular subtypes, to confer more power to the GWA studies.

FGFR2

FGFR2 is a member of a receptor tyrosine kinase gene superfamily, which contributes to the cell growth, invasiveness, motility and angiogenesis (Ricol *et al.*, 1999). Overexpression of *FGFR2*, one of the common low-penetrance susceptibility genes, is observed in breast tumor tissues (Adnane *et al.*, 1991) and in BC cell lines (Tannheimer *et al.*, 2000). Its expression is associate with ER+ tumors (Luqmani *et al.*, 1992), suggesting a hormone-dependent action of this gene. Recently, gene expression studies have shown increasing *FGFR2* expression levels associated with the rare homozygote genotype and functional studies identified the OCT1/RUNX2-binding site as the main determinant of the increased expression levels (Meyer *et al.*, 2008). Aberrant expression of alternatively spliced isoforms of FGFR2 transforms BC cells by sustained signal transduction (Moffa and Ethier, 2007). The *FGFR2* gene, located at chromosome 10q26, contains at least 22 exons (Ingersoll *et al.*, 2001).

Several mutations and common SNPs within or flanking the *FGFR2* gene have been identified. A number of studies have been conducted to investigate the association between *FGFR2* polymorphisms and the risk of BC in humans. The association is restricted to SNPs in the LD block covering intron 2. In particular, three polymorphic variants, rs1219648 (A>G), rs2420946 (C>T) and rs2981582 (C>T) are more investigated for their closed correlation with BC. Easton *et al.* (2007) showed that rs2981582 had a clear relevance to BC.

Gold *et al.* (2008) confirmed the previously reported results for *FGFR2* locus. Recently, a further GWAS study confirmed the correspondence between *FGFR2* susceptibility loci and BC risk. In particular, the per-allele odds ratio was higher for ER-positive rather than for ER-negative BC (Ahmed *et al.*, 2009). This finding is consistent with the involvement of FGFR2 in estrogen-related breast carcinogenesis (Tamaru *et al.*, 2004), and with higher levels of FGFR2 expression in ER+ than ER− cell lines and tumors (Zhang *et al.*, 1999). Stacey *et al.* genotyped ~300 000 SNPs in 1600 Icelandic individuals with BC and 11 563 controls. They found that 25% of individuals of European descent are homozygous for allele A of rs13387042 on chromosome 2q35 and have an estimated 1.44-fold greater risk than noncarriers. Risk from both alleles was confined to ER-positive tumors.

The variant in the 5p12 region, which is close to the FGFR2 ligand FGF10, also shows strong evidence of an association primarily with ER+ tumors (Stacey *et al.*, 2008).

TNRC9

The locus on 16q includes a gene *TNRC9* and a hypothetical gene *LOC643714*. *TNCR9* (also known as *TOX3*) is a gene of uncertain function containing a tri-nucleotide repeat motif and encoding a member of the high-mobility group family of non-histone chromatin proteins. The presence of a putative high-mobility group box motif suggests that it might function as a transcription factor (Easton *et al.*, 2007). Several studies have shown that susceptibility loci at *TNCR9* predispose to sporadic BC. Rs3803662, located near the 5′ end of *TNRC9*, has been shown to be the SNP with the strongest association with BC. The SNP rs3803662 is related to both ER+ and ER− tumors (McInerney *et al.*, 2009).

The associations of rs3803662 with other SNPs seem to be not significant. Other two SNPs (rs12443621 and rs8051542) with important evidence of association are located in an LD block containing the 5′ end of *TNRC9*. Furthermore, Hunter *et al.* (2007) showed that there is only one SNP significant (rs8049226) within 200 kb of *TNRC9*. In contrast, the coding region of *TNCR9* contains SNPs showing no evidence of association. The A allele of rs13387042 located on chromosome 2q35 (A-rs13387042) and the T allele of rs3803662 on 16q12 (T-rs3803662) confer increased risk of BC for ER-positive tumors. Any interaction was observed between the 2q35 and 16q12 loci. Moreover, no known gene or human RNA was found for the LD block containing rs13387042. The rs3803662 SNP located on 16q12 occurs in the fourth exon of a poorly characterized mRNA. In BC, the q arm of chromosome 16 is frequently lost, therefore, it is likely that one or more tumor suppressor genes are present in the same region. Differences in stage, grade or histopathological subtype were not significantly correlated with the low-penetrance susceptibility alleles, and there was no significant difference in allele frequencies between *in situ* and invasive carcinoma. In African Americans, T allele of the SNP rs3803662 was significantly protective and, thus, it was not associated with increased BC risk (Stacey *et al.*, 2007). Three susceptibility alleles (rs2981582, rs3803662 and rs13281615) also have shown an evidence of association with family history of BC. In fact, each of these SNPs was more frequent in women with a first-degree relative with the disease than in those without. Furthermore, an evidence of association with breast *in situ* carcinoma has been shown by three SNPs (rs2981582, rs3803662 and rs889312; Easton *et al.*, 2007). Increased expression of *TNRC9* indicates a major

susceptibility to metastasis of BC to bone. ER positivity is predictive of bone metastases. The possible effects of the correlation between rs3803662, *TNRC9*, bone metastases and ER positivity remain to be explicated.

Many association studies have shown that SNPs in *FGFR2*, *TNRC9* and *MAP3K1* increase the BC risk in *BRCA2* mutation carriers with a similar relative risk to that seen in the general population. In contrast, in *BRCA1* mutation carriers only the rs3803662 SNP was associated with an increased BC risk (Easton and Eeles, 2008).

MAP3K1

MAP3K1 (MEKK1) encodes the MAPK protein that phosphorylates and activates the MAPK kinase (MAPK2) that in turn phosphorylates the MAPK/ERK to produce downstream signaling effects on a variety of cancer genes. MAP3K1 forms part of the MAPK cell signaling pathway implicated in cellular response to mitogens. The MAPK pathway is strongly linked to HER2 receptor activity and activating mutations in the MAPK pathway have been associated with HER2+ breast tumors (Bild *et al*., 2006; Creighton *et al*., 2006). *MAP3K1* was identified by Easton *et al*. (2007) to have a per-allele odds ratio effect of 1.13 (95% confidence interval: 1.09–1.18). MAP3K1 effects were found to be relevant in ER+ and PR+ tumors to a greater degree than in ER− or PR− tumors (Garcia-Closas and Chanock, 2008). MAP3K1 is differentially expressed in different BC subtypes (Nordgard *et al*., 2007). Hunter *et al*. (2007) found only one SNP (rs726501) with a P value in the range of $P<0.01$ by allele test. Gold in the Memorial Sloan–Kettering Cancer Center study and Stacey, in the DeCode study, did not see significant SNPs between individuals with BC and controls. GWA studies conducted by Garcia-Closas and Chanock identified that the rs889312 variant is in a LD block containing the *MAP3K* (Garcia-Closas *et al*., 2008).

The Consortium of Investigators of Modifiers of BRCA1/2 has recently evaluated whether variants in *FGFR2* (rs2981582), *TNRC9* (rs3803662) and *MAP3K1* (rs889312) are associated with the risk of BC in over 10 000 *BRCA1* and *BRCA2* mutation carriers from 23 studies (Antoniou *et al*., 2008). The evidence of association with SNP rs889312 in *MAP3K1* was weaker and was restricted to *BRCA2* mutation carriers, however, this SNP was not associated with an increased risk in *BRCA1* carriers.

LSP1 and H19

LSP1 gene (also known as WP43) encodes an F-actin bundling cytoskeletal protein expressed in hematopoietic and endothelial cells. *LSP1* has been implicated in malignant lymphoma and Hodgkin's disease (Marafioti *et al*., 2003), and other variants in this gene have been associated with risk of developing non-Hodgkin's lymphoma (Cerhan *et al*., 2007). The most important GWASs reported different conclusion about the role of *LSP1* gene in BC susceptibility. Easton *et al*. (2007) reported one SNP (rs3817198) lies in intron 10 of *LSP1* gene with P values in the range 10^{-5}–10^{-9}; Gold *et al*. (2008) found two SNPs (rs3817198, rs498337), near the *LSP1* region, with P values in the range of $P<0.01$ by allele test, where Hunter *et al*. (2007) provided evidence for one SNP (rs7120258) in the region with a P value 0.01. Recent study identified that *LSP1* minor allele of rs3817198 was associated with increased BC risk only for BRCA2 mutation carriers (Antoniou *et al*., 2009). A further SNP, rs2107425, located just 110 kb from rs3817198, was also identified (overall $P=0.00002$). rs2107425 is within the *H19* gene, an imprinted maternally expressed untranslated messenger RNA closely involved in regulation of the insulin growth factor gene 2 (Easton *et al*., 2007). In *H19* region on chromosome 11p, Easton *et al*. (2007) reported P values in the range 0.01–10^{-5}, Gold *et al*. saw no signal, whereas Hunter *et al*. (2007) found two SNPs (rs7120258, rs7578974), with association P values in the range of 0.01, with one additional SNP, rs217228, with a P value in the range of 0.02.

Recently discovered BC susceptibility loci

New susceptibility allelic variants associated with BC risk were recently discovered through large replication studies in combination with the original GWAS data. The combination between these studies and GWAS data allowed to identify three SNPs: rs4973768, rs4132417 and rs6504950. There is a strong evidence for additional susceptibility loci on 3p and 17q. The region 3p24 includes two potential causative genes, *SLC4A7* and *NEK10*. SLC4A7 (solute carrier family 4, sodium carbonate cotransporter, member 7) is a potential tyrosine kinase protein whose expression is reduced in BC specimens and cell lines. NEK10 (never-in mitosis-related kinase 10) belongs to a family of 11 never in mitosis a-related kinases that are involved in cell cycle regulation. However, unlike other NEKs, no role has been associated to NEK10. A 300-kb LD block on 17q23.2 includes rs6504950 that lies in intron 1 of *STXBP4* (syntaxin-binding protein 4), codifying an insulin-regulated STX4-binding protein involved in the regulation of GLUT4 vesicle translocation and glucose transport. The rs6504950 allelic variant showed no association with age or family history, but a stronger association in ER-positive disease versus ER-negative disease. The same LD block includes other genes as *COX11* (cytocrome C assembly protein 11), that is located 10 kb upstream of rs6504950, and *TOM1L1* (target of myb-1-like1). In lymphocytes, the rs6504950 risk allele is correlated with higher expression of *COX11* levels, but no association has been shown with expression levels of either *TOM1L1* or *STXBP4*. Allele frequency studies in European populations have revealed that rs4973768 and rs6504950 could explain

respectively 0.4% and 0.07% of the familial risk of BC. These susceptibility loci together with those previously identified in original GWAS would give rise to 5.9% of BC familial risk (Ahmed *et al*., 2009). Further genome-wide linkage studies have revealed three putative BC susceptibility regions of interest, located on 3q25, 6q24 and 21q22. Moreover, it has been observed that the allelic variants on both chromosomes 21 and 3 were correlated with a higher percentage of bilaterality and a higher number of familial cases (Rosa-Rosa *et al*., 2009).

A recent GWAS has identified a novel polymorphic variant rs11249433 within the 1p11.2 region associated with BC risk.

This association is stronger in ER-positive than ER-negative tumors, is correlated with mRNA expression of *NOTCH2* gene and is highest in breast tumors without *TP53* mutations.

Further studies are needed to evaluate the possible role of rs11249433 in *NOTCH2* regulation and BC development (Fu *et al*., 2010). Other variants can significantly modify the BC risk in *BRCA1* and *BRCA2* mutations carriers. The rs6138178 in *SNRPB* and rs6602595 in *CAMK1D* show a strongest association in *BRCA1* carriers, whereas rs9393597 in *LOC134997* and rs12652447 in *FBXL7* in *BRCA2* carriers.

These loci appeared to interact multiplicatively for BC risk in *BRCA1*/*BRCA2* carriers, therefore, these SNPs together with other genetic and environmental factors may improve the BC risk assessment in these populations (Wang *et al*., 2010).

Recently, a new study (Black Women's Health Study) has been conducted in a population of African American Women (886 BC cases versus 1089 controls) to identify genetic variants associated with risk of BC. As in the original study (Stacey *et al*., 2008), it has been confirmed the strong association of rs4415084 on 5p12 with overall risk and ER-positive tumors. No association was observed for ER- and PR-negative tumors. Other susceptibility allelic variants identified from BWHS are rs6451770, rs12515012, rs13156930 and rs16901937. A 21% increase in risk was associated with each copy of the rs16901937 G-allele. The closest gene to these regions is *MRPS30*, involved in apoptosis and encoding a component of the mitochondrial ribosome. Moreover, *MRPS30* is involved in a gene expression profile that allows to discriminate ER-positive from ER-negative tumors (Ruiz-Narvaez *et al*., 2010). Other BC susceptibility alleles could be identified through large-scale replication studies in combination with previous GWAS. However, these analyses have still a limited power.

In a recent work, the BC risk association with eight susceptibility loci identified by GWAS was investigated in relation to specific breast tumor subtypes. A strong association was identified between ER + tumors and six of eight loci identified by GWAS: rs2981582 (10q26), rs3803662 (16q12), rs13281615 (8q24), rs13387042 (2q35), rs4973768 (3p24) and rs6504950 (17q23). A most strongly relation was observed between two candidate loci, *CASP8* (rs1045485, rs17468277) and *TGFB1* (rs1982073) and PR tumors. Four loci were associated with triple negative tumors ($P \leqslant 0.016$): rs3803662 (16q12), rs889312 (5q11), rs3817198 (11p15) and rs13387042 (2q35) but only two of them (16q12 and 2q35) were associated with tumors with the core basal phenotype ($P \leqslant 0.002$). This study identifying novel risk factors associated with BC subtypes could allow a better tumor stratification resulting in prevention, early detection and treatment improvement (Broeks *et al*., 2011).

The power of GWAS

The GWAS represents a new powerful approach to identify lower penetrance alleles that cannot be detected by genetic linkage studies. The risk conferred by these alleles individually is too weak, generally 1.3-fold or less, but the combined effects may be useful for risk prediction (Easton *et al*., 2007). This would promote the development of novel methodologies for analysis of data generated by large-scale SNP studies. In recent years, the research and identification of low-penetrance susceptibility loci played a key role in the etiology of BC and, in particular, of those BCs that have estrogen and progesterone receptors. The combination of BC susceptibility alleles together with other risk factors may be important clinically and it may explain an appreciable fraction of the genetic variance in BC risk. The identification of the causative variants can be extremely problematic but the use of GWAS from multiple populations with different patterns of LD can reduce the difficulty of analysis. The power of GWAS may be increased by enlarging the number of samples in both the cases and the controls, and by identifying clinical and molecular subtypes (Kristensen and Borresen-Dale, 2008). However, the GWA experiments need the effort of several research groups to collect a sufficient number of patients for large multistage studies and they require large amounts of money. The allele frequency of the variant and the risk conferred by it will determine the number of cases to be genotyped. There is a common scepticism toward these new approaches because it is not known the mechanism by which the novel allelic variants cause the susceptibility. Furthermore, some differences were found between different studies. This could be due to population stratification, sample-size differences or genetic heterogeneity in the setting of different genotyping platforms (Perlegen, Mountain View, CA, USA; Affymetrix, Santa Clara, CA, USA; Illumina, San Diego, CA, USA) and different algorithms to filter data (Gold *et al*., 2008).

Recently, a novel multi-SNP GWAS analysis method called Pathways of Distinction Analysis was developed. This method uses GWAS data and pathway-gene and gene-SNP associations to identify pathways that could permit the distinction of cases from controls. Therefore, relating a pathway with the disease risk, for the SNPs associated with a pathway, the cases will be similar to other cases than to controls. This method provides a new analytical tool that could enrich the power of GWAS in BC risk prediction (Braun and Buetow, 2011).

In conclusion, the recently discovered data could open up new streets for basic research. In future, a new

generation of large-scale association studies, in combination with replication analyses and multiple scans could be able to identify many more loci.

Conflict of interest

The authors declare no conflict of interest.

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Journal of Cellular Physiology

miR-20b Modulates VEGF Expression by Targeting HIF-1α and STAT3 in MCF-7 Breast Cancer Cells

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MicroRNAs (miRNAs) are small non-coding RNAs that regulate the expression of different genes, including genes involved in cancer progression. A functional link between hypoxia, a key feature of the tumor microenvironment, and miRNA expression has been documented. We investigated whether and how miR-20b can regulate the expression of vascular endothelial growth factor (VEGF) in MCF-7 breast cancer cells under normoxic and hypoxia-mimicking conditions ($CoCl_2$ exposure). Using immunoblotting, ELISA, and quantitative real-time PCR, we demonstrated that miR-20b decreased VEGF protein levels at 4 and 24 h following $CoCl_2$ treatment, and VEGF mRNA at 4 h of treatment. In addition, miR-20b reduced VEGF protein expression in untreated cells. Next, we investigated the molecular mechanism by which pre-miR-20b can affect VEGF transcription, focusing on hypoxia inducible factor 1 (HIF-1) and signal transducer and activator of transcription 3 (STAT3), transcriptional inducers of VEGF and putative targets of miR-20b. Downregulation of VEGF mRNA by miR-20b under a 4 h of $CoCl_2$ treatment was associated with reduced levels of nuclear HIF-1α subunit and STAT3. Chromatin immunoprecipitation (ChIP) assays revealed that HIF-1α, but not STAT3, was recruited to the VEGF promoter following the 4 h of $CoCl_2$ treatment. This effect was inhibited by transfection of cells with pre-miR-20b. In addition, using siRNA knockdown, we demonstrated that the presence of STAT3 is necessary for $CoCl_2$-mediated HIF-1α nuclear accumulation and recruitment on VEGF promoter. In summary, this report demonstrates, for the first time, that the VEGF expression in breast cancer cells is mediated by HIF-1 and STAT3 in a miR-20b-dependent manner.

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MicroRNAs (miRNAs) are a group of non-coding regulatory RNAs, 20–25 nucleotides in length, which are known to regulate different cellular processes such as proliferation, differentiation, apoptosis, cell metabolism, angiogenesis (Bartel, 2004; Fabbri et al., 2008). miRNAs modulate gene expression either directly through translational repression, or by mRNA degradation, depending on partial or perfect complementarity to the 3′untranslated region of their targets (Bartel, 2004). In humans, it is estimated that 20–30% of all genes are targeted by miRNAs (Krek et al., 2005; Lewis et al., 2005). Several hundred miRNAs have been identified to date and each is thought to have hundreds of targets, accounting for the great complexity of their functions (Krek et al., 2005).

miRNAs expression profiles (miRNome) revealed specific miRNAs expression patterns in different cancers (Volinia et al., 2006). Recent evidences suggest that intratumoral hypoxia might be a master regulator of cancer-associated miNA expression (Kulshreshtha et al., 2007; Ivan et al., 2008). Under hypoxia, expression of a subset of miRNAs is transiently altered in colon and breast cancer cell lines (Kulshreshtha et al., 2007), possibly reflecting adaptive changes enabling survival of cancer cells by increased invasion and resistance to chemo- and radiotherapy (Blouw et al., 2003; Bertout et al., 2008).

Hypoxia inducible factor 1 (HIF-1) acts as a master regulator of the hypoxia response. HIF-1 is composed of alpha and beta subunits. In normoxia, HIF-1α is degraded by proteasomal pathways, while upon induction of hypoxia, HIF-1α is rapidly stabilized, dimerizes with HIF-1β subunit, translocates to the nucleus and binds specific consensus sequences—hypoxia response elements (HRE) within promoters of target genes (Iliopoulos et al., 1996; Ivan et al., 2001).

