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Epigenetic changes and nuclear factor-κB activation, but not microRNA-224, downregulate Raf-1 kinase inhibitor protein in triple-negative breast cancer SUM 159 cells

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Abstract. Raf-1 kinase inhibitor protein (RKIP) is a tumor suppressor and metastasis inhibitor, which enhances drug-induced apoptosis of cancer cells. Downregulation of RKIP may be significant in the biology of highly aggressive and drug-resistant tumors, for example triple-negative breast cancers (TNBCs). Potential causes for the low levels of RKIP expressed by SUM 159 TNBC cells were investigated in the present study. Bisulphite modification, methylation specific-polymerase chain reaction (PCR) and a TransAM NF-κB assay were performed and the results suggested that various mechanisms, including methylation of the gene promoter, histone deacetylation and nuclear factor-κB (NF-κB) activation, but not targeting by microRNA-224 (miR/miRNA-224), as determined by transfection of pre-miR-224 miRNA precursor or anti-miR-224 miRNA inhibitor, may downregulate RKIP in these cells. Furthermore, reverse transcription-quantitative PCR, western blotting, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium cell growth assay and flow cytometry revealed that in SUM 159 cells, the demethylating agent 5-aza-2'-deoxycytidine (5-AZA), the histone deacetylase inhibitor trichostatin A (TSA) and the NF-κB inhibitor dehydroxymethylepoxyquinomicin (DHMEQ) enhanced RKIP expression and resulted in significant cell growth inhibition and induction of apoptosis. 5-AZA and TSA mainly produced additive antitumor effects, while the combination of DHMEQ and TSA exhibited significant synergy in cell growth inhibition and induction of apoptosis assays. Increasing evidence that aberrant activation of NF-κB signaling is a frequent characteristic of TNBC highlights the fact that this transcription factor may be a useful target for treatment of such tumors. In addition to DHMEQ, proteasome inhibitors may also represent valuable therapeutic resources in this context. Notably, proteasome inhibitors, in addition to the inhibition of NF-κB activation, may also restore RKIP levels by inhibiting proteasome degradation of the ubiquitinated protein. The current results contribute to the understanding of the molecular mechanisms of RKIP downregulation in TNBC and suggest possible novel therapeutic approaches for the treatment of these types of cancer.

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Key words: triple-negative breast cancer, Raf-1 kinase inhibitor protein, epigenetic changes, microRNA-224, nuclear factor-κB

Introduction

A major challenge in the field of breast cancer is the identification and exploitation of useful therapeutic targets for the most aggressive forms. Among these, the triple-negative breast cancers (TNBCs), characterized by a lack of estrogen, progesterone and human epidermal growth factor receptor-2 (HER2) receptors, are a highly heterogeneous group of tumors which account for ~15% of all breast cancers. TNBCs are often associated with epithelial-mesenchymal transition and a high propensity for early metastasis (1). To date, no molecularly-targeted therapeutic agents are clinically available for TNBCs, therefore these tumors, which are frequently resistant to cytotoxic chemotherapy, remain challenging to treat. Nevertheless, progress is being made in the subtyping of TNBCs following the identification of molecular alterations, which current therapeutic efforts are focused towards (2).

With regard to the identification of molecular alterations in TNBCs, various studies have indicated the potential importance of the dysregulation of the Raf-1 kinase inhibitor protein (RKIP) signaling pathway in breast cancer metastasis and the biology of TNBCs (3-5). RKIP inhibits the Raf-1/mitogen-activated protein kinase kinase interaction and thus the oncogenic activities of the mitogen activated protein kinase pathway (6). RKIP also affects the activation of nuclear factor-κB (NF-κB) and signal transducer and activator of transcription-3 (7,8), antagonizing their pro-oncogenic effects. In its phosphorylated state, RKIP downregulates numerous G protein-coupled

receptors and may also be involved in regulation of the partitioning of chromosomes and progression through mitosis (9,10). Overall, RKIP is a tumor and metastasis suppressor, which is downregulated in numerous types of cancer, including breast cancer, and is correlated with a more severe prognosis (11). Furthermore, RKIP is able to promote drug-induced apoptosis in cancer cells (12), underscoring the need for novel approaches for the rescue of RKIP expression levels in neoplastic tissues.

Unfortunately, the causes underlying RKIP downregulation in these tumors remain to be elucidated. In the case of breast cancer, the reduction in RKIP has been associated with transcriptional repression by the NF-κB/Snail pathway (13,14) or the polycomb protein enhancer of zeste homolog 2 (15), as well as silencing by microRNA-224 (miR-224) (16).

The present study aimed to examine the potential relevance of various mechanisms underlying the low levels of RKIP exhibited by the SUM 159 TNBC cell line.

Materials and methods

Reagents. The demethylating agent 5-aza-2'-deoxycytidine (5-AZA) and the histone deacetylase (HDAC) inhibitor trichostatin A (TSA) were purchased from Sigma-Aldrich Srl, Milan, Italy. The NF-κB inhibitor dehydroxymethylepoxyquinomicin (DHMEQ) was provided by Professor Kazuo Umezawa (Department of Applied Chemistry, Faculty of Science and Technology, Keio University, Yokohama, Japan).

