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HIF-1α induces downregulated expression of Aurora A in breast cancer cell lines

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ABSTRACT

Background: Aurora A (AURKA) is an oncogenic serine/treonine kinase that play a critical role during mitosis, governing the correct distribution of genetic material to the daughter cells. AURKA is overexpressed in many types of human tumors including breast cancer and its deregulated expression, inducing alteration of mitotic spindle, chromosomal segregation and aneuploidy can lead to malignant transformation of cells.

Aims: 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 was to obtain new insights into *AURKA* regulation in breast cancer cell lines cultured under normoxic and hypoxic conditions.

Materials and Methods: Microarray analysis, using Affymetrix platform, was performed in MCF7, MDA-MB-231 and SKBr3 cells, in order to compare the differential gene expression profile in response to hypoxia. This analysis showed *AURKA* downregulation in all breast cancer cell lines analyzed and the reduction of both mRNA levels and related protein were confirmed by Real-Time RT-PCR and Western Blotting. The HIF-α involvement in transcriptional control of *AURKA* were assess by ChiP assay and siRNA against HIF-1α was used to inhibit the HIF-1α induction during hypoxia.

Results: Our data showed that hypoxic condition induces a reduction of *AURKA* expression suggesting a possible direct involvement of HIF-1α in this downregulation.

Conclusions: These results suggest a new mechanism of *AURKA* regulation that might be able to suppress the proliferation, lead to the apoptosis of breast cancer cell lines and play a key role in the realization of new possible therapeutic approaches.

Introduction

INTRODUCTION

Breast cancer is the most common malignancy affecting women worldwide, second only to lung malignancies in cancer mortality .
Recent epidemiological studies showed that breast cancer is one of the most frequent tumor in the Western World, with more than 1.000.000 cases/year, while in Europe, 115/100.000 cases of this tumor are diagnosed annually[1].
In the last decade, the mortality rate for breast cancer has decreased, probably as a result of a more intensive program of mammographic screening that leading to earlier diagnosis and consequently a higher probability of survival for patients.

Breast Cancer Types

Each breast contain 15 to 20 sections of glandular tissue called lobes that are further divided into smaller lobules that produce milk during pregnancy and breast-feeding. The lobes and lobules are connected by thin tubes, called ducts (Figure 1).

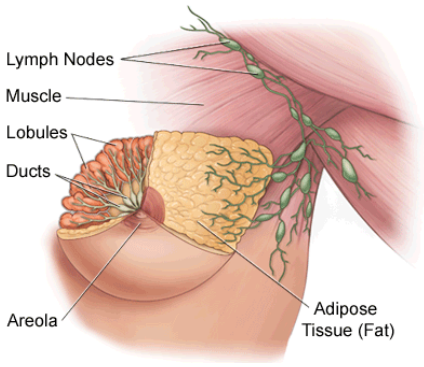

Figure 1: Anatomy of breast

The most common type of breast cancer is ductal cancer that is found in the cells of the ducts. Breast cancer that starts in lobes or lobules is called lobular cancer. Ductal and lobular breast cancer are classified as non- invasive (in situ) and invasive (infiltrating).
Ductal carcinoma in situ (DCIS) is the main type of non-invasive breast cancer while lobular carcinoma in situ (LCIS) have higher ability to spread invading either breast. However invasive lobular carcinoma makes up a small portion of all breast cancers. Other rare types of breast cancer are medullary, mucinous and tubular carcinoma.

Breast Cancer Risk Factors

Every woman is at risk for developing breast cancer. The “established” risk factors for breast cancer are female gender, age, previous breast cancer, benign breast disease, hereditary factors (family history of breast cancer), early age at menarche, late age at menopause, late age at first full-term pregnancy, postmenopausal obesity, low physical activity, race/ethnicity and high-dose exposure to ionizing radiation early in life.
The “speculated” risk factors for breast cancer include never having been pregnant, having only one pregnancy rather than many, not breast feeding after pregnancy, use of postmenopausal estrogen replacement therapy or postmenopausal hormone (estrogen/progestin) replacement therapy, use of oral contraceptives, alcohol consumption, tobacco smoking, and abortion.
Epidemiological studies have shown that in women with a family history of breast cancer, the risk of breast tumor is increased two- to threefold. Studies have also shown that there are families in which breast cancer risk is inherited in an autosomal-dominant fashion (‘hereditary breast cancer’).
In particular, it has been shown that germline mutations in the *BRCA1* and *BRCA2* genes account for a large proportion of cases of hereditary breast cancer [2].

Microarray analysis in Breast Cancer

Breast cancer is a heterogeneous group of different tumor that vary in prognosis and response to therapy. This heterogeneity has spawned an era of molecular assays striving to classify and thus predict outcome to guiding the future in targeted personalized treatment strategies.
In particular, advances in molecular characterization of tumor by microarray profiling, allowed has to identify five distinct molecular subtypes of breast cancer such basal-like, ERBB2 overespressing, normal breast-like and luminal A/B (Figure 2). This molecular classification, based on difference in gene expression of breast cancer, has led to the identification of a large number of novel targets and, in parallel, the develop of multiple approaches to anticancer therapy [3] .

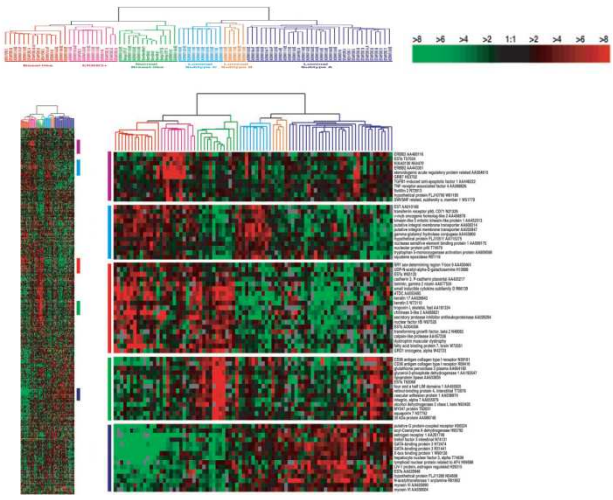

Figure 2: Gene expression patterns of five subtypes analyzed by hierarchical clustering. The cluster dendrogram showing the six subtypes of tumors are colored as: luminal subtype A, dark blue; luminal subtype B, yellow; luminal subtype C, light blue; normal breast-like, green; basal-like, red; and ERBB2, pink.

Cell cycle and Aurora kinase family

The cell cycle is a genetically controlled process, consisting of a series of events coordinated and interdependent, which ensuring the correct division of eukaryotic cells [4].

Specific cellular processes and checkpoints through the inter-mitotic phases are crucial to control the correct entry and progression in the cell cycle. Accordingly aberration in genetic pathways controlling cell cycle, cause abnormally cell growth, senescence and apoptosis inducing malignant transformation of cells [5] (Figure 3).

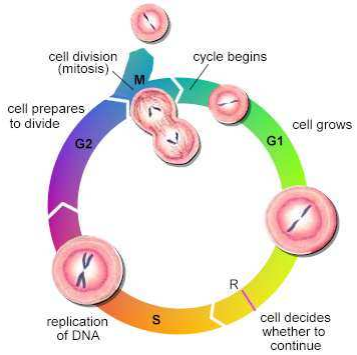

Figure 3: Overview of the cell cycle.

A key step that govern the distribution of genetic material to the daughter cells is the mitosis and a multigenic family of mitotic serine/threonine kinases called Aurora kinases, play essential roles during G2/M transition [6].
Aurora kinases is a family of mitotic serine/threonine kinases involved in different process of cell cycle control [7]. In mammals, three members of this kinases were found: Aurora A (AURKA), -B (AURKB) and -C (AURKC) [8]. AURKA is mainly involved in centrosome maturation, spindle assembly and chromosome segregation [9]. AURKB participates in chromatin modification, microtubule-kinetochore attachment, spindle checkpoint and cytokinesis. AURKC does not seem to have a role in mitosis in the majority of normal cells and its expression is limited to testes [10].
AURKA localizes to centrosomes and spindle poles and drive centrosome maturation, separation, bipolar spindle assembly and cytokinesis in accordance with its localization at the central spindle and midbody in anaphase and telophase of mitosis [11] . AURKA is associated with several co-activators that during cell division drive the localization, activation and substrate preference of the kinases. These cofactors are BORA, TPX2, ajuba (JUB), growth arrest and DNA damage-inducible 45 α (GADD45 α) [12].
AURKA play a critical role in mitotic event and its aberrant expression and activity leading to alteration of microtubule dynamics, mitotic spindle and chromosomal segregation, induces aneuploidy that result in genetic instability and transformation of cells [13] [14].

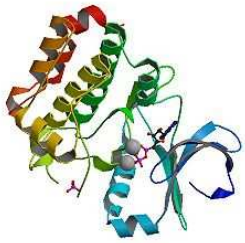

Figure 4: AURKA protein three-dimensional structure

AURKA is overexpressed in many types of human malignancies, such as breast, bladder, ovarian, colorectal, pancreatic and gastric cancers [15].
This overexpression correlates with tumorigenesis, metastasis and chemoresistance, confirming the AURKA pro-survival function in cancer cells. In particular, the 20q13 region where AURKA maps, is amplified in 40% of breast cancer cell lines and in 12 to 18% breast primary tumors with an increased of incidence to 60% in breast cancer with familial predisposition [16]. AURKA overexpression correlate with invasive disease and genomic instability in breast cancer [17], while the gene amplification showed high

association with poor prognosis in human bladder cancer [18]. AURKB is also expressed at high level in primary human colorectal cancer and other tumor cell lines. This deregulated expression inducing centrosome amplification and errors in chromosome segregation, with a consequent increased ploidy and altered cytokinesis, may play a role in carcinogenesis.

Furthermore, both AURKA and AURKB deregulation, inducing inappropriate cellular division, can lead to cancer development.

In recent years, small molecules with an inhibition action towards Aurora kinase family are developed. This molecules can act blocking the protein–protein interaction between the Aurora kinase and their substrates or blocking the ATP-binding site of the serine threonine kinase [19].

Several Aurora kinase inhibitors are currently undergoing various Phase I-II evaluations for different human cancers [20]. Among these AT-9283, a small-molecule multi-targeted kinase inhibitor, in patients with hematological malignancies, such as leukemias, myelodysplastic syndrome, myeloproliferative disease, chronic myeloid leukemia, lymphomas, multiple myeloma and solid tumors, has been show a good safety and efficacy in phase I clinical trials, placing with a possible therapeutic agent in several human cancer [21].

Hypoxia and cancer

A typical feature of several solid tumors microenvironment is the hypoxia [22]. During the rapid cellular proliferation, tumors outstrip their blood supply and consequently the availability of oxygen and nutrients is limited. In this contest, the transcription factor hypoxia-inducible factor-1 (HIF-1) appears play a crucial role. HIF-1 is a heterodimeric protein, consisting of two subunits: HIF-1α that is expressed constitutively and HIF-1β that stabilized under hypoxia, dimerized with HIF-1α and lead to transcriptional activation of target genes via binding to hypoxia responsive elements (HREs) [23, 24].

HIF-1 induces the expression of more than 100 genes [25], among them the angiogenesis mediator vascular endothelial growth factor (VEGF), glycolytic enzymes (Hu et al., 2003), and various genes involved in proliferation and cell cycle progression to ensure the survival of cancer cells [26, 27] (Figure 5).

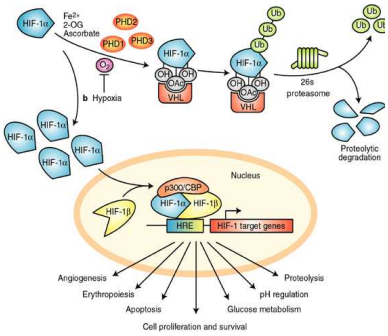

Figure 5: Role of HIF-1

In a recent work *AURKA* overexpression has been correlated at the reduced partial pressure of oxygen inside tumors [28] .