Vascular endothelial growth factor (VEGF) is the most prominent angiogenic factor promoting tumor progression and a recognized therapeutic target (Ferrara and Kerbel, 2005). VEGF promoter contains a potential HIF-1α binding site located at position −975 (Forsythe et al., 1996) and a signal transducer and activator of transcription 3 (STAT3) binding site at position −848 (Niu et al., 2002; Wei et al., 2003). It has been shown that upon induction of hypoxia, both factors bind respective regulatory sequences ensuring strong increase in VEGF transcription rate (Gray et al., 2005; Jung et al., 2005). Moreover, it has been recently demonstrated that STAT3 is involved in both hypoxia or growth signal induced HIF-1α stabilization (Xu et al., 2005; Jung et al., 2008).

Recently several miRNAs have been found to regulate angiogenic processes (Wang et al., 2008), including VEGF expression (Hua et al., 2006; Wu et al., 2009). miRNA-20b (miR-20b) belongs to the miRNA 106a-363 cluster, which

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together with mirR 17-92 and miR 106b-25 clusters form a large family of highly similar miRNAs called miR-17 family (Tanzer and Stadler, 2004). miR-20b share high homology with miR-17-5p and miR-20a from the miR-17-92 cluster, as well as miR-106b and miR-93 from miR-106b-25 cluster (Tanzer and Stadler, 2004). Members of miR 17 family are frequently overexpressed in many human cancers including lung cancer and leukemias (Hayashita et al., 2005; Landais et al., 2007; Matsubara et al., 2007; Ventura et al., 2008; Inomata et al., 2009). Recently, miR-20a has been reported as a negative regulator of HIF-1α in lung cancer (Taguchi et al., 2008), while miR-93 targets STAT3 in mouse embryo (Foshay and Gallicano, 2009). Bioinformatic approaches suggested that miR-20b might targets both HIF-1α and STAT3 transcription factors (Lewis et al., 2005).

Here, we studied if and how miR-20b can be involved in the regulation of VEGF in breast cancer cells.

Materials and Methods

Cell culture and treatments

MCF-7 cells were routinely grown in Gibco DMEM:F12 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 50 μg/ml streptomycin. Seventy percent confluent cells were stimulated with 300 μM $CoCl_2$ for 4 or 24 h.

Quantitative real-time PCR to determine the expression of miR-20b and VEGF in MCF-7 cells

Total cellular RNA was isolated using the miRNeasy Mini Kit (Qiagen Inc, Valencia, CA) and quantified through 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Ten nanograms of total RNA were reverse transcribed using Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The obtained cDNA was amplified using Taqman miR-20b or miR-20a MicroRNA assay (Applied Biosystems). For VEGF mRNA detection, 5 μg of total RNA was reverse transcribed using the high capacity cDNA archive kit (Applied Biosystems), according to vendor's instructions, and 5 μl of the RT products were used to amplify VEGF mRNA sequence using the Hs00900054_m1 VEGF TaqMan gene expression assay (Applied Biosystems). To normalize quantitative real-time PCR reactions, parallel reactions were run on each sample for RNU6B snRNA or Cyclophilin A. Changes in the target mRNA content relative to RNU6B or Cyclophilin were determined using the comparative C_t method to calculate changes in C_t, and ultimately fold and percent change. An average C_t value for each RNA was obtained for replicate reactions.

Western blotting (WB)

The cells transfected with both pre- or anti-miR-20b were stimulated with 300 μM $CoCl_2$ for 4 or 24 h, or left untreated. Then, the cells were lysed to obtain total proteins using complete Lysis-M reagent (Roche, Mannheim, Germany) or cytoplasmic and nuclear protein fractions using NE-PER Nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL). The expression of proteins was analyzed in 120 μg of total protein lysates, 80–50 μg of cytoplasmic and nuclear protein fractions. The following antibodies (Abs) were used for WB: anti-HIF-1α mouse monoclonal Ab (1:1,000; BD Transduction Laboratories, Heidelberg, Germany), anti-STAT3 (C-20 X) rabbit polyclonal Ab (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA), anti-VEGF (147) rabbit polyclonal Ab (1:1,000; Santa Cruz Biotechnology), anti-GAPDH (6C5) mouse monoclonal Ab (Santa Cruz Biotechnology), anti-C23 (MS-3) mouse monoclonal Ab (Santa Cruz Biotechnology). The proteins were separated on an 8% polyacrylamide gel under denaturing conditions, and the specific signal was detected with ECL WB substrate (Pierce Biotechnology Inc., Rockford, IL).

Pre-miR-20b and anti-miR-20b transfections

Transfections were performed with either pre-miR20b or anti-miR20b (Ambion, Austin, TX). MCF-7 cells were seeded at 7×10^5 in 60 mm culture dish or at 4×10^5 in six-well culture plates. After 24 h (30–40% confluence), the cells were transfected with pre-miR20b (50 nM) or anti-miR20b (100 nM) using siPort Neo Fx transfection agent (Ambion), according to manufacturer's instructions. The mixture was transfected into MCF-7 cells for 24 h in conditioned medium. After that, medium was replaced and the cells were stimulated with 300 μM $CoCl_2$ for 4 or 24 h, or left untreated. Non-specific pre-miR and anti-miR (Ambion) were used as negative controls together with mock control. The success of transfection was confirmed by quantitative real-time PCR.

Knockdown of STAT3 using small interfering RNA (siRNA)

STAT3 knockdown was achieved using small interfering RNA (siRNAs; Dharmacon Inc., Lafayette, CO), according to manufacturer's instructions. MCF-7 cells were seeded at 4×10^5 in 60 mm culture dish in antibiotic-free medium. Twenty-four hours following transfection, the cells were placed in fresh complete medium and then stimulated with 300 μM $CoCl_2$ for 4 h, or left untreated. Non-specific siRNA (Dharmacon Inc.) was used as a negative control.

Immunofluorescence

A total of 5×10^4 MCF-7 cells were plated in four-well Labtek II chamber slides (Nunc, Rockester, NY). After 24 h, the cells were transfected with pre-miR20b (50 nM) for 48 h and/or treated with 300 μM $CoCl_2$ for 4 h. Then, the cells were extensively washed with PBS and fixed for 20 min at 4°C in 4% paraformaldehyde. Next, the cells were permeabilized with 0.2% Triton X-100 for 20 min, and unspecific binding was blocked in 7.5% BSA fraction V for 1 h at room temperature. HIF-1α expression was detected using anti-HIF-1α (28b) mouse monoclonal Ab (1:50; Santa Cruz Biotechnology) or anti-STAT3 (C-20 X) rabbit polyclonal Ab (1:100; Santa Cruz Biotechnology) and FITC-conjugated goat anti-mouse IgG (1:1,000; Santa Cruz Biotechnology) or Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:1,000, Molecular Probes; Invitrogen). In control experiments, primary Abs were replaced by non-immune serum. The slides were covered with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA) to allow visualization of cell nuclei. The abundance of nuclear HIF-1α and STAT3 was assessed using Axioscope-40 microscope and Zeiss axiovision LE software (Carl Zeiss, Jena, Germany).

Chromatin immunoprecipitation (ChIP)

ChIP was performed using the Chromatin Immunoprecipitation Assay kit (Upstate, Temecula, CA), according to manufacturer's instructions. MCF-7 cells were treated with 300 μM $CoCl_2$ and/or 50 nM Pre-Mir-20b, 100 nM anti-Mir-20b for 4 h, or left untreated. Next, the cells were cross-linked with 1% formaldehyde and chromatin was collected and sonicated. Soluble chromatin was immunoprecipitated with the following Abs: anti-HIF-1α (28b) mouse monoclonal Ab (Santa Cruz Biotechnology), anti-STAT3 (C-20 X) rabbit polyclonal Ab (Santa Cruz Biotechnology). DNA–protein immune complexes were eluted, reverse cross-linked and DNA was extracted with phenol/chloroform and precipitated. The presence of the VEGF promoter domain containing HRE motifs in immunoprecipitated DNA was identified by PCR using the following primers: VEGF (region −1,041 to −750) forward 5′-CAGGAACAAGGGCCTCTGTCT-3′, reverse 5′-TGTCCCTCTGACAATGTGCCATC-3′. The PCR conditions for the VEGF promoter region were: 1 min at 94°C, 1 min at 60°C, 1 min at 72°C. The amplification of the VEGF promoter region was analyzed after 35 cycles. In control samples, the primaries Abs were

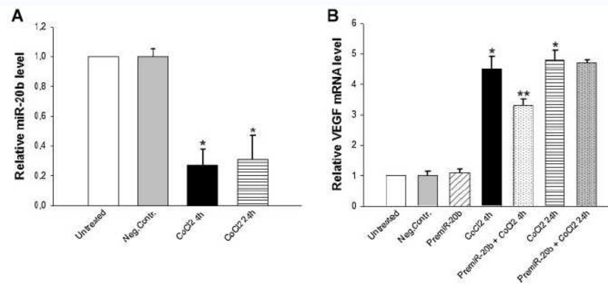
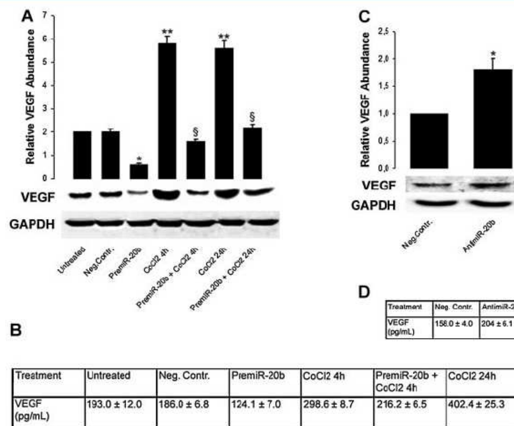


Fig. 1. **MiR-20b is downregulated during hypoxia and regulates VEGF mRNA expression. The abundance of miR-20b (A) and VEGF mRNA (B) were studied with quantitative real-time PCR. MCF-7 cells were treated with $CoCl_2$ for 4 or 24 h, and/or transfected with pre-miR-20b for 48 h or left untreated. To normalize miR-20b and VEGF quantitative real-time PCR reactions, parallel reactions were run on each sample for RNU6B or Cyclophilin A, respectively. The graphs represent respectively the increase of miR-20b and VEGF mRNA relative to untreated ± SD; *Columns*, mean; *bars*, SD. $^*P < 0.05$, control versus $CoCl_2$; $^{**}P < 0.05$, $CoCl_2$ versus miR-20b + $CoCl_2$.**



Treatment	Neg. Contr.	AntimiR-20b
VEGF (pg/mL)	158.0 ± 4.0	204 ± 6.1

Treatment	Untreated	Neg. Contr.	PremiR-20b	CoCl2 4h	PremiR-20b + CoCl2 4h	CoCl2 24h	PremiR-20b + CoCl2 24h
VEGF (pg/mL)	193.0 ± 12.0	186.0 ± 6.8	124.1 ± 7.0	298.6 ± 8.7	216.2 ± 6.5	402.4 ± 25.3	294.0 ± 12.2

Fig. 2. **VEGF protein levels are decreased by transfection with pre-miR-20b and increased by miR-20b silencing. A: The abundance of VEGF total protein was determined by WB in 120 µg of total proteins, as decribed in Materials and Methods Section. The graph represents the increase of VEGF protein levels relative to untrasfected cells ± SD; *columns*, mean; *bars*, SD. $^*P < 0.05$, control versus pre-miR-20b; $^{**}P < 0.05$, control versus $CoCl_2$; §$P < 0.05$, $CoCl_2$ versus miR-20b + $CoCl_2$. B: The secreted protein levels were determined by ELISA. A total of 7×10^5 MCF-7 cells were treated as described Materials and Methods Section. The concentrations represent pg of VEGF/ml of conditioned medium from 4×10^5 cells. C: The abundance of VEGF proteins was studied by WB in 120 µg of total proteins in MCF-7 cells transfected with synthetic oligonucleotides targeting miR-20b (anti-miR-20b) or with negative control anti-miR molecules, as described in Materials and Methods Section. The graph represents the increase of VEGF protein levels relative to negative control ± SD; *columns*, mean; *bars*, SD. $^*P < 0.05$ control versus anti-miR-20b. D: The concentration of secreted VEGF was measured by ELISA in cells transfected with anti-miR20b or with negative control anti-miR. The concentrations represent pg VEGF/ml conditioned medium from 4×10^5 cells.**

replaced with non-immune rabbit IgG. All experiments were repeated at least three times.

VEGF detection by ELISA

A total of 7×10^5 (pre-miR-20b transfections) or 4×10^5 (antimiR-20b and siSTAT3 transfections) MCF-7 cells were treated, as described before. VEGF protein was measured in conditioned medium using the Human Quantikine ELISA kit (R&D, Systems, Minneapolis, MN) with the lowest detection limit of 5 pg/ml, with intra-assay precision of <4.8% (or better than 2.5% and <4.8%) and inter-assay precision of <7.2% (or better than 4.7% and <7.2%). All points were done in triplicate, and the experiments were repeated three times. The range of curve standards was 0, 15.6, 31.2, 62.5, 125, 250, 500, 1,000 pg/ml; all VEGF concentrations in samples were within the range of curve standard. Linear regression analysis was performed to create the standard curve.

Statistical analysis

The correlations were studied by Student's *t*-test. *P*-values of <0.05 were considered statistically significant.

Results

Effects of miR-20b on VEGF expression under hypoxia-mimetic conditions

Since miR-20b has been suggested to act as a negative regulator of VEGF in nasopharyngeal carcinoma epithelioid (CNE) cells (Hua et al., 2006), we set out to study the effects of this miRNA on VEGF expression in breast cancer cells under hypoxia-mimetic conditions. First, we verified how treatment of cells with $CoCl_2$, a hypoxia-mimetic agent, affects miR-20b and VEGF expression. We found that $CoCl_2$ downregulated miR-20b expression at 4 and 24 h by 3.3- and 3.0-fold, respectively, relative to untreated cells (Fig. 1A). The specific downregulation of miR-20b was verified by running parallel reactions for miR-20a detection, where no change in miR-20a level under $CoCl_2$ was observed (data not shown). In parallel, $CoCl_2$ treatment induced VEGF mRNA expression by 4.5-fold compared to untreated cells (Fig. 1B).

Next, we investigated the effects of miR-20b on VEGF protein expression in breast cancer cells. VEGF intracellular protein levels were reduced in pre-miR-20b transfected cells by 2.5-fold related to untransfected cells (Fig. 2A), while VEGF basal mRNA levels were not significantly affected (Fig. 1B). Stimulation with $CoCl_2$ for 4 or 24 h increased intracellular VEGF protein levels by 2.9- and 2.7-fold, respectively (Fig. 2A). However, transfection of MCF-7 cells with pre-miR-20b, reduced a 4 h $CoCl_2$-induced stimulation of VEGF protein by 3.3-fold (Fig. 2A) and VEGF mRNA levels by 1.2-fold (Fig. 1B). In cells treated with $CoCl_2$ for 24 h, pre-miR-20b inhibited VEGF intracellular protein levels by 2.7-fold (Fig. 2A), but VEGF mRNA expression was not affected (Fig. 1B).

The effects of miR-20b on the secreted VEGF protein mirrored the above findings. As expected, $CoCl_2$ treatment for 4 and 24 h induced extracellular VEGF protein by 60% and 105%, respectively (Fig. 2B). Consistent with previous results, transfection with pre-miR-20b inhibited 4 and 24 h $CoCl_2$-mediated induction of extracellular VEGF by 30% and 25%, respectively, relative to negative control (Fig. 2B), while basal level of secreted VEGF were reduced by 32%.

In further validate the impact of miR-20b on VEGF expression, we used a synthetic oligonucleotide (anti-miRs) designed to target the mature forms of miR-20b and thereby inhibit their expression. Following miR-20b silencing, intracellular VEGF proteins levels increased by 1.5-fold (Fig. 2C), while the amount of secreted VEGF were elevated by 20% (Fig. 2D).

MiR-20b downregulates HIF-1α and STAT3

Next, we investigated the molecular mechanism by which miR-20b can regulate VEGF mRNA expression under $CoCl_2$ treatment. We hypothesize that this miRNA could exert its activity by targeting both HIF-1α and STAT3.

Transfection of pre-miR-20b reduced 4 h $CoCl_2$-dependent HIF-1α cytosolic and nuclear accumulation by 1.5- and 1.8-fold (Fig. 3A). Next, we tested whether miR-20b could downregulate STAT3. Cytosolic levels of STAT3 are not significantly affected by pre-mir-20b transfection. STAT3 nuclear accumulation was strongly inhibited by miR-20b relative to $CoCl_2$-treated cells for 4 h (Fig. 3A). At 24 h $CoCl_2$ treatment, miR-20b did not modulate nuclear accumulation of HIF-1α and STAT3 proteins (Fig. 3B), despite it retains the ability to downregulate HIF-1α, but not STAT3, in the cytoplasm (Fig. 3B).

Changes in nuclear accumulation and cytoplasmic downregulation of HIF-1α and STAT3 were confirmed by immunofluorescence analysis, which revealed that HIF-1α levels were considerably reduced in both cytoplasm and

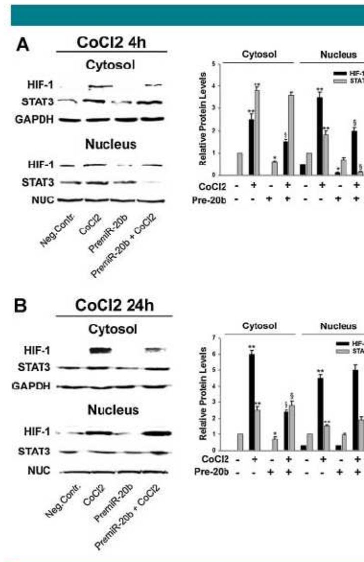


Fig. 3. MiR-20b modulates HIF-1α and STAT3 protein levels. The abundance of HIF-1α and STAT3 protein levels were studied by WB, as described in Materials and Methods Section. MCF-7 cells were treated with $CoCl_2$ for 4 (A) or 24 h (B), and/or transfected with pre-miR-20b for 48 h or left untreated. Protein loading was controlled by re-probing WB filters for the expression of a nuclear marker nucleolin (NUC) or cytosolic protein GAPDH. The graphs represent increase of HIF-1α and STAT3 levels relative to negative control ± SD. *Columns*, mean; *bars*, SD. $^*P<0.05$, control versus pre-miR-20b; $^{**}P<0.05$, control versus $CoCl_2$; §P<0.05, $CoCl_2$ versus pre-miR-20b + $CoCl_2$.

nucleus of cells transfected with miR-20b and stimulated with 4 h $CoCl_2$ compared to the cells treated with $CoCl_2$ alone (Fig. 4A). Interestingly, as previously indicated by WB (Fig. 3A), miR-20b inhibited STAT3 nuclear accumulation rather than cytosolic. Moreover, immunofluorescence analysis shows that transfection with pre-miR-20b under normoxic conditions slightly downregulates both HIF-1α and STAT3 compared to negative control (Fig. 4A).

Finally, MCF-7 cells were transfected with the specific anti-miR-20b molecule or with anti-miR negative control. When the cells were transfected with anti-miR-20b, both cytoplasmic protein levels of HIF-1α and STAT3 were slightly increased, while the levels were increased by twofold and threefold, respectively, in the nucleus (Fig. 4B).

HIF-1 and STAT3 binding to VEGF promoter is modulated by miR-20b under $CoCl_2$ treatment

We hypothesized that miR-20b might be involved into the regulation of HIF-1α or STAT3 binding to VEGF regulatory sequences. Using ChIP assay, we found that the HIF-1α binding to VEGF promoter significantly decreased by fourfold in cells transfected with pre-miR-20b and treated with $CoCl_2$ for 4 h, compared to untreated cells (Fig. 4C). However, HIF-1α binding was no longer affected by miR-20b following 24 h $CoCl_2$ treatment (data not shown).