Cell cultures. The BT 549, MCF 7, MCF 7R and MDA MB 231 cell lines were cultured in RPMI-1640, the MDA MB 468 cell line was cultured in Dulbecco's modified Eagle's medium (DMEM), the SUM 159 and SUM 149 cell lines were cultured in DMEM/F-12 supplemented with insulin (5 μg/ml), and the MCF 10A cell line was cultured in DMEM/F-12 supplemented with insulin (0.01 mg/ml), epidermal growth factor (20 ng/ml) and hydrocortisone (500 ng/ml). All media were supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (all reagents were from EuroClone S.p.A., Milan, Italy; GE Healthcare Life Sciences, Logan, UT, USA). The cells were cultured in a humidified atmosphere at 37˚C in 5% CO_2. After obtaining the cells, the first passage carried out was assigned passage number 1. Cells with a narrow range of passage number (4-6) that were routinely tested for Mycoplasma contamination were used for all experiments. The breast carcinoma MCF-7 line was purchased from the American Type Culture Collection (Manassas, VA, USA) and its multi-drug resistant variant MCF-7R was established by treating the wild-type MCF-7 cells with gradually increasing concentrations of doxorubicin. Non-malignant MCF 10A breast epithelial cells were provided by Dr. Agata Giallongo (Institute of Biomedicine and Molecular Immunology, National Research Council, Palermo, Italy) and TNBC cell lines were provided by Dr. Elda Tagliabue (Molecular Targeting Unit, Department of Experimental Oncology and Molecular Medicine, Fondazione Institute of Hospitalization and Scientific Care, National Cancer Institute, Milan, Italy).

Cell growth assays. The cells were seeded at $2x10^4$ cells/well onto 96-well plates and incubated overnight at 37˚C. At time 0, the medium was replaced with fresh complete medium supplemented with 5-AZA, TSA, DHMEQ or combinations of AZA + TSA or DHMEQ + TSA at the indicated concentrations. Following 72 h of treatment, 15 μl commercial solution obtained from Promega Corp. (Madison, WI, USA) containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) and phenazine ethosulfate was added. The plates were incubated in a humidified atmosphere at 37˚C in 5% CO_2 for 2 h, and the bioreduction of MTS dye was evaluated by measuring the absorbance of each well at 490 nm using a microplate absorbance reader (iMark Microplate Reader; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cell growth inhibition was expressed as a percentage of the absorbance of the control cells. For the combinations, to evaluate the type of interaction between the agents, dose-effect curves were analyzed according to the Chou and Talalay method (17) using CalcuSyn® software (version 2.1; Biosoft, Cambridge, UK) as a non-constant ratio combination. The combination index (CI) indicates a quantitative measure of the degree of drug interaction in terms of synergistic (CI<1), additive (CI=1) or antagonistic (CI>1) effects.

Evaluation of cell death by flow cytometry. SUM 159 cells were treated with the agents alone or in combination for 48 h. Subsequently, the cells were washed twice with ice-cold phosphate-buffered saline (PBS; EuroClone S.p.A.) and then suspended at a density of $1x10^6$ cells/ml in a hypotonic fluorochrome solution containing 50 μg/ml propidium iodide in 0.1% sodium citrate with 0.03% (v/v) Nonidet P-40 (Sigma-Aldrich Srl). Following incubation in this solution for 1 h, the samples were filtered through a 40-μm mesh nylon cloth, and fluorescence was evaluated as single-parameter frequency histograms using a FACSort instrument (BD Biosciences, San Jose, CA, USA). The data were analyzed using CellQuest™ software (version 3.1; BD Biosciences). Cell death was measured by determination of the percentage of events accumulated in the pre-G0-G1 position.

DNA extraction and bisulphite modification. Ten breast cancer samples were obtained from the Anatomic Pathology Unit, University Hospital 'Paolo Giaccone' (Palermo, Italy) between 2013 and 2014. This study was approved by the ethics commitee of the University of Palermo (Palermo, Italy) and written informed consent was obtained from all patients. Two 8-μm sections microdissected from each of the ten paraffin-embedded samples. Cell lysis and DNA extraction were performed on the ten samples, as well as SUM 159 cells, using a QIAamp DNA mini kit (Qiagen GmbH, Hilden Germany) according to the manufacturer's protocol. Extracted genomic DNA was diluted in 50 μl distilled water. Sodium bisulphite conversion was performed using the EpiTect Plus Bisulfite Conversion kit (Qiagen GmbH) using 500 ng DNA, according to the manufacturer's protocol.

Immunohistochemical analysis. Evaluation of the positivity for Ki-67, ER and PR, HER2 and RKIP protein was performed by immunohistochemistry, as previously described (18,19). Briefly, tissue samples were fixed in 10% buffered formalin and paraffin-embedded. Tissue sections (4 μm) were then deparaffinized and rehydrated. The sections were pretreated with Tris/EDTA buffer (pH 9.0; Novocastra Reagents, Milton

Table I. RKIP and miR-224 expression levels in various breast cancer cell lines.

