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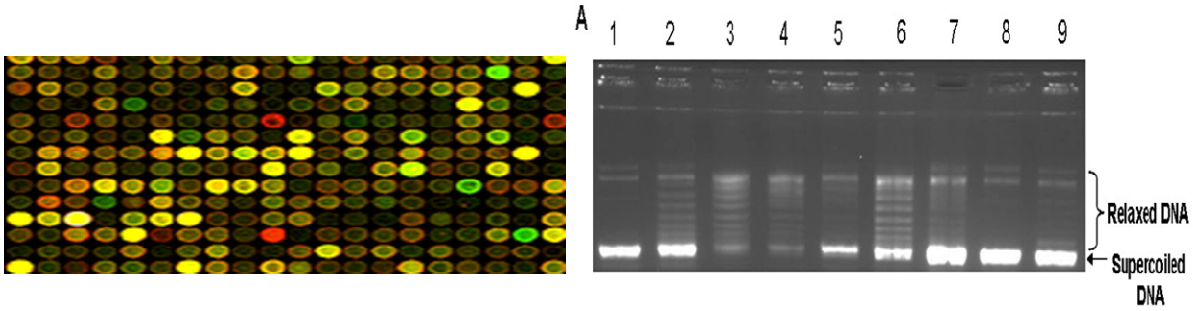

Gene expression profiling identifies novel key players involved in the cytotoxic effect of Artesunate on pancreatic cancer cells

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Abstract

Pancreatic cancer is one of the most aggressive human malignancies, with an extremely poor prognosis. The paucity of curative therapies has translated into an overall 5-year survival rate of less than 5%, underscoring a desperate need for new therapeutic options. Artesunate (ART), clinically used as anti-malarial agent, has recently revealed remarkable anti-tumor activity. However, the mechanisms underlying those activities in pancreatic cancer are not yet known. Here we evaluated the antitumor activity of Artesunate and the possible underlying mechanisms in pancreatic cancer. MiaPaCa-2 (poorly differentiated) and BxPC-3 (moderately differentiated) pancreatic cancer cell lines were treated with Artesunate and the effect was monitored by a tetrazolium-based assay (MTT) for evaluating cell viability and by flow cytometry and caspase 3/7 activation for apoptosis evaluation. In addition cDNA arrays were used to identify differentially expressed genes. The microarray data were then validated by RT-PCR and western blotting. Moreover, pathways associated with these expression changes were identified using the Ingenuity Pathway Analysis. The expression analysis identified a common set of genes that were regulated by Artesunate in pancreatic cancer. Our results provide the first in vitro evidence for the therapeutic utility of Artesunate in pancreatic cancer. Moreover, we identified Artesunate as a novel topoisomerase IIα inhibitor that inhibits pancreatic cancer growth through modulation of multiple signaling pathways. The present analysis is a starting point for the generation of hypotheses on candidate genes and for a more detailed dissection of the functional role of individual genes for the activity of Artesunate in tumor cells.

1. Introduction

Pancreatic cancer remains a devastating and poorly understood malignancy with increasing incidence worldwide. Cancer of the exocrine pancreas is the fourth most common malignancy in the United States. The annual incidence rate is almost identical to the mortality rate. Currently, surgery is the only treatment, although due to its late presentation, only 9% to 15% of patients are suitable for surgery [1].

Gemcitabine, the standard of care since 1997 [2], is the current drug of choice for treatment of pancreatic cancer. It has been shown to improve the clinical outcome and survival compared with 5-fluorouracil [3], however, the median survival for all pancreatic cancer stages is still ~ 3 to 5 months from diagnosis [4] and the five-year survival rate remains less than 5% [5]. Therefore, new therapeutic strategies are necessary to combat this deadly disease. To obtain an effective regimen, a careful and well-designed combination of multi-therapeutic agents with different modes of action will be required and that was a goal in our study.