Next, we addressed STAT3 loading on VEGF promoter. We did not detect STAT3 binding to VEGF promoter after 4 h treatment with $CoCl_2$. Finally, RNA Polymerase II (RNAPII)

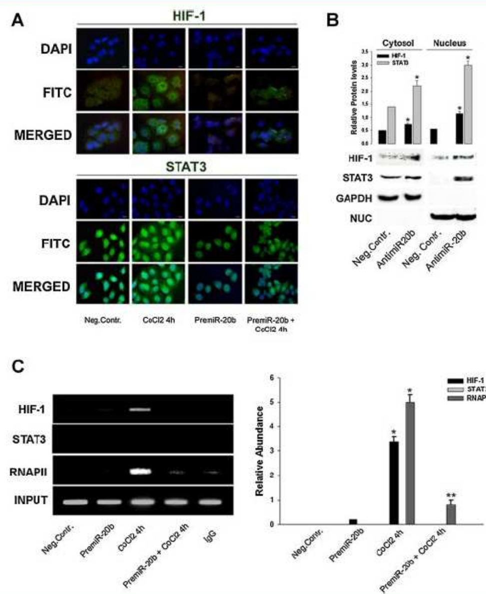


Fig. 4. miR-20b decreases HIF-1α and STAT3 nuclear accumulation and affects HIF recruitment on VEGF promoter. A: The expression of HIF-1α and STAT3 (green fluorescence) was assessed by immunostaining with specific Abs and fluorescence microscopy, as detailed in Materials and Methods Section. Cell nuclei were stained with DAPI (blue fluorescence). *Scale bar*, 10 μm. B: HIF-1α and STAT3 protein levels in anti-miR-20b or negative control transfected cells were studied by Western blotting. Protein loading and purity of fractions were controlled by re-probing WB filters for the expression of a cytoplasmic protein GAPDH and nucleolin (NUC). The graphs represent increase of HIF-1α and STAT3 levels relative to negative control ± SD. *columns*, mean; *bars*, SD. $^*P < 0.05$, control versus anti-miR-20b. C: miR-20b inhibits the recruitment of HIF-1 and RNAPII on VEGF promoter. ChIP experiments were performed as described in Materials and Methods Section. To test whether miR-20b could modulate the loading of HIF-1α, STAT3, and RNAPII transcription factors on the VEGF promoter. The graph represents relative abundance of HIF-1α, STAT3, or RNAPII on the VEGF promoter, relative to negative control ± SD; *columns*, mean; *bars*, SD. $^*P < 0.05$, control versus $CoCl_2$; $^{**}P < 0.05$, $CoCl_2$ versus miR-20b + $CoCl_2$.

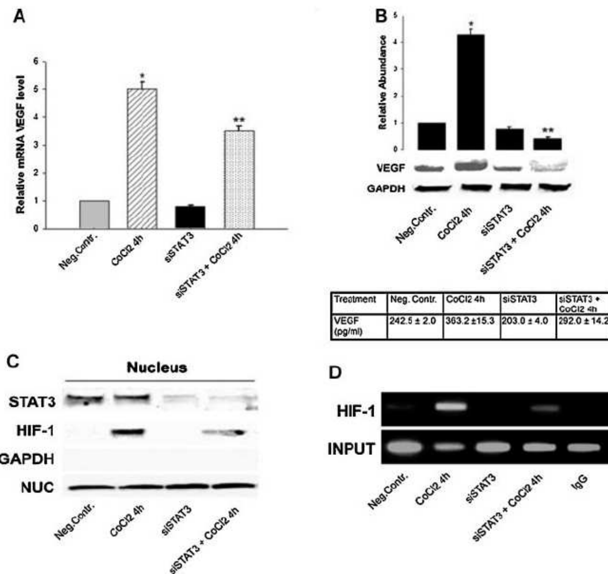
binding to VEGF promoter was investigated to assess whether HIF-1α loading promotes assembly of a functional transcription complex. Similar to HIF-1α, RNAPII binds the VEGF promoter upon treatment with $CoCl_2$ while pre-miR20b transfection inhibits RNAPII binding in $CoCl_2$-treated cells by 3.5-fold (Fig. 4C).

$CoCl_2$-dependent modulation of VEGF by miR-20b requires STAT3

Finally, we investigated the role of STAT3 in miR-20b-modulated VEGF transcription. Specifically, we tested whether STAT3 could modulate loading of HIF-1α on the VEGF promoter, indirectly affecting VEGF transcriptional activation.

First, transcriptional regulation of VEGF by STAT3 was determined by targeting STAT3 with RNA-duplex interference. Targeting STAT3 did not significantly decrease basal levels of VEGF (Fig. 5A). However, siSTAT3 downregulated the 4 h $CoCl_2$-induced VEGF expression by 1.5-fold, relative to control. Following STAT3 silencing, VEGF protein levels were reduced by 20% relative to negative control, and by 80% in siSTAT3 plus $CoCl_2$, relative to $CoCl_2$ only treated cells (Fig. 5B). Negative (control) target RNA did not interfere with VEGF mRNA and protein expression (data not showed). ELISA assay confirmed a 20% decrease in secreted VEGF protein level in siSTAT3 cells, compared to negative control, and a 20% decrease in siSTAT3- and $CoCl_2$-treated cells, compared to $CoCl_2$-treated cells (Fig. 5B).

Immunoblot analysis revealed a significant decrease of 4 h $CoCl_2$-dependent HIF-1α nuclear accumulation under STAT3 silencing (Fig. 5C). Finally, we assessed whether STAT3 was able to regulate HIF-1 transcriptional activity. STAT3 silencing



Treatment	Neg. Contr.	CoCl2 4h	siSTAT3	siSTAT3 + CoCl2 4h
VEGF (pg/ml)	242.5 ± 2.0	363.2 ±15.3	203.0 ± 4.0	292.0 ± 14.2

Fig. 5. Knockdown of STAT3 interferes with HIF-1 trascriptional activity and reduces VEGF expression. STAT3 knockdown was achieved using siRNA, as described in Material and Methods Section. MCF-7 cells were transfected with siSTAT3 for 48 h and/or treated with $CoCl_2$ for 4 h to mimic hypoxia or left untreated. Non-specific siRNA was used as a negative control. A: VEGF mRNA expression levels were determined by quantitative real-time PCR. The graphs represent increase in VEGF mRNA relative to negative control ± SD; *columns*, mean; *bars*, SD. $^{*}P < 0.05$, control versus $CoCl_2$; $^{**}P < 0.05$, $CoCl_2$ versus siSTAT3 + $CoCl_2$. B: The expression of VEGF protein was determined by WB in 80 μg of cytosolic proteins. GAPDH was used to normalize protein levels. Cells were transfected with STAT3 siRNA and/or treated for 4 h with $CoCl_2$ or left untreated. The graph represent increase in VEGF protein relative to negative control ± SD; *columns*, mean; *bars*, SD. $^{*}P < 0.05$, control versus $CoCl_2$; $^{**}P < 0.05$, control versus siSTAT3; §$P < 0.05$, $CoCl_2$ versus siSTAT3 + $CoCl_2$. The abundance of secreted VEGF (pg/ml) in conditioned medium was determined by ELISA, as described in Materials and Methods Section. A total of 4×10^5 cells were treated as described above. C: STAT3 silencing efficiency was confirmed by both quantitative real-time PCR (data not shown) and Western blot. The expression of HIF-1α and STAT3 proteins was analyzed in 50 μg of nuclear cell lysates. Nucleolin was used as nuclear loading control. D: In STAT3-knockdown MCF-7 cells, the modulation of HIF-1 binding on VEGF promoter was assessed by ChIP assay.

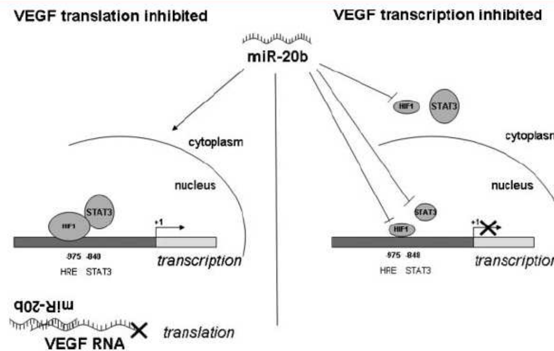


Fig. 6. Model of translational and transcriptional regulation of VEGF expression by miR-20b. (Left) pre-mir-20b transfection decreases VEGF protein levels. (Right) pre-mir-20b control VEGF transcription through HIF and STAT3 factors.

diminished HIF-1α recruitment to the VEGF promoter under $CoCl_2$ treatment, similar to that caused by miR-20b (Fig. 5D). In summary, these results indicate that STAT3 modulates HIF-1α binding to VEGF promoter at 4 h of hypoxia-mimetic treatment (Fig. 6).

Discussion

In the present study, we identified VEGF and its two main hypoxia-inducible transcriptional activators, HIF-1α and STAT3, as targets for miR-20b in MCF-7 breast cancer cells. One previous report identified miR-20b as a negative regulator of VEGF protein, and recently a reciprocal regulation of miR-20b and HIF-1 has been suggested in H22 cells (Hua et al., 2006; Lei et al., 2009). Here, we confirmed that VEGF is a direct target of miR-20b in breast cancer. First, we found that transfection with pre-miR-20b reduced the levels of both cytoplasmic and secreted VEGF protein under hypoxia-mimicking and normoxia conditions in MCF-7. On the other hand, transfection with synthetic anti-miR-20b increased cytoplasmic and secreted VEGF protein level significantly, even though the effect of miR-20b inhibition appears to be lower compared to pre-miR-20b activity. This is due to a decreased transfection efficiency.

In addition, we observed that transfection with pre-miR-20b decreased VEGF mRNA levels, suggesting that miR-20b can modulate VEGF transcription. According to our data, this effect is mediated by miR-20b-dependent inhibition of both HIF-1α and STAT3 nuclear accumulation under a 4 h $CoCl_2$ treatment. We hypothesize that this could be related to miR-20b-induced HIF-1α degradation in the cytoplasm and reduction of STAT3 nuclear translocation, perhaps via interference with its phosphorylation. Notably, miR-20b action on VEGF mRNA expression was noticeable only under a short $CoCl_2$ treatment (4 h), as prolonged $CoCl_2$ treatment (24 h) did not interfere with VEGF mRNA induction or nuclear accumulation of HIF-1α and STAT3. We hypothesize that miR-20b can directly target moderate amounts of HIF-1α in the cytoplasm (4 h $CoCl_2$), but is not able to compete with the enhanced accumulation of this protein under prolonged hypoxia-mimicking conditions (24 h $CoCl_2$; Bertout et al., 2008). Subsequent ChIP analyses revealed that miR-20b inhibited binding of HIF-1α on the VEGF promoter, which could explain the diminished VEGF mRNA transcription (Gray et al., 2005; Jung et al., 2005), we did not find STAT3 on VEGF promoter in MCF-7 cells at 4 h. Since it is known that transcription factors bind chromatin in an ordered and cyclical manner, which is dependent on cell type (Metivier, 2003; Cascio et al., 2007) we hypothesize that observed discrepancies could be related to cell-specific dynamics of STAT3 interactions the VEGF promoter.

Finally, our siRNA-mediated knockdown experiments indicated that VEGF expression can be controlled by STAT3 under hypoxia-mimicking conditions. Specifically, STAT3 downregulation inhibited HIF-1α nuclear accumulation and binding to the VEGF promoter in response to 4 h $CoCl_2$ treatment. This could be caused by reduced STAT3 interaction with HIF-1α, which normally increases HIF-1α stability by interfering with von Hippel–Lindau protein (pVHL) factor (Jung et al., 2008).

In summary, this report demonstrates for the first time that the VEGF expression in breast cancer cells is mediated by HIF-1α and STAT3 in a miR-20b-dependent manner. Our work also implies that angiogenesis of breast cancer can depend on the intricate circuit involving VEGF, STAT3, HIF-1α, and miR-20b, all of which can become novel targets in breast cancer treatment.

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cancer
TREATMENT
REVIEWS

Searching for the Target in Oncology

12th National GOIM (Gruppo Oncologico dell'Italia Meridionale) Annual Meeting

Guest Editors:
Saverio Cinieri, Brindisi
Giuseppe Colucci, Bari

Co-Guest Editors:
Evaristo Maiello, S. G. Rotondo
Antonio Russo, Palermo
Nicola Silvestris, Bari

Methods: Retrospective analysis of 17 patients with SCC due to metastatic breast cancer treated from 2005 to 2009. The patients have been submitted to 4 different RT schedule: 2000 cGy (400 cGy × 5), 3000 cGy (300 cGy × 10) and a split-course regimen of 5 Gy × 3, 4 days rest, and then 3 Gy × 5, and a short-course regimen of 8 Gy, 7 days rest, and then 8 Gy.
Results: At presentation 6 patients were ambulant with mild neurological deficit, 8 patients were paraparetic and 3 patients were paraplegic. Diagnosis was established by CT-scan or MRI of the spine, 15 patients presented dorsal or lumbar pain requiring opioid treatment on average 25 days before onset of neurological symptoms (range 10–230 days). All patients underwent steroid treatment; the 14 patients underwent radiotherapy alone and 3 radiotherapy and laminectomy. Overall 10/17 patients were ambulant after treatment. 2 out of 3 patients treated by laminectomy and radiotherapy were ambulant after treatment versus 8 out of 14 patients treated by radiotherapy alone. 14 patients died during follow-up with a median survival of 3.7 months (2 weeks to 41 months), while 3 patients were alive at the last control. No patient complained of spinal cord morbidity.
Conclusions: The patients' prognosis with spinal cord compression from metastatic breast cancer is poor. Cord compression should be treated promptly, late cases with loss of ambulation and sphincter function is associated with poor prognosis and poor outcome. The golas of treatment are (4Ps): a) preservation or recovery of neurological function, b) palliation of pain, c) prevention of recurrence, d) preservation of spinal stability. Besides if treatment is started within 24 to 48 hours of onset of symptoms neurological damage may be reversible. Heightened awareness of the significance of back pain is the most important factor in successful treatment of cord compression. Efforts must be concentrated on early diagnosis and on prevention of spinal cord compression: Moreover patients with spinal cord compression from metastatic breast cancer who develop persistent back pain should undergo imaging studies (bone scan, spine CT-scan or MR) to the purpose to identify precocious lesions and to begin the radiant treatment.

31
HIGH ACTIVITY OF SEQUENTIAL COMBINATION OF LOW DOSE CHEMO-MODULATING TEMOZOLOMIDE (TMZ) + FOTEMUSTINE (FM) IN METASTATIC MELANOMA (MM). A FEASIBILITY STUDY

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Background: MM is an incurable and chemoresistant cancer with poor prognosis. Preclinical and clinical experiences, support the concept that continuous exposure to alkilating agent, can effectively deplete cells of the DNA repair enzyme O^6-methylguanine DNA methyltransferare which is the primary mechanism of tumor resistance to alkylating agents like nitrosurea analogs. Our study was finalized to verify this hypothesis using a sequential combination of low dose chemo-modulating TMZ with FM. Primary endpoints were safety and tumor response evaluation.
Methods: 14 consecutive MM pts were enrolled into two well balanced cohorts of 7 pts each using 2 schedules of TMZ+FM (Cohort A: TMZ *os* 100 mg/m^2 d1,2; 7,8; FM *iv* 100 mg/m^2 d2, 8, 4 h after TMZ, every 4 weeks for 2 cycles; then every 3 weeks for further 6 cycles. Cohort B: TMZ+FM at the same dose but every 3 weeks for a total of 9 cycles).
Results: Main results are reported in the table.

Cohort	Schedule	Toxicity profile G3–G4			Response	
		Neutrop.	Thrombop.	Anemia	Number	Site
Cohort A	1,8,28	1/7 pts	4/7 pts	1/7 pts	1 CR, 2 PR, 2 SD	LN, soft tissue, bowel
Cohort B	1,21	1/7	1/7	0/7	2 PR, 3 SD	LN, soft tissue, adrenal gland, liver

Conclusions: sequential combination of low dose TMZ and FM demonstrated a high activity in our pts population. d1–21 schedule showed a more acceptable toxicity with respect to d1–8-28 schedule maintaining his antitumoral activity. Thus schedule d1–21 has been used in our phase II ongoing study.

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PAIN MANAGEMENT AND QUALITY LIFE IN BONE METASTASIS FROM BREAST CANCER: ROLE OF RADIOTHERAPY

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Background: In this study we have evaluated the impact on the control of the pain and on the quality of life of different schemes of dose fractions and total dose of radiotherapy in patients affected by bone metastasis.
Methods: Between 2003 and 2008, 78 patients, with bone metastasis from breast cancer were treated at "Hospital Pugliese-Ciaccio", Catanzaro. At December 2008, 78 patients with a median age at diagnosis of 64 years (range 35–74 years) were analyzed, of these, 57 (73%) were males and 21 (27%) females. Forty-five patients introduced multiple metastasis while in the remaining patients the bone metastasis was unique. The intensity of pain was assessed by WHO Criteria. The average follow-up time was 7 months (range 2–45 months).
Results: All patients manifested moderate (23/78) or severe (55/78) pain and were in treatment with transdermic fentanyl (25, 50, 75 mg/h) in association or less to FANS. In sixty-seven patients we have used a scheme of dose fractions of 300 cGy × 10 fractions (total dose, TD 3000 cGy), in 9 patients a schemes of dose fractions of 400 cGy × 5 fractions (TD, 2000 cGy) and in two patients a schemes of dose fractions of 600 cGy × 2 fractions (TD, 1200 cGy), in all patients was associated treatment with Zoledronic acid. Fifty five patients, at the end of the treatment, have obtained an improvement in the intensity of the pain, in ten the total disappearance of the same. For fifteen patients has been necessary to perform a new treatment after a median of 6.5 months (range 2–9 months).
Conclusions: In summary, our data confirm the results of literature on the control of the pain and on the improvement of the quality of life of the patients with bone metastases treated with radiotherapy and Zoledronic acid. Moreover, with regard the different schemes of radiotherapy dose fractions and total dose, the short course regimen (600 cGy × 2) can become the treatment of choice for the majority of patients with bone metastasis.

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EFFECT OF miR-21, miR-182 AND let-7i ON TSP-1 EXPRESSION IN COLON CANCER CELL LINE

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Background: MicroRNAs (miRNAs) are small non-coding RNAs that regulate the expression of different genes, including genes involved in cancer progression, angiogenesis and metastasis. Thrombospondin-1 (TSP-1) has been shown to contrast angiogenesis in vivo. TSP-1 expression levels are inversely correlated with tumor vascularity and metastasis in colon cancer. Bio-informatic statistical analysis indicated that TSP-1 is hypothetical target of miR-21, miR-182, overexpressed in CRC, and let-7i which expression is down-regulated in this tumor. In this work we investigated whether TSP-1 expression could be regulated by miR-21, mir-182 and let-7i in HT29 colon cancer cell line.
Methods: To investigated whether miR-21, mir-182 and let-7i directly modulates TSP-1 expression, we transfected HT29 cell line with pre-mir21, pre-mir182 and pre-let7i by using siPortNeo FX tranfection agent and after 48h we evaluated TSP-1 mRNA using Quantitative Real Time-PCR, and intracellular and secreted protein level performed by Western blotting and ELISA. To confirm the modulation of TSP-1 by miRNAs we transfected HT29 cell line with anti-mir to target the mature form of miR-21, miR182 and let-7i.
Results: Using Real-Time PCR we did not find any variation of TSP-1 mRNA expression levels after transfection with pre-miRs in HT29 cell line, but we observed a down-regulation of cytosolic and secreted protein by Western blot and ELISA. In cells transfected with pre-mir182 we did not observe any down-regulation both

of note, OS was significantly longer in pts with higher expression of IGF1R if compared with those with normal/lower expression (17 vs. 8 months; $p = 0.011$).

Conclusions: As known, *KRAS/BRAF* mutations are the strongest negative predictive marker of response to cetuximab in mCRC. cMET overexpression could represent a new predictive marker of resistance to cetuximab and a prognostic factor in mCRC. Interestingly, IGF1R overexpression is not associated with resistance to cetuximab but it seems to represent a favorable prognostic factor in mCRC.