Cell line	RKIP protein levels	RKIP mRNA levels	miR-224 levels
MDA 231	0.717	0.500	0.815
MDA 468	0.980	0.768	0.013
BT 549	0.686	0.136	0.101
SUM 149	1.131	0.866	3.964
SUM 159	0.559	0.402	0.923
MCF 10A	1.063	1.000	1.000
MCF 7	1.667	1.130	0.001
MCF 7R	1.069	0.390	0.000

MCF 10A cell line was used as a control reference for the evaluation of RKIP mRNA and miR-224 expression levels by quantitative polymerase chain reaction. For the evaluation of RKIP protein expression, immunoblots were quantified by densitometry and Pearson's correlation coefficients were determined. Results were then expressed as arbitrary units (RKIP/β-actin). RKIP, Raf-1 kinase inhibitor protein; miR-224, microRNA-224; mRNA, messenger RNA.

Keynes, UK) and Cell Condition Solution (Ventana Medical Systems, Inc., Tucson, AZ, USA) in a PT Link pre-treatment system (Dako UK Ltd., Ely, UK) at 98˚C for 30 min. Subsequently, the sections were washed in PBS at room temperature. The endogenous peroxidase was neutralized using 3% H_2O_2 and the membranes were blocked using 4% casein (Novocastra Reagents). The samples were then incubated for 1 h at room temperature with the following primary antibodies: Polyclonal rabbit anti-human RKIP (1:100; cat. no 4742; Cell Signaling Technology Inc., Danvers, MA, USA), monoclonal rabbit anti-human estrogen receptor, (clone SP1; 1:100; cat. no. 790-4324; Ventana Medical Systems), monoclonal rabbit anti-human progesterone receptor (clone 1E2; 1:100; cat. no. 790-2223; Ventana Medical Systems), rabbit anti-human monoclonal Ki-67 (clone 30-9; 1:500; cat. no. 790-4286; Ventana Medical Systems). Staining was detected using a polymer detection kit (Novocastra Reagents) and 3,3'-diaminobenzidine substrate-chromogen (Novocastra Reagents) and the slides were counterstained using Harris hematoxylin (Novocastra Reagents).

Methylation-specific polymerase chain reaction (PCR). Methylation of the RKIP gene promoter was investigated using methylation-specific PCR. The reaction was performed in a total volume of 50 μl, comprised of 6 μl bisulphite modified DNA, 0.2 μM each sense and anti-sense primers (Invitrogen Life Technologies, Carlsbad, CA, USA), 1 μl dNTPs (0.2 mM each), 1,5 mM $MgCl_2$, 1X PCR buffer and 1 unit Platinum Taq DNA Polymerase (Invitrogen Life Technologies). The cycling conditions were as follows: Initial denaturation at 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 1 min, annealing at 52˚C for 1 min and extension at 72˚C for 1 min, followed by a final extension step at 72˚C for 10 min. To differentiate between the methylated DNA (204 bp PCR product) and the unmethylated DNA (205 bp PCR product), the following specific primers were used: Unmethylated RKIP promoter forward, 5'-TTTAGTGATATTTTTTGAGATATGA-3' and reverse, 3'-CACTCCCTAACCTCTAATTAACCAA-5'; methylated RKIP promoter forward, 5'-TTTAGCGAT ATTTTTTGAGATACGA-3' and reverse, 3'-GCTCCCTAA CCTCTAATTAACCG-5' (Applied Biosystems Life Technologies, Foster City, CA, USA).

CpGenome universal methylated DNA (Chemicon International, Inc., Temecula, CA, USA) was used as a methylated positive control. Blood DNA obtained from a healthy, young individual was used as an unmethylated negative control following receipt of written informed consent.

Cell transfection with anti-miR microRNA (miRNA) inhibitor and pre-miR miRNA precursor. SUM 159 cells were transfected with 30 nM pre-miR-224 miRNA precursor, anti-miR-224 miRNA inhibitor and relative random sequences (pre-miR negative control) as negative controls, using siPORT™ Amine Transfection Agent (all these reagents were from Ambion Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions.

Extraction of cellular RNA and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from clinical samples and cell lines using TRIzol reagent (Invitrogen Life Technologies). For the evaluation of miR-224 levels, mature miRNA was reverse transcribed using miRNA-specific stem-loop primers (TaqMan MicroRNA Reverse Transcription kit; Applied Biosystems Life Technologies) prior to qPCR, which was conducted according to the manufacturer's instructions (TaqMan MicroRNA assay; Applied Biosystems Life Technologies) on a StepOne RealTime PCR System (Applied Biosystems Life Technologies). The PCR cycling conditions were as follows: Denaturation at 50˚C for 2 min, annealing at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and extension at 60˚C for 60 min. Samples were normalized to RNU6B small RNA.

For the evaluation of RKIP expression, RNA was reverse transcribed using a high capacity complementary DNA (cDNA) reverse transcription kit (Applied Biosystems Life Technologies). The resulting cDNAs were subjected to real-time RT-PCR using the TaqMan Gene Expression Master Mix kit (Applied Biosystems Life Technologies) in triplicates. The PCR cycling conditions were as follows: Denaturation

Table II. Characteristics of ten breast cancer samples.