Artemisinin, a sesquiterpene isolated from *Artemisia annua* L., is used in traditional Chinese medicine for the treatment of fever and chills [6]. Artesunate (ART) is a semisynthetic derivative of artemisinin. ART and other artemisinin derivatives are promising novel drugs in the treatment of malaria [7]. Large clinical studies with malaria patients have shown that ART is well tolerated, with a few and insignificant side effects [8-10]. In addition to the well known anti-malarial activity of ART, recently a cytotoxic action of ART against cancer cell lines of different tumor types is identified [11-16]. Until now, the accepted antitumor mechanism is similar to the antimalarial mechanism; the artemisinin structure contains an endoperoxide bridge that reacts with an iron atom to form free radicals [17-19], which causes macromolecular damage and cell death [20].

Previous studies showed that ART induces ROS-mediated apoptosis in doxorubicin-resistant T leukemia cells [21], attenuates the growth of human colorectal carcinoma, inhibits hyperactive Wnt/β-catenin pathway [15], exhibits anticancer growth activities in human ovarian cancer cells [22], inhibits nitric oxide [23] and induces DNA damage and repair [24]. Despite the great efforts that have been done, the effect of ART on pancreatic cancer and its possible molecular mechanisms are not yet known.

In the present study, we established that ART induces growth arrest and apoptosis in pancreatic cancer cell lines and its effect depends on the differentiation stage, being more effective against the poorly differentiated cells. In addition, our results suggest that ART potentiates the antitumor effects of gemcitabine in pancreatic cancer. Moreover, to the best of our knowledge, this is the first study demonstrating ART as a novel topoisomerase IIα (TOP2A) inhibitor, and indicating that Proliferating cell nuclear antigen (PCNA), DNA-damage-inducible transcript 3 (DDIT3), Non steroidal anti-inflammatory drug-activated gene (NAG-1) and Ribonucleotide reductase 2 (RRM2) are among the novel markers modulating ART effect on pancreatic cancer cells.

2. Materials and Methods

2.1. Cell lines and treatment. The human pancreatic cancer cell lines, MiaPaCa-2 (poorly differentiated) and BxPC-3 (moderately differentiated), were obtained from the American Type Culture Collection (Rockville, USA). BxPC-3 cells maintained in RPMI 1640 containing 100 units /ml penicillin and 100 µg /ml streptomycin and supplemented with heat-inactivated 10% fetal bovine serum (FBS). MiaPaCa-2 cells were maintained in DMEM containing 100 units /ml penicillin, 100 µg/ml streptomycin and 10% FBS (Invitrogen, Carlsbad, CA).Cultured cells were maintained in a humidified environment at 37°C with 5% CO_2. Artesunate (purity ≥99%) was purchased from Saokim Pharma (Hanoi,Vietnam) and a stock solution in DMSO at 1 M was prepared. Gemcitabine was kindly provided by Eli Lilly (Gemzar®, Bad Homburg, Germany). The concentration of DMSO was kept at or below 0.1 % in all experiments.

2.2. MTS cell proliferation assay. To assess cell proliferation, CellTiter 96® Aqueous Non-radioactive Cell Proliferation Assay (Promega, Mannheim, Germany) was used according to the manufacturer's instructions. The assay tests cellular viability and mitochondrial function. Briefly, cells were grown in tissue culture flasks, and then harvested by treating the flasks with 0.025% trypsin and 0.25mM EDTA for 5 min. Once detached, cells were washed, counted and an aliquot ($5x10^3$ cells) was placed in each well of a 96-well cell culture plate in a total volume of 100 µl. Cells were allowed to attach overnight and then treated with or without increasing concentrations of ART. After 24, 48, and 72 h, 20 µl MTS solution was added to each well and the plates were incubated at 37°C for 3h. The absorbance of the product formazan, which is considered to be directly proportional to the number of living cells in the culture, was measured at 490 nm and at 650 nm using a Precision Microplate Reader (Molecular Devices, Sunnyvale, CA). The same routine was done after treating cells

for 48 h with increasing concentrations of gemcitabine in absence or presence of 10 μM ART, respectively.

2.3. Flow Cytometry analysis of apoptotic cells. Cells treated with gradient concentrations of ART were harvested and washed with ice-cold phosphate-buffered saline (PBS; Invitrogen) and resuspended in 150 μl hypotonic fluorochrome solution [50 μg/ml propidium iodide, 0.1% (w/v) sodium citrate (pH 7.4) and 0.1% (v/v) Triton X-100]. The cells were incubated in the dark at 4°C overnight before performing FACS analysis. The propidium iodide fluorescence of individual nuclei was measured using a FACS-Calibur cytometer (BD Biosciences, Heidelberg, Germany). Data were analysed with the CellQuess Pro V5.2.1 software (BD Biosciences). For each condition, at least three independent experiments were performed.