4

SORAFENIB PLUS CISPLATIN AND GEMCITABINE IN THE TREATMENT OF ADVANCED HEPATOCELLULAR CARCINOMA (HCC): A PHASE II STUDY BY THE GRUPPO ONCOLOGICO DELL'ITALIA MERIDIONALE (PROT. GOIM 2705)

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Background: sorafenib is the standard treatment in advanced HCC. The combination of cisplatin and gemcitabine demonstrated to be active and well tolerated in tumors with a similar poor outcome such as pancreatic and biliary-tract cancers. Considering these data, the GOIM started a phase II trial aiming to evaluate the activity and safety of the combination of sorafenib, gemcitabine and cisplatin in advanced HCC.

Methods: patients affected by advanced HCC, not suitable for surgery or loco-regional procedures, with measurable disease (Recist criteria), age ≥18 years, clip-score ≤3, Ecog performance status ≥60 (K.fsky), adequate bone marrow reserve and renal and hepatic function and who signed written informed consent, were enrolled and received cisplatin at 40 mg/mq iv plus gemcitabine at 800 mg/mq iv bi-weekly, while sorafenib was orally administered at the dosage of 400 mg bid continuously. A maximum of 6 cycles of chemotherapy was planned; a maintenance with sorafenib was permitted for not progressing patients. The evaluation of activity was performed every three cycles. A Simon's two stage, two steps study design was applied: at the first step, at least 3PR had to be observed among the first 28 patients to continue the enrollment. Up to now, 17 patients have been enrolled. Their main characteristics were: sex (male/female) 13/4, median age: 67, median PS 80, main site of disease liver 16, lymph-nodes 5, lung 1, other 1.

Results: among the first 11 evaluable patients we observed 1CR, 3PR, 3SD and 4PRO for an ORR of 4/11 (36%) and a tumor control of 7/11 (63%). Fifteen patients are evaluable for safety.

The main observed side effects (%G1–2/G3–4) (NCI criteria) were: hand-foot skin reaction (HFSR) 0/13, mucositis 6/6, diarrhea 33/13, nausea/vomiting 33/0, leucopenia 13/0, anemia 13/0, thrombocytopenia 13/6, asthenia 6/6, hepatic 6/6, cardiovascular 0/6.

Conclusion: our preliminary data seems to demonstrate that the combination of cisplatin, gemcitabine and sorafenib is active and well tolerated in advanced HCC patients. The accrual is ongoing.

5

PANITUMUMAB PLUS CHEMOTHERAPY AS SALVAGE TREATMENT IN PRETREATED ADVANCED COLORECTAL CANCER PATIENTS: A SINGLE INSTITUTION'S EXPERIENCE

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Background: Panitumumab, a fully human monoclonal antibody direct against the epidermal growth factor receptor (EGFr) demonstrated to be active in pretreated advanced colorectal cancer patients. No data exists regarding its activity when employed in combination with chemotherapy in pretreated patients who previously received a cetuximab-based therapy. So the GOIM analyzed the activity and the safety of the combination of panitumumab plus chemotherapy in pretreated patients who previously received both cetuximab and bevacizumab-based regimens.

Methods: patients affected by metastatic colorectal cancer, who previously received both cetuximab and bevacizumab-based regimens, with measurable disease (Recist criteria), age >18 yrs, Ecog performance status ≤1, adequate bone-marrow reserve and renal and hepatic function and who signed a written informed consent, were treated with a combination of panitumumab at 6 mg/kg on day 1 plus chemotherapy (5 pts with oxaliplatin 85 mg/mq on day 1 plus capecitabine 1500 mg/mq on days 1–7 every two weeks, 5 pts with oxaliplatin at 85 mg/mq on day 1 plus tomudex 2 mg/mq on day 1 every two weeks, 2 pts with irinotecan-based regimens). The disease evaluation was performed every 3–4 cycles.

Results: up to now, 12 patients have been treated. Their main characteristics were as follows: sex (male/female) 8/4, median performance status 1 (range 0–2), median age 68 (range 54–72), main sites of disease liver 11, lymph-nodes 3, lung 4, others 6. Among the first 7 evaluable patients we observed 1PR, 3SD and 3PRO disease for an ORR of 1/6 (17%) and a tumor control of 4/6 (67%).

The main observed side-effects (% G1–2/G3–4) NCI criteria in eleven patients who received at least one cycle, were: skin 73/0, leucopenia 9/0, anemia 0/9, thrombocytopenia 9/0, mucositis 18/0, diarrhea 18/9, nausea/vomiting 18/0.

Conclusions: our preliminary data seem to demonstrate that panitumumab plus chemotherapy is active and well tolerated in cetuximab and bevacuzumab pretreated advanced colorectal cancer patients. The safety profile seems to be not different from that of cetuximab-based regimens.

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***C-KIT* MUTATIONS IN GASTROINTESTINAL STROMAL TUMORS**

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Background: Gastrointestinal Stromal Tumors (GISTs) are specific, generally Kit (CD117)-positive, mesenchymal tumors of the gastrointestinal tract encompassing a majority of tumors previously considered gastrointestinal smooth muscle tumors. The majority of C-KIT Immuno-histochemically positive tumors present mutations in *C-KIT* gene, but a minor percentage (less than 5%) can have mutations in *PDGFRa* gene.

Most Kit-mutant proteins are sensitive to Imatinib. We report our consecutive series of mutation analysis in GIST.

Methods: Samples were collected. DNA was extracted by paraffin embedded tissues section of GIST (after qualifying examination by pathologist), and Polymerase Chain Reaction (PCR) assay was performed and DNA was purified. Samples were sequenced by Direct Squencing (Abiprism 3100). According with data reported in literature we examined the four different "hot-spot" regions of *KIT* found to be mutated in sporadic GISTs (respectively in decreasing order of frequency: exon 11, exon 9, exon 13, and exon 17), and the 3 most frequent mutation regions for *PDGFRa* gene (located in exon 18, 12, 14).

Results: From 30 cases analyzed, 9 (30%) were wild type (Wt), 18 (60%) mutated in exon 11, 3 (10%) in exon 9 of *C-KIT* gene, independently from the tumor location. Of the Wt samples 2 had indeed a mutation in exon 18 of *PDGFRa* gene. The most frequent mutations in exon 11 of *C-KIT* gene were aminoacidic substitutions located at the 5′ of the coding region of the gene, followed by deletions and insertions.

Conclusions: *C-KIT* mutational status is fundamental for the target therapy in GIST, and this is underlined by the very high frequency of mutations in this tumors, expecially in exon 11 of *C-KIT*, and by the good prognosis of patients reporting genetic alteration in this exon, unlike other patients carrying mutations in other *C-KIT* exons or in *PDGFRa* gene, as the D842V *PDGFRa* mutation. According with literature, the most frequent *C-KIT* genetic alterations founded, were located in exon 11, specifically in codons 549–550 and 556–558.

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that hypoxia inhibits the DNA repair process and promotes genomic instability in human cancers. Very little is known regarding the functional consequences of hypoxia in the expression of proteins involved in DNA double-strand break repair in human breast cancer.
Therefore the aim of our studies is to evaluate the effects of hypoxia on genomic stability in breast cancer cell lines to obtain new insights on role of the hypoxic tumor microenvironment on DNA repair and on genetic instability.
Methods: A microarray analysis, using Affymetrix platform, was performed in MCF7, MDA-MB-231 and SKBr3 breast cancer cell lines, cultured under normoxia and hypoxia for 24 and 48 hours, to identify genes showing a differential gene expression profile in the examined conditions.
Among all the genes, we selected those involved in DNA repair mechanisms to obtain new knowledge about the process that regulate genomic instability in response to hypoxia.
Results: MCF-7, MDA-MB-231 and SKBr3 breast cancer cell lines have shown a downregulated expression of *BRCA2* and other genes involved in DNA repair process. By focusing our attention on *BRCA2*, our results were confirmed evaluating the reduction of mRNA levels and the related protein by Real-Time PCR and Western Blotting. In the three breast cancer cell lines there was a reduction of the protein levels after 48 hours, but no particular difference after 24 hours.
Conclusions: Our data suggest that the hypoxia, decreasing the DNA repair capacity by downregulated expression of *BRCA2* and other genes involved in the same pathway, could be responsible for the continuous changes that affect the DNA during the process of tumorigenesis favoring the progression to stage more advanced of breast cancer.

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ANTIANGIOGENIC PROPERTIES OF IMMUNOMODULATORY DRUG LENALIDOMIDE IN ENDOTHELIAL CELLS OF PATIENTS WITH ACTIVE MULTIPLE MYELOMA

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Background: The immunomodulatory drug lenalidomide (Revlimid®) belongs to a novel class of small molecules, structurally related to thalidomide, with more potent and less toxic anti-inflammatory and anti-tumor activities, successfully used for the treatment of hematological cancers. It has shown impressive response rates in patients with relapsed/refractory multiple myeloma (MM), resulting in improved disease-free survival and overall survival. Its anti-tumor activity in MM is due to a dual mechanism: *i*) direct cytotoxic effect on MM plasma cells, through inhibition of plasma cell growth and induction of apoptosis, *ii*) indirect effect on their survival, by interfering with several components of the bone marrow microenvironment. Lenalidomide, indeed, inhibits the support of bone marrow stromal cells to plasma cells, by impairing cell adhesion, as well as the expression and secretion of the pro-angiogenic factors (VEGF and bFGF), and of other growth signals (TNF-α and IL-6) that promote bone marrow angiogenesis. It also stimulates T-cell and NK cell activities to plasma cells. However, its role in bone marrow endothelial cells of patients with MM (MMECs), remain still undefined.
Here we investigated whether lenalidomide can directly inhibit angiogenesis of bone marrow ECs of patients with MM in active phase, and sought to elucidate the molecular mechanisms involved.
Methods: We evaluated by *in vivo* experiment the angiogenic pathway through the chorioallantoic membrane (CAM) assay, in the interstitial fluid of patients daily treated with lenalidomide. The evaluation of angiogenic pathway was performed also by *in vitro* experiments. Real-Time PCR was performed to evaluate the drug effect on the expression of key genes closely related to angiogenesis, and western blotting and comparative proteomic analysis were performed to confirm the obtained data.
Results: We showed that 1.75 μM lenalidomide, i.e. the concentration reached in the interstitial fluid of patients daily treated with 25 mg, induces a significant inhibition of angiogenesis *in vivo* in the chorioallantoic membrane (CAM) assay. *In vitro*, lenalidomide inhibited angiogenesis and migration of MMECs, but not of ECs of patients with monoclonal gammopathies of undetermined significance (MGECs), while had no effect on MMECs proliferation, apoptosis and adhesion. Real-Time RT-PCR revealed that the drug strongly down-regulates the expression of key genes closely related to angiogenesis (VEGF, bFGF, CCL2, CXCL12, BNIP3, IER3, SEPW1). Finally, western blotting and comparative proteomic analysis showed that lenalidomide markedly affects VEGF/VEGFR2-mediated downstream signaling pathways involved in the motility process, such as mitogen activated protein kinase (MAPK) extracellular signal regulated kinase-1/2 (Erk-1/2), Src kinase, vascular endothelial (VE)-cadherin and NF-κB, and several other proteins controlling ECs invasiveness, cell-shape, cytoskeleton remodelling and energy metabolism as well.
Conclusions: Overall data provide evidence that lenalidomide exerts an antiangiogenic activity *in vivo* and *in vitro* on MMECs, and earmark new avenues for enhancing therapeutic activity in MM patients.

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EXPRESSION ANALYSIS OF *AURKA* UNDER HYPOXIA IN BREAST CANCER CELL LINES

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Background: *AURKA* is an oncogenic serine/treonine kinase that is highly misregulated in several types of human tumors, including breast cancer. Its overexpression inducing aneuploidy and centrosome amplification has been correlated with chromosomal instability and clinically aggressive disease.
Since hypoxia is a typical tumoral condition which influences the expression of various proteins involved in proliferation and cell cycle progression, aim of our study is to identify the mechanisms involved in *AURKA* expression, evaluating the possible HIF-1 role in its transcriptional control.
Methods: A microarray analysis, using Affymetrix platform, was performed in MCF7, MDA-MB-231 and SKBr3 breast cancer cell lines cultured under normoxia and hypoxia in order to compare the differential gene expression profile in response to hypoxia. A set of genes involved in cell cycle progression, angiogenesis and tumor pathogenesis was selected.
Results: We found a reduced expression of *AURKA* in all breast cancer cell lines analyzed and we confirmed this results showing a reduction of both mRNA levels and related protein, by Real-Time PCR and Western Blotting. The involvement of HIF-1 in the transcriptional control of *AURKA* expression was demonstrated by ChIP assay.
Conclusions: Our data suggest a new mechanism of *AURKA* regulation and, in discordance with previous reports, we hypothesize that this specific downregulation of *AURKA* might be able to suppress the proliferation and lead to the apoptosis of breast cancer cell lines.

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ThinPrep® CYTOLOGICAL SPECIMENS ARE OFTEN MORE SUITABLE THAN HISTOLOGICAL SPECIMENS TO DETECT EGFR AND K-RAS MUTATIONS IN NSCLC AND COLORECTAL CARCINOMA

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Background: *KRAS* (exon 2) and *EGFR* (exons 19–21) mutations have to be investigated before setting a target therapy in colorectal and lung cancer, respectively (NCCN guidelines v2.0, ...0).

Unfortunately, core biopsies not always permit to obtain intact DNA and the amplicons are not sufficient for direct sequencing. Furthermore, the histological material could not be quantitatively appropriate for all biomolecular analysis or could be not easily available.
Methods: To overcome this problem, ThinPrep® preparations, after cytological diagnosis, have been routinely stored at −20°C. Samples with more that 70% of neoplastic cells have been then used for molecular analyses. DNA has been extracted by commercial kit (Qiagen DNAmicro kit) and analyzed for *KRAS* and *EGFR* mutations by direct sequencing.
Results: Formalin-fixed paraffin-embedded (FFPE) tissues permitted *KRAS* analysis on 91% of 232 colon carcinomas and 82% of 49 NSCLC patients, while fine needle aspiration biopsy (FNAB) was suitable for 100% of 9 colon carcinomas and 83% of 12 patients with NSCLC. In particular, patient whose primary tissue DNA resulted unsuitable for molecular analyses, could receive the right therapy thanks to the availability of cytological metastatic specimen. Using this method it was possible to identify some patients with *EGFR* 19–21 mutations that have successfully been treated with tyrosine kinase inhibitor (TKI).
Conclusions: Fine-needle samples stored in ThinPrep® at −20°C are very good material for molecular detection directed to therapy setting. In addition, it is feasible to adopt a strategy of storing excess FNAB material to create cellular banks that will be invaluable for future genetic studies.

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COPY NUMBER VARIATION IN MALE BREAST CANCER

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Background: Male breast cancer (MBC) is a rare disease and little is known about its etiopathogenesis. Comparative genomic hybridization (CGH) is a technique by which it is possible to detect and map genetic changes that involve gain or loss of segments of genomic DNA. Microarray formats of CGH provide copy number information at thousands of locations distributed throughout the genome. The aim of this study was to investigate DNA imbalances by aCGH and compare them with a female breast cancer dataset.
Methods: The pathological tissues of 25 male breast cancer patients enrolled at the NCC of Bari were hybridized on high-density oligonucleotide aCGH arrays. aCGH was performed using the Agilent Human Genome CGH Microarray Kit (Agilent Technologies, Santa Clara, California, USA).
Data analysis was performed with Nexus Copy Number 5.0 software (Biodiscovery, Inc., El Segundo, CA, USA). This software uses the Rank Segmentation algorithm, a proprietary variation much faster at processing, on Circular Binary Segmentation (CBS) together with the statistical Significance Testing for Aberrant Copy number (STAC) method, to identify non random genomic amplifications and deletions across multiple experiments.
To integrate our analysis, we compare our dataset with a female breast cancer dataset deposited with the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/projects/geo//query/acc.cqi?acc5GSE12659), applying the same algorithms.
Results: All the 25 male and 16 female breast cancer samples displayed some chromosomal instability. Male and female datasets presented an average of 69 and 111 aberrations per patient, respectively. However, male presented a lower frequency of genetic alterations both in terms of loss and gains. The genomic aberration profile is quite different among the two datasets with very few common regions among male and female.
Conclusions: aCGH is an effective tool for analysis of cytogenetic aberrations in MBC, which involves different biological processes than female. Male most significant altered regions contained genes involved in cell communication, cell division and immunological response, while female cell-cell junction maintenance, regulation of transcription and neuron development.

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ANALYSIS OF MOLECULAR MECHANISMS AND ANTI-TUMORAL EFFECTS OF ZOLEDRONIC ACID IN BREAST CANCER CELLS

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Background: Zoledronic acid (ZOL) is a strong amino-bisphosphonate (N-BPs), that reduce skeletal complications in cancer treatment. N-BPs inhibit enzyme farnesyl diphosphate synthase, required for prenylation of small GTP-ases, such Rho, Rac and cdc42, implicated in key cellular processes and cancer development including breast cancer.
The aim of this study will be to identify, describe and summarize evidences on molecular basis, still unclear, of ZOL anti-tumoral effects, studying apoptosis, proliferation, adhesion, invasion and migration in breast cancer cells.
Methods: We observed gene expression profile by Microarrays analysis in MCF7, SkBR3, MDA-MB-231 breast cancer cell lines treated with ZOL for 24 hours. Quantitative Real Time-PCR (qRT-PCR) and Western Blotting confirmed differential gene expression and relative proteins. Involvement of cellular pathways, cell cycle regulation or possible transcription activation is examined by relative expression proteins (Western Blotting).
Results: Results revealed increased expression of TGFβ2, cytokines and cytoskeletal component similarly Actin and Fibronectin 1 (FN1), but reduced Bcl-2, receptor growth factors and solute carriers. In culture treated cells show morphologic alterations and inhibition of cell proliferation likely due to cell cycle arrest. Activation of TGFβ2 and possible consequences seems to be independent of MAPK and AKT pathways because their phosphorilation levels have been observed strongly reduced.
Conclusions: In light of these preliminary results we can speculate that a possible mechanism of action of ZOL may be due to its ability in migration cancer cells modulating attachment of tumor cells to extracellular matrix proteins. Furthermore we raised the hypothesis that treatment with zoledronate induces cytoskeletal modification by variation of FN1 and Actin expression, probably after TGFβ2-signalling, recently showed implicated in regulation of matrix proteins.