Sample	Histotype	Grading	Ki-67	ER	PR	HER2	Methylation of *RKIP* gene promoter	RKIP mRNA levels	RKIP protein	Lymph node metastasis
T1	Luminal A	G1	Pos<5%	+	+	-	+	0.058	-	-
T2	Luminal A	G2	Pos<10%	+	+	-	+	0.279	-	-
T3	Luminal A	G2	Pos<10%	+	+	-	+	0.352	-	+
T4	Luminal B	G2	Pos<10%	+	+	+	+	0.426	-	-
T5	Luminal A	G2	Pos<10%	+	+	-	+	0.355	-	-
T6	TN	G3	Pos<10%	-	-	-	-	1.068	+	+
T7	Luminal B	G3	Pos>10%	+	+	+	-	0.477	+	-
T8	TN	G3	Pos>10%	-	-	-	-	0.493	+	-
T9	TN	G3	Pos>10%	-	-	-	-	0.627	+	+
T10	Basal-like CK 5/6+	G3	Pos>50%	-	-	-	-	0.494	+	+

A healthy sample was used as control reference for the analysis of RKIP mRNA expression levels by quantitative polymerase chain reaction. TN, triple negative; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor-2; RKIP, Ras-1 kinase inhibitor protein; mRNA, messenger RNA; Pos, positive expression; CK, cytokeratin.

Table III. RKIP mRNA and protein levels in SUM 159 cells following treatment with 5-AZA, TSA or a combination of the two for 72 h.

Conditions	RKIP mRNA	RKIP protein
Control	1.000±0.000	0.642±0.093
5-AZA	1.454±0.111[b]	1.010±0.107[b]
TSA	1.476±0.206[a]	1.184±0.085[b]
5-AZA+TSA	1.507±0.099[b]	1.011±0.145[a]

Untreated SUM 159 cells were used as control reference for the determination of RKIP mRNA expression levels by quantitative polymerase chain reaction. For RKIP protein, immunoblots were quantified by densitometry and results were expressed as arbitrary units (RKIP/β-actin). 5-AZA and TSA were used at concentrations of 2 mM and 1 μM, respectively. Data are expressed as the mean ± standard deviation of three independent experiments. [a]P<0.05 and [b]P<0.01 vs. control. RKIP, Ras-1 kinase inhibitor protein; mRNA, messenger RNA; 5-AZA, 5-aza-2'-deoxycytidine; TSA, trichostatin A.

Table IV. Results of cell growth assays of SUM 159 cells following treatment with various concentrations of 5-AZA, TSA or DHMEQ.

Treatment	Cell growth inhibition, %
5-AZA, mM	
0.5	1.1±0.2[b]
0.75	6.0±2.0[b]
1.0	5.2±1.9[a]
2.0	40.1±4.9[b]
TSA, μM	
0.5	5.8±2.9[a]
0.75	19.2±3.7[b]
1.0	24. 5±4.9[b]
1.5	43.1±4.4[b]
2.0	83.1±2.1[b]
DHMEQ, μg/ml	
5.0	0.0
7.5	0.2±0.2
10	0.3±0.5
15	2.5±2.0
20	2.0±0.7[b]

[a]P<0.05 and [b]P<0.01 vs. control. Data are expressed as the mean ± standard deviation of three independent experiments. 5-AZA, 5-aza-2'-deoxycytidine; TSA, trichostatin A; DHMEQ, dehydroxymethylepoxyquinomicin.

at 50˚C for 2 min, annealing at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and extension at 60˚C for 60 min. The running of the samples and data collection were performed on a StepOne AB Real Time PCR system (Applied Biosystems Life Technologies). β-actin was used as an internal standard. The specific primers used were as follows: hsa-miR-224, 002099; RNU 6B, 001093; RKIP, Hs01110783_g1; and β-actin, Hs99999903_m1 (Applied Biosystems Life Technologies).

Relative expression was calculated using the comparative Ct method [$\Delta Ct=Ct_{(target\ gene)}-Ct_{(housekeeping\ gene)}$]. Where Ct was the fractional cycle number at which the fluorescence of each sample passed the fixed threshold. Fluorescence was measured at 515-518 nm using StepOne AB Real Time PCR System software (Applied Biosystems Life Technologies). The ΔΔCt method for relative quantification of gene expression was used to determine miRNA or gene expression levels. ΔΔCt was calculated using the formula: $\Delta\Delta Ct=\Delta Ct_{(each\ sample)}-\Delta Ct_{(reference\ sample)}$. Fold change was calculated using the $2^{-\Delta\Delta Ct}$ equation.

Table V. Cell growth inhibition and combination indices in SUM 159 cells following combination treatment with various concentrations of 5-AZA, TSA and DHMEQ.