The authors demonstrate that in hypoxic conditions, the stabilization of HIF-1α, well as induce the expression of genes involved in angiogenesis, glucidic metabolism, proliferation and survival, is involved in *AURKA* overexpression in hepatoblastoma cell lines.

Hypoxia is a typical tumoral condition that influence the expression of various proteins involved in proliferation and cell cycle progression but little is known about the molecular mechanisms thought AURKA is regulated in response to hypoxic condition in breast cancer cell lines.

Therefore aim of our study is to obtain new insights into AURKA regulation in breast cancer cell lines cultured under hypoxic environment by evaluating the HIF-1α role in transcriptional control of *AURKA* expression.

Aims of the thesis

The aim of this study was to obtain new insights into AURKA regulation in breast cancer cell lines cultured in normoxic and hypoxic condition. The main goal is to identify new mechanism of transcriptional control of this kinase that could help in the realization of novel therapeutic strategies.

Hypoxia is a typical tumoral condition, that influence the expression of several genes involved in cell cycle, angiogenesis, glucidic metabolism, and survival.

Concerning this initially, a microarray analysis, using Affymetrix platform, was performed in MCF-7, MDA-MB-231 and SKBr3 breast cancer cell lines, cultured in normoxic and hypoxic condition, in order to identify, comparing the differential gene expression profile a panel of several differential expressed genes, involved in cellular adaptation to hypoxic environment.

Focusing on *AURKA* expression, was found a statistically significant downregulation ($P \leq 0.01$) in all breast cancer cell lines cultured in hypoxia and the reduction of mRNA levels and related protein were confirmed by Real-Time RT-PCR and Western Blotting.

Since HIF-1α play a critical role during hypoxic condition, a ChiP assay and siRNA against HIF-1α was used to evaluate the possible HIF-1α involvement in transcriptional control of *AURKA* expression.

Materials and Methods

MATERIALS AND METHODS

Cell culture, small-interfering RNA, and transfection

Human breast cancer cell lines, MCF-7, MDA-MB-231 and SK-Br3, purchased from the American Type Culture Collection (Rockville, MD, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM:F12) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin and 50 mg/mL streptomycin) (Invitrogen, Carlsbad, CA, USA).

Eighty % confluent cell lines were cultured in a normoxic atmosphere of 16% O2, 79% N2 and 5% CO2 (by volume) for 24h. Then medium was renewed and cells were further cultured under normoxia or hypoxia (3% O2, 87% N2, 5% CO2, by volume) at two different time-points, 24 and 48 hours. The small-interfering RNA (siRNA) oligonucleotides specific for HIF-1α were purchased from Thermo Scientific Dharmacon®. The cells were cultured in 6-well culture dishes in 2 ml of DMEM medium, transfected by adding DharmaFECT® 4 according to the recommendations in the respective siRNA transfection protocol and 5 μM siRNAs. After 3-4 hours of incubation with siRNA and trasfection reagent, the cells were exposed to hypoxia for 24 and 48 hours.

Western Blotting (WB)

Cells were lysed using complete Lysis-M reagent set (Roche, Mannheim Germany). Protein concentration was measured using Quick Start Bradford (Bio-Rad Laboratories, Hercules, CA). 80-100 μg of total protein lysate were separated on 10% polyacrilamide gel under denaturating conditions and immunoblotted into nitrocellulose membrane. The following antibodies (Abs) were used: anti-HIF-1α rabbit affinity purified Ab (Bethyl Laboratories, Montgomery, USA); anti-Phospho-Aurora-A (Thr288) (CD39D8) rabbit (Cell Signaling Technology, Boston, MA, USA); anti-VEGF(147) rabbit polyclonal IgG; anti-ARK1(N-20) goat polyclonal IgG; anti-GAPDH(6C5) mouse monoclonal IgG1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Quantitative real-Time PCR (qRT-PCR) and RT-PCR

Total cellular RNA was extracted using RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA). Then, the RNA was controlled through 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and quantified through the spectrophotometer NanoDrop ND-1000 (CELBIO). For AURKB, AURKA and HIF-1α mRNAs detection, 2 ng of total RNA were reverse transcribed in to single-stranded cDNA using High Capacity cDNA Reverse Trancription Kit (Applied Biosystems, Foster City, CA, USA) according to vendor's instructions. Gene-primers for AURKB, AURKA and HIF-1a were purchased from Applied Biosystems (TaqMan gene expression

assay). Quatitative real- time PCR (qRT-PCR) was performed with the ABI PRISM 7900 sequence detection system (Applied Biosystems, Foster City, CA, USA) using SDS software version 2.1. The reactions were performed in triplicate and the results were normalized using Human Cyc Pre-Developed TaqMan assay reagents (Applied Biosystems). Changes in the target mRNA content were determined using a comparative CT method (ABI User Bulletin no. 2). An average CT value for each RNA was obtained for triplicate reactions. It was also performed a RT-PCR in order to confirm the results previously obtained with qRT-PCR. For this aim there were used the AURKA primers.The results were evaluated in 2% agarose gel.

Chromatin Immunoprecipitation (ChIP)

Immunoprecipitation was performed using Chromatin Immunoprecipitation (ChIP) Assay Kit (Millipore, Temecula, CA) according to manufacturer's protocol. 1x106 cells were cultured on a 10cm culture dish under normoxia and hypoxia conditions (24h and 48h). The cells were then treated with a solution of PBS and 1% formaldehyde and incubated for 8 minutes at 37°C in order to cross-link histones to DNA. The cells were pelletted (4 minutes, 4°C, 4000rpm) and sonicated in order to shear DNA to lengths between 200-1000 basepairs. Soluble chromatin was immunoprecpitated with 5mg of HIF-1ąantibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. DNA-protein immune complexes were eluted, reverse crosslinked and DNA was recovered by phenol/chloroform extraction and ethanol precipitation. The binding of HIF-1a to the AURKA promoter region -336/-236 that contains the three putative HREs (HRE-1: -336/-332; HRE-2: -323/-319; HRE-3: -240/-236) was determined though PCR using the following primers: forward 5'-AGTCGTTTCTGTGTTTTCTC-3' and reverse 5'-GAGATAAAGTCCAAGGAGGTGAAC-3'. The PCR conditions were: 5 min at 95°C; 30 sec at 95°C, 30 sec at 60°C, 40 sec at 72 °C for 35 cycles; 7 min at 72°C.

Microarray Analysis

Microarray analysis was performed as previously described (Federico et al, 2010). Total RNA was extracted according to the manufacturer's protocol (Affymetrix, Santa Clara, CA). Fragmented cRNA was hybridized using a human oligonucleotide array U133 Plus 2.0 (Genechip Affymetrix, Santa Clara, CA). Washing and staining were performed through Affymetrix GeneChip Fluidic Station 450. Probe arrays were scanned using Affymetrix GeneChip Scanner 3000 G7. For statistical analysis, the background subtraction and normalization of probe set intensities was performed using the method of Robust Multiarray Analysis (RMA) described by Irizarry et al. (Irizarry et al., 2003). To identify DEGs, gene expression intensity was

compared using a moderated t test and a Bayes smoothing approach developed for a low number of replicates (Smyth, 2004). To correct for the effect of multiple testing, the false discovery rate, was estimated from p values derived from the moderated t test statistics (Benjamini et al., 2001). The analysis was performed using the affylmGUI Graphical User Interface for the limma microarray package (Wettenhall, 2006).

KEGG and Biocarta Analysis

Differentially expressed genes were analyzed according to predefined pathways annotated by KEGG (Kyoto Encyclopedia of Genes and Genomes) (Kanehisa and Goto, 2000) and Biocarta (BioCarta Pathways [http://www.biocarta.com/genes/index.asp]) using the Gene Set Analysis Toolkit. For an over-represented KEGG or Biocarta Pathway, a cutoff P value of 0.01 has been selected.

Results

AURKA downregulation in breast cancer cell lines cultured in hypoxic condition

Considering that hypoxia is a condition that during tumoral growth influences the expression of several genes involved in proliferation and cell cycle progression [29], we first performed a microarray analysis, using Affymetrix platform, to compare the differential gene expression profiles of MCF-7, MDA-MB-231 and SKBr3 breast cancer cell lines, cultured in normoxic and hypoxic conditions for 24 and 48 hours.

The scan and subsequent analyses of the probe arrays processed by GCOS and other software of statistical analysis (Console, Genespring, Toolkit), allowed to obtain three lists of differentially expressed genes ($P< 0.005$), that include 15,157 genes (8,059 down-regulated and 7,098 up-regulated) for MCF-7; 14,337 genes (8,079 down-regulated and 6,258 up-regulated) for MDA-MB-231; and 18,641 genes (9,664 down-regulated and 8.977 up-regulated) for SKBr3 cell lines, after 24 and 48 hours of hypoxic exposure.

A study of the three lists of genes obtained was conducted in order to verify the involvement of some of them in cell pathways related to tumoral pathogenesis. In particular, for this analysis, the list of genes were screened considering as significative only the genes with fold change (M) >2 and with statistical difference of expression of each gene was at least $P < 0.001$.

The following tables shows a partial list of genes differentially expressed in MCF-7, MDA-MB-231 and SKBr3 breast cancer cell lines, cultured in hypoxic environment for 24 hours respect to controls cultured in normoxia. The data regarding the cells cultured for 48 hours in hypoxia are not shown (Table 1).

The gene commons differentially expressed ($P < 0.001$ and M >2), in response to hypoxic exposure for 24 and 48 hours, in all tree breast cancer cell lines analyzed were grouped in a hierarchical clustering (Figure 6).

Symbol	MCF7 N1	MCF7 N2	MCF7 H1	MCF7 H2	M	P.Value
	Signal					
CDC25A	6.34	6.36	3.14	3.66	-7.70	1.79E-04
CDC20	10.09	10.08	7.51	7.60	-5.77	6.49E-05
TACC1	8.76	8.61	6.17	6.63	-4.88	0.00031
CDC2	10.88	10.90	8.59	8.71	-4.73	8.13E-05
CDC25B	9.91	9.96	7.71	7.93	-4.33	0.00013
CCNA2	9.24	9.24	7.12	7.22	-4.20	9.34E-05
GTSE1	6.77	6.69	4.80	4.84	-3.75	0.000112
TGFB2	6.67	6.75	4.93	4.73	-3.69	0.000164
MPHOSPH9	8.19	8.41	6.42	6.48	-3.61	0.000189
CDC6	7.86	7.80	5.86	6.11	-3.60	0.000205
CCNE2	8.45	8.47	6.54	6.72	-3.56	0.000159
TPX2	9.08	9.23	7.25	7.42	-3.52	0.000195
AURKB	8.20	8.36	6.35	6.60	-3.49	0.000266
TUBG1	10.25	10.21	8.42	8.47	-3.45	0.000127
AURKA	11.28	11.22	9.47	9.65	-3.23	0.000197
CDK2	9.74	9.89	7.99	8.33	-3.15	0.000485
BRCA2	7.45	7.27	5.99	5.43	-3.15	0.001519
CCND1	10.37	10.38	8.69	8.76	-3.14	0.000153
BRCA1	7.95	8.16	6.43	6.61	-2.89	0.000396
ESPL1	8.94	9.10	7.37	7.63	-2.86	0.000475
JUB	9.58	9.69	8.18	8.09	-2.83	0.000229
AKT1	10.20	10.21	8.86	8.87	-2.53	0.000241
CDC25C	8.12	8.13	6.85	6.86	-2.41	0.000284
HRAS	8.24	8.30	7.00	7.14	-2.30	0.000467
ANAPC1	7.40	7.11	6.27	5.88	-2.26	0.003301
E2F5	7.24	7.33	6.25	6.27	-2.04	0.000619
ATR	7.10	7.10	6.20	6.16	-2.02	0.000816
WEE1	9.51	9.60	8.83	9.16	-2.01	0.024842
EIF1	11.73	11.65	12.77	12.69	2.05	0.00067
MDM2	6.42	6.40	7.46	7.44	2.05	0.000537
PPP4R1L	6.07	5.77	7.03	6.88	2.05	0.002167
MARS	6.31	6.38	7.27	7.49	2.05	0.001145
GTF3C4	8.37	8.41	9.51	9.36	2.06	0.000778
IL1RL1	4.10	3.77	4.95	5.00	2.06	0.002127
BCL10	6.34	6.30	7.48	7.24	2.06	0.001165
FOS	7.19	7.31	8.82	8.76	2.91	0.000208
JUN	6.42	6.52	8.74	8.57	4.55	0.000112
DDIT3	7.82	7.69	10.15	9.85	4.73	0.000169
VEGFA	6.36	6.47	9.21	9.26	7.07	6.15E-05