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CLINICAL SIGNIFICANCE OF INTRONIC VARIANTS OF *BRCA* GENES OF SICILIAN PATIENTS WITH HEREDITARY BREAST/OVARIAN CANCERS

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Background: Germline mutations in *BRCA1/2* genes confer a high risk to breast and/or ovarian cancer. Only mutations causing frameshifts and premature stop codons are generally assumed to be pathogenic. About 8% of all *BRCA1* and *BRCA2* alterations reported to the BIC database are intronic variants probably involved in splice sites and a subset of these variant are located in intronic sequences. These alterations may be defined as variant of uncertain/unknown significance (VUS), pathological and polymorphism.
Methods: One hundred and forty one patients with breast and/or ovarian cancer were screened for germline mutations in *BRCA1* and in *BRCA2* at the " Regional Reference Center for the Characterization and Genetic Screening of Hereditary Tumors" at the University of Palermo. In our study we performed a molecular analysis of the complete coding sequence and the exon-intron boundaries of *BRCA* genes, using dHPLC as pre-screening and then the identification of different sequence variants was done by automatic direct sequencing. Moreover, we collected a control population consisting of 50 index cases without a family history of cancer and we analysed this control group for the presence of VUS.
Results: During *BRCA1/2* molecular screening of this group of patients, we identified 7 intronic variants of which 1 (IVS7–34 C>T) in *BRCA1* gene and 6 (IVS24–16T>C, IVS25–12T>G, IVS2–7T>A, IVS 14+6G/A, IVS11–19delAT, IVS11+80del4) in *BRCA2* gene. The

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BIOLOGICAL CHARACTERIZATION OF MC70, AS POTENT INHIBITOR OF ABC TRANSPORTERS INVOLVED IN MULTIDRUG RESISTANCE

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Background: Multidrug resistance (MDR) is a major limitation in the cancer chemotherapy. The most important mechanism involved in MDR is the overexpression and the increased activity of several ATP-dependent efflux pump namely ATP-Binding Cassette (ABC) transporters, such as Pglycoprotein (P-gp), Breast Cancer Resistant Protein (BCRP) and MultiDrug Resistant Proteins (MRPs).
Methods: We characterized one potent P-gp inhibitor MC70, a 6,7 dimethoxytetrahydroisoquinoline derivative, in MCF7/ADR and Caco-2, a breast and a colon cancer cell line, respectively. Initially, we investigated P-gp inhibition by our compound in MCF7/Adr cells, analyzing its ability to interfere with Rhodamine-123 active efflux which was highly inhibited by MC70 probably through P-gp blockage. In the same cells, our compound was not able to induce consistent cell death or cell growth inhibition, conversely, it strongly enhanced the citotoxicity induced by doxorubicin when cells were pre-exposed to our P-gp inhibitor, with an increase of cell growth inhibition of about 66%. This strong activation of doxorubicin activity probably was not dependent, only, from P-gp inhibition and other cellular targets should be involved.
Results: To identify the mode of action of our compound, microarray analysis, cell cycle determination and western blotting were carried out. Cells were exposed to 20 μ MC70 for 2 days, mRNA was extracted and processed on Affymetrix GeneChip Human Gene 1.0 ST. Data suggested that this agent did not modulate mRNA expression probably acting at a post-transcriptional step. Cell cycle modulation by MC70 evidenced an early increase of cell accumulation in G2/M phase that seemed to recover the baseline cell cycle rate after 2 days drug exposure. Western blot analysis demonstrated that our agent stimulated Akt activation without affecting p-ERK1/2 phosphorylation. In Caco-2 cell, MC70 behaviour was quite different. This compound showed a strong antiproliferative activity when given alone and conversely it did not enhance doxorubicin cytotoxicity. The cell cycle perturbation by MC70 evidenced an accumulation of cells in S phase after 1 day exposure instead of G2/M phase, and its ability to stimulate Akt activation was confirmed. Furthermore, preliminary data suggested that MC70 was able to modulate other MDR transporter than P-gp as BCRP.
Conclusions: These evidences show that this agent, designed and synthesized as a pure P-gp inhibitor, enhanced conventional chemotherapeutics efficacy also through other mechanisms.

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ACTIVATING MUTATION OF KRAS ONCOGENE AS A PREDICTIVE BIOMARKER OF RESPONSE TO FIRST-LINE CONVENTIONAL CHEMOTHERAPY: A RETROSPECTIVE ANALYSIS

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Background: In metastatic colorectal cancer (mCRC) the concept of KRAS as a marker for resistance to anti-EGFR antibodies has been validated. However, new challenges emerged: the complete understanding of the crucial and central role of KRAS in processes of tumor growth and the development of new treatment strategies for KRAS mutant tumors. Since the combination of an anti-EGFR therapy with oxaliplatin (despite irinotecan) causes a significant worsening in mutated KRAS patients, we supposed that KRAS mutational status could also affect response to oxaliplatin.
Methods: We performed a retrospective analysis of 76 patients with mCRC. All patients received FOLFOX-6 schedule and FOLFIRI schedule+Bevacizumab, in first or second line therapy. We retrospective analyzed the efficacy of the FOLFOX-6 in terms of RR and PFS; then, we compared these parameters of effectiveness according to KRAS status and retrospectively analyzed the efficacy of front-line chemotherapy (FOLFOX-6 vs FOLFIRI) as RR and PFS. Person chi-square and log-rank test were respectively used to determine statistical sygnificance.
Results: All patients were in excellent conditions: Performance Status (ECOG) was 0. Among 76 patients, 34 were KRAS wild-type (wt KRAS) and 43 were KRAS mutated (mKRAS). 25 mKRAS patients received front-line FOLFOX-6 chemotherapy and 18 received FOLFIRI-B. CR+PR were 4 in wtKRAS (25%) and 19 in mKRAS (76%), in first line with FOLFOX-6 (p 0.006). PFS was 6.8 mts in wtKRAS and 10.7 in mKRAS (p 0.013), in first line. Considering mKRAS population, RR was 72% among patients receiving FOLFOX and 22% among those receiving FOLFIRI-B (p 0.0012). PFS was 10 months for FOLFOX group and 6 for FOLFIRI one. Once again a significantly better outcome was recorded for patients receiving FOLFOX (p 0.004).
Conclusion: Our data suggest that KRAS activating mutation could be predictive biomarker of response to oxaliplatin. Evaluation of molecular pathways, involved in oxaliplatin metabolism and potentially interfering with KRAS, is ongoing. A prospective comparison between FOLFOX and FOLFIRI is warranted in mutated KRAS patients.

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EFFECTS OF PPARγ AGONIST CIGLITAZONE ON VEGF EXPRESSION IN BREAST CANCER CELLS

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Background: PPARγ are ligand-activated transcription factors, members of the nuclear receptor superfamily. PPARγ can be activated by its natural ligand, the prostanoid 15-deoxy-prostaglandin J2, as well as by its synthetic ligands, such as thiazolidinediones (TZDs) class of antidiabetic drugs, including ciglitazone (CGZ). There is evidence that PPARγ is overexpressed in different cancers, including breast, prostate, pancreas and colon. However the role of PPARγ in cancer progression remains controversial. While some reports demonstrated anti-proliferative role of PPARγ ligands in cellular and animal models of human cancer, other documented that activation of PPARγ can induce cell growth and tumor proliferation, depending on the dose and duration of treatment. Interestingly, PPARγ agonists have been shown to stimulate angiogenesis in Huvec cells through upregulation of VEGF and other yet unidentified pro-angiogenic factors. Here we studied whether or not peroxisome proliferator-activated receptor (PPARγ) ligand CGZ can affect the expression of VEGF in breast cancer cells.
Methods: With XTT, we first tested the effects of PPARγ activation with 0.5 μM of CGZ on cell growth in MDA-MB-231 and MCF-7 breast cancer cells. In the second part, using Real-time Quantitative PCR, western blot and ELISA we assessed the effects of CGZ on VEGF mRNA expression, protein production and secretion.
Results: we found that submolar concentrations of CGZ induce cell proliferation in both MCF-7 and MDA-MB-231 breast cancer cells; this effect corresponds with an increase of VEGF mRNA, protein synthesis and secretion.
Conclusion: Although anti-cancer activity of PPARγ ligands has been observed in several in vitro studies our results, together with the observations of proliferative and angiogenic effects of PPARγ agonists observed with low doses of these compounds should open a debate about the safety of PPARγ targeting drugs.

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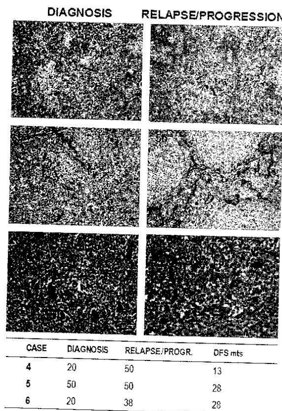
several authors reporting possible correlations among macrophagic infiltration, neovascularity and prognosis.
The aims of this study are the analysis of neoangiogenic pattern of indolent NHL patients (pts) and the role of stromal cells in neovascularization at diagnosis and relapse/progression disease, in order to identify a subset of pts that could benefit by integrated anti-angiogenic treatment.
Methods: Nodal and bone marrow biopsies from 6 follicular (FL) and 7 small lymphocytic (SLL) NHL's pts were selected and studied at diagnosis and disease relapse/progression (9 males and 5 females; mean age at diagnosis 59.8 yrs; 13/13 stage III/IV; low/intermediate FLIPI 7/13 pts, high risk FLIPI 6/13 pts, 8 relapsed pts with median PFS 33.37 months and 5 refractories).
We evaluated both neoangiogenesis by immunohistochemistry (anti-CD34, vascular hot spots/field), confirmed by morphometric analysis, and monocytic-macrophagic infiltrate (anti-CD68).
Results: At diagnosis we observed an angiogenic activity in all nodal samples with an homogeneous vascular distribution in SLL and perifollicular in FL (mean of 27.50 vascular hot spots (vhs)/field; median of 20 vhs/field in nodal biopsies).
Moreover, at onset patients with low/intermediate prognostic risk showed a higher vhs/field. The number of vhs/field increased in all cases at relapse/progressive disease (mean 39,6; median 44 vhs/field) with statistical significance (T-test $p = 0.0049$; Wilcoxon signed-rank test $p = 0.03$) (Fig 1). On the contrary, no significant angiogenic activity was reported in bone marrow neither at diagnosis nor at relapse/progression. The distribution of monocytic-macrophagic infiltrate was heterogeneous in nodal samples with a focal or diffuse pattern and with a different match with the neovessels The percentage of monocytic-macrophagic infiltrate showed a poor increment at relapse/progression and seemed to lack any correlation with clinical features.
Conclusions: These preliminary data could justify the employment of angiogenesis analysis in the prognostic stratification of indolent NHL and hypothesize the use of anti-angiogenic drugs in patients relapsed or refractory to treatment. Larger series are warranted to confirm these data.



CASE	DIAGNOSIS	RELAPSE/PROGR.	DFS mts
4	20	50	13
5	50	50	28
6	20	38	28

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FIRST-LINE MODIFIED SCHEDULE OF GEMCITABINE INDUCES HIGH CLINICAL BENEFIT RATE WITHOUT SIGNIFICANT TOXICITY IN ELDERLY OR NOT ELDERLY ECOG PS 2 PATIENTS WITH ADVANCED NON-SMALL CELL LUNG CANCER

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Background: Monochemotherapy with gemcitabine (Gem) is often the treatment of choice in elderly or poor performance status (PS) patients with advanced non-small cell lung cancer (NSCLC). Our study was aimed to assess the efficacy and tolerability of a modified schedule of Gem using a lower dose than standard.
Methods: From May 2009 through April 2010, thirty-one patients (25 males and 6 females with a median age of 75 years ranging from 64 to 84) with advanced NSCLC (stage IIIB 38.8% and IV 61.2%) were enrolled. Histology was: squamous 38.8%, adenocarcinoma 29%, large cell 6%, undifferentiated 3%, undetermined 24.8%. Only six patients (19.3%) had a WHO PS 0 whereas fourteen (45.2%) were PS 1 and eleven (35.5%) PS 2. All patients received first-line chemotherapy with 6 cycles of Gem 1000 mg/sq on days 1 and 8 every 4 weeks.
Results: At the time of analysis 26 patients were evaluable for response. Partial response (PR) was achieved in 4 patients (15.4%), stable disease >12 weeks (SD) in 11 (42.3%) whereas 11 had progressive disease (42.3%). Importantly, the clinical benefit rate (PR+SD) was 57.7%. Tumour markers (CEA and NSE) were high in 21 patients with a reduction in their values observed in 8 of them (38%). Both pain and PS improved in 4 patients (15.4%) whereas 16 (53%) had an improvement in pain with no worsening of PS. We observed only grade 2 WHO haematological toxicities including anemia, leucopenia, neutropenia and trombocytopenia. Not-neutropenic fever occurred in 4 patients (13.3%). Overall, we did not observe any not-haematological treatment-related event.
Conclusions: Our data show that a modified schedule of Gem with a lower dose intensity than standard may be beneficial in terms of both disease control and tolerability when employed in elderly or PS 2 patients with advanced NSCLC.

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VUS VARIANTS IN *BRCA* GENES OF HEREDITARY BREAST/OVARIAN CANCER

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Background: Germline mutations in *BRCA1/2* genes are responsible for a large proportion of hereditary breast and/or ovarian cancers (HBOC syndrome). Many highly penetrant predisposition alleles have been identified and include frameshift or nonsense mutations which lead to the translation of a truncated protein. Other alleles contain missense mutations which result in amino acid substitution and intronic variant with splicing effect. The finding of variant of uncertain/unclassified significance (VUS) is a possible result that can complicate rather than improve the risk assessment process. VUSs are mainly missense mutations but also include a number of intronic variants and in-frame deletions and insertions.
Methods: A total of 141 unrelated families affected with breast and/or ovarian cancer proved to be eligible for a inclusion in an ongoing study and were screened for germline mutations in *BRCA1* and *BRCA2* at the "Regional Reference Center for the Characterization and Genetic Screening of Hereditary Tumors" at the University of Palermo using automatic direct sequencing. Moreover we collected a control population consisting of 50 index cases without a familial history of cancer and we analyzed this control group for the presence of VUS.
Results: We analyzed *BRCA1* and *BRCA2* genes and we found five VUS in the *BRCA1* gene (A521T, Y179C, N550H, V740L, A622V) and twelve in *BRCA2* gene (A22T, Y42C, A 2466V, 3010I, T200I, IVS24–16T>C, R2034C, IVS25–12T>G, IVS 2–7T>A, P2639A, IVS

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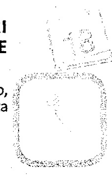

VARIANTI GENOMICHE DI SIGNIFICATO INCERTO: CRITERI PER LA CARATTERIZZAZIONE E L'IDENTIFICAZIONE DELLE CLASSI DI RISCHIO

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ABSTRACT

Mutazioni germinali nei geni BRCA1/2 sono responsabili della maggior parte dei tumori ereditari della mammella e dell'ovaio. Molte varianti alleliche ad alta penetranza sono state identificate e comprendono mutazioni frameshift o nonsenso che causano la formazione di proteine tronche.

Altri alleli contengono mutazioni missenso più rare, rappresentate da singole sostituzioni aminoacidiche e varianti introniche che interferiscono con il processo di splicing. Queste, denominate varianti di significato clinico incerto (VUS) possono rendere più complicata la stima del rischio durante il consultorio oncogenetico.

La maggior parte delle varianti di significato clinico incerto sono mutazioni missenso ma possono includere anche varianti introniche e piccole inserzioni o delezioni in frame.

Più di 2000 sono le varianti missenso identificate nei geni BRCA1/2, localizzate lungo l'intero gene e riportate nel BIC (Breast Cancer Information Core). Di queste il 10-20% sono riportate come varianti di significato incerto.

Ad oggi, molti metodi sono stati proposti e differenti strategie di studio sono utilizzate da diversi laboratori per classificare le VUS in varianti deleterie/ad alto rischio o neutre/a basso rischio. Tra questi, vi sono l'analisi di co-segregazione della variante all'interno della famiglia, l'influenza della mutazione sull'attività della proteina, la conservazione aminoacidica nelle diverse specie, l'analisi di un gruppo controllo, saggi biochimico-funzionali etc.

Più recentemente, l'utilizzo di modelli integrati comprendenti dati epidemiologici diretti ed indiretti, unitamente a dati basati su modelli matematici, fisici e statistici, ha contribuito all'interpretazione del ruolo clinico e funzionale delle VUS. Bisogna però sottolineare che non sempre tutte le variabili sono disponibili e, nonostante l'utilizzo dei modelli integrati dia un notevole contributo all'interpretazione del significato clinico, non può predire la patogenicità con assoluta certezza.

La creazione di un sistema di classificazione standard comprendente diverse Classi di rischio potrebbe aiutare i clinici a proporre, a tutti i portatori di VUS, le più adeguate strategie di sorveglianza.

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... age. There was no significant ... respect to ER or Her2 status among ...

... Our results show considerable ... in CNA in G3N0 breast cancer, some of ... associated with younger and older groups of patients. Other studies have suggested that breast cancer in elderly women is more indolent than in younger patients, although few have dissected this as a function of histological grade. Further studies breaking down these differences may result in better targeting of therapy in pathologically similar BC, and may lead to differing treatment options based on age-associated changes in biology.

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TWIST1 ONCOGENE: COMPARATIVE STUDIES, SEQUENCE VARIATIONS AND MRNA EXPRESSION IN CAT MAMMARY GLAND CARCINOMAS

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Feline mammary carcinoma (FMC) is highly aggressive, mainly hormone receptor-negative cancer, proposed as a model for poor prognosis human breast cancer (HBC). Germline mutations in *TWIST1* may predispose to breast cancer and when increased in breast cancer cells it has been shown to promote metastatic ability in *in vivo* animal models. The aims of this study were to partially isolate the *TWIST1* gene in *Felis catus*, perform comparative studies, to screen spontaneous FMC for sequence variations and evaluate its mRNA expression.
Primer combinations were selected based on the alignments of homologous DNA sequences. After PCR amplification using cat gDNA, 3 bands were obtained (around 300, 800 and 1000 bp), purified and sequenced. Several bioinformatic tools were used to perform comparative studies. 30 spontaneous FMC were screened for polymorphisms. To evaluate the *TWIST1* expression in 7 FMC, RNA extraction/purification and cDNA synthesis were performed. Primers were designed and hybridization probes selected according to major homology for each transcript.
There is a higher similarity between the isolated *TWIST1* gene in *Felis catus* and *Homo sapiens* (86%) than between *Homo sapiens* and *Rattus norvegicus* or *Mus musculus* (75%). The partial amino acid sequence showed no change in these four species. This inferred coding sequence presented high similarity (~96%) between *Homo sapiens* and *Felis catus*. No sequence variations were identified in all tumours analyzed regarding the predicted coding region. *TWIST1* was downregulated in all carcinomas.
We believe that this investigation is the first one to study the *TWIST1* gene in cat regarding all the aspects here reported. The comparative studies evidenced a higher similarity between cat and man than between man and other widely used animal models such as rat or mouse. These results suggest that cat is as an attractive model, at least for *TWIST1* studies, and that may be used instead of the classical animal models. *TWIST1* downregulation in all carcinomas is, however, an unexpected result. Nevertheless numbers are small suggesting future directions for further investigations. In conclusion, although we present here some challenging results, we also give the first insights regarding the *TWIST1* gene in cat that may contribute to establish a feline spontaneous model to study HBC.

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HYPOXIA INDUCES DOWNREGULATED EXPRESSION OF SERINE/THREONINE KINASE-15 (STK15) IN BREAST CANCER CELL LINES

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Background: STK15 (Aurora A/BTAK) is an oncogenic serine/treonine kinase playing a key role in centrosome separation and in mitotic bipolar spindle formation during mitosis. This role appears consistent with the high incidence of its misregulation in cancer; it is highly expressed in many types of human tumors including breast cancer, and its overexpression induces aneuploidy and centrosome amplification. Several studies suggested that the hypoxia is a determinant factor involved in *STK15* up-regulation in hepatoblastoma cell lines. Since the hypoxia is a

expression of HO-1 induced a great resistance to radiotherapy ($P < 0.05$ vs HO-1 negative group).
In this study, we demonstrated the expression of HO-1 in HNSCCs, and HO-1 seems to be a useful index in identifying patients with well response to radiotherapy. These data indicate a new therapeutic for HNSCCs by inhibiting HO-1 activity, which warrants further investigation.