2.4. Caspase-Glo 3/7 assays. Caspase-Glo™ 3/7 Assay (Promega) was used to detect Caspase 3/7 activities of MiaPaCa-2 cancer cell lines triggered by ART. This test provides a proluminescent caspase-3/7 substrate, which contains the caspase-3 specific tetrapeptide sequence DEVD in a reagent optimized for cell lysis and determination of caspases and luciferase activity. MiaPaCa-2 cells cultured in DMEM were seeded in 96-well plates and treated with ART. Six hours after treatment, cellular caspase 3/7 activity was determined according to the manufacturer's protocol. Luminescence was measured using Mithras LB 940 (Berthold Technologies, Bad Wildbad, Germany). Cellular apoptosis was expressed as percentage of the untreated medium control.

2.5. Microarray gene expression profiling. MiaPaCa-2 and BxPC-3 cell lines were treated with 25 μM ART or with DMSO alone (control) for 48 h. Total-RNA from each sample was isolated with the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The integrity of the isolated RNA was checked on an Agilent Bioanalyser 2100 using the RNA 6000 Nano Kit (Agilent Technologies, Palo Alto, USA). Fluorescently

labelled cDNA samples were prepared from 15 µg total-RNA. Cy3- or Cy5-labelled dCTP was directly incorporated during first-strand synthesis [25] Microarrays were produced and processed as described in detail previously [26], representing a well-defined subset of some 7,000 genes that are highly associated with the occurrence of pancreatic cancer including apoptotic and oncogenic genes, growth factors, angiogenic, cell cycle, metastasis-associated, and housekeeping genes. Hybridization was done in TeleChem Chambers at 62°C overnight. After washing, fluorescence signals were detected with a confocal ScanArray 5000 scanner (Packard Bioscience, USA) and analyzed with GenePix Pro 6 (Axon Instruments, Union City, USA).

2.6. Real-time Reverse Transcription-PCR. Total RNA was extracted and purified from cells treated with 0 or 25 µM ART for 48 h using RNeasy kit (Qiagen) according to the manufacturer's instructions. Total RNA was converted to cDNA by reverse transcriptase (Invitrogen) with random hexamer primers. The cDNAs were quantified by real-time PCR using the QuantiTect SYBR Green PCR Kit (Qiagen) and the Light Cycler 480 instrument (Roche Diagnostics), PCR was done by initial denaturation at 95°C for 15 min and 40 cycles of strand separation at 94°C for 15 s, annealing at 56°C for 20 s and extension at 72°C for 20 s. Expression levels were normalised relative to the transcription level of β-actin. All samples were run in triplicate.

2.7. Western blotting. Cells were seeded as noted above. 24 h after incubation, the cells were treated with 0 or 25 µM ART for 48 h. Total protein was extracted using Qproteome mammalian protein preparation kit (Qiagen) according to the manufacturer's instructions. Protein samples were resolved on SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). After blocking, membranes were incubated with primary antibodies against the following proteins: TOP2A (Millipore, Schwalbach, Germany), PCNA (Biozol, Eching, Germany), AREG,

NAG-1 and DDIT3 (Sigma-Aldrich, Munich, Germany) at 4°C overnight. After incubation with secondary antibody, bands were detected by chemiluminescence using the ECL Western Blotting Detection System (Amersham) and images were acquired with western blot documentation instruments LAS-3000 (Fuji Film, Tokyo, Japan) and the signal intensities were quantified using the Image Gauge software (ver. 4.23; Fujifilm). Data are presented as the mean ± S.E.