Symbol	MDA N1	MDA N2	MDA H1	MDA H2	M	P.Value
	Signal					
JUB	11.9	11.84	8.33	8.66	-10.4	0.000086
CDC25A	6.34	6.36	3.14	3.66	-7.70	1.79E-04
CDC20	10.09	10.08	7.51	7.60	-5.77	6.49E-05
CDC25C	6.66	6.96	4.58	4.30	-5.16	0.00016
TACC1	8.76	8.61	6.17	6.63	-4.88	0.00031
CDC2	10.88	10.90	8.59	8.71	-4.73	8.13E-05
CDC25B	9.91	9.96	7.71	7.93	-4.33	0.00013
CCNA2	9.24	9.24	7.12	7.22	-4.20	9.34E-05
GTSE1	6.77	6.69	4.80	4.84	-3.75	0.000112
TGFB2	6.67	6.75	4.93	4.73	-3.69	0.000164
MPHOSPH9	8.19	8.41	6.42	6.48	-3.61	0.000189
CDC6	7.86	7.80	5.86	6.11	-3.60	0.000205
CCNE2	8.45	8.47	6.54	6.72	-3.56	0.000159
TUBG1	10.25	10.21	8.42	8.47	-3.45	0.000127
AURKA	11.28	11.22	9.47	9.65	-3.23	0.000197
AURKB	8.41	8.21	6.82	6.45	-3.18	0.000266
CDK2	9.74	9.89	7.99	8.33	-3.15	0.000485
BRCA2	7.45	7.27	5.99	5.43	-3.15	0.001519
CCND1	10.37	10.38	8.69	8.76	-3.14	0.000153
BRCA1	7.95	8.16	6.43	6.61	-2.89	0.000396
ESPL1	8.94	9.10	7.37	7.63	-2.86	0.000475
AKT1	10.20	10.21	8.86	8.87	-2.53	0.000241
HRAS	8.24	8.30	7.00	7.14	-2.30	0.000467
ANAPC1	7.40	7.11	6.27	5.88	-2.26	0.003301
E2F5	7.24	7.33	6.25	6.27	-2.04	0.000619
ATR	7.10	7.10	6.20	6.16	-2.02	0.000816
TPX2	9.08	8.99	8.09	7.95	-2.01	0.019
WEE1	9.51	9.60	8.83	9.16	-2.01	0.024842
VEGFA	7.92	7.81	8.92	8.85	2.03	0.00086
EIF1	11.73	11.65	12.77	12.69	2.05	0.00067
MDM2	6.42	6.40	7.46	7.44	2.05	0.000537
PPP4R1L	6.07	5.77	7.03	6.88	2.05	0.002167
MARS	6.31	6.38	7.27	7.49	2.05	0.001145
GTF3C4	8.37	8.41	9.51	9.36	2.06	0.000778
IL1RL1	4.10	3.77	4.95	5.00	2.06	0.002127
BCL10	6.34	6.30	7.48	7.24	2.06	0.001165
FOS	7.19	7.31	8.82	8.76	2.91	0.000208
JUN	6.42	6.52	8.74	8.57	4.55	0.000112
DDIT3	7.82	7.69	10.15	9.85	4.73	0.000169

Symbol	SKBr3 N1	SKBr3 N2	SKBr3 H1	SKBr3 H2	M	P.Value
	Signal					
CDC25A	6.34	6.36	3.14	3.66	-7.70	1.79E-04
CDC20	10.09	10.08	7.51	7.60	-5.77	6.49E-05
TACC1	8.76	8.61	6.17	6.63	-4.88	0.00031
CDC2	10.88	10.90	8.59	8.71	-4.73	8.13E-05
CDC25B	9.91	9.96	7.71	7.93	-4.33	0.00013
CCNA2	9.24	9.24	7.12	7.22	-4.20	9.34E-05
GTSE1	6.77	6.69	4.80	4.84	-3.75	0.000112
TGFB2	6.67	6.75	4.93	4.73	-3.69	0.000164
MPHOSPH9	8.19	8.41	6.42	6.48	-3.61	0.000189
CDC6	7.86	7.80	5.86	6.11	-3.60	0.000205
AURKA	10.52	10.85	8.87	8.82	-3.57	0.00011
CCNE2	8.45	8.47	6.54	6.72	-3.56	0.000159
TUBG1	10.25	10.21	8.42	8.47	-3.45	0.000127
CDK2	9.74	9.89	7.99	8.33	-3.15	0.000485
BRCA2	7.45	7.27	5.99	5.43	-3.15	0.001519
CCND1	10.37	10.38	8.69	8.76	-3.14	0.000153
BRCA1	7.95	8.16	6.43	6.61	-2.89	0.000396
ESPL1	8.94	9.10	7.37	7.63	-2.86	0.000475
AKT1	10.20	10.21	8.86	8.87	-2.53	0.000241
HRAS	8.24	8.30	7.00	7.14	-2.30	0.000467
ANAPC1	7.40	7.11	6.27	5.88	-2.26	0.003301
CDC25C	8.98	8.93	7.94	7.78	-2.13	0.0445
TPX2	9.54	9.80	8.56	8.64	-2.10	0.00086
AURKB	6.00	6.61	5.20	5.30	-2.08	0.0058
E2F5	7.24	7.33	6.25	6.27	-2.04	0.000619
ATR	7.10	7.10	6.20	6.16	-2.02	0.000816
WEE1	9.51	9.60	8.83	9.16	-2.01	0.024842
EIF1	11.73	11.65	12.77	12.69	2.05	0.00067
MDM2	6.42	6.40	7.46	7.44	2.05	0.000537
PPP4R1L	6.07	5.77	7.03	6.88	2.05	0.002167
MARS	6.31	6.38	7.27	7.49	2.05	0.001145
GTF3C4	8.37	8.41	9.51	9.36	2.06	0.000778
IL1RL1	4.10	3.77	4.95	5.00	2.06	0.002127
BCL10	6.34	6.30	7.48	7.24	2.06	0.001165
FOS	7.19	7.31	8.82	8.76	2.91	0.000208
JUN	6.42	6.52	8.74	8.57	4.55	0.000112
DDIT3	7.82	7.69	10.15	9.85	4.73	0.000169
VEGFA	6.89	6.29	10.1	10.1	11.7	3.52E-05

Table 1: Patterns of differential expressed genes in response to hypoxia. Partial list of
of genes differentially expressed in MCF-7, MDA-MB-231 and SKBr3 breast cancer cell lines exposed to hypoxia for 24 hours (P < 0.001; fold change M >2).
Symbol: gene symbol; Signal: A quantitative measure of the relative abundance of a transcript. N: Normoxia. H:Hypoxia. N1 and N2: biological replicates under normoxia. H1 and H2: biological replicates under hypoxia.

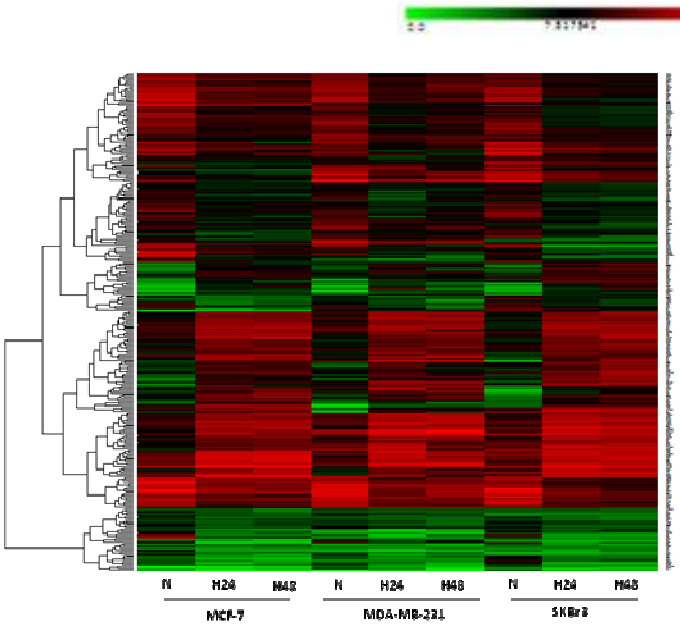

Figure 6 : Hierarchical clustering of the common statistically significant differential expression genes in MCF-7, MDA-MB-231 and SKBr3 breast cancer cell lines exposed to hypoxia for 24 and 48 hours. (P < 0.001; fold change M >2). Each horizontal line represents a particular gene and the columns the experimental conditions.. The colored bar on the top represent the gene expression variations.

Focusing our analysis primarily on genes involved in proliferation and response to hypoxia, the genes involved in cell cycle regulation, AURKA signaling and HIF-1α network, that changed in a statistically significant manner (P < 0.001) after hypoxic exposure to 24 and 48 hours, were grouped in the heat maps (Figure 7).

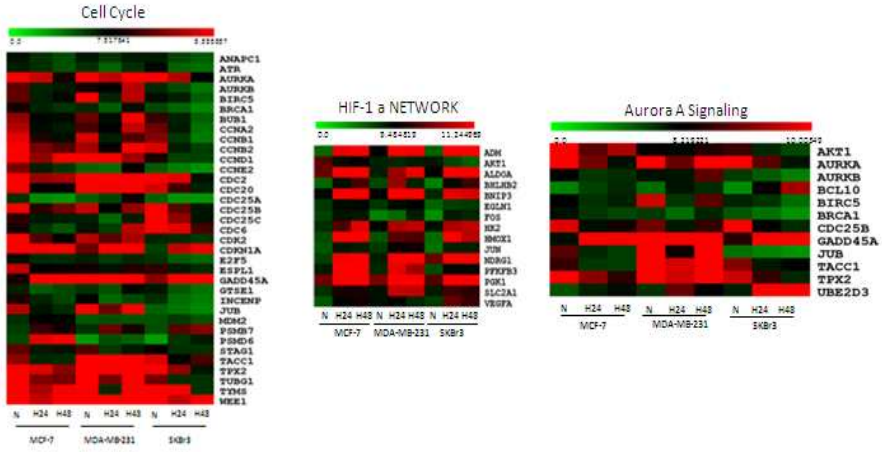

Figure 7: Corrected microarray signal values of genes involved in cell cycle regulation, AURKA signaling and HIF-1α network of MCF-7, MDA-MB- 231 and SKBr3 breast cancer cell lines exposed to hypoxia for 24 and 48 hours in comparison to control cells ($P < 0.001$; fold change M >2).