Treatment	Cell growth inhibition, %	Combination index
5-AZA (0.5 mM)+TSA (0.5 μM)	20.2±2.4[a]	0.941
5-AZA (0.5 mM)+TSA (1.0 μM)	34.9±4.1[a]	1.175
5-AZA (0.5 mM)+TSA (1.5 μM)	61.9±5.8[a]	1.105
5-AZA (0.75 mM)+TSA (0.5 μM)	21.3±3.2[a]	1.089
5-AZA (0.75 mM)+TSA (1.0 μM)	34.3±2.6[a]	1.327
5-AZA (0.75 mM)+TSA (1.5 μM)	69.8±5.3[a]	1.053
5-AZA (1.0 mM)+TSA (0.5 μM)	17.1±3.0[a]	1.376
5-AZA (1.0 mM)+TSA (1.0 μM)	47.3±2.3[a]	1.206
5-AZA (1.0 mM)+TSA (1.5 μM)	80.0±3.7[a]	0.936
5-AZA (2.0 mM)+TSA (0.5 μM)	54.8±6.0[a]	1.129
5-AZA (2.0 mM)+TSA (1.0 μM)	87.2±3.3[a]	0.810
5-AZA (2.0 mM)+TSA (1.5 μM)	99.3±0.7[a]	0.342
DHMEQ (7.5 μg/ml)+TSA (0.5 μM)	35.1±2.9[a]	0.662
DHMEQ (7.5 μg/ml)+TSA (1.5 μM)	64.9±6.7[a]	0.716
DHMEQ (10 μg/ml)+TSA (0.5 μM)	41.7±6.5[a]	0.659
DHMEQ (10 μg/ml)+TSA (1.0 μM)	77.2±5.7[a]	0.608
DHMEQ (15 μg/ml)+TSA (0.5 μM)	72.0±2.7[a]	0.482
DHMEQ (15 μg/ml)+TSA (1.0 μM)	87.9±2.3[a]	0.507
DHMEQ (20 μg/ml)+TSA (0.5 μM)	88.5±1.5[a]	0.366
DHMEQ (20 μg/ml)+TSA (1.0 μM)	84.2±3.1[a]	0.628

[a]P<0.01 vs. control. Data are expressed as the mean ± standard deviation of three independent experiments. 5-AZA, 5-aza-2'-deoxycytidine; TSA, trichostatin A; DHMEQ, dehydroxymethylepoxyquinomicin.

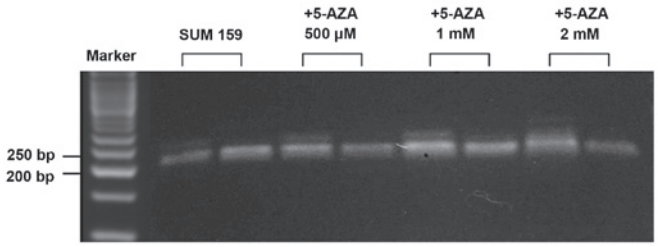

Figure 1. Effect of treatment with 5-AZA (500 μM, 1 and 2 mM for 72 h) on methylation of the Raf-1 kinase inhibitor protein gene promoter in SUM 159 cells. The results are representative of two independent experiments with comparable outcomes. 5-AZA, 5-aza-2'-deoxycytidine; U, unmethylated; M, methylated.

Western blotting. Whole-cell lysates were obtained from breast cancer cells using radioimmunoprecipitation assay buffer (Santa Cruz Biotechnology Inc., Dallas, TX, USA) and 25 μg protein was subjected to 10% SDS-PAGE and transferred to Hybond-P membranes (GE Healthcare Europe GmbH, Freiburg, Germany). Filters were incubated with primary antibodies raised against β-actin (1:10,000; Sigma-Aldrich Srl) or RKIP (1:1,000; Cell Signaling Technology, Inc.). Immunoblots were quantified by densitometry and results were expressed as arbitrary units (RKIP/β-actin).

Cell invasion assay. Cell invasion assays were performed using BD BioCoat Matrigel Invasion Chambers (BD Biosciences). SUM 159 cells were seeded at a density of $4x10^5$ cells/well onto 6-well plates. Following 24 h of incubation, 5-AZA (1.5 mM) or TSA (1 μM) was added. Following 16 h of treatment, the cells were trypsinized (EuroClone S.p.A.) and transferred to the upper Matrigel chamber in 500 μl medium at a density of $2.5x10^4$. Medium supplemented with 10% fetal bovine serum (EuroClone S.p.A.) was added to the lower chamber. Following 48 h of incubation, the non-migrated cells in the upper chamber were carefully removed with a cotton tip and the adherent cells present on the lower surface of the Matrigel insert were fixed with 100% methanol and stained with Giemsa solution (1:10; Sigma-Aldrich Srl). Migrated cells were counted microscopically (CK2 microscope; Olympus, Tokyo, Japan) in four randomly selected fields of the membrane at a magnification of x40. Each invasion experiment was carried out in duplicate and repeated at least twice. Data are expressed as a percentage of invasion through the Matrigel matrix, relative to that of the untreated controls. In parallel, cell growth assays were performed under identical conditions.

NF-κB activation. The DNA-binding capacity of NF-κB (p65 subunit) was determined in the nuclear extracts of SUM 159 cells using the TransAM™ NF-κB and Nuclear Extract™ kits (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. The SUM 159 cells were treated with 10 or 20 μg/ml DHMEQ for 8 or 16 h. Briefly, the determination of binding capacity was based on a 96-well plate, upon which an oligonucleotide containing the NF-κB consensus binding site

Table VI. Flow cytometric analysis of apoptosis in SUM 159 cells following treatment with 5-AZA, TSA, DHMEQ or a combination of these.