2.8. Topoisomerase II Assays. Inhibition of topoisomerase II activity by ART was measured by a supercoiled DNA relaxation assay using a topoisomerase II drug screening kit (TopoGEN, FL, USA). The kit allows detection of two kinds of topoisomerase inhibitors: those that antagonize topoisomerase II action on the DNA (relaxation assay) and those that stimulate formation of cleavable complexes (stabilization assay). Briefly, 0.25 μg super-coiled DNA (pRYG) was suspended in a reaction buffer. ART, etoposide or a solvent control was added to the mixture before the reaction was started by topoisomerase II enzyme addition. After 30 minutes incubation at 37 °C, the reaction was stopped by adding 0.1 volumes of 10% SDS. The DNA-bound protein (topoisomerase IIα) was digested by proteinase K (50 μg/mL) at 37 °C for 15 min. The proteinase K was removed by chloroform/isoamyl alcohol (24:1, v/v) treatment. DNA samples were then analyzed by 1% agarose gel electrophoresis. The gel did not contain ethidium bromide and was stained by 0.5 μg/mL ethidium bromide before UV photography. The stabilization of topoisomerase IIα cleavage complex was studied by using pRYG plasmid, and 1% agarose gel containing 0.5 μg/mL ethidium bromide. The other procedures were the same as the relaxation assay.

2.9. Identification of signalling pathways. The Ingenuity Pathway Analysis software (IPA) (Ingenuity Systems, Mountain View, USA) was utilized to identify networks of interacting genes and other functional groups. A cut-off ratio of 1.5 was used to define genes. Using the

IPA Functional Analysis tool, we were able to associate biological functions and diseases to the experimental results. Moreover we used a biomarker filter tool and the Network Explorer for visualizing molecular relationships.

2.10. Data analysis. Microarray data quality assessment, normalisation and correspondence cluster analysis were performed with the MIAME-compatible analysis and data warehouse software package M-CHiPS [27, 28] (www.mchips.org). Signal intensities of repeated hybridizations were normalised and significance levels assessed by two stringency criteria. The highly stringent 'min- max separation' is calculated by talking the minimum distance between all data points of two conditions. The less stringent criterion 'standard deviation separation' is defined as the difference of the means of the two data sets diminished by one standard deviation. Only variations with a P-value of less than 5% were taken into account. Cluster analysis was performed using correspondence analysis [26].

3. Results

3.1. Artesunate inhibits growth and proliferation of pancreatic cancer cell lines.

Recently, it was shown that ART has profound cytotoxic activities against cancer of different tumor types. To determine its effect on pancreatic cancer cells, MiaPaCa-2 and BxPC-3 cells were cultured in a monolayer and treated with ART for 24, 48, and 72 h. The MTS assay was performed to assess the rate of proliferation, and the resulting growth curves showed that ART has a concentration and time-dependent inhibitory effect (Fig. 1). In comparison of differentiation stage with the decrease in cell viability, the moderately differentiated BxPC-3 cell line (Fig. 1B) were less sensitive to ART treatment than the poorly differentiated MiaPaCa-2 cells (Fig. 1A)

3.2. Artesunate induces apoptosis in a dose-dependant manner. To analyze the mechanism of cell death induced by ART, flow cytometry analysis following 48 hr treatment with ART (0-100 μM) was performed. ART significantly increased the percentage of cells with hypo-diploid or sub-G1 peaks, the hallmark of apoptosis [29], in a concentration dependant manner (Fig. 2A) and the apoptotic effect was more prominent in the poorly differentiated MiaPaCa-2 cells compared to the moderately differentiated BxPC-3 cells (Fig. 2B).

3.3. Artesunate induces caspase 3/7 activation. It is well established that the induction of the apoptotic cascade is one of the main mechanisms of chemotherapy- induced cell death [30]. To determine whether the chemosensitizing effect of Artesunate demonstrated above is secondary to its ability to activate the apoptotic cascade, MiaPaCa-2 cells were treated with ART. Six hours after treatment, the activity of caspase 3/7 were measured using the Caspase-Glo 3/7 assay. Fig. 2C shows that ART caused significant increase in activation of caspase-3/7 in a dose dependent manner. These results suggest that ART-induced apoptosis is, in part, due to activation of caspases 3/7.

3.4. Microarray Analysis identifies novel Artesunate targets significantly up- and down-regulated in their expressions. For the identification of possible targets and mechanisms of action of ART; MiaPaCa-2 and BxPC-3 pancreatic cancer cell lines were treated with 0 or 25 µM ART for 48 h. RNA preparation, hybridization, data quality assessment, filtering, normalization and subsequent analysis were performed as described before [26, 28] by procedures that meet or exceed the MIAME-criteria of microarray analysis [31]. In MiaPaCa-2 and BxPC-3 cells the expression of 1161 genes, was found to be significantly regulated ($p<0.05$) (for a detailed list see supplemental table 1).