The most significant changes were observed in key genes involved in progression and regulation of cell cycle and in pathway of response to hypoxic condition. In particular, in all three breast cancer cell lines analyzed was observed a downregulation of *AURKA* (fold change: -3.23 and -3.57 in MCF-7, -3.23 and -2.46 in MDA-MB-231, and -3.57 and -4.53 in SKBr3, respectively to 24 and 48 hours) and a significant decrease in the expression level of the two positive regulator of this kinase, such TPX2 (fold change: -3.52 and -2.62 in MCF-7, -2.02 and +1.66 in MDA-MB-231 and -2.10 and 4.55 in SKBr3 respectively to 24 and 48 hours) and JUB (fold change: -2.83 and -3.03 in MCF-7, - 5.4 and -2.23 in MDA-MB-231 and -1.50 and -2.65 in SKBr3 respectively to 24 and 48 hours).

Moreover, significant changes were observed in HIF-1 α target genes, including the up-regulation of VEGF (fold change: + 7.07 and +8.43 in MCF-7, +2.03 and + 5.08 in MDA-MB-231 and + 11.7 and + 12.76 in SKBr3). Since the previously analysis allowed has to obtain three lists of differentially expressed genes that showed a downregulation of *AURKA* in the three breast cancer cell lines, the next step was to validate the results and correlate the reduction of gene expression level of this kinase with the hypoxic condition. Using RT-PCR and QRT-PCR was confirmed that hypoxia induces downregulation of *AURKA* mRNA expression, with an increased reduction of expression levels of this kinase after 48 hours of hypoxic exposure in MCF-7 and SKBr3 and with an initial decreased (after 24 h) followed by a partial expression recover after 48 hours in MDA-MB-231, respect to normoxic condition (Figure 8)

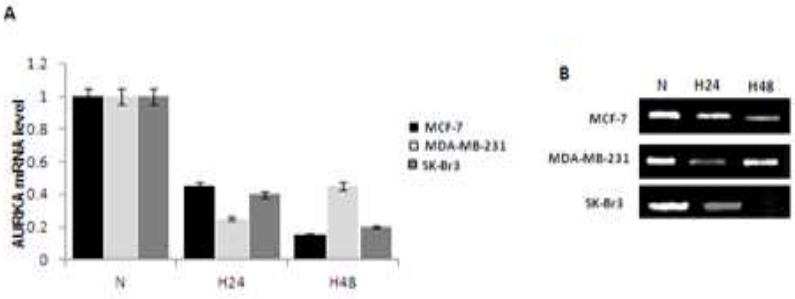

Figure 8: Effect of hypoxia on AURKA mRNA expression. A. mRNA expression of AURKA, as quantified by real time PCR in MCF-7, MDA-MB-231 and SKBr3 cells expose to hypoxia for 24 and 48 hours. B. AURKA mRNA expression as quantified using RT-PCR.

Interestingly, in parallel to *AURKA* downregulation, was found a reduction of AURKA protein levels in MCF-7 and SKBr3 after 24 and 48 hours of hypoxic exposure, compared to control condition.
Differentially MDA-MB-231 cells, as observed by microarray analysis, showed a reduction of *AURKA* mRNA levels and relative protein after 24 hours with an increased expression after 48 hours of exposure to hypoxia.
The reduction of AURKA protein is paralleled to an increase in HIF-1α and VEGF protein levels. Consistent with our findings, together with a reduction of AURKA protein under hypoxia, we detected a decrease in the phosphorylation level of AURKA(Thr288) (Figure 9).

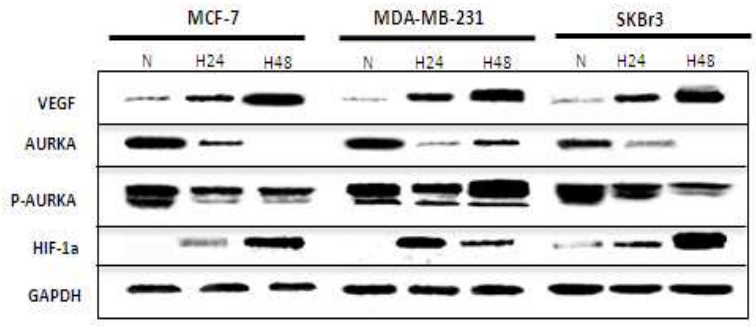

Figure 9: Effect of hypoxia on the protein levels of VEGF, AURKA, p-AURKA and HIF-1α. MCF-7, MDA-MB-231 and SKBr3 were cultured in hypoxic environment for 24 and 48 hours and protein expression were examined by Western blot developing with the enhanced chemoluminescence reagent (ECL). The house-keeping protein GAPDH was used as loading control.

In contrast, these effects of hypoxic exposure were reversed silencing HIF-1α mRNA expression. Using HIF-1α-siRNA in MCF-7, MDA-MB-231 and SKBr3 cell lines exposed to hypoxia for 24 and 48 hours, was observed a significant increased both AURKA mRNA levels, evaluated by RT-PCR and QRT-PCR, together with an increase in AURKA phosphorylation and AURKA protein levels (Figure 10).

According to these results we suggested that HIF-1α could act directly or indirectly as a possible negative regulator of *AURKA* expression in breast cancer cell lines cultured in hypoxia.

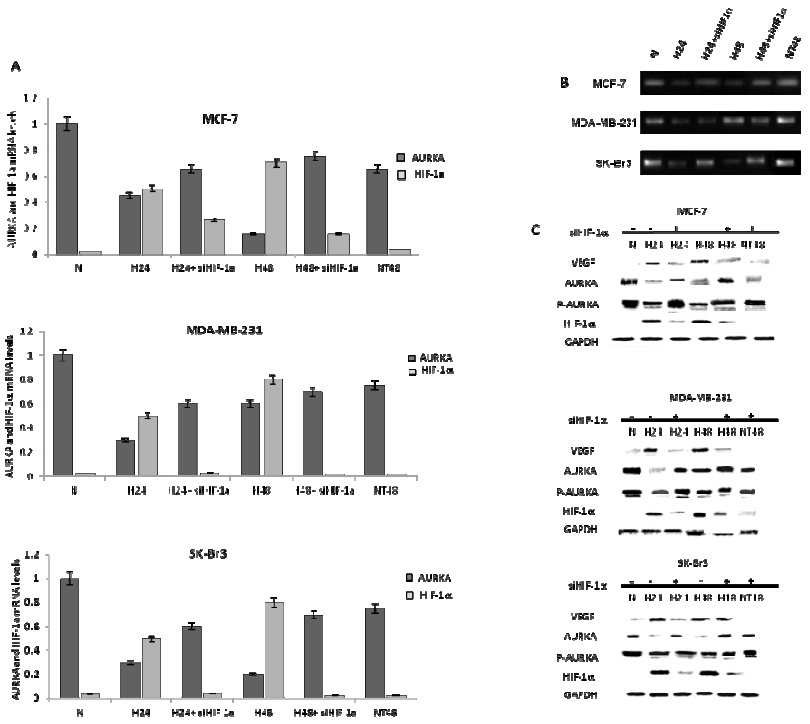

Figure 10: A. Effect of HIF-1αsiRNA in MCF-7, MDA-MB-231 and SKBr3 cell lines exposed to hypoxia for 24 and 48 hours. A. mRNA expression levels of AURKA and HIF-1α as quantified by real time PCR. B. Protein expression levels of VEGF, AURKA, p-AURKA and HIF-1α examined by Western blot. Each membrane was also probed with GAPDH to confirm equal loading.

HIF-1α involvement in transcriptional regulation of *AURKA*

HIF-1α regulates the transcription of its target genes via binding to HREs. Sequence analysis have showed three HREs sites into AURKA proximal promoter (HRE-1: -336/-332; HRE-2: -323/-319; and HRE-3: -240/-236).

Therefore, to investigate whether HIF-1α could regulate *AURKA* expression, a Chromatin Immunoprecipitation (ChIP) assay was performed to evaluate the effect of hypoxia on HIF-1α binding to the HREs into the proximal AURKA promoter region.

This analysis allowed has to observe that in MCF-7, SKBr3 and MDA-MB-231 breast cancer cell lines, HIF-1α does not bind AURKA promoter under normoxia but an increased binding at 24 and 48 hours of hypoxia exposure was observed in MCF-7 and SKBr3 cells. Differentially, in MDA-MB-231 cells was obtain an enhanced HIF-1α HREs binding after 24 hours following by a decreased after 48 hours of hypoxia exposure (Figure 11).

Contrary to earlier findings, we hypothesize a new possible mechanism where under hypoxia, HIF-1α rather than induce transcriptional activation, can promote *AURKA* downregulation by its HREs promoter binding.

The reduced HIF-1α binding in AURKA promoter region observed after 48 hours of hypoxia in MDA-MB-231, supports our hypothesis because in response to a lower HIF-1α promoter binding, we observed an increase of gene expression and protein levels, as well AURKA activation status (Figure 8 and 9). Therefore these results can suggest a new possible transcriptional role of HIF-1α that could be determine a negative control of *AURKA* expression in breast cancer cell lines.

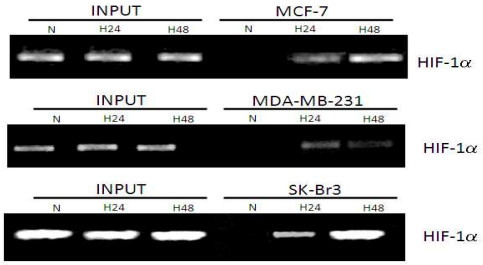

Figure 11: Effect of hypoxia on HIF-1α binding into proximal AURKA promoter region. Interaction between HIF-1α and AURKA promoter region containing HRE-1, HRE-2 and HRE-3 as observed by Chromatin Immunoprecipitation (ChIP) assay in MCF-7, SKBr3 and MDA-MB-231 breast cancer cell lines cultured in hypoxic conditions for 24 and 48 hours.

Discussion

Breast cancer continues to be one of the most common cancers and a major cause of death among women worldwide. Since oncologists have several options available (chemotherapy, hormone-therapy and biologic agents such as anti-angiogenic and anti-HER2 drugs), breast cancer is still responsible for 18 percent of cancer deaths in women and is second only to lung cancer.

Analysis of gene expression profiling has recently categorized breast carcinomas into 5 distinct subtypes; luminal A, luminal B, normal breastlike, human epithelial growth factor receptor 2 (HER2) overexpressing, and basal-like, allowing to identify most suitable personalized therapeutic approach.

The Aurora family kinases, especially Aurora A, play a particularly important role in cell cycle and its deregulated expression is involved in many types of human malignancies. During mitosis, Aurora A is involved in maturation and separation of the centrosomes of the bipolar spindle and correct gene expression levels of this kinase are needed for a correct cytokinesis.

Several studies showed that Aurora A overexpression correlates with tumorigenesis, metastasis and chemoresistance, confirming the AURKA pro-survival function in cancer cells.

AURKA overexpression induces centrosome amplification (Katayama et al., 2001), which may result in chromosomal instability and aneuploidy (Zhou et al., 1998; Miyoshi et al., 2001). Thus the understanding of the regulatory pathways mediating a reduced transcription of *AURKA* could be important in order to identify new possible mechanism that can be unravel the mechanisms regarding not only breast cancer progression, but also other tumors associated with *AURKA* overexpression.

Tumor hypoxia is known to be an important regulator for the expression of many genes involved in tumorigenesis and cell cycle regulation. During this condition, therefore, the stabilization of the transcription factor HIF-1 appears to be a key regulatory factor for ~ 100 genes, of which VEGF, PGI, c-MET, and CXCR4, CDC2, RB1, or PAI-1 are known to play a pivotal role in tumorigenesis and cancer metastasis.