Poster session 18: Tumor Biology and Response to Therapy

P116
SPECIFIC MICROCELL DEVELOPING MECHANISM IN HeLa CANCER CELL LINE

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Carefully explored cancer stem cell developing mechanism could show the way how to treat cancer effectively. The aim of our study was to investigate specific cells (called microcells) properties and development in HeLa cancer cells. The HeLa cells were grown in DMEM medium at 37 degrees for 3-4 days and irradiated with Hg UV lamp behind UFC-1 filter. The cell samples were supravitaly stained with acridine orange, water soluble CdSe/ZnS nanoparticles, Indian ink, carmine red. Expression of embryonic stem cell antigens (SSEA-1, SSEA-3, SSEA-4, Nanog, Oct-4) in HeLa cells and mesenchymal stem cell antigens (CD29, CD54) in HeLa and human bone marrow samples were initiated using "Millipore" monoclonal antibodies. Snapshots of cell samples were taken using Leica DM6000B microscope connected with DFC 490 digital camera. Microscope images were analyzed and measured with "Media Cybernetics" image analyzing program IPP 5.0. We found that different markers accumulate more in specific morphological states of cancer cells called microcells. High mean fluorescence intensity indicates more intense uptake and higher endocytosis capability in early microcell development stages. All used supravital staining methods allow detecting increased endocytosis ability in young cancer cells. We noticed that after UV irradiation in early microcell development phases (while microcells are still in mother cells) stem cell markers SSEA-1, SSEA-4 and OCT4b are expressed more, but mesenchymal cell markers CD29 and CD54 were expressed later in already detached young cancer cells. In conclusion we suggested that microcell developing mechanism is cancer stem cell arousing source via selection process.

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CIGLITAZONE MODULATES LEPTIN EXPRESSION IN BREAST CANCER CELLS

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Background: The obesity hormone leptin, initially discovered as a cytokine controlling food intake and energy balance, has recently emerged as a potent regulator of different physiological and pathological processes, including cancer development and progression. The importance of leptin signaling in breast tumorigenesis has been confirmed by the fact that breast tumors overexpress both leptin and its receptor, both of which correlate with higher tumor grade and worse prognosis. In vitro studies demonstrated that breast cancer cells are able to synthesize leptin in response to obesity-related stimuli, like hyperinsulinemia and hypoxia. This process is mediated through interactions of Sp-1, a nuclear factor that mediates the effects of insulin and/or HIF-1, the master transcription factor in cellular response to oxygen deficiency, with specific motifs within the leptin gene promoter. Considering that in adipocytes leptin promoter is regulated by the activation of peroxisome proliferator activated receptor (PPAR) γ, we studied whether or not ciglitazone, a PPAR-γ ligand, used for treatment of patients with diabetes and obesity and a potential anti-neoplastic agent, can modulate the expression of leptin mRNA in breast cancer cells.
Methods and results: Using chromatin immunoprecipitation (ChIP), we found that treatment of MCF-7 and MDA-MB-231 breast cancer cells with submolar concentrations of ciglitazone induced binding of PPAR-γ to the proximal portion of the leptin promoter, while it decreased the association of Sp-1 with this DNA region. Results obtained with Real Time PCR, Western blotting as well as growth experiments confirmed that these effects coincided with elevated leptin mRNA expression, protein synthesis and increased cell proliferation. The

[illegible] tumoral condition which influences the [illegible] of various proteins involved in proliferation [illegible] cell cycle progression, the aim of our study was to [illegible] new insights into STK15 regulation *in vitro*, evaluating the possible HIF-1 role in its transcriptional control of expression.

Methods: A microarray analysis using Affymetrix platform, was performed in MCF7, MDA-MB-231 and SKBr3 breast cancer cell lines cultured in normoxic and hypoxic conditions in order to compare the differential gene expression profile in response to hypoxia. This analysis allowed us to obtain a statistically significant ($p < 0.05$) differential expression genes, which made it possible to select a set of genes involved in cell cycle progression, angiogenesis and tumor pathogenesis.

Results: By focusing our attention on genes involved in cell cycle progression, we found a downregulated expression of *STK15* gene in MCF-7, MDA-MB-231 and SKBr3 breast cancer cell lines. We confirmed this downregulation of the *STK15* gene, showing a reduction of mRNA levels and of related protein, by means of Real-Time PCR and Western Blotting. We investigated the HIF-1 role in the transcriptional control of *STK15* expression by means of the ChIP assay.

Conclusions: Our data suggest that hypoxia induces a reduction of *STK15* expression and in discordance with previous reports, we hypothesize that this specific downregulation of the *STK15* gene might be able to suppress its proliferation and lead to the apoptosis of breast cancer cell lines.

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HYPOXIA AND HUMAN GENOME STABILITY:DOWNREGULATION OF BRCA2 IN BREAST CANCER CELL LINES

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Background: Hypoxia is a key tumor microenviromental factor. Many studies have demonstrated that hypoxia inhibit the DNA repair process and promotes genetic instability in human cancers. Very little is known regarding the functional consequences of hypoxia in the expression of proteins involved in DNA double-strand break repair in human breast cancer. Therefore aim of our studies is to evaluate the effects of hypoxia on genomic stability in breast cancer cell lines, to obtain new insights on role that the tumor microenviromental have on DNA repair and on genetic instability in breast cancers.

Methods: A microarray analysis, using Affymetrix platform, was performed in MCF7, MDA-MB-231 and SKBr3 breast cancer cell lines, cultivated in normoxic and hypoxic conditions, to identify genes showing a differential gene expression profile in the examined conditions. Among all the genes, we selected those involved in DNA repair mechanisms, to obtain new knowledge about the mechanisms that regulate genomic instability in response to hypoxia.

Results: We found a downregulated expression of *BRCA2* and other genes involved in DNA repair process both MCF-7, MDA-MB-231 and SKBr3 breast cancer cell lines. Focusing on *BRCA2* our results were confirmed evaluating the reduction of mRNA levels and the related protein by Real-Time PCR and Western Blotting.

Conclusions: Our data suggest that the hypoxia, decreasing the DNA repair capacity by downregulated expression of *BRCA2* and other genes involved in DNA repair, could be responsible for the continuous changes that affect the DNA during the process of tumorigenesis favoring the progression to stage more advanced of breast cancer.

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QUANTITATIVE REAL-TIME PCR OF BASAL MARKER EXPRESSION IN FORMALIN-FIXED PARAFFIN EMBEDDED BREAST CANCER SECTIONS

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Background: Gene expression measurement of biomarkers on RNA level is valuable for prognosis and prediction of treatment response in primary breast cancer. In addition, distinct transcript patterns have been shown to be characteristic for intrinsic subtypes. However, the infrastructure required for collecting and processing fresh tumor samples yielding in high quality RNA remains challenging for clinical routine. RNA

[illegible] These data suggest that one of the [illegible] overexpression in breast tumors [illegible] the activation of PPAR-γ with submolar [illegible] ciglitazone.

P118
QUANTITATIVE ANALYSIS OF EARLY EFFECTS OF RADIOTHERAPY IN A HUMAN TUMOUR XENOGRAFT MODEL

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[illegible] Exploring early response to radiotherapy (RT) is thought to be important to reach more efficient treatment schemes and fewer side effects. The aim of this study was to quantify in vivo changes in water diffusion and perfusion effects in human tumor xenografts before and after RT.
Methods: 27 nude rats were co-transplanted subcutaneously with non small cell lung cancer cells (A549) and rat glomerular endothelial (RGE) cells. Tumors were examined 24 h before and 36 h after RT (15 Gy, 14 rats) or without RT (13 rats, control) using a 1.5T clinical MRI scanner. Tumor volume was analyzed in T2- weighted magnetic resonance imaging (MRI). Apparent diffusion coefficient (ADCdiff) and perfusion-influenced ADCperf were calculated from high and low b-value diffusion-weighted (DW) MRI, respectively. Two minutes after intravenous injection of 2.5 μM fluorescent beads, rats were sacrificed; tumors and organs were collected and analyzed by fluorescent microscopy.
Results: It was found that 36 h after RT, tumor volume increased 2.1 times in comparison to the control group. ADCdiff increased by 70% and ADCperf decreased by 6.4% (vs. 0.1% in the control group) in irradiated tumors. Fluorescent imaging evaluated areas of high vascularity and vessel permeability suitable for fusion with MRI findings.
Discussion: RT damages endothelial cells in small tumor vessels, leading to an increased permeability and increased diffusion of free water quantifiable by DW-MRI. Destroyed vessels may also lead to reduced perfusion causing hypoxia, cell swelling and enlargement of the extracellular space in the tumor represented by increased tumor volume. These changes in the tumors vasculature happen during the first days after irradiation and might be useful for the evaluation early response to RT.

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Poster session 19: Experimental Therapy

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CANCER STEM CELLS PLASTICITY: A TWO EDGED SWORD

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To date, one of the most contentious issues in biology is the existence of stem cell (SC) plasticity. The term plasticity refers to the capacity of tissue derived SCs to exhibit a phenotypic potential that extends beyond the differentiated cell phenotypes of their resident tissue. Although the classic definition of cell plasticity taken from stem cell biology implies the ability of SCs to differentiate into various cell lineages, the term is also currently applied to the ability of a given cell type to reciprocally dedifferentiate, redifferentiate, and/or transdifferentiate in response to specific stimulation. Furthermore, a great deal of studies have been shown that a growing tumor is a heterogeneous mix of mostly differentiated cancer cells and cancer stem cells (CSCs), a rare population that have stem-like properties and having capability of self-renewal and multi-potency of differentiation . The differentiated cancer cells exhibit the characteristic rapid proliferation, while the CSCs are much slower at dividing, making them resistant to conventional therapy. Since CSCs have stem like properties as major features of plasticity, hence, on the one hand, plasticity potential of CSCs can considers as a strategy for tumor-derived CSCs therapy (e.g. differentiation therapy) and on the other hand it can be considered as a pitfall since CSCs may transdifferentiate to endothelial-like cells causing resistance to current anti-angiogenic therapies. In this respect, cancer stem cells plasticity might be seen as a two-edge sword, its bright side being represented by new strategies for cancer therapies (i.e. differentiation therapy), its dark side by

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Oncology

EFFECT OF EGF ON VEGF EXPRESSION IN COLON CANCER CELL LINE

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Background: Epidermal growth factor (EGF) is a key regulating cell survival and several different studies confirmed this role in the pathogenesis of human cancer. Through its binding to epidermal growth factor receptor (EGFR), EGF activates an extensive network of signal transduction pathways. Moreover, this growth factor might be associated with synthesis and secretion of several different angiogenic growth factors, like vascular endothelial growth factor (VEGF). Infact, in several cancer cell lines EGF as well as abnormal activation of EGFR induce VEGF expression. VEGF plays a major role in tumor angiogenesis, infact it is up-regulated in different types of cancer and its expression is inversely correlated with patient survival in many human cancers, including colon carcinomas. Signal transducer and activator transcription 3 (STAT3) has been identified as a major regulator of VEGF expression in glioblastoma and prostate cancer.
Methods: We investigated whether the treatment with EGF in HT-29 cells could induce an increase of VEGF expression like in glioblastoma and prostate cancer cell line. We measured the effects of EGF on the VEGF mRNA levels by Quantitative Real Time-PCR (qRT-PCR) and in parallel we measured secreted VEGF levels by Enzime-Linked Immunosobent Assay (ELISA). Subsequently, we examined the abundance of nuclear STAT3 in HT-29 treated with EGF by Western Blotting and we conduced Chomatin Immunoprecipitation (ChIP) to assess STAT3 binding to specific motifs in the VEGF promoter. Finally, to confirm STAT3 involvement in EGF-induced VEGF mRNA production, we silenced STAT3 expression using RNA interference (siRNA). Moreover, using LY294002, an inhibitor of the phosphoinositide 3-kinase, we investigated whether PI3K pathway is required for VEGF transcriptional regulation.
Results: We found that EGF up-regulates VEGF expression. Our results suggested, also, that STAT3 binds consensus motifs within VEGF promoters under EGF stimulation in colon cancer cells. All these EGF effects were significantly blocked when HT-29 cells were treated with LY294002 or with small interfering RNA (siRNA) targeting STAT3.
Conclusions: This study identified the EGF/PI3K/STAT3 signaling as an essential pathway regulating VEGF expression in EGF-responsive colon cancer cells. This suggests that STAT3 pathways might constitute attractive pharmaceutical targets in colon cancer patients where anti-EGF receptor drugs are ineffective.

DETECTION AND DISTRIBUTION OF CANCER STEM CELLS IN SOLID TUMOURS

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Cancer Stem cells (CSCs) hypothesis supports that only a small subset of cells within a tumour is capable of both tumour initiation and sustaining tumour growth. In this preliminary study, we analyzed stemness and differentiation phenotype in 6 types of human cancer such as: breast, head and neck, lung, gastric cancer, melanoma and sarcoma. For breast (40 samples) and head and neck (16 samples) cancer, we used CD44 and CD24 antigens, for lung (133 samples) and gastric (35 samples) cancer, melanoma (16 samples) and sarcoma (20 samples), we used CD133 by flow cytometry as reported in literature. We started from fresh samples obtained from surgery compared to stabilized cell lines. The tissue samples were disaggregated mechanically and immediately tested by flow cytometry, while another part of tissue was digested in a digestive solution (collagenase/dispase) at 37°C for 3-4 hours in order to obtain a cell line. Calu1, A549 and LC31 are stabilized cell lines from Non Small Cell Lung Cancer (NSCLC), Colo 38 is a stabilized cell line from melanoma, MCF-7 is a stabilized cell line from breast cancer, MKN28 and AGS from gastric cancer and MG63 and HT1080 are stabilized cell lines from sarcoma. The results showed, that, after the disaggregation, in breast cancer, the mean percentage of cells $CD44^{+}CD24^{low}$ were 5,8%, in lung cancer the mean percentage of CD133 marker was 6%, in gastric cancer the mean percentage of CD133 was 7%, in melanoma, the mean percentage of CD133 was 2% and in sarcoma was 3% of total cell population. The same results were obtained for stabilized cell lines.
Our data showed that, in the cancers analysed, there was a small cell subpopulation with stemness phenotype, ind[illegible]cating that the tumour can be originated starting fro[illegible] cancer stem cell.

PHENOTYPIC CHARACTERIZATION OF HUMAN PULMONARY BLASTOMA CELL LINE

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BACKGROUND. Sarcomatoid carcinomas are po[illegible] ferentiated carcinomas with a sarcomatoid com[illegible] and characterized by the epithelium-mesenchyma [illegible]tion. They include different cancers such as spi[illegible] carcinomas, giant cell carcinomas and lung pu[illegible] blastomas. In our study, we have isolated and c[illegible] ized a human pulmonary blastoma primary [illegible] termed LC114.
METHODS. The tissue has been partially disa[illegible] and digested in a solution of collagenase/d[illegible] obtain a stabilized cell line, three mediums [illegible] IMDM, BEBM and IMDM/BEBM (2:1). Moreove[illegible] acterize the phenotype of LC114 cell populat[illegible]

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were analyzed for CD133 and CXCR4 expression. Flow cytometry showed significative $CD133^+CXCR4^+$ cells in OVCAR-4 and OVCAR-5 cell lines; these data were confirmed by Western Blotting and immunocytochemistry. Sorted OVCAR-5/$CD133^+$ cells exhibited higher proliferation, self-renewal, colony-forming ability and forming sphere-clusters in serum-free medium with a high clonogenic efficiency in comparison with OVCAR-5/$CD133^-$. In addition it was possible to isolate the side population profile in $CD133^+$ and $CXCR4^+$ ovarian cell lines and expression of ABCG2 transporters. Furthermore, OVCAR-5/$CD133^+$ overexpressed CXCR4 compared to CD133 negative population. OVCAR-5/$CD133^+$ cells exhibit enhanced resistance to platinum-based therapy, drugs commonly used as first-line agents for the treatment of ovarian cancer.
CONCLUSIONS: We described $CD133^+CXCR4^+$ cells in ovarian cell lines and primary tumours. OVCAR-5/$CD133^+$ cells exhibit stem cell-like features such as high proliferation, self-renewal ability and are characterized by higher resistance to chemotherapy. Strategies aimed at modulating the SDF-1/CXCR4 axis may have important clinical applications to inhibit metastasis of cancer stem cells.

EGF DOWNREGULATES EXPRESSION OF CDC25A GENE IN BREAST CANCER CELL LINES

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Background: The phosphatase Cdc25A is a major regulator of both G1/S and G2/M transitions during cell cycle progression.
This role appears consistent with the high incidence of its misregulation in cancer; it has been shown that Cdc25A is overexpressed in primary breast tumors and this overexpression is correlated with an increased cell proliferation and with a poor prognosis in patients with breast cancer.
In a previous work the authors have suggested that EGF treatment induced a modest effect on cell proliferation and a transitional G1 arrest in MCF-7 cells.
To evaluate this hypothesis, aim of our study was to identify, through the analysis of gene expression, the main factors involved in this process of cell cycle slowing in breast cancer cell lines.
Methods: A microarray analysis, using Affymetrix GeneChip expression arrays, are performed inMCF-7 and SKBR3 breast cancer cell lines stimulated with epidermal growth factor (EGF), to compare the differential gene expression profile of breast cancer cells treated and untreated controls.
This analysis allowed us to obtain a statistically significant (p-value < 0.05) differential
expression genes, and we selected a set of genes involved in cell cycle progression
and tumor pathogenesis.
Results: We found a down-regulation of *CDC25A* and *E1,E2,D3 cyclins* genes, known to be involved in the G1 phase, both MCF-7 and SKBR3 breast cancer cell lines.
Focusing on *CDC25A* gene, we showed a reduction of mRNA levels and of related protein, by Real-Time RT-PCR and Western Blotting, with a greater reduction in the gene expression and protein levels, higher in MCF-7 cells.
Conclusions: These data suggest that EGF treatment induced a reduction of *CDC25A* expression [illegible] viously demonstrated, we hypothesize a te[illegible] cycle arrest in the G1 phase, that seems to [illegible] this downregulation.
Therefore, if our results are confirmed by [illegible] cytofluorimetric analysis, in the future [illegible] *CDC25A* could be an important therapeu[illegible] breast cancer and play a key role in the new [illegible] strategies.

CYTOMETRY AND DNA PLOIDY: CLINICAL USES AND MOLECULAR PERSPECTIVE

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Flow cytometry is one of the most powerful [illegible] methods for the integrated study of the mo[illegible] morphological events occurring during cell pr[illegible] and many methods have been described for [illegible] this process. Many cell cycle regulators co[illegible] correct entry and progression through the ce[illegible] altered in tumors. Infact, in most, if not all, [illegible] cers show a deregulated control of G1 phase [illegible] sion, a period when cells decide to start prolife[illegible] stay quiescent. Moreover, clinical studies focuse[illegible] the rapidly growth of tumor, determined by frac[illegible] liferant cancer cells relative to normal cells. [illegible] (flow and image) is capable to analyze DN[illegible] thanks the use of same "molecule" conjugates [illegible] orochrome that permits to identify DNA conten[illegible] cell in a sample. We have reviewed the mos[illegible] results of studies on DNA ploidy during the last [illegible] have seen that analyses of DNA ploidy in ca[illegible] provide clinically useful information on diagno[illegible] peutic and prognostic aspect. Infact, aneuplo[illegible] has a high proliferative activity and a metasta[illegible] sive potential, markers of a poor prognosis. [illegible] many proliferation markers and oncogene produ[illegible] been discovered and their application in clinica[illegible] seems to be very promising. Multiparametric f[illegible] etry should allow the contemporaneous determ[illegible] morphology, phenotype, intracellular protein ex[illegible] and status of chromatin and of DNA. Evaluate [illegible] ular protein is responsible of aggressiveness of [illegible] if it is responsible of alteration of DNA conten[illegible] activated state is the cause of quickly growth [illegible] cells, is an important result that can help clinica[illegible] to patients.

CANCER STEM CELLS-LIKE MARKERS IN SO[illegible] TUMORS

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Cancer stem cell-like (CSCs-like) or initiating tumor cells, cell subpopulations with stem cells properties, was found in many solid tumors using cell surface markers and side-population by flow cytometry. Numerous putative markers are under investigation with the most significant body of work in brain, breast, colon, liver, lung, prostate cancer; to characterize and compare the specific markers that have been found to be present on CSCs is important for the future directions in this intriguing new research field. Moreover, surface markers were analized also in vitro tumor cells-sphere and in trsplantation experiments in NOD-SCID mouse and cells capable of forming sphere or tumor in vivo were characterized by the same specific markers. Some markers commonly used are CD34, CD133, CD24, and CD90, CD117, CD20, CD29, CD31 often in association; $CD44^{+}$, $CD24^{-/low}$, (with worse outcome: $CD44^{+}$, $CD24^{-}$, PROCR+), CD29, CD133+ in breast tumors, $CD133^{+}$ and CD166, CD44, CD49f, ESA in colorectal tumors, $CD133^{+}$, CD34, CD24 in lung tumors; in many case the tumors that formed from these cells recapitulated the histologic characteristics of primary tumors. Our data in solid tumors are concordant with the work that proposed CD 133 as important marker in prostate, breast and lung tumors.