For further interpretation, the results were subjected to correspondence cluster analysis [26, 27]. It was apparent that the two cell lines exhibited markedly different expression profiles and form distinct clusters (data not shown). Replicate experiments of the two cell lines always fell in the same respective cluster, demonstrating the high degree of experimental reproducibility. The analysis documented clearly, that the principle difference (distance between clusters along the vertical axis) in expression between the two cell lines exhibits correlation with the differentiation status of the respective cells.

3.5. Verification of Microarray Results. For independent verification of the expression variations, RT-PCR and/or western blotting were performed on some genes selected during the analysis process (e.g. TOP2A, COX-2, NAG-1, PCNA, DDIT3, RRM2, AREG and FOS). All results were in accordance with the array results (Fig. 3 and 4)

3.6. Functional classification of microarray regulated genes. In addition to the purely statistical analysis of the correspondence analysis, we employed the Ingenuity Pathway Analysis Knowledge database (www.ingenuity.com) to improve further the understanding of the biological consequences of ART treatment. Among the differentially expressed genes, 1161 genes were in the Ingenuity Pathway Analysis (version 6.5) database, and 1155 genes mapped to genetic networks as defined by the IPA tool. 42 and 12 networks were found to be

highly significant in that they had more of the identified genes present than would be expected by chance in MiaPaCa-2 and BxPC-3 cell lines respectively (Supplemental table 2 A, 2 B, 2 C and 2 D). These networks were associated with cancer, cell cycle, cell death, cellular growth and proliferation. The tool then associates these networks with known biological pathways (Fig. 5). Moreover the gene ontology analysis showed that 51 and 56 relevant biological functions and diseases were identified as high-level functions (data not shown), of which, the top functions were cell death, cell cycle, and cellular growth and proliferation, and the top diseases were cancer, gastrointestinal diseases, immunological and inflammatory diseases in MiaPaCa-2 and BxPC-3 cell lines, respectively. Moreover we carried out a biomarker analysis, which allows identifying and prioritizing the most relevant and promising molecular biomarker candidates from datasets from nearly any step of the drug discovery process or any type of disease research. Using the Biomarker Comparison Analysis, Moreover, we identified 48 common biomarkers between the 2 cell lines that are common to ART response (Fig. 6) and 133 unique potential specific biomarkers to MiaPaCa-2 cells and 21 unique potential biomarkers to BxPC-3 cells that discriminate between ART responses (Fig.7).

3.7. Artesunate Targets Topoisomerase IIα. Agents that target Top2, involving etoposide and doxorubicin, are among the most effective anticancer drugs used in the clinic. Top2A is essential for cell proliferation and is highly expressed in vigorously growing cells [32]. Here, our microarray gene expression results (Supplemental table 1) showed that ART significantly down-regulates Top2A expression which was confirmed also by RT-PCR (Fig. 3) and by western blotting (Fig.4 A and B). Down-regulation was higher in case of Miapaca-2 cells. In addition, to test whether ART could antagonize topoisomerase IIα action on the DNA, using etoposide as a drug positive control, pRYG and topoisomerase IIα as a non solvent control and pRYG, topoisomerase IIα plus DMSO as a solvent control, in a cell-free topoisomerase

IIα relaxation assay, we found that 50 -100 μM ART to 50- 100 μM etoposide indeed inhibited the relaxation of supercoiled plasmid DNA by topoisomerase IIα (Fig. 8A), and the inhibition was dose dependent, indicating that ART is a as potent topoisomerase IIα inhibitor as etoposide.