In a previous work, *AURKA* overexpression has been correlated at the reduced partial pressure of oxygen inside tumors in hepatoblastoma cell lines (Klein A et al 2008). The authors demonstrate that in hypoxic conditions, the stabilization of HIF-1α is involved in *AURKA* overexpression by its binding at *AURKA* promoter in hepatoblastoma cell lines.

In breast cancer cell lines, the molecular mechanisms thought AURKA is regulated in response to hypoxic condition are unknown.

Therefore the overall goal of this investigation was to obtain new insights into AURKA regulation in breast cancer cell lines cultured under normoxia and hypoxia, evaluating the possible HIF-1α role in transcriptional control of *AURKA.*

A microarray analisys, using Affymetrix platform, was performed to compare the differential gene expression profile in MCF-7, MDA-MB-231 and SKBr3 breast cancer cell lines cultured in normoxic and hypoxic condition. Focusing on *AURKA* expression, was found a statistically significant downregulation ($P \leq 0.01$) in all breast cancer cell lines cultured in hypoxia and a reduction of mRNA levels and related protein were confirmed by Real-Time RT-PCR and Western Blotting. In order to identify a new possible mechanism of response to hypoxic condition, a ChiP assay and siRNA against HIF-1α was used, to evaluate the HIF-1α involvement in transcriptional control of *AURKA* expression.

Conclusions

The overall goal of this study was to identify a downregulation of *AURKA* in MCF-7, MDA-MB-231 and SKBr3 breast cancer cell lines cultured in hypoxic condition for 24 and 48 hours.

Contrary to earlier findings, our result showed that HIF-1α rather than induce transcriptional activation, can promote *AURKA* downregulation by its HREs promoter binding in breast cancer cell lines analyzed.

The reduced HIF-1α binding into the proximal AURKA promoter region observed after 48 hours of hypoxia in MDA-MB-231 cell lines, supports our hypothesis because in response to a lower HIF-1α promoter binding, was observed an increase of gene expression and protein levels, as well AURKA activation status.

This result suggest a new possible transcriptional role of HIF-1α that can determine a negative control of *AURKA* expression in breast cancer cell lines exposed to hypoxic environment.

Aim of subsequent research will be to deepen the molecular bases of this *AURKA* downregulation evaluating if HIF-1α alone is directly involved in transcriptional control or if other co-repressor proteins are involved in this negative transcriptional control of *AURKA.*

The evaluation of this mechanisms, in the future, could play a fundamental role in the realization of new possible therapeutic approaches in breast cancer.

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BOOKS, PAPERS AND ABSTRACTS PUBLISHED DURING THE PHD COURSE

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Appendix

Expert Opinion

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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

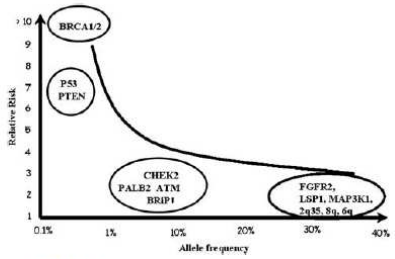

Figure 1 Allelic variants at low frequency tend to be associated with higher relative risk of BC (for example, BRCA1, BRCA2), high-frequency allelic variants are associated with lower RR (for example, FGFR2, LSP1, and so on) configuring an inverse correlation.

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 10000 *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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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

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 biotinylated 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

LOW RESOLUTION FIG

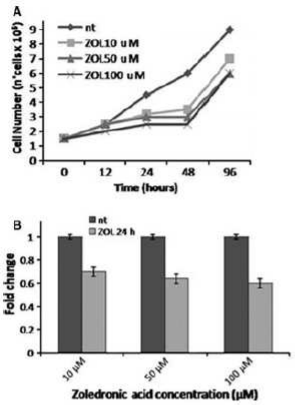

Fig. 1 A, B ZOL inhibits cell growth *in vitro*. About 10^5 and 10^4 cells were cultured in 6-well and 96-well tissue culture plates and exposed to ZOL at a concentration ranging from 10 to 100 μM for different times. Cellular viability was analyzed by cellular count (**A**) and MTT assay (**B**).

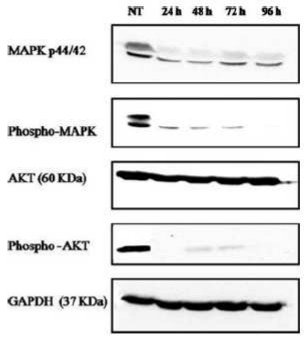

Fig. 2 Effects of ZOL addition on MAPK and Akt-dependent pathways on MCF-7 cells. Cells were treated with 10 μM ZOL for 24, 48, 72 and 96 hrs. Thereafter, both the expression and activity of MAPK p44/42 and AKT were evaluated. Determination of the expression and phosphorylation of MAPK p44/42 and AKT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

LOW RESOLUTION FIG

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

LOW RESOLUTION FIG

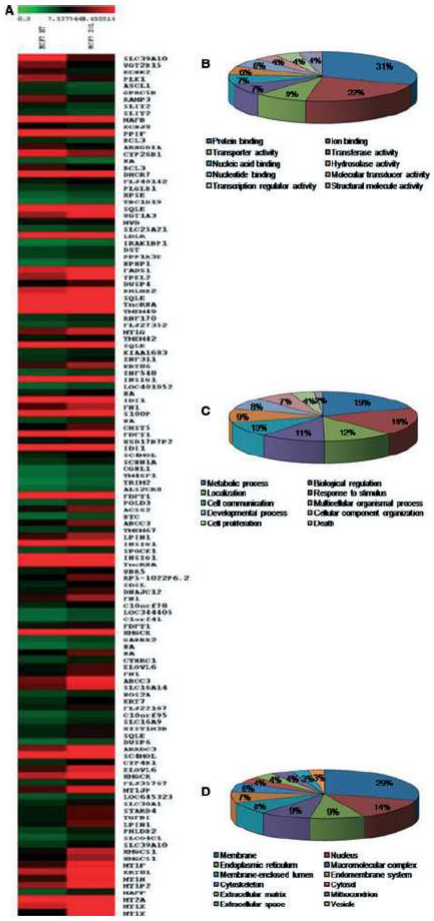

Fig. 3 (A) Treatment with ZOL globally affects gene expression profile in MCF-7 cells. **(B, C, D)** Corrected microarray signal values of genes involved in different biological process, clustered by specific functions (Biological process, Cellular function, Cellular component) of MCF-7 cells treated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

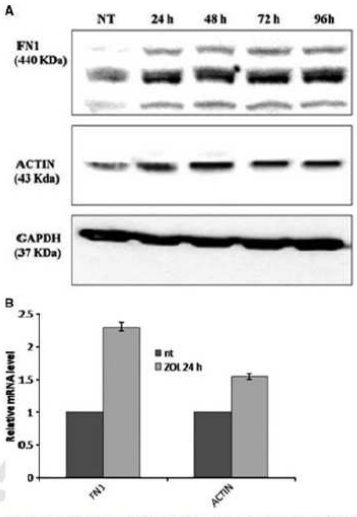

Fig. 4 Effect of ZOL on the mRNA expression and protein levels of FN1 and ACTIN. (**A**) Effect of ZOL 10 μM on the mRNA expression of FN1 and ACTIN, as quantified by real time PCR in MCF-7 cells. (**B**) Effect of ZOL on FN1 and ACTIN protein levels. MCF-7 cells were incubated with low concentration 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

LOW RESOLUTION FIG

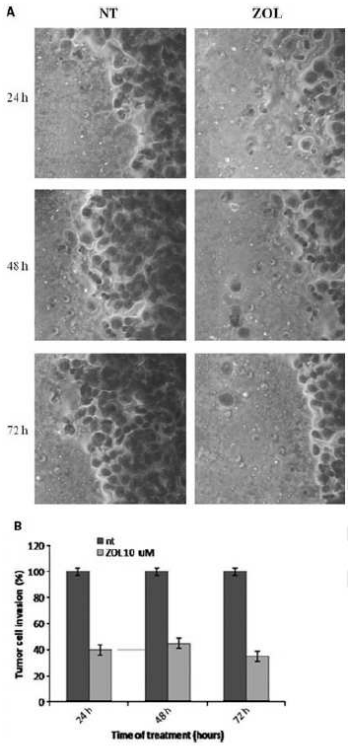

Fig. 5 ZOL decrease the invasive potential of human breast cancer cells. Effect of ZOL on the invasion of MCF-7 cells. Treated or not with ZOL 10 μM for 24 hrs, were plated onto Matrigel invasion chambers as described in 'Materials and Methods', and the cell invasion was evaluated. The invaded cells 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%).

LOW RESOLUTION FIG

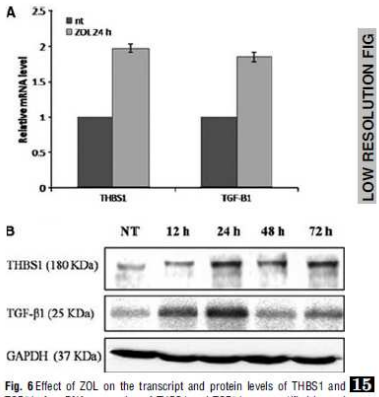

Fig. 6 Effect of ZOL on the transcript and protein levels of THBS1 and TGFβ1. A. mRNA expression of THBS1 and TGFβ1, as quantified by real time PCR in MCF-7 cells treated. (**B**) Effect of ZOL on THBS1 and TGFβ1 protein levels. MCF-7 cells were incubated with low concentration of ZOL for different times, 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.

LOW RESOLUTION FIG

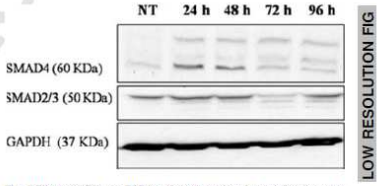

Fig. 7 Effects of ZOL on TGF-β1-dependent pathway and Smad protein expression. Cells were treated with 10 μM ZOL for 24, 48, 72 and 96 hrs. Determination of the expression SMAD2/3 and SMAD4 evaluated after blotting with specific antibodies, as described in 'Materials and Methods'. Expression of the house-keeping 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).

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

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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Prognostic vs predictive molecular biomarkers in colorectal cancer: is KRAS and BRAF wild type status required for anti-EGFR therapy?

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SUMMARY

An important molecular target for metastatic CRC treatment is the epidermal growth factor receptor (EGFR). Many potential biomarkers predictive of response to anti-EGFR monoclonal antibodies (cetuximab and panitumumab) have been retrospectively evaluated, including EGFR activation markers and EGFR ligands activation markers. With regard to the "negative predictive factors" responsible for primary or intrinsic resistance to anti-EGFR antibodies a lot of data are now available. Among these, KRAS mutations have emerged as a major predictor of resistance to panitumumab or cetuximab in the clinical setting and several studies of patients receiving first and subsequent lines of treatment have shown that those with tumors carrying KRAS mutations do not respond to EGFR-targeted monoclonal antibodies or show any survival benefit from such treatments. The role of B-RAF mutations, mutually exclusive with KRAS mutations, in predicting resistance to anti-EGFR mAbs is not yet consolidated. It therefore appears that BRAF mutations may play a strong negative prognostic role and only a slight role in resistance to anti-EGFR Abs.