A, 5-AZA and TSA

Conditions	Apoptosis, %	Expected, %
Control	3.7±0.1	
5-AZA, mM		
2.0	16.9±1.3	
3.0	41.2±2.0	
TSA, μM		
1.0	18.3±0.6	
2.0	41.2±1.8	
Combinations		
5-AZA (2.0 mM)+TSA (1.0 μM)	37.2±2.5	31.5±0.6
5-AZA (2.0 mM)+(TSA 2.0 μM)	79.8±5.4	54.4±3.0[a]
5-AZA (3.0 mM)+TSA (1.0 μM)	64.5±1.6	56.0±2.7
5-AZA (3.0 mM)+TSA (2.0 μM)	81.3±2.8	78.9±0.3

B, DHMEQ and TSA

Conditions	Apoptosis, %	Expected, %
Control	3.8±0.1	
DHMEQ, μg/ml		
15	15.2±1.1	
20	18.5±0.1	
TSA, μM		
1.0	19.8±1.2	
1.5	25.4±0.5	
Combinations		
DHMEQ (15 μg/ml)+TSA (1.0 μM)	59.4±1.9	31.1±2.4[b]
DHMEQ (15 μg/ml)+TSA (1.5 μM)	62.2±1.7	36.8±1.8[b]
DHMEQ (20 μg/ml)+TSA (1.0 μM)	64.3±0.8	34.5±1.2[b]
DHMEQ (20 μg/ml)+TSA (1.5 μM)	74.6±2.4	40.1±0.5[b]

[a]P<0.05 and [b]P<0.01 vs. observed. Data are expressed as the mean ±standard deviation of two independent experiments. Expected value: Sum of the effects of the agents alone minus that of the untreated cells. 5-AZA, 5-aza-2'-deoxycytidine; TSA, trichostatin A; DHMEQ, dehydroxymethylepoxyquinomicin.

(5'-GGGACTTTCC-3') was immobilized. Activated NF-κB contained in the extracts is able to specifically bind to this nucleotide. NF-κB bound to the oligonucleotide may subsequently be detected using an antibody directed against an epitope on p65 (polyclonal rabbit anti-human; cat. no. 40096; 1:1,000; Active Motif), accessible only when NF-κB is bound to its target DNA.

Subsequently, the addition of a horseradish peroxidase-conjugated secondary antibody provided a sensitive colorimetric readout that may be quantified by densitometry (iMark Microplate Reader; Bio-Rad Laboratories, Inc.). The specificity of the assay was confirmed by simultaneous incubations in the presence of excess, non-immobilized consensus

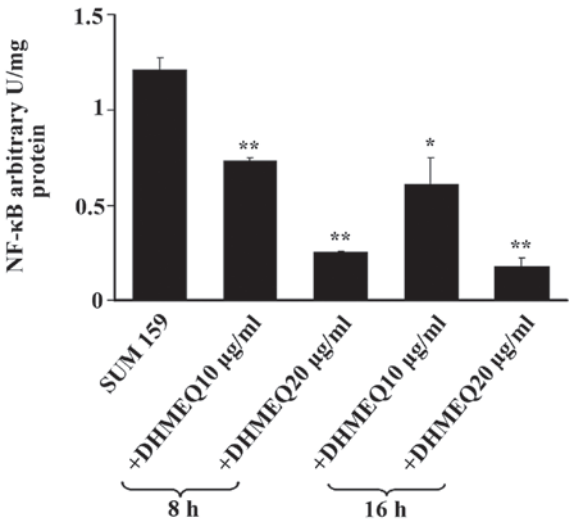

Figure 2. NF-κB (p65 subunit) DNA binding capacity in nuclear extracts of SUM 159 cells. The cells were treated for 8 or 16 h with DHMEQ (10 or 20 μg/ml). Results are expressed as arbitrary units/μg protein of SUM 159 nuclear extracts. Results are presented as the mean ± standard deviation of a representative experiment carried out in duplicate. *P<0.05, **P<0.01 vs. control. NF-κB, nuclear factor-κB; DHMEQ, dehydroxymethylepoxyquinomicin.

oligonucleotides, as a competitor, or of a mutated consensus oligonucleotide. The results were expressed as arbitrary units: one unit indicated the DNA binding capacity exerted by 2.5 μg whole cell extract from Jurkat cells (positive control for NF-κB p65 activation; Active Motif) (stimulated with 12-*O*-tetradecanoylphorbol-13-acetate and calcium ionophore) per microgram of protein from the nuclear extracts.

Statistical analysis. Results are presented as the mean ± standard deviation. The significance of differences between means was evaluated by Student's t-test for unpaired samples. The association between miR-224 and RKIP mRNA or protein levels (Table I) was evaluated by calculating Pearson's correlation coefficient (r). P<0.05 indicated a statistically significantly difference.