3.8. Effect of Artesunate on the Topoisomerase II-mediated DNA cleavage-religaion reaction. Several potent and clinically relevant anti-neoplastic agents stabilize the topoisomerase II-DNA cleavage complex by inhibiting the topoisomerase II-mediated religation reaction [33]. When this stabilization occurs, the DNA fragments resulting from the double-strand breaks appear in the gel as linear species [34]. To test if ART traps topoisomerase IIα in its DNA cleavage complex form, we used the pRYG plasmid as supercoiled DNA substrate and detected linear DNA formation after incubation with topoisomerase enzyme in presence of either DMSO (solvent control), ART or etoposide. and also we used linear DNA as a marker. As can be seen in (Fig. 8B), when topoisomerase II was incubated in the cleavage assay in the presence of ART (25 – 100 μM), no linear pRYG DNA was produced. We also found that ART at 100 times the concentration that inhibits topoisomerase II activity in the relaxation assay was also unable to stabilize the topoisomerase II-mediated DNA cleavage-religation complex (data not shown). Linear DNA was produced clearly from pRYG DNA by topoisomerase II enzyme when the incubation was carried out in the presence of 150 μM etoposide (drug control), an agent that stabilizes the cleavage complex (Fig. 8B). These results suggest that topoisomerase IIα is a major intracellular target of Artesunate.

3.9. Artesunate potentiates growth inhibition induced by gemcitabine. Ribonucleotide reductase 2 (RRM2) was among the significantly down-regulated genes in our microarray data. Previous studies showed that over-expression of RRM2 was associated with resistance to gemcitabine in patients with pancreatic cancer [35]. Also, it was demonstrated that

systemic delivery of siRNA-based therapy can enhance the efficacy of gemcitabine [36]. Accordingly, we hypothesized that Artesunate could potentiate the growth inhibitory effects of gemcitabine on pancreatic cancer cells. MiaPaCa-2 (Fig. 9A) and BxPc-3cells (Fig. 9B) were either treated with gemcitabine alone or in combination with Artesunate (10 μM), and the number of viable cells were evaluated 48 h post-treatment by MTS assay. Data presented here showed that BxPc-3 cells were more sensitive to gemcitabine compared to MiaPaCa-2 cells and that Artesunate potentiates the growth inhibitory effect of gemcitabine on both cell lines in a dose-dependent manner and the effect was sometimes additive or synergistic depending on gemcitabine doses.

4. Discussion

Natural products have been a continuous source of novel compounds for the treatment of numerous diseases, with natural products and their synthetic derivatives comprising over 60% of the approved anticancer drug candidates developed between 1981 and 2002 [37]. In order to observe an effect using compounds like Artesunate (ART) in clinical trials, the identification of potential responders would greatly increase the power of such trials. Potential responders are those patients whose tumors express molecular characteristics that match the molecular effects of Artesunate, and this was our motivation to understand the effects of Artesunate on pancreatic cancer cells.

Artemisinin and its derivative Artesunate, distinguish themselves as a new generation of anti-malarial drugs with a few and insignificant side effects [8-10]. Recently it has been reported that they also possess antitumor activity. Although the anti-tumor effects of Artesunate have been previously investigated *in vitro* and *in vivo*, the effect of Artesunate and its possible mechanisms of action have not been studied with regard to pancreatic cancer. Gene expression profiling using cDNA microarray has been widely used in screening drug targets. In order to investigate the antitumor potential of Artesunate, we treated MiaPaCa-2 (poorly differentiated) and BxPC-3 (moderately differentiated) pancreatic cancer cells with Artesunate and the expression of cancer related-genes were monitored using microarray technology. Differentially expressed genes were then organized into functionally annotated networks.

Pharmacokinetic studies indicate that the concentration of Artesunate applied clinically for the treatment of malaria (e.g. 2 mg/kg intravenously) results in peak plasma drug concentration of 2640 ± 1800 µg/l (6.88 ± 4.69 mM) [11, 38].The doses used in our investigation were approximately two orders of magnitude lower than those used clinically.

Therefore, the selected concentrations that we used for gene expression profiling and for other experiments were much lower than the clinically relevant molar concentrations.

At the cellular level, our data shows that Artesunate inhibited growth and induced apoptosis of human pancreatic cancer cells and the effect was more prominent with the poorly differentiated MiaPaCa-2 cells.

At the molecular level, a network analysis was done on the basis of expression profiling data, in order to discover relevant connections and pathways among the regulated genes.