Introduction

Colorectal cancer (CRC) is the third most common cause of cancer-related death, with an incidence of almost a million cases annually in both males and females.[1] Despite the fact that recent progress in diagnosis and treatment has increased the number of patients who have been completely cured at an early stage of the disease, the prognosis for advanced forms of this cancer is still very poor, with treatment limited to palliation for the vast majority of patients.[2]

The development of colorectal cancer (CRC) is a multistep process brought about by the accumulation of several genetic alterations, including chromosomal abnormality, gene mutations and epigenetic modifications involving several genes regulating proliferation, differentiation, apoptosis and angiogenesis.[3,4]

Of the various genetic alterations, only a few are involved in cell growth and will lead to cancer development. This phenomenon therefore, known as 'oncogene addiction' might represent a rationale for molecular target therapy,[5] possibly helping to develop new and targeted treatment options in patients with metastatic CRC.[6]

An important molecular target for metastatic CRC treatment is the epidermal growth factor receptor (EGFR). EGFR is a member of the HER (ErbB) family of receptor tyrosine kinases involved in a variety of signal transduction pathways which are able to promote tumor cell proliferation, angiogenesis, invasion and metastasis in different epithelial malignancies.[7]

In more specific terms, the binding of the epidermal growth factor (EGF) to the extracellular binding site of EGFR activates three major signal transduction pathways, including the RAS–RAF mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and phospholipase C pathways, causing downstream change in the gene expression profile leading to cancer development.[8]

EGFR is expressed on normal human cells but higher levels of expression of the receptor have also been correlated with malignancy in a variety of cancers, including CRC.[9]

Two predominant classes of EGFR inhibitors have been developed including monoclonal antibodies (mAbs), which target the extracellular domain of EGFR, such as cetuximab, and small molecule tyrosine kinase inhibitors (TKIs), which target the receptor catalytic domain of EGFR, such as gefitinib and erlotinib.[10] Although both classes of agents show clear antitumor activity, only anti-EGFR

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monoclonal antibodies such as Cetuximab and Panitumumab have been approved for clinical use in metastatic CRC.[11]

These costly and potentially toxic treatments are, however, efficient in only a small percentage of patients, and it is therefore extremely important to identify specific factors which will lead to a clearer definition of those patients who will benefit from anti-EGFR treatments.

Potential positive predictive biomarkers

The major potential predictive factors of response to cetuximab and/or panitumumab evaluated up till now in literature are molecular factors involved more or less directly in the EGFR signaling pathway. Among these, EGFR protein expression, EGFR gene copy number, EGFR gene mutations, and overexpression of EGFR ligands (such as epiregulin and amphiregulin) have been evaluated in order to select patients who may benefit from EGFR-targeted treatment.[12]

Several clinical trials have been performed in patients with metastatic CRC to relate the level of EGFR protein expression, as determined by immunohistochemistry with sensitivity to anti-EGFR antibodies. The results have demonstrated a lack of association between EGFR detection by immunohistochemistry, and response to EGFR-targeted treatment. Objective responses have been observed in patients with low or high EGFR level expression and since this biomarker has proved to be poorly associated with sensitivity to anti-EGFR antibody, it cannot therefore be considered as an inclusion criterion for patients undergoing treatment with cetuximab.[13]

Activating mutations in the EGFR catalytic domain play an important role in determining responsiveness to anti-EGFR treatment in lung cancer; these alterations are however, rare or absent in CRC and are not significantly associated with clinical response of metastatic CRC to the anti-EGFR monoclonal antibodies.[14]

In a small fraction of CRCs, the overexpression of *EGFR* is frequently associated with amplification of the gene. The evaluation of the *EGFR* gene copy number evaluated by quantitative PCR does not seem to correlate with the clinical outcome of patients, whereas the analysis by fluorescence *in situ* hybridation (FISH) appears to be associated with an increase of treatment response.[15] The predictive value is, however, uncertain and further studies are therefore required to assess the increase of *EGFR* gene copy number as a predictive marker of response to anti-EGFR treatment.

The overexpression of alternative EGFR ligands, such epiregulin and amphireguline may promote tumor growth and survival by an autocrine loop.[16] Several studies have correlated the expression of these ligands with sensitivity to cetuximab monotherapy. The results showed a statistically longer progression free survival (PFS) among patients with high expression of epiregulin. The exclusive use of amphiregulin or epiregulin gene expression profile does not, however, result in the selection of patient populations benefiting from cetuximab treatment.[17]

In order to increase the power of patient selection for anti-EGFR therapy, several studies involving the identification of alternative predictive molecular biomarkers have been conducted.

Biomarkers downstream to EGFR

EGFR-mediated signaling involves two main intracellular cascades: KRAS, which, activating BRAF, triggers in its turn the mitogen-activated protein kinases (MAPKs) and the membrane localization of the lipid kinase PIK3CA, which counteracts with PTEN and promotes AKT1 phosphorylation, thereby activating a parallel intracellular axis.[18]

KRAS is a proto-oncogene encoding a small 21kD guanosine triphosphate (GTP)/guanosine diphosphate (GDP) binding protein involved in the regulation of cellular response to many extracellular stimuli.[19] After binding and activation by GTP, RAS recruits the oncogene RAF, which phosphorylates MAP2K (mitogen-activated protein kinase kinase), initiating the MAPK signaling leading to the expression of the protein involved in cell proliferation, differentiation and survival.[20] PIK3CA is an oncogene encoding for the p110 subunit of PI3K, which can be activated via interaction by the RAS protein.[21]

The constitutive activation of signaling pathways downstream of the EGFR by mutations in *KRAS*, *BRAF* and *PI3KCA* and the interaction between these pathways, drive the growth and progression of CRC and provide an escape mechanism which allows the tumors to overcome the pharmacological blockade induced by anti-EGFR molecules.[22]

KRAS is the mostly commonly mutated gene in this pathway. It is mutated in 35–45% of colorectal adenocarcinomas and this alteration is an early event in colon tumorigenesis.[23] Up to 90% of activating *KRAS* gene mutations are detected in codons 12 (70%) and 13 (30%), and less in codon 61. Frequently these mutations result in an exchange of different amino acids at the catalytic sites which induce the glicine-to-valine substitution associated with a more aggressive tumor growth.[24]

Negative predictive role of *KRAS* gene mutations

KRAS mutations have emerged as a major predictor of resistance to panitumumab or cetuximab in the clinical setting and several studies of patients receiving first and subsequent lines of treatment have shown that those with tumors carrying *KRAS* mutations do not respond to EGFR-targeted monoclonal antibodies or show any survival benefit from such treatments.

Several studies have been conducted in order to explore the role of *KRAS* mutations as a predictive biomarker of tumors from patients with metastatic CRC treated with anti-EGFR monoclonal antibody (with or without chemotherapy).[25]

The first study evaluating the correlation between *K-RAS* mutational status in primary tumors and absence of response to treatment with cetuximab or panitumumab, was that of *Lievre et al.*, which involved a cohort study of 30 patients, and reported a link between *KRAS* mutations and lack of response of metastatic patients to EGFR-targeted monoclonal antibodies.

In this study *K-RAS* mutations were observed in 13 of the 30 patients enrolled in the study and these mutations were closely associated with response to treatment; none of the mutated tumors responded to cetuximab. Among responders none (0/11) presented *KRAS* mutations, while a mutational status of *KRAS* was found in 68.4% (13/19) of patients who were non-responders ($p = 0.0003$). The overall survival of *K-RAS* wild type patients (WT) was significantly higher compared with those with mutated *KRAS* (median OS: 16.3 vs 6.9 months, $p = 0.016$).[26]

Amado et al., confirmed the negative predictive value of *KRAS* mutations in a randomized phase III study, comparing the effect of panitumumab monotherapy with best supportive care (BSC) in patients with chemotherapy-refractory metastatic CRC.

The treatment effect on PFS in the WT *KRAS* group was significantly greater (P 0.0001) than in the mutant group. Median PFS in the WT *KRAS* group was 12.3 weeks for panitumumab and 7.3 weeks for BSC. The Authors therefore concluded their study confining the panitumumab monotherapy efficacy in metastatic CRC to patients with WT *KRAS* tumors.[27] In a randomized study of 572 patients, *Karapetis et al.* examined the role of *KRAS* mutations in treatment response, comparing the effect of cetuximab monotherapy with best supportive care (BSC) in patients with chemo-refractory metastatic CRC.

VARIABLES	Disease-Free Survival			Overall Survival		
	HR	95% CL	P	HR	95% CL	P
Ki-RAS mut. codon 12						
Valine	**1.30**	**1.09-1.54**	**< 0.01**	**1.29**	**1.08-1.55**	**< 0.01**
Aspartate	**1.04**	**0.89-1.22**	**NS**	**0.94**	**0.79-1.11**	**NS**
Cysteine	**1.11**	**0.85-1.46**	**NS**	**1.26**	**0.93-1.62**	**NS**
Serine	**1.42**	**1.04-1.93**	**NS**	**1.20**	**0.86-1.70**	**NS**
Alanine	**1.21**	**0.89-1.66**	**NS**	**1.35**	**0.98-1.87**	**NS**
K-RAS mut. codon 13						
Aspartate	**0.94**	**0.79-1.12**	**NS**	**0.93**	**0.78-1.12**	**NS**

Fig. 1. The RASCAL study: results of the multivariate analysis.

Cetuximab treatment in WT *KRAS* patients compared to BSC resulted in a significant increase of the OS (median OS: 9.5 vs 4.8 months; HR = 0.55, 95% CI: 0.41–0.74, P < 0.001) and PFS (median PFS: 3.7 vs 1.9 months; HR = 0.40, 95% CI: 0:30 to 0:54, p < 0.001) while among *KRAS* mutated patients the differences were not significant in either PFS or OS (p = 0.96 p = 0.89, respectively).[28]

Reports regarding *KRAS* data from large randomized trials have recently been published, including the first-line phase II study OPUS (Oxaliplatin and Cetuximab in First-Line Treatment of metastatic CRC) and the first-line phase III study CRYSTAL (Cetuximab Combined With Irinotecan in First-Line Therapy for Metastatic Colorectal Cancer).[29] These results show that the *KRAS* mutated patients do not benefit from addition of cetuximab to conventional chemotherapy. Both PFS and OS were similar for cetuximab and control groups in patients carrying tumors with *KRAS* mutations (progression-free interval = 1.8 vs 1.8 months [HR = 0.99, 95% CI = 0.73 to 1.35, *P* = 0.96]; overall survival = 4.6 vs 4.5 months [HR = 0.98, 95% CI = 0.70 to 1.37, *P* = 0.89]). In the wild-type *KRAS* patients, however, cetuximab treatment was associated with statistically significantly (*P* < 0.001) longer survival than control treatment (progression-free interval = 3.7 vs 1.9 months [HR = 0.40, 95% CI = 0.30 to 0.54]; overall survival = 9.5 vs 4.8 months [HR = 0.55, 95% CI = 0.41 to 0.74, *P* < 0.001].[29,30]

Retrospective data from the OPUS and CRYSTAL studies indicate that from the addition of cetuximab to first-line FOLFOX (folinic acid, fluorouracil, and oxaliplatin)[30] or FOLFIRI (folinic acid, fluorouracil and irinotecan)[29] chemotherapy does not benefit patients with *KRAS* mutations. In fact, the OPUS study indicates that addition of EGFR-targeted treatment to chemotherapy may even be detrimental in such patients.[30] In some cases the addition of cetuximab or panitumumab to standard chemotherapy may be not useful even in KRAS wt patients.

In the PACCE (Panitumumab Advanced Colorectal Cancer Evaluation) study, the addition of panitumumab to bevacizumab and chemotherapy was associated with shortening of the progression free interval among patients with tumors carrying WT *KRAS* (11.5 months in the chemotherapy–bevacizumab arm vs 9.8 months in the panitumumab–chemotherapy–bevacizumab arm).[31]

In the CAIRO-2 (CApecitabine, IRinotecan, and Oxaliplatin trial) study, the addition of cetuximab to capecitabine, oxaliplatin, and bevacizumab as first-line treatment in patients with metastatic CRC had no effect on progression-free interval among those with tumors carrying WT *KRAS* (10.6 months in the chemotherapy–bevacizumab arm vs 10.5 months in the combined cetuximab arm).[32]

In the large COIN trial the addition of cetuximab to Oxaliplatin-based CT did not improve OS or PFS with increased nonhematological toxicity in KRAS wt patients, even if the very advanced disease in the COIN population may be the reason for the negative results..[33]

Prognostic role of *KRAS* gene mutations

The collaborative RASCAL II studies have been conducted with the aim of investigating the prognostic role of *KRAS* mutations in CRC progression.