These markers have facilitated CSCs-like identification in multiple tumor sites and metastasis, but the impact of tissue digestion on marker specificity must be evaluated; moreover cell surface molecules analized by flow cytometry, may not ideal for histochemical analysis. Nevetherless, the explosion of new data, in this exciting field, but no single marker in any sites has emerged as definitive solution and many works support an important role of inhibitors of signaling pathways involved in self-renewal, growth and survival of these cells (Hedgehog, Wnt/B-catenin, Notch, ABC multidrug efflux transporters et al.). If indeed cancer stem cell-like are mediators of recurrence and metastasis, in fact several experimental data suggest that CSCs-like can be resistant to therapy, methods to identify these cells will represent a significant advance in cancer therapy with new strategies target signaling pathways that are involved in the self-renewal processes of CSCs.

ANALYSIS OF GERMLINE GENE COPY NUMBER VARIANTIONS IN PATIENTS WITH SPORADIC PANCREATIC ADENOCARCINOMA

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Background: The rapid fatality of pancreatic cancer is, in large part, the result of a diagnosis at an advanced stage in the majority of patients. Identification of individuals at risk of developing pancreatic adenocarcinoma would be useful to improve the prognosis of this disease. There is presently no biological or genetic indicator allowing detection of patients at risk of developing sporadic pancreatic cancer.

Methods: We analyzed gene copy number variations (CNVs) in leucocyte DNA from 31 patients (24 Europeans and 7 Japanese) with sporadic pancreatic adenocarcinoma and from 93 matched controls. Genotyping was performed with the use of the GeneChip Human Mapping 500K Array Set (Affymetrix). The HapMap database was used as the reference set.

Results: Our main goal was to identify CNVs common to all patients with sporadic pancreatic cancer. We identified 431 SNP probes with abnormal hybridization signal present in the DNA of all 31 patients. Of these SNP probes, 284 corresponded to 3 or more copies and 147 corresponded to 1 or 0 copies. Several cancer-associated genes such as CDC14B, CENPE, EIF2S2, FGF20, FZD10, GTF3C3, KLHL1, NOTCH3, RAB21, TULP3, VSNL1 and ZWINT were amplified in all patients. In addition, several genes supposed to oppose cancer development such as ASH1L, CD9, GRB14, IER3, LPXN, MAP3K7, MDC1, MINK1, SGPL1 and VRK1 were present as single copy in the genome of all 31 patients. Other genes involved in cancer such as BMP1, EGFL11, FLT4, FOSB, KIT, MAP4K4, MYB, PDGFRA, TGFA, AKT3 and KRAS were found amplified in almost all patients, whereas only one allele of the Myc inhibitor PAK2 and ARRB2 was detected in the majority of these patients. The set of the 431 SNP probes with abnormal hybridization signal of patients with sporadic pancreatic cancer was checked in the 93 control patients. None of them showed more than 5% match.

Conclusions: These data suggest that the set of 431 CNVs detected in the DNA of patients with sporadic pancreatic adenocarcinoma is associated to the disease. This CNV set could be used for early diagnosis of individuals with a genetic predisposition to develop a sporadic pancreatic cancer, for understanding the physiopathology of this disease and also to target these genes in a preventive strategy.

FUNCTIONAL ACTIVITY OF CXCL8 RECEPTORS, CXCR1 AND CXCR2, ON HUMAN MALIGNANT MELANOMA PROGRESSION

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We examined the autocrine/paracrine role of interleukin 8 (CXCL8) and the functional significance of CXCL8 receptors, CXCR1 and CXCR2, in human malignant melanoma proliferation, migration, invasion and angiogenesis. We found that a panel of seven cell lines, even though at different extent, secreted CXCL8 protein, and expressed CXCR1 and CXCR2 independently from the CXCL8 expression, but depending on the oxygen level. In fact, hypoxic exposure increases the expression of CXCR1 and CXCR2 receptors. The cell proliferation of both M20 and A375SM lines, expressing similar levels of both CXCR1 and CXCR2 but secreting low and high amounts of CXCL8, respectively, was significantly enhanced by CXCL8 exposure and reduced by CXCL8, CXCR1 and CXCR2 neutralizing antibodies, indicating the autocrine/paracrine role of CXCL8 in melanoma cell proliferation. Moreover, an increased invasion and migration in response to CXCL8 was observed in several cell lines, and a further enhancement evidenced under hypoxic conditions. A CXCL8-dependent *in vivo* vessel formation, evaluated through a matrigel assay, was also demonstrated. Furthermore, when neutralizing antibodies against CXCR1 or CXCR2 were used, only the involve-

action of Cetuximab we have focused on its in vivo impact on both the peripheral blood lymphocyte and dendritic cell (DC) immunophenotype in metastatic colorectal cancer (mCRC) pts.
Methods - The peripheral blood lymphocytes and DC subsets we analyzed by multicolor FCM in 18 pts (M/F:12/6, median age: 63 yrs; range 43-78) treated with Cetuximab-based therapy, in absence of clinically relevant infections. Baseline data were compared with reference values obtained by 50 healthy subjects (M/F: 25/25, median age: 43 yrs, range 21-65).
Results - With respect to normal donors in our pts at baseline we observed a significant lower level of the absolute lymphocyte number (p=0.0001), B cells (p=0.0002), T cells (0.001), NK cells (p=0.04) and DCs with their subsets (p=0.0002; p=0.002; p=0.001), while activated T cells showed a higher level (p=0.03). After 3 courses of chemotherapy + Cetuximab, a trend was shown toward a progressive increase of all the lymphocyte subsets, of total DCs and of their subsets. This trend was confirmed after 6, 9 courses and at the time of disease evaluation.
Conclusions - These data show that Cetuximab seems to improve in vivo the T-cell mediated immune response in pre-treated in mCRC pts. This provides new insight into its possible additional antitumor mechanism and may be helpful in the design of combination therapy for mCRC pts.

EFFECTS OF CIGLITAZONE, PPARγ AGONIST, ON LEPTIN EXPRESSION IN MCF-7 AND MDA-MB-231 BREAST CANCER CELLS

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Background: Leptin, a hormone produced mostly by the adipose tissue, in addition to its well-documented role in the control of appetite and energy homeostasis, is known to regulate various physiological and pathological processes in the peripheral organs. Of note is the accumulating evidence that leptin can induce growth and progression of different cancer types and data obtained in cellular and animal cancer models demonstrated that can act as a mitogen as well as antiapoptotic transforming and motogenic factor.
The importance of leptin signaling in breast tumorigenesis has been confirmed by the fact that breast tumors overexpress both leptin and its receptor, both of which correlate with higher tumor grade and worse prognosis. In vitro studies demonstrated that breast cancer cells are able to synthesize leptin in response to obesity-related stimuli, like hyperinsulinemia and hypoxia. This process is mediated through interactions of Sp-1, a nuclear factor that mediates the effects of insulin and/or HIF-1, the master transcription factor in cellular response to oxygen deficiency, with specific motifs within the leptin gene promoter.
Considering that in adipocytes leptin promoter is regulated by the activation of peroxisome proliferator activated receptor (PPAR) γ, we studied whether or not ciglitazone, a PPAR-γ ligand, used for treatment of patients with diabetes and obesity and a potential anti-neoplastic agent, can modulate the expression of leptin mRNA in breast cancer cells.
Methods and results: Using chromatin immunoprecipitation (ChIP), we found that treatment of MCF-7 and MDA-MB-231 breast cancer cells with submolar concentrations of ciglitazone induced binding of PPAR-γ to the proximal portion of the leptin promoter, while it decreased the association of Sp-1 with this DNA region. Results obtained with Real Time PCR, Western blotting as well as growth experiments confirmed that these effects coincided with elevated leptin mRNA expression, protein synthesis and increased cell proliferation. The mitogenic effects of ciglitazone were not observed when higher doses of the drug were used.
Conclusions: These data suggest that one of the mechanisms of leptin overexpression in breast tumors might involve activation of PPAR-γ with submolar concentrations of ciglitazone.

IN VIVO TUMOR TARGETING BY IDIOTYPE-SPECIFIC PEPTIDES

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In order to limit the adverse side effects of cancer therapy, it is necessary to design new strategies of drugs delivery into tumor cells. Peptides are promising tools to deliver radionuclides or therapeutic drugs to tumor cells. The idiotypic determinants of surface immunoglobulins of neoplastic B cells have unique amino acid sequences and can be regarded as highly specific tumor markers. This suggests that idiotype-specific peptides (Id-peptides) for the BCR (B cell receptor) of neoplastic B cells could target selectively transformed B cells. In this work, we evaluated the ability of Id-peptides for B-lymphoma cells selected by screening Random Peptide Libraries (RPLs) as a tool for the specific delivery of a therapeutic cargo into tumor cells.
Results can be summarized as follows:
a) We selected three phage clones by screening three distinct RPLs with immunoglobulins purified from the murine B lymphoma cell line A20;
b) Synthetic peptides, corresponding to the insert of phage clones, maintained their antigenic properties;
c) Id-peptides were internalized into target tumor cells by BCR-mediated endocytosis;
d) When inoculated in tumor-bearing mice, Id-peptides targeted specifically tumor cells;
e) Id-peptides were able to specifically deliver a cargo protein (GFP) or radionuclides into target tumor cells both *in vitro* and *in vivo*.
These results show that Id-peptides are powerful tools for *in vivo* targeting of tumorigenic B cell lymphoma and to deliver therapeutic drugs selectively into tumor cells.

CHARACTERIZATION OF A HUMAN MYXOID LIPOSARCOMA (MLS) CELL LINE MADE RESISTANT TO TRABECTEDIN (T; ET-743)

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Recent clinical data have shown that the myxoid liposarco-

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1
EGF Induces STAT3-Dependent VEGF Expression in HT-29 Colon Cancer Cells

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Background: Angiogenesis, the sprouting of blood vessels, is a fundamental biological process for tumor growth and metastatic spread. Vascular endothelial growth factor (VEGF) plays a major role in tumor angiogenesis, in fact it is up-regulated in different types of cancer and its expression is inversely correlated with patient survival in many human cancers, including colon carcinomas. In several cancer cell lines VEGF expression is induced by epidermal growth factor (EGF) as well as abnormal activation of epidermal growth factor receptor (EGFR). The VEGF gene promoter contains several regulatory motifs as ERE, CRE, SP-1, AP-2, HRE, and SRE (STAT3-responsive element). Signal transducer and activator transcription 3 (STAT3) has been identified as a major regulator of VEGF expression in glioblastoma and prostate cancer. **Methods:** We investigated the molecular mechanisms by which VEGF expression is regulated in colon cancer cells by EGF. First, we measured the effects of EGF on the VEGF mRNA levels by Quantitative real-time-PCR (qRT-PCR) and in parallel we measured secreted VEGF levels by Enzyme-Linked Immunosorbent Assay (ELISA). Secondary, by Western Blotting, we examined the abundance of nuclear STAT3 in HT-29 treated with EGF and then, to assess STAT3 binding to specific motifs in the VEGF promoter, we conducted Chromatin Immunoprecipitation (ChIP). Finally, to confirm STAT3 involvement in EGF-induced VEGF mRNA production, we silenced STAT3 expression using RNA interference (siRNA). Moreover, using LY294002, an inhibitor of the phosphoinositide 3-kinase, we investigated whether PI3K pathway is required for VEGF transcriptional regulation. **Results:** We found that EGF up-regulates VEGF expression. Our results suggested, also, that STAT3 binds consensus motifs within VEGF promoters under EGF stimulation in colon cancer cells. All these EGF effects were significantly blocked when HT-29 cells were treated with LY294002 or with small interfering RNA (siRNA) targeting STAT3. **Conclusions:** This study identified the EGF/PI3K/STAT3 signaling as an essential pathway regulating VEGF expression in EGF-responsive colon cancer cells. This suggests that STAT3 pathways might constitute attractive pharmaceutical targets in colon cancer patients where anti-EGF receptor drugs are ineffective.

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Changes in CpG Islands Promoter Methylation Patterns during Ductal Breast Carcinoma Progression

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Background: Aberrant promoter hypermethylation of several known or putative tumor suppressor genes occurs frequently during carcinogenesis. **Methods:** We examined the changes in methylation patterns of 9 genes by QMSP. Synchronous pre invasive lesions (ADH and/or DCIS) and invasive ductal breast carcinoma from 48 patients, together with pure lesions from 27 patients and 32 normal breast tissues were analyzed. **Results:** Aberrant promoter methylation was detected in both pre invasive and invasive lesions for genes APC, CDH1, CTNNB1, TIMP3, ESR1, GSTP1. However, only APC, CDH1, and CTNNB1 promoter regions showed an increase in frequency and levels of methylation in pathological samples when compared with normal breast tissues. CDH1 showed higher methylation levels in invasive tumors as compared with pre-invasive lesions ($p < 0.04$). The analysis of APC, CDH1 and CTNNB1 methylation status was able to distinguish between normal and pathological samples with a sensitivity of 67% (95%CI 60–71%) and a specificity of 75% (95%CI 69–81%). **Conclusions:** Our data point to the direct involvement of APC, CDH1, and CTNNB1 promoter methylation in the early stages of breast cancer progression and suggest that they may represent a useful tool for the detection of tumor cells in clinical specimens.

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BRCA 1 and BRCA2 Variants of Uncertain Clinical Significance and Their Implications for Genetic Counseling

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Background: Germline mutations in BRCA1/2 genes are responsible for a large proportion of hereditary breast and/or ovarian cancers (HBOC). About one-third of the genetic variant in BRCA1 and 50% of those found in BRCA2, reported by the Breast Cancer Information Core are considered variants of unknown clinical significance, also known as unclassified variants (UVs), because of the uncertainty about their cancer risk. This is often the case for missense variations or when the nucleotide change affects or creates a putative splice-site. In families affected by HBOC syndrome with only an unknown variant identified, it is difficult to determine whether the variant is or not causally linked to predisposition and so it is uninformative for genetic counselling and predictive testing purposes. Presyntomatic testing is not possible in family with an UV, and surveillance can only be based upon the severity of the cancer family history. Actually the methods to discriminate deleterious/high-risk from neutral/low-risk unclassified variants are based on informations about the cosegregation in families of the UVs, comparison of sequence conservation across multiple species, loss of heterozigosity in the tumor, histopathologic characteristic and functional assay, in addiction to biochemical and epidemiological criteria. **Methods:** 141 Sicilian patients with Breast and/or Ovarian Cancer were submitted to both counselling and genetic testing. 121 of these patients were screened for BRCA1 and 86 of them also for BRCA2 mutations in all coding exons and exon-intron boundaries of the genes by automatic direct sequencing. Moreover, we collected a control population consisting of 50 index cases without a familial history of cancer and we analysed this control group for the presence of UVs. **Results:** During these mutational screening we identified five variants of BRCA1 gene (Y179C, F486L, A521T, N550H, V740L.) and ten of BRCA2 gene (A22T, Y42C, A2466V, T3013I, T200I, IVS24–16T>C, R2034C, IVS25–12T>G, IVS 2–7T>A, P2639A), classified in BIC database as UVs. Fifty healthy individuals were analyzed but any UVs did not found, suggesting no association of UVs with HBOC. **Conclusions:** We identified fifteen UVs in the BRCA1/2 genes that could contribute to the early onset of breast and/or ovarian cancer. The study of this variants in fifty healthy individuals confirms this hypothesis. For this reason it is difficult to inform carriers about the finding of UVs because of the actual lack of clinical significance. Classification of UVs into deleterious/high risk or neutral/low clinical significance is essential to identify individuals with high risk to develope a breast and/or ovarian tumor. Likely some of UVs detected may have a functional relationship with breast/ovarian cancer development, but this remains to be further explored.

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BRCA1 and *BRCA2* Germline Mutations in Sicilian Breast and/or Ovarian Cancer Families and Their Association with Familial Profiles

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Background: Germline mutations in *BRCA1* and *BRCA2* genes account for the majority of familial and hereditary cases of breast and/or ovarian cancer (BC and OC). Many highly penetrant predisposition alleles have been identified and include frameshift or nonsense mutation which lead to the translation of a truncated protein. Other alleles contain missense mutations which result in amino acid substitution and intronic variant with splicing effect. The features that indicate increased likelihood of having BRCA mutations are multiple cases of early onset BC, OC (with family history of BC or OC), BC and OC in the same woman, bilateral BC and male BC. We evaluate the contribution of germline *BRCA1/2* mutations in the incidence of hereditary and familial BC and/or OC in Sicilian patients and identify a possible association between the higher frequency of *BRCA1/2* mutations and a specific familial profile. **Methods:** One hundred and forty one BC and OC families were screened for germline mutations in *BRCA1* and eighty-six for germline mutations in *BRCA2* at the 'Regional Reference Center for the Characterization and Genetic Screening of Hereditary Tumors' at the University of Palermo. Each case was selected for personal and/or familial tumoral history according to selection criteria for genetic testing of American Society of Clinical Oncology. In our study we performed a molecular analysis complete coding sequence and the exon-intron boundaries of *BRCA* genes using automatic direct sequencing. **Results:** 141 patients were selected. 121 of them had BC (among which 7 were bilateral), 10 had OC and 5 both tumors. We detected eight pathological mutations (C61G, Y101X, 633delC, 916deTT, R1443X, 5083de19, 4843delC, 5149del4) that lead not functional truncated proteins, identified in 13/141 (9%) families. In eighty-six patients analyzed for *BRCA2* gene we found 8 *BRCA2* pathological mutations (IVS 14 + 6G/A, 6079del4, Q2042X, IVS11–19delAT, 9254del5, V211T, 6079del4, 6310del5) identified in 9/86 (10%) families. The frequency of *BRCA1/2* mutations is 25% (22/86) in our Sicilian population group. According to the analysis of the different familial profiles and also taking into consideration the II degree, the HBOC profile had a major frequency

[illegible] MBC profile. **Conclusions:** This is the first study that analyzes the incidence of germinal mutations in *BRCA1/2* gene in patients with familial hereditary of BC/OC in Sicilian population. Both genes contribute to identify 25% of families with HBOC syndrome. From familial profile analysis it result that family history of BC and OC would be a good predictor to identify *BRCA1* mutation carriers in the Sicilian population, but identification of specific features should address BC/OC patients and their families to genetic counseling and *BRCA1/2* mutational analysis.

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Case Report: First-Line Sorafenib in Metastatic Kidney Cancer

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Background: Here we describe three cases in which the tyro[illegible]kinase inhibitor sorafenib was given as first-line medication [illegible]tients with metastatic clear cell renal cancer and good or in[illegible]mediate risk according to the revised MSKCC prognostic scor[illegible]system. **Methods:** Patients all underwent radical nephrecto[illegible] diagnosis and were considered not eligible for sunitinib or [illegible]kines due to age and/or co-morbidities whereas use of beva[illegible]mab was not yet approved in Italy for this disease. The first [illegible]nt was 70 years old with multiple pulmonary metastasis. She suffered of blood hypertension, ischemic cardiopathy, HCV+ [illegible]nic hepatitis and thyroid dysfunction. The second was 68-[illegible]old woman with only a single histology-proven pancreatic [illegible]stasis associated to insulin-dependent diabetes. The young[illegible]tient was 63 years old and he was one with intermediate risk [illegible]ned by presence of more than one metastatic site (lung and [illegible]) and time from diagnosis to treatment less than 1 year. He suffered of hypertensive cardiopathy. **Results:** All three pa[illegible] received sorafenib at standard dose of 400 mg bidpo. Pro[illegible]on free survival was 12, 10 and 6 months, respectively. Im[illegible]tly, no significant toxicities were observed and prolonged dose reduction or suspension were not required. Quality of [illegible] co-morbidities did not worsened. Overall, the outcome of [illegible] good risk patients is in line with the average reported in [illegible]ure for standards first line therapy with sunitinib or beva[illegible]b plus IFNa. On the contrary, the intermediate-risk pa[illegible] experienced a lesser benefit. **Conclusions:** We believe that [illegible]ct role of sorafenib in the first-line setting of good risk [illegible]tic renal cancer deserves further studies.