Our gene expression analysis identified many significantly up- and down-regulated genes, some of them are already established to be involved directly in the apoptotic pathway and others recently identified either as a potential pre-apoptotic and apoptotic genes, cancer prognostic agents, oncogenes, cancer drug targets or genes responsible for drug resistance mechanisms but their actual rules remains to be elucidated.

DNA topoisomerase II is a ubiquitous nuclear enzyme that alters the topological structure of DNA and chromosomes through a transient DNA double-strand break (DSB) and subsequent religation of the DSB. The enzyme has been implicated in many aspects of DNA metabolism, including DNA replication, repair, transcription, and chromosome condensation /segregation. Topoisomerase II-targeting agents, including etoposide, doxorubicin, and mitoxantrone, are among the most effective and widely used anticancer drugs in cancer chemotherapy [32, 39]. Topoisomerase-targeting anticancer drugs can be divided into two broad classes that vary widely in their mechanisms of action. Class I drugs include acridines, anthracyclines, actinomycins and quinolones act by stabilizing covalent topoisomerase-DNA complexes that are the intermediates during the catalytic cycle of the enzyme. They are also referred to as `topoisomerase poisons' because they transform the enzyme into a potent cellular toxin. Class II drugs; by contrast, interfere with the catalytic function of the enzyme

without trapping the covalent complex. The drugs in this class are referred to as `topoisomerase inhibitors'. The main topoisomerase inhibitors are coumarin antibiotics and fostriecin analogues [40].

The results presented here clearly demonstrate for the first time that Artesunate down-regulates expression TOP2A at both mRNA and protein levels, Moreover, we showed that 100 μM Artesunate completely inhibited the activity of topoisomerase IIα. However, ART was unable to trap topoisomerase IIα in its cleavage complexes, demonstrating that Artesunate is a novel topoisomerase inhibitor but not a topoisomerase poison. The concentrations required for Artesunate (100 μM) and etoposide (100 μM) to maximally inhibit topoisomerase IIα activity in our cell-free topoisomerase II relaxation assay could be higher than those needed actually in vivo. One reason may be that the DNA damage threshold to evoke checkpoint response is far less than which can be detected by relaxation of supercoiled DNA by topoisomerase IIα in this cell-free assay. In addition, the cell-free assay generates nearly total conversion of the plasmid substrate, whereas fewer lesions may be detected by the checkpoint sensors.

Apoptosis or programmed cell death is a key regulator of physiological growth control and regulation of tissue homeostasis. Killing of tumor cells by most anticancer strategies currently used in clinical oncology, such as chemotherapy or immunotherapy, has been linked to activation of apoptosis signal transduction pathways in cancer cells such as the intrinsic and/or extrinsic pathway [41]. Our results demonstrate that apoptosis induction was among the mechanisms triggering Artesunate inhibitory effects on pancreatic cancer cell lines, Where Artesunate induces sub-G1 phase (Fig. 2A), Caspase 3/7 activation (Fig. 2C) and up-regulates a variety of important and well-known apoptotic and proapoptotic molecules including APAF1, BAX, BAK and caspases 2, 3, 4, 5, 6, 8, 9 and 10 [41] as shown in the gene expression results (Supplemental table 1).

GADD153, also known as DDIT3 (DNA-damage-inducible transcript 3) has been shown to be involved in growth arrest and apoptosis following DNA damage and a variety of stress conditions, such as nutrient deprivation and treatment with anticancer agents [42]. Here, we demonstrate that DDIT3 is up-regulated in MiaPaCa-2 and BxPC-3 cell lines upon treatment with Artesunate which could explain, in part, the cytotoxic activities of Artesunate.

Proliferating cell nuclear antigen (PCNA) plays important roles in nucleic acid metabolism. The protein is essential for DNA replication and has been shown to be involved in RNA transcription [43]. Recent work has shown that PCNA expression is significantly higher in pancreatic cancer [44, 45]. Down-regulation of PCNA by Artesunate at mRNA and protein level in pancreatic cancer cell lines implies that Artesunate inhibits their growth, in part, by decreasing the PCNA level.