To explore the effect of *KRAS* mutations at different stages of CRC, 3493 patients were recruited in this multivariate analysis. The results obtained suggest that of the 12 possible mutations on codons 12 and 13 of *KRAS*, only the substitution glycine to valine on codon 12, found in 8.6% of all patients, had a statistically significant impact on PFS (P = 0.0004, HR 1.3) and OS (P = 0.008, HR 1.29) (Fig. 1). Furthermore, these mutations have a greater impact on outcome in Dukes'C cancers (failure-free survival, P = 0.008, HR 1.5; OS P = 0.02 HR 1.45) than in Dukes'B cancers (failure-free survival, P = 0.46, HR 1.12; OS P = 0.36 HR 1.15).

The RASCAL studies therefore show that tumors carrying mutated *KRAS* might have an effect on the survival rate of CRC patients, and that the specific codon 12 glycine/valine mutation not only might play a role in the tumor progression, but this alteration might also predispose to more aggressive biological behavior in patients with advanced CRC.[23]

Not all identified mutations of *KRAS*, however, necessarily have the same biological, biochemical and functional role.[34]

The prognostic role of *KRAS* mutations in CRC progression is still controversial. In a recent work *Roth el al.* in accordance with data from smaller retrospective studies (PETACC-3, EORTC 40993, SAKK 60-00) sustain the lack of prognostic value for *KRAS* mutation status for PFS and OS in patients with CRC. The prognostic significance of *KRAS* mutations observed in the multivariate analysis of the RASCAL studies might have been overestimated as a result of the number of subset analyses. Larger studies are therefore required in order to confirm whether a specific *KRAS* mutation might lead to a clinically relevant prognostic effect in patients with CRC.[35]

Predictive and prognostic role of *BRAF* gene mutations

The most frequent *BRAF* mutation observed is a DNA missense mutation leading to a valine to glutamic acid amino acid substitution (V600E). It is functionally the most important mutation involved in the receptor-independent aberrant activation of the MEK-ERK pathway and CRC carcinogenesis. Mutations of BRAF, kinase located downstream of K-RAS in the EGFR signal transduction pathway, are found in colorectal tumors with a relatively low frequency (approximately 10%). These alterations have been studied in recent years in order to reach a better understanding of its possible role in predicting response to anti-EGFR drugs. Assuming that *BRAF* mutations may have, in *K-RAS* wild type tumors, a predictive/prognostic role, Di Nicolantonio et al. have retrospectively analyzed RR, TTP (time to progression), OS and mutational status of *K-RAS* and *BRAF* in 113 patients

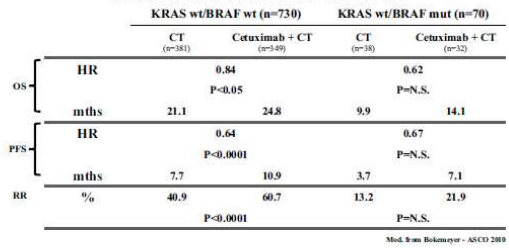

Fig. 2. B-RAF mutational status in K-RAS Wt patients: pooled analysis of CRYSTAL and OPUS studies.

with mCRC treated with cetuximab or panitumumab. The *BRAF* mutation V600E was the only one present in the cohort of analyzed patients. Its presence appears inversely related to the activity of the treatment, especially since some of the mutated patients (11 out of 79 *K-RAS* w.t.) responded to the drug administration and conversely none of the responders presented the mutation in question ($p = 0.029$). In addition, *BRAF*-mutated patients compared with w.t obtained a significantly shorter PFS ($p = 0.011$) and OS ($p < 0.0001$). The authors concluded by supporting the need to have *BRAF* w.t. in order to obtain response from treatment with EGFR inhibitors.[36] Furthermore, in *BRAF*-mutated colorectal cancer cell lines, the sensitivity to EGFR inhibitors may be restored by means of the multikinase inhibitor sorafenib. Several studies have confirmed the negative prognostic role of *BRAF* mutations. In *K-RAS* wild-type patients, *BRAF*-mutated individuals have shown a worse outcome in terms of PFS and OS. Furthermore, *BRAF* is prognostic for OS, especially in patients with microsatellite instability (MSI) low (MSI-L) and stable (MSI-S) tumors. In the MSI-H (high) subpopulation no prognostic value of *K-RAS* and *BRAF* mutation status was found for RFS and OS.[35] The relatively low frequency of this genetic alteration in colorectal cancer makes it rather difficult to draw absolute conclusions also based on *post-hoc* analysis of the Phase II and III studies recently published. The retrospective analysis of the B-RAF mutational status has been performed in pts in the CRYSTAL and OPUS studies (Fig. 2). The analysis of the OS and PFS in pts with wt KRAS/wt BRAF showed a significant difference between the two treatment arms. This difference was not significant in the mutated BRAF/KRAS wt patients. Nevertheless, these patients seem to benefit from the addition of Cetuximab, with an increase of OS and a doubling of PFS rates. Furthermore, there is clearly a worse outcome in mutated BRAF patients independently of treatment with Cetuximab, which supports the hypothesis of a possible negative prognostic role of BRAF mutations.[37] In the CAIRO-2 study, a similar pattern was observed in a large series of mCRC patients treated with chemotherapy and Bevacizumab with or without Cetuximab. It was seen that the BRAF mutation is associated with a worse outcome, both in terms of PFS and of OS, independently of the addition of Cetuximab to the treatment..[38] To date, therefore, the negative value of mutations of *BRAF* is only suggested by some reports,[39] while the significant negative prognostic value seems to be now established.[40]

PTEN-PI3K-AKT-mTOR pathway alterations

In addition to KRAS and BRAF, the HER family of receptors also activates the PI3K signaling pathway, which in turn can be oncogenically deregulated either by activating mutations in the PIK3CA p110 subunit or by inactivation of the PTEN phosphatase. The role of deregulated PIK3CA/PTEN signaling on the response to targeted therapy has therefore been investigated in breast cancer,[41] glioblastoma[42] and also mCRC. Mutation constitutive activation of the PI3K signaling pathway has been reported to occur in ~30% of colon tumors, primarily due to activating mutations in exons 9 and 20 of the *PIK3CA* gene[43,44] and, to a lesser extent, due to inactivating *PTEN* mutations or *PTEN* promoter methylation.[45] PTEN is a tumor suppressor that acts as a negative regulator of PI3K signaling by converting PIP3 to PIP2, and truncating mutations which result in loss of *PTEN* expression, reported in ~20% of MSI colon cancers.[46–51]

The molecular alteration of PTEN is often caused by epigenetic mechanisms,[45] supporting the detection of the intact protein by IHC as a better diagnostic tool than gene sequencing, as it potentially covers more mechanisms of alteration. *PIK3CA* mutation and *PTEN* expression status predicts response of colon cancer cells to the EGFR inhibitor cetuximab distinguishing drug sensitive and resistant cell lines. Colon cancer cell lines with activating *PIK3CA* mutations or loss of *PTEN* expression (*PTEN* null) were more resistant to cetuximab than *PIK3CA* wild type (WT)/*PTEN* expressing cell lines. Furthermore, cell lines that were *PIK3CA* mutant/*PTEN* null and Ras/BRAF mutant were highly resistant to cetuximab compared with those without dual mutations/*PTEN* loss, indicating that constitutive and simultaneous activation of the Ras and PIK3CA pathways confer maximal resistance to this agent. On the other hand, these patients may be suitable candidates for treatment with newer targeted drugs currently involved in clinical trials, which inhibit signaling mediators further downstream, including PI3K, AKT, or mTOR inhibitors and Ras, Raf, or MEK inhibitors. A possible mechanism of resistance to cetuximab of these cell lines may be the existence of alternate mutations in the Ras/BRAF and or PIK3CA/PTEN pathway. Increased sensitivity to cetuximab was observed in PIK3CA WT lines. Likewise, breast cancers with either activating mutations in PIK3CA or with loss of PTEN expression respond poorly to treatment with the Her2/Neu targeting antibody, trastuzumab.[52] Consistent with the present findings, Frattini et al. recently reported that colon tumors with loss of PTEN expression have significantly reduced response to cetuximab.[48] Furthermore, PIK3CA mutations and PTEN loss in colorectal tumors are statistically and significantly associated with lack of response to panitumumab or cetuximab treatment.[18] PIK3CA mutations and/or loss of PTEN expression are negatively associated with PFS, and loss of PTEN expression is also linked with poorer OS. *A priori* screening of colon tumors for PTEN expression status and PIK3CA and Ras/BRAF mutation status could help stratify patients likely to benefit from this therapy.[53] Razis et al.[54] reported that normal PTEN protein expression was associated with a higher response rate and longer time to progression in patients treated with cetuximab-based therapy, despite a 50% response rate observed in patients who had lost PTEN protein expression. Loupakis et al. performed a retrospective analysis on the status of PTEN in a cohort of

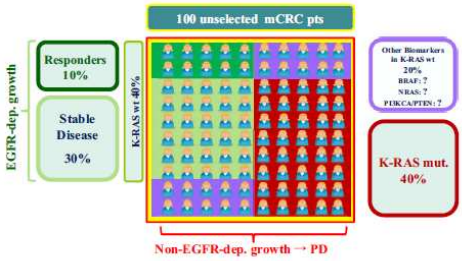

Fig. 3. Activity of anti EGFR-Ab monotherapy in chemorefractory mCRC patients.

55 metastases from patients with irinotecan refractatory mCRC treated with irinotecan and cetuximab: 12 (36%) of 33 patients with PTEN-positive metastases were responders compared with one (5%) of 22 who had PTEN-negative metastases.[50] Patients with PTEN-positive metastases and KRAS wild type had longer PFS compared with other patients.

Collectively, these findings show that colon cancer cell lines with constitutively active PI3K signaling are refractory to cetuximab. These data imply that colon cancer cell lines which acquire mutations that result in constitutive activation of the PI3K pathway have a diminished dependence on canonical EGFR ligand-induced signaling for their growth and are, therefore, more resistant to EGFR targeted therapies. Collectively, these studies provide additional clinical evidence that the mutation status of the PI3K signaling pathway should be considered before treatment with EGFR family antagonists. Colon cancer cell lines mutant for PIK3CA/PTEN null are significantly more resistant to cetuximab compared with PIK3CA/PTEN WT lines. Furthermore, cell lines with both constitutively active PIK3CA and Ras/BRAF signaling were highly refractory to cetuximab. These studies suggest that combining mutation analysis for K-RAS and PIK3CA (loss of PTEN and/or PIK3CA mutation) could identify up to 70% of patients with metastatic colorectal cancer who are unlikely to respond to treatment with an EGFR-targeted monoclonal antibody.[18]

Conclusions

EGFR and its downstream K-RAS/B-RAF and PTEN-PI3K-AKT-mTOR pathways play an important role in tumorigenesis and tumor progression of CRC.