Results

RKIP expression levels are low in the SUM 159 breast cancer cell line. The RKIP content of the non-malignant MCF 10A breast epithelial cells and of several breast cancer cell lines, including MCF 7 and its multi-drug resistant variant MCF 7R, as well as the TNBC MDA MB 231, MDA MB 468, BT 549, SUM 149, SUM 159 and MCF 10A cell lines were evaluated (Table I). Together with BT 549, the SUM 159 cell line was found to express low levels of RKIP, at the mRNA and protein level, and therefore this cell line was selected for further analysis of the potential mechanisms involved in the repression of RKIP. SUM 159 cells are highly invasive, originate from a primary anaplastic, grade 4 carcinoma and belong to the basal B mesenchymal stem-like subtype according to the classification outlined by Neve *et al* (20).

Demethylating agent 5-AZA demethylates the RKIP gene promoter, increases RKIP expression and inhibits invasion

Table VII. RKIP mRNA and protein levels of SUM 159 cells following treatment with DHMEQ for 8 or 16 h.

Conditions	Duration, h	RKIP mRNA	RKIP protein
Control	0	1.000	0.654
DHMEQ, μg/ml			
10	8	1.218	0.983
20	8	1.302	0.870
10	16	1.375	1.780
20	16	1.285	1.537

Untreated SUM 159 cells were used as control reference for the determination of RKIP mRNA expression levels by quantitative polymerase chain reaction. For RKIP protein expression analysis, immunoblots were quantified by densitometry and results are expressed as arbitrary units (RKIP/β-actin). The data shown are the results of a representative experiment. A repeat experiments produced very similar results. RKIP, Ras-1 kinase inhibitor protein; DHMEQ, dehydroxymethylepoxyquinomicin; mRNA, messenger RNA.

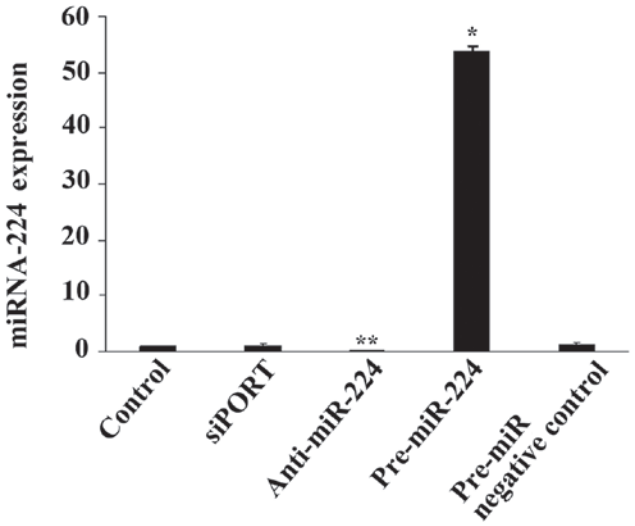

Figure 3. Reverse transcription-quantitative polymerase chain reaction analysis of miR-224 expression in the SUM 159 cell line following no treatment (Control), treatment with siPORT Amine Transfection Agent, anti-miR-224 miRNA inhibitor (30 nM), pre-miR-224 miRNA precursor (30 nM) or its negative control (30 nM) for 24 h. Relative quantification ($2^{-\Delta\Delta CT}$) values normalized against RNU6B small RNA are shown on the y axis on a linear scale. The data shown are the mean ± standard error of the mean of a representative experiment carried out in duplicate. *P<0.05, **P<0.01 vs. control. miR-224, microRNA-224; miRNA, microRNA.

of SUM 159 cells. It has been debated whether epigenetic changes, including methylation of the RKIP gene promoter, may be responsible for RKIP downregulation in colorectal and other types of cancer (11,21-24).

Methylation-specific PCR was performed on 10 primary ductal infiltrating breast cancers of various subtypes and with differing grades of RKIP expression (Table II) and in SUM 159 cells (Fig. 1). Gene promoters were considered methylated if the intensity of methylated bands was >10% of their respective unmethylated bands (25). Five of the clinical tumors exhibited RKIP gene promoter hypermethylation, which was associated with the low expression of RKIP protein and mRNA levels. In addition, SUM 159 cells demonstrated hypermethylation of the RKIP promoter (Fig. 1). The SUM 159 cells were subsequently exposed to various concentrations (500 μM, 1 and 2 mM) of demethylating agent 5-AZA for 72 h. The 5-AZA reagent was able to demethylate the RKIP gene promoter in a dose-dependent manner (Fig. 1) and to increase RKIP expression at the mRNA and protein level (Table III). Conversely, in cell invasion assays, 5-AZA, at a concentration of 1.5 mM, which was only marginally cytotoxic (3.7±0.2% inhibition of cell growth), markedly inhibited (82.5±2.1%) the invasion of SUM 159 cells.

HDAC inhibitor TSA enhances RKIP expression and inhibits invasion in SUM 159 cells. It is well known that another epigenetic mechanism that affects gene expression is histone acetylation (26). Notably, the HDAC inhibitor TSA was able to increase the mRNA and protein expression levels of RKIP (Table III) in SUM 159 cells, as well as exert inhibitory effects in the specific assays determining their invasion ability (45.9±3.1% at 1 μM, which induced 12.2±1.6% cell growth inhibition).