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Case Report: Metronomic Capecitabine in Metastatic Gasric Cancer

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Background: Metronomic chemotherapy (MC) may represent the paradigm of effective/low-toxic anticancer therapy. The challenge is the right selection of patients for whom this kind of treatment may be beneficial. **Methods:** A 72-years-old man underwent total gastrectomy for G3 diffuse type adenocarcinoma of the stomach infiltrating the adventia (pT3) with 10/13 lymph nodes involved (pN2). Pre-operative CA-19–9 was 97 Ul/ml whereas a CT scan performed soon after surgery showed one single distant metastasis to the liver. Due to age, poor performance status (ECOG 2) and anemia (HBG 9 gr/dl) patient was not admitted to first line triplet cytotoxic chemotherapy with epirubicin plus cisplatin or oxaliplatin plus fluoruracil or capecitabine. We decide to start oral MC with capecitabine 1,500 mg daily. **Results:** After 2 months of treatment a CT scan showed a complete regression of the liver metastasis, CA 19–9 dropped to normal levels, ECOG performance status was 0 and HGB raised to 11.8 g/dl. At 8 months patient was still on metronomic capecitabine without any sign of disease progression or major toxicity. A new CT scan confirmed the complete remission, capecitabine was stopped and patient began follow up achieving a progression free survival of 13 months. **Conclusions:** Impossibility of adopting standard maximum tolerable dose cytotoxic chemotherapy regimens due to aspects related to the individual patient, such as elderly age, poor performance status and/or important co-morbidities, is part of our daily clinical practice and may be a first selection criterion to choose alternative treatments. Here we have reported a case in which the decision to start MC has been taken under this circumstance.

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Use of Prulifloxacin in Addition to Granulocyte-Colony-Stimulating-Factor (GCSF) in Order to Prevent Neutropenia Complications in Cancer Patients

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Background: Among patients with cancer submitted to chemotherapy, 20 to 40% suffer of G4 leukopenia. These patients are generally treated with G-CSF, antibiotics or a combination of both, depending on patient PS, comorbilities, leukopenia duration and physician preferences. Fluoroquinolones are among the most used drugs in this setting. Prulifloxacin is a prodrug, that converts in the body to the active form ulifloxacin which is active in various bacterial diseases including respiratory and urinary tract infections, skin infections, infectious enteritis, and cystitis. **Methods:** Since January 2009, we have treated 28 patients affected

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Downregulated Expression of Cdc25A Gene in MCF-7 Breast Cancer Cell

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Background: The phosphatase Cdc25A plays an important role in cell cycle regulation both in G1/S and G2/M transitions. This role appears consistent with the high incidence of its misregulation in cancer; it has been shown that Cdc25A is overexpressed in primary breast tumors and that this overexpression is correlated with biological behaviour of breast tumors and with poor survival. A previous work suggests that EGF induced a modest effect on cell proliferation and a transitional G1 arrest in MCF-7 cells. Therefore aim of our study was to identify, through the analysis of gene expression, the main factors involved in the process of cell cycle slowing. **Methods:** We performed a microarray analysis in MCF-7 breast cancer cells, using Affymetrix GeneChip expression arrays, to compare the gene expression profile of MCF-7 treated with epidermal growth factor (EGF) and untreated controls. This analysis allowed us to obtain a statistically significant (p value < 0.05) differential expression of 706 genes, and we selected a set of genes involved in cell cycle progression and tumor pathogenesis. **Results:** We found a down-regulation of *CDC25A, E1,E2,D3 cyclins* genes, known to be involved in the G1 phase. Focusing on *CDC25A* gene, we showed a reduction of mRNA level and of related protein by Real-Time RT-PCR and Western Blotting. **Conclusions:** These data suggest that EGF treatment induced a reduction of *CDC25A* expression and, as previously demonstrated, we hypothesize a temporary cell cycle arrest in the G1 phase, that seems to depend on this downregulation. Therefore this indicates that in the future phosphatase *CDC25A* could be an important therapeutic target in breast cancer and play a key role in the new therapeutic strategies.

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Different Impact of Hematologic Neoplasms and Solid Tumors in Burden of Caregivers in the Southern of Italy

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Background: Cancer places both physical and emotional burdens on the family members and caregivers. The aim of this study was to explore the burden of caregivers related to the different impact of hematologic neoplasms and solid tumors. **Methods:** Caregivers were interviewed with the Family Strain Questionnaire that assess perceived caregiving-related problems. It consists of a brief semi-structured interview and 44 dichotomic items that evaluate five factors: emotional burden (F1), problems of social involvement (F2), need for knowledge of the disease (F3), family relationships (F4) and death thoughts (F5). High scores indicate the presence of problems except in F4 (high score indicates good quality family relationships). Five questions on relationship and communication with health personnel were added as supplement of evaluation. Data were analyzed by statistic t test. **Results:** Participants included 52 caregivers (70% females, 30% males) of patients with cancer receiving a chemotherapeutic treatment at Medical Oncology (62%) or Hematology (38%) Unit in Lecce. The median age of caregiver was 46 $\pm$ 12.73 years. The caregiver was spouse in 27% of cases, parent in 21%, son/daughter in 33% and other in 19%. Our sample presented scores significantly lower than the reference norms for factor 1 ($p < 0.05$) and significantly higher for factors 2, 3 and 5 ($p < 0.05$). Results of t test indicate statistically significant differences between onco-hematological and oncological caregiver burdens in relation to F1 and F5 ($p < 0.05$). Both dimensions appear more burden on caregivers of onco-hematological patients. No statistically significant differences were reported about F2 and F3 ($p > 0.05$). In addition, responses to the supplement questionnaire showed that the caregivers try anger and frustration with the medical staff in relation with poor communication and information. **Conclusions:** The recognition of the profound impact of cancer on the family and caregivers needs to be considered with differences between onco-hematological and oncological caregiver burdens. Enhancing communication among patients, families and health care providers and psychosocial support for family caregivers should be part of global care.

observed. Non-haematological toxicities were usually mild to moderate. **Conclusions:** These results, although premature, suggest that the combination of sorafenib plus mitomycin-C is active, synergic, well tolerated and able to confer prolong stabilizations and therefore, might be of value in further investigation in larger clinical trial.

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Analysis of Germline Gene Copy Number Variants of Patients with Sporadic Pancreatic Adenocarcinoma Reveals Specific Variations

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Background: The rapid fatality of pancreatic cancer is, in large part, the result of a diagnosis at an advanced stage in the majority of patients. Identification of individuals at risk of developing pancreatic adenocarcinoma would be useful to improve the prognosis of this disease. There is presently no biological or genetic indicator allowing detection of patients at risk of developing sporadic pancreatic cancer. **Methods:** We analyzed gene copy number variations (CNVs) in leucocyte DNA from 31 patients (24 Europeans and 7 Japanese) with sporadic pancreatic adenocarcinoma and from 93 matched controls. Genotyping was performed with the use of the GeneChip Human Mapping 500K Array Set (Affymetrix). The HapMap database was used as the reference set. **Results:** Our main goal was to identify CNVs common to all patients with sporadic pancreatic cancer. We identified 431 SNP probes with abnormal hybridization signal present in the DNA of all 31 patients. Of these SNP probes, 284 corresponded to 3 or more copies and 147 corresponded to 1 or 0 copies. Several cancer-associated genes such as CDC14B, CENPE, EIF2S2, FGF20, FZD10, GTF3C3, KLHL1, NOTCH3, RAB21, TULP3, VSNL1 and ZWINT were amplified in all patients. In addition, several genes supposed to oppose cancer development such as ASH1L, CD9, GRB14, IER3, LPXN, MAP3K7, MDC1, MINK1, SGPL1 and VRK1 were present as single copy in the genome of all 31 patients. Other genes involved in cancer such as BMP1, EGFL11, FLT4, FOSB, KIT, MAP4K4, MYB, PDGFRA, TGFA, AKT3 and KRAS were found amplified in almost all patients, whereas only one allele of the Myc inhibitor PAK2 and ARRB2 was detected in the majority of these patients. The set of the 431 SNP probes with abnormal hybridization signal of patients with sporadic pancreatic cancer was checked in the 93 control patients. None of them showed more than 5% match. **Conclusions:** These data suggest that the set of 431 CNVs detected in the DNA of patients with sporadic pancreatic adenocarcinoma is associated to the disease. This CNV set could be used for early diagnosis of individuals with a genetic predisposition to develop a sporadic pancreatic cancer, for understanding the physiopathology of this disease and also to target these genes in a preventive strategy.

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Early Stage Nasal Vestibule Tumors: Safety and Efficacy of HDR Brachytherapy in Elderly Patients

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Background: Squamous cell carcinoma of the nasal vestibule is a rare disease representing less than 1% of all head and neck tumors and occurs mainly in the elderly. In early stage disease, radiotherapy and in particular low dose rate brachytherapy (LDR BRT), is able to achieve the same local control results than surgery but a better cosmetic outcome. The only drawback of a LDR BRT treatment is that patient's hospitalization is required. To overcome this aspect, high dose rate brachytherapy (HDR) could be considered, since it is able to deliver the same nominal dose of a LDR BRT treatment, in a shorter time. This aspect is tremendously advantageous in elderly patients, but just a limited amount of data are present in literature, probably because the concern that such a 'dense dose' could cause excessive toxicity and affect the cosmetic result. Aim of this work is assess local control and toxicity rates in a cohort of elderly and older elderly patients. **Methods:** Between 2006 and 2008 a total number of 26 patients with nasal vestibule squamous carcinoma have been treated at our institution. Seventeen of them were older than 65 years and have been considered for this study. The median age at the time of patient' referral to Radiotherapy department was 79 years and in particular 4 were young old (65–74 years), 9 patients were older old (75–84 years) and 4 were oldest old (85 years and older). All patients had one or more cardiovascular or metabolic co-morbidities. Eight and nine patients had respectively a Wang stage I or a stage II disease. All patients except one had received a surgical resection that had been judged not radical at the pathological examination. Five patients had undergone multiple surgical procedures and were referred to radiation oncology department after the surgical removal of the 2nd or 3rd local relapse. All patients were treated as outpatients with Ir 192 HDR implants that delivered a nominal dose of 35 Gy corresponding to a biologic effective dose (BED_{10}) of 47 Gy to the tumor and 75 Gy to the late responding tissues (BED_3). **Results:** After a median follow up of 26 months (15–36 range) no patients experienced a local relapse. The cosmetic result was judged satisfying. Treatment related toxicity was extremely mild mainly consisting in crustae and 'dry nose'. No respiratory functional abnormalities were reported and

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Determination HER2 Status In Breast Cancer Patients by Two Methods: Real-Time Quantitative PCR (QPCR) and Immunoenzimatic Assay (EIA)

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Background: Over expression and/or amplification of the HER2 oncogene, occur in up to 20–30% of breast cancer patients and genomic alterations are associated with poor prognosis and more aggressive tumor phenotype. Actually, HER2 determination on tumour tissues is reliably performed by immunochemistry assay. However the development of non invasive procedure to determine HER2 status may represent a powerful method for monitoring disease progression and response to the treatment in breast cancer patients. **Methods:** Serum samples and RNA from peripheral blood were evaluated in 85 breast cancer patients and 22 healthy controls. Of those patients 49 were HER2 positive and 36 were negative. HER2 mRNA levels were measured by real time quantitative PCR (QPCR) and serum HER2 protein by immunoenzimatic assay (EIA). ROC curve analyses were used to determine the optimal cut off values. **Results:** A statistically significant difference was detected for both QPCR and EIA in HER2 positive patients as compared with both healthy controls and HER2 negative tumours. QPCR showed a 91% (CI95%: 84–98%) specificity and a 78% (CI95%: 68–88%) sensitivity for an optimal cut off value of 4.74. The optimal cut off value for EIA was 22 ng/ml yielding a 95% (CI95%: 90–100%) specificity and a 59% (CI95%: 48–70%) sensitivity. The QPCR assay was slightly less specific than EIA in discriminating HER2 positive breast cancers from HER2 negative tumours, but it was more sensitive. **Conclusions:** Our results indicate that QPCR is suitable alternative method for the determination of HER2 status in peripheral blood of breast cancer patients. Thus, QPCR could be used as diagnostic tool when primary tumour samples are unavailable or to monitor the outcome of the disease and the response to therapy during follow up of breast cancer patients.

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Case Report: Maintenance Bevacizumab in Metastatic Colorectal Cancer

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Background: Presence of synchronous hepatic metastasis is a common feature in colorectal cancer. Best treatment is surgery of both primary and metastatic tumors followed by cytotoxic chemotherapy. Concomitant use of anti-angiogenic drugs, such as bevacizumab, has been recently demonstrated to be of great added value. **Methods:** Here we describe the case of 48-years-old male who underwent left radical emicolectomy and right epatectomy for stage IV pT3N1(3/14)M1 G2 colorectal adenocarcinoma with synchronous hepatic metastases. Unfortunately, postsurgical PET scan showed a residual single hepatic metastasis at the I segment. **Results:** Patient performance status was 0 according to ECOG scale and he was admitted to first line chemoregimen with FOLFIRI plus bevacizumab obtaining a complete response (CR) after 12 cycles. At this point chemotherapy was stopped and treatment prolonged with only bevacizumab. After two years maintenance bevacizumab patient is still in CR as documented by PET scan. Importantly, no significant toxicities were observed and quality of life was normal. **Conclusions:** In conclusion, this case report underscore the usefulness of bevacizumab as maintenance therapy to stabilize responses achieved with conventional cytotoxic chemotherapy.

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The Proximal Leptin Gene Promoter is Regulated by Pparγ Agonist in MCF-7 and MDA-MB-231 Breast Cancer Cells

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Background: The obesity hormone leptin, initially discovered as a cytokine controlling food intake and energy balance, has recently emerged as a potent regulator of different physiological and pathological processes, including cancer development and progression. The importance of leptin signaling in breast tumorigenesis has been confirmed by the fact that breast tumors overexpress both leptin and its receptor, both of which correlate with higher tumor grade and worse prognosis. In vitro studies demonstrated that breast cancer cells are able to synthesize leptin in response to obesity-related stimuli, like hyperinsulinemia and hypoxia. This process is mediated through interactions of Sp-1, a nuclear factor that mediates the effects of insulin and/or HIF-1, the master transcription factor in cellular response to oxygen deficiency, with specific motifs within the leptin gene promoter. Considering that in adipocytes leptin promoter is regulated by the activation of peroxisome proliferator activated receptor (PPAR) γ, we studied whether or not ciglitazone, a PPAR-γ ligand, used for treatment of patients with diabetes and obesity and a potential anti-neoplastic agent, can modulate the expression of leptin mRNA in breast cancer cells. **Methods:** We used chromatin immunoprecipitation (ChIP), to treat of MCF-7 and MDA-MB-231 breast cancer cells with submolar concentrations of ciglitazone. Real Time PCR, Western blotting as well as growth experiments were used to confirm previous experiments. **Results:** Using chromatin immunoprecipitation (ChIP), we found that treatment of MCF-7 and MDA-MB-231 breast cancer cells with submolar concentrations of ciglitazone induced binding of PPAR-γ to the proximal portion of the leptin promoter, while it decreased the association of Sp-1 with this DNA region. Results obtained with Real Time PCR,

Western blotting as well as growth experiments confirmed that these effects coincided with elevated leptin mRNA expression, protein synthesis and increased cell proliferation. The mitogenic effects of ciglitazone were not observed when higher doses of the drug were used. **Conclusions:** These data suggest that one of the mechanisms of leptin overexpression in breast tumors might involve activation of PPAR-γ with submolar concentrations of ciglitazone.

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Oncosuppressor Methylation in Metastatic Colorectal Cancer

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Background: Several evidences demonstrated that colorectal cancer (CRC), as other tumor types, is the result of an accumulation of genetic and epigenetic alterations. Aberrant DNA methylation is an early epigenetic change in carcinogenesis associated with the inactivation of gene expression of tumor suppressor genes. CpG islands methylation seems to precede genetic mutations, mostly investigated in CRC, such as K-ras and BRAF gene mutation. The detection of these last alterations is essential to set a therapy with anti-EGFR antibodies (cetuximab and panitumumab) in CRC. We hypothesized the potential role of tumor suppressor methylation in inhibiting EGFR signaling cascade thus blocking anti-EGFR therapeutic effect and the role of epigenetic alteration in metastatic progression. **Methods:** Primary tumor and liver metastatic tissues of 50 patients affected by mCRC have been characterized for promoter methylation of p16, RASSF1A and RAR beta suppressor genes by Quantitative Methylation Specific PCR (QMSP). **Results:** RARbeta, RASSF1A and p16 genes resulted methylated in 50%, 60 and 30% of primary tumors, respectively. RARbeta promoter methylation mean content resulted significantly higher in k-ras mutated with respect to k-ras wt tumors (209.96 vs. 0.44 respectively, $p < 0.01$). Interestingly, the percentage of RARbeta methylation in metastatic sites of K-ras wt tumors is significantly higher than that of the related primary tumors (20 vs. 100% respectively, $p < 0.05$). RASSF1A and p16 resulted significantly higher methylated in metastatic than in primary site ($p < 0.01$). **Conclusions:** These evidences support the role of oncosuppressor methylation in both colon tumorigenesis and progression suggesting that the epigenetic events have to be taken into account when biological therapies have to be set.

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Drug-Drug Interaction between Antineoplastics Drug-Based Protocols and General Medications: A Novel Methodological Approach in the Clinical Practice

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Background: Patients with cancer are usually treated with drugs for comorbilities while they have anti-neoplastic and biological agents. The probability of drug-drug interactions (DDI) is associated with the number of such drugs. Despite this, little is known about DDI between general medications and anti-neoplastic drugs in particular with antineoplastic protocols. Currently, no instruments are available to predict DDI between general medications and antineoplastic protocols in the clinical practice. **Methods:** The data were extracted from Drugs.com powered by Micromedex™, Cerner Multum™, Wolters Kluwer™ and others, PubMed National Library of Medicine. Data elaboration were performed by using Excell electronic datasheet. We focused our observation on antifungal, antiviral and antibiotic drugs. **Results:** The visual support emerging from our research, consists of a table reporting possible interactions ordered in three grade of significance, from absolutely prohibited (red colour), allowed with relevant limitations (orange colour) to allowed associations (yellow colour). The contra-indications to certain types of association for the risk of DDI is overcome by changes in dose or route of administration for such drugs. From this analysis emerges that beta-lactam antibacterials are among the safest when associated with antiblastic regimens; a moderate-severe interaction is with methotrexate due to the synergistic renal toxicity of the two classes of drugs. There are contraindications to the co-administration of platinum derivatives, temsiroliums, and methotrexate regiments with the antiviral cidofovir, or of irinotecan-based regimens with the antiviral atazanavir and antifungal drugs. **Conclusions:** For the first time it has been addressed the problem of interactions between most common cancer protocols used in solid tumours and antimicrobial drugs. Our table is rationally created because of the use of different colours in order to provide rapid and effective means of consulting the data. Data will be available in electronic format, implemented and updated annually with other classes of drugs such as NSAIDs, antihypertensives, hypoglycaemic and other drugs used in combination with antiblastics.
The authors gratefully acknowledge EISAI for supporting the DDI program.

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