Only in a fraction of patients with mCRC (10% according to RECIST criteria that are not adequate for the evaluation of tumor response to new molecular agents) anti-EGFR mAbs (cetuximab and panitumumab) have shown remarkable efficacy (Fig. 3). Forty percent of patients with mCRC obtain clinical benefit from monotherapy with anti-EGFR antibodies, which means that they present EGFR-dependent tumoral growth. The remaining 60% of the patients are Non Responders, that is, they present tumoral growth which does not depend on the EGFR block mediated by the anti-EGFR Abs. This limitation seems linked mainly to oncogenic KRAS mutations in codon 12 and 13, which implies its continuous activation and signal transduction to the nucleus, even when the EGFR is blocked. KRAS mutation seems to be responsible in 35–45% cases of resistance to anti-EGFR Abs. The potential bias associated with a retrospective evaluation of the mutational status of KRAS in the CRYSTAL and OPUS studies seems not relevant today, since in both studies the difference between the ITT population and the population assessable for KRAS is negligible.

Another group of patients, representing 10% of the total number of patients, carry the BRAF mutation that is mutually exclusive with *KRAS* mutations. It therefore appears that BRAF mutations may play a strong negative prognostic role and only a slight role in resistance to anti-EGFR Abs, since even pts with mCRCs treated without Cetuximab have significantly reduced PFS and OS when the tumor presents a BRAF mutation. The investigation of other biomarkers such as EGFR copy number and expression levels of EGFR ligands, phosphatase and tensin homolog (PTEN) loss or *NRAS* mutation may be useful to further refine the responder population. Nevertheless, up till now, clinical evidence all points towards the identification of the KRAS mutation as the only evaluated and reproducible predictive factor of resistance to anti-EGFR antibodies.

Conflict of interests

All authors have no conflict of interest to declare.

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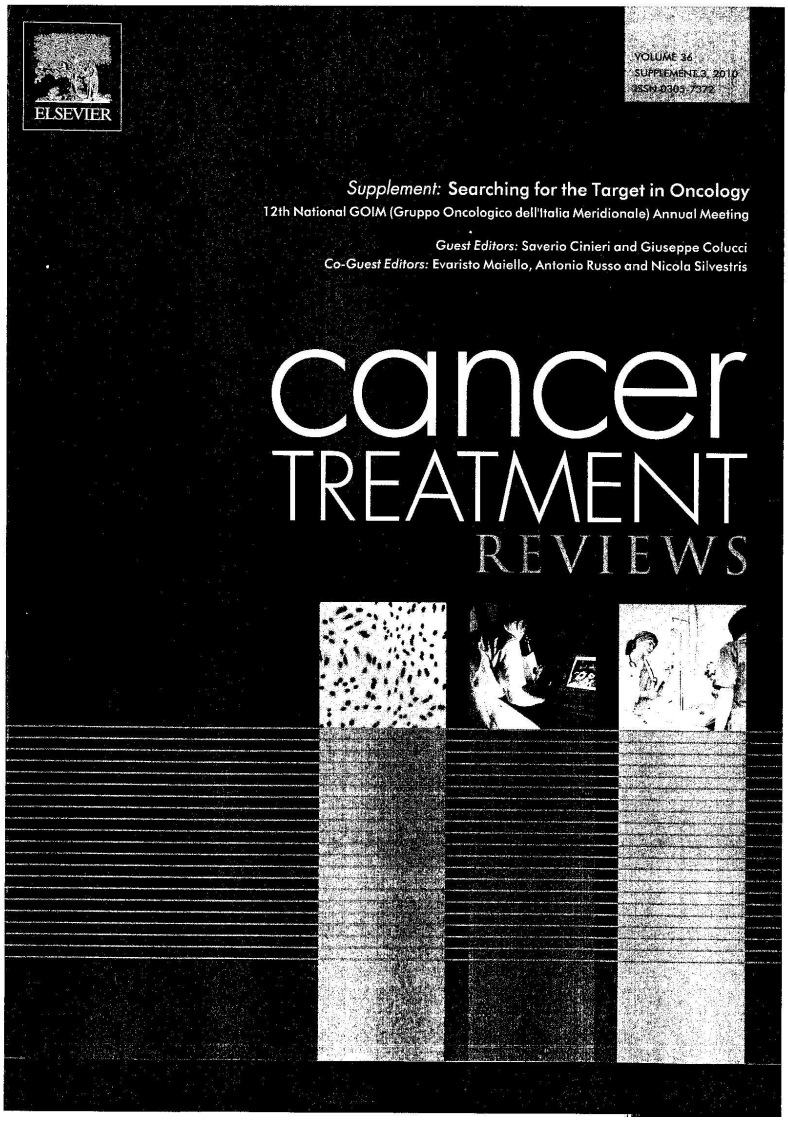
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of note, OS was significantly longer in pts with higher expression of IGF1R if compared with those with normal/lower expression (17 vs. 8 months; p = 0.011).
Conclusions: As known, *KRAS/BRAF* mutations are the strongest negative predictive marker 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.

TSP-1 mRNA and cytosolic and secreted protein. Finally, we did not find any variation of TSP-1 level in cells transfected with let-7i. Results were confirmed by transfection with anti-mir21, anti-mir182 and anti-let7i and, using the same method, we evaluated TSP-1 expression.
Conclusions: Data suggest that mir-182 induces degradation of TSP-1 mRNA in HT29 cell line, whereas mir-21 affects probably by blockage of TSP-1 translation. Let-7i does not seem involved in regulation of TSP-1 expression in HT29 cells. Understanding the molecular mechanism by which miRNAs regulate TSP-1 expression could be used to restore TSP-1 expression to contrast angiogenic events in colon cancer.

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AZD1152 PLUS GEMCITABINE FOR PANCREAS CANCER TREATMENT: *IN VITRO* AND *IN VIVO* STUDY

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Background: AZD1152 is a prodrug that, after activation in AZD1152-HQPA, impairs cytokinesis by inhibition of the activity of its specific target Aurora B kinase. Aurora B kinase is known to be involved to determining the correct chromosome alignment, kinetochore-microtubule biorientation, and activation of the spindle assembly checkpoint. In this report, we verify the possibility of combine this novel drug with gemcitabine widely used in chemotherapy for pancreas cancer patients.
Methods: Pancreatic (MiaPaCa-2) cancer cells were used and the capability of the drug to enhance gemcitabine effectiveness has been evaluated as cell growth inhibition, apoptosis induction and cell cycle perturbation.
Results: Our results showed that AZD1152-HQPA strongly modifies cell structure and activity, with an increase in cell size, in polyploidia and chromosome numbers. Its activity was through the inhibition of Histone 3 phosphorylation even if it also seemed to modulate other signal transduction pathways, such as survival one with the implication of p53.
Kinetic experiments evidenced that AZD1152-HQPA was an enhancer of gemcitabine effectiveness in MiaPaCA-2 cells and the best schedule was that in which our aurora kinase B inhibitor was given before the chemotherapeutic drug, with a gain of about 20–30% of efficacy.
Then, the promising *in vitro* combination of AZD1152 with gemcitabine has been tested *in vivo* with MiaPaCa-2 xenografts in CD *nu/nu* male mice. At the appearance of a measurable subcutaneous tumor (~100 mm^3), mice were grouped randomly and treated as follows: *i)* control (vehicle alone), *ii)* AZD1152 alone (25 mg/kg daily for four days), *iii)* gemcitabine alone (120 mg/kg four times at 3-day intervals) and *iv)* the sequential combination of AZD1152 and gemcitabine. AZD1152 and gemcitabine alone significantly inhibit tumour growth in absence of toxicity. When mice were treated sequentially with the two compounds, the tumor growth was delayed and the inhibition of both tumor volumes and weights was markedly enhanced.
Conclusions: In conclusion, our results suggest that AZD1152, a novel selective inhibitor of Aurora kinase B, could be a promising therapeutic approach in combination with gemcitabine in pancreas cancer treatment.
AZD1152 and AZD1152-HQPA are trademarks of the AstraZeneca group of companies.

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DNA DOUBLE STRANDS BREAK REPAIR GENES EXPRESSION ANALYSIS REVEAL RAD51 AS A NEW POTENTIAL BIOMARKER IN BREAST CANCER.

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Background: We determined expression for genes that play key roles as sensors, modulators or effectors in this pathway. We analyzed Mrna expression of 15 DSB related genes from 20 breast cancers in order to classify them into homogeneous clusters. For genes *ATR*, *G22P1/ku70* and *RAD51* was developed a mRNA relative quantification method that was used to analyze additional 55 cases.
Methods: *RAD51* protein expression was determined by immunohistochemestry on 58 tumours represented on a commercial available tissue microarray. Hierarchical clustering analysis of the DSB repair genes analyzed identified *ATR*, *G22P1/ku70* and *RAD51* as differentially expressed among the breast cancer cases.
Results: The analysis of the additional 55 tumours for these three genes indicate an association between *RAD51* increased mRNA levels and ER-positive/PR-negative breast cancers ($P = 0.09$). This result was confirmed at protein expression level when a tissue microarray including 58 breast cancers was analyzed by immunohistochemestry ($P = 0.003$).
Conclusions: Our results indicate that the *RAD51* gene is differentially expressed in breast cancer characterized by different steroid hormone receptor status and may represent a novel potential breast cancer biomarker.

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DETECTION OF KRAS MUTATIONS IN COLORECTAL CARCINOMA PATIENTS WITH AN INTEGRATED PCR/SEQUENCING AND REAL TIME PCR APPROACH

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Background: Patients with metastatic colorectal carcinoma (mCRC) carrying activating mutations of the *KRAS* gene do not benefit of treatment with anti-epidermal growth factor receptor (EGFR) monoclonal antibodies. Therefore, *KRAS* mutation testing of mCRC patients is mandatory in the clinical setting for the choice of appropriate therapy.
Methods: We developed a cost/effective approach for the determination of *KRAS* mutations in codons 12 and 13 in clinical practice based on a sensitive PCR/sequencing technique and the commercially available Real-Time PCR-based Therascreen kit (DxS).
Results: The PCR/Sequencing test was able to detect 10% mutant DNA in a background of wild-type DNA. By using this assay, we determined the mutational status of *KRAS* in 527/540 (97.6%) formalin-fixed paraffin-embedded (FFPE) tissues from mCRC patients. PCR/sequencing was not conclusive in 13 cases in which low-intensity peaks suggestive of potential mutations were identified. DxS, which showed a sensitivity of 1%, identified mutations in 11/13 inconclusive cases. Interestingly, 5 of these 11 cases showed high levels of DNA fragmentation. No significant difference was found in the ability of PCR/sequencing and DxS to identify *KRAS* mutations within 160 cases with >30% tumor cells. However, in 24 samples with ≤30% tumor cells DxS showed an higher sensitivity.
Conclusion In conclusion, our findings suggest that PCR/sequencing can be used for mutational analysis of the majority of tumor samples that have >30% tumor cell content, whereas more sensitive and expensive tests should be reserved for inconclusive cases and for samples with a low amount of tumor cells.

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HYPOXIA INDUCES DECREASED EXPRESSION OF *BRCA2* IN BREAST CANCER CELL LINES

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Background: The hypoxic tumor microenvironment is a key factor that induces genetic instability. Several studies have demonstrated

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.cgi?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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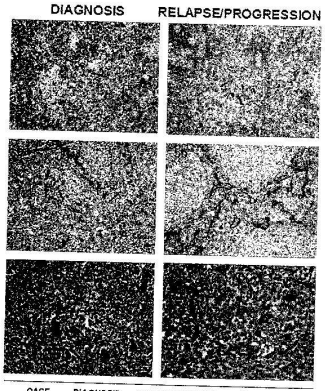
Background: Germline mutations in *BRCA1/2* genes confer a high risk to breast and/or ovarian cancer. Only mutations causing frameshifts and premature stop codons are generally assumed to be pathogenic. About 8% of all *BRCA1* and *BRCA2* alterations reported to the BIC database are intronic variants 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

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

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 o 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.

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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/m² d1,2; 7,8; FM *iv* 100 mg/m² 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	3,8,28	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-mir21 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

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.

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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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