The combination of 5-AZA and TSA analyzed was not able to further increase the effects of either agent on RKIP mRNA and protein expression levels (Table III). The effects of 5-AZA and TSA alone or in combination on the inhibition of cell growth were evaluated using MTS assays (Tables IV and V). 5-AZA and TSA treatment both inhibited cell growth in a dose dependent manner (Table IV). The combinatory effects were found to be substantially additive, as indicated by the combination indices (Table V). Furthermore, flow cytometric analysis of the induction of apoptosis revealed that, overall, the combination of 5-AZA+TSA produced only modest increases in apoptosis with respect to the expected sum, based on the effects of the agents alone (Table VIA).

DHMEQ reduces constitutive activation of NF-κB and increases RKIP levels in SUM 159 cells. Previous studies have indicated that activated NF-κB is able to downregulate RKIP expression via Snail induction (13,14,27). Evaluation by TransAM® assays revealed that SUM 159 cells exhibited a marked constitutive activation of NF-κB, which was reduced in a concentration-dependent manner by DHMEQ, an inhibitor of the nuclear translocation of this transcription factor (Fig. 2) (28,29). Furthermore, identical treatments with DHMEQ induced marked increases in RKIP mRNA and protein expression in SUM 159 cells (Table VII).

Notably, combined treatment with DHMEQ and TSA produced synergistic effects on the inhibition of cell growth (Table V) and induction of apoptosis (Table VIB).

miR-224 does not alter RKIP expression levels. Upregulation of miRNAs has previously been associated with cancer progression, via the inhibition of tumor suppressor genes (30,31). Furthermore, a previous study (16) demonstrated that miR-224 was able to inhibit RKIP gene expression by directly targeting the 3'-untranslated region of the highly invasive MDA MB 231 breast cancer cell line.

However, by calculating the Pearson's correlation coefficient, a significant inverse association between miR-224 and

RKIP levels could not be found in the breast cancer cell lines evaluated in the present study (Table I).

Furthermore, SUM 159 cells were transfected with pre-miR-224 miRNA precursor, anti-miR-224 miRNA inhibitor or siPORT Amine Transfection Agent alone or with a negative control for 24 h. The increase or reduction in miR-224 (Fig. 3) induced by the precursor or inhibitor, respectively, did not alter RKIP expression at the mRNA or protein level (data not shown).

Discussion

Overall, the present results suggest that various mechanisms, including methylation of the gene promoter, histone deacetylation and NF-κB activation, but not targeting by miR-224, may be responsible for the downregulation of RKIP gene expression in the highly invasive SUM 159 TNBC cell line. However, previous studies of hepatocellular carcinoma cell lines (11,32) ruled out the role of gene methylation, histone acetylation and miR-224 in the reduction of RKIP expression. These results highlight the complexity of the regulation of this tumor suppressor gene, which may be differentially affected depending on the type of cancer. From a therapeutic perspective, the demethylating agent 5-AZA, the HDAC inhibitor TSA and the NF-κB inhibitor DHMEQ, in addition to inducing an increase in RKIP expression, appeared to be able to generate significant cytotoxicity, induce apoptosis and, in the case of 5-AZA and TSA, reduce cell invasion. Results in other cell models have demonstrated that the combination of DNA demethylating agents and HDAC inhibitors, for example 5-AZA and TSA, may be synergistic in the reactivation of specific genes (33,34). However, this did not appear to be the case for RKIP in SUM 159 cells. Furthermore, 5-AZA and TSA generated mainly additive effects on cell growth inhibition and induction of apoptosis when the cells were treated with the two agents together. The combination of DHMEQ and TSA exhibited significant synergy in cell growth and induction of apoptosis assays. This was unsurprising, since, besides influencing RKIP expression, 5-AZA, TSA and DHMEQ may distinctly influence additional genes, and therefore their respective properties may determine their respective capabilities of synergizing with other agents, with regard to the inhibition of cell growth, survival and invasive and metastatic ability. Concurrently, it was previously demonstrated that TSA and DHMEQ were able to diversely alter gene expression and synergize with conventional chemotherapeutic drugs, including doxorubicin and cisplatin, in hepatocellular cancer cells (29,32).

The present results, in addition to confirming the potential significance of NF-κB activation in the downregulation of RKIP expression (13,14,27), suggest that this transcription factor may be a useful target for the treatment and chemosensitization of the most aggressive forms of breast cancer. There is increasing evidence that aberrant activation of NF-κB signaling is a frequent characteristic of TNBC cells, although the underlying causes of this activation have remained largely elusive (35,36). Apart from DHMEQ, which at preclinical levels appears to be a promising agent for anticancer, anti-inflammatory and immunosuppressive treatments (28), other valuable therapeutic resources in this context may include the proteasome inhibitors, which, in addition to inhibiting NF-κB activation, may also restore RKIP levels via inhibition of proteasome degradation of the ubiquinated protein (11,37).

In conclusion, the present findings, in addition to other potential causes of RKIP downregulation, require further investigation and validation in a larger number of TNBC cell models.

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