Non steroidal anti-inflammatory drug-activated gene (NAG-1) was another key gene among the up-regulated genes. NAG-1 is a distant member of the transforming growth factor super family. NAG-1 has been identified as an anti-tumorigenic and pro-apoptotic protein and its expression is able to be induced by NSAIDs and several other anti-cancer compounds [46, 47]. Furthermore, over-expression of NAG-1 is reported to induce caspase-dependent apoptosis in prostate cancer cell line DU-145 [48] which is consistent with our results.

Ribonucleotide reductase (RRM) mediates the rate-limiting step in DNA-synthesis because it is the only known enzyme that converts ribonucleotides to deoxynucleotides. The enzymatic activity of ribonucleotide reductase is modulated by the levels of RRM type 2 (RRM2) [49]. Previous studies showed that over-expression of RRM2 was associated with resistance to gemcitabine in patients with pancreatic cancer [35], and demonstrated that systemic delivery of siRNA-based therapy can enhance the efficacy of gemcitabine [36]. Here, we demonstrate that RRM2 is significantly down-regulated in both pancreatic cancer cells by Artesunate treatment, which is to our knowledge the first study to identify RRM2 as

a marker for the cytotoxic effect of Artesunate on pancreatic cancer. Moreover, we proved that Artesunate at lower molecular concentration significantly potentiates the growth inhibitory effects of gemcitabine on both pancreatic cancer cell lines.

In combination, our data demonstrate that Artesunate inhibits cell growth and promotes apoptosis in pancreatic cell lines through the modulation of multiple signaling pathways. The mechanisms driving Artesunate-induced growth arrest of human pancreatic cancer cells were dependent, in part, on the differentiation stage of the cells. At the molecular level, many genes related to apoptosis, cell cycle, angiogenesis, metastasis and differentiation are significantly regulated upon Artesunate treatment. Moreover, we showed for the first time that the common mechanisms modulating Artesunate effect may be mediated through down-regulation of TOP2A, RRM2 and PCNA and up-regulation of NAG-1 in both cell lines. In addition we introduced Artesunate for the first time to be a novel topoisomerase IIα inhibitor. The present analysis is a starting point for the generation of hypotheses on candidate genes and for a more detailed dissection of the functional role of individual genes for the activity of Artesunate in tumor cells. Moreover, our results provide new insights into Artesunate-related signalling activities, which may facilitate the development of Artesunate-based anticancer strategies and/or combination therapies. Further preclinical and clinical investigations are required to elucidate the full potential of Artesunate as a powerful cytotoxic agent for treatment of pancreatic cancer.

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Figure and Table legends

Fig.1. Growth inhibition of human pancreatic cancer cells by ART. Cultures of exponentially growing MiaPaCa-2 (A) and BxPc-3 cells (B) were grown in 96-well microtiter plates in the presence of Artesunate (ART) for 24, 48 and 72 h. The MTS assay was performed to determine the number of viable cells as described in Materials and Methods section. The data shown represent the mean value ± SE obtained from 8 replica wells each of three independent experiments.

Fig.2. ART induces apoptosis in pancreatic cancer cells. MiaPaCa-2 and BxPc-3 cells were grown with various concentrations of ART. The cells were then analyzed by flow cytometry.Panel (A) shows typical results for MiaPaCa-2 after 48 h. The percentage of sub-G1 phase cells (M1) was determined based on the DNA content histogram. Panel (B); shows that the apoptotic effect was more prominent in the poorly differentiated MiaPaCa-2 cells compared to the moderately differentiated BxPc-3 cells. Again, the mean ± SE of three independent experiments is shown for each cell line. (C) Enzymatic activity of caspase 3 after 6 h treatment of Miapaca-2 cells. The activity of caspase 3 is expressed as percentage % relative to untreated cells.

Fig.3. Results of real-time reverse transcriptase PCR analysis. BxPc-3 cells were treated with 25 μM ART for 48 h, Transcriptional changes are expressed relative to β actin. The mean value ± S.D. of three independent experiments is shown.

Fig.4. Western blot analysis. (A) In order to confirm changes in Top2A, AREG, NAG-1, DDIT3 and PCNA protein levels in MiaPaCa-2 cells after treatment with ART (25μM) for 48 h. (B) Bands were quantified and the results represent the mean value ± S.E. of the mean.

Fig.5. Functional assignment of genes significantly transcribed in MiaPaCa-2 (A) and BxPc-3 cells (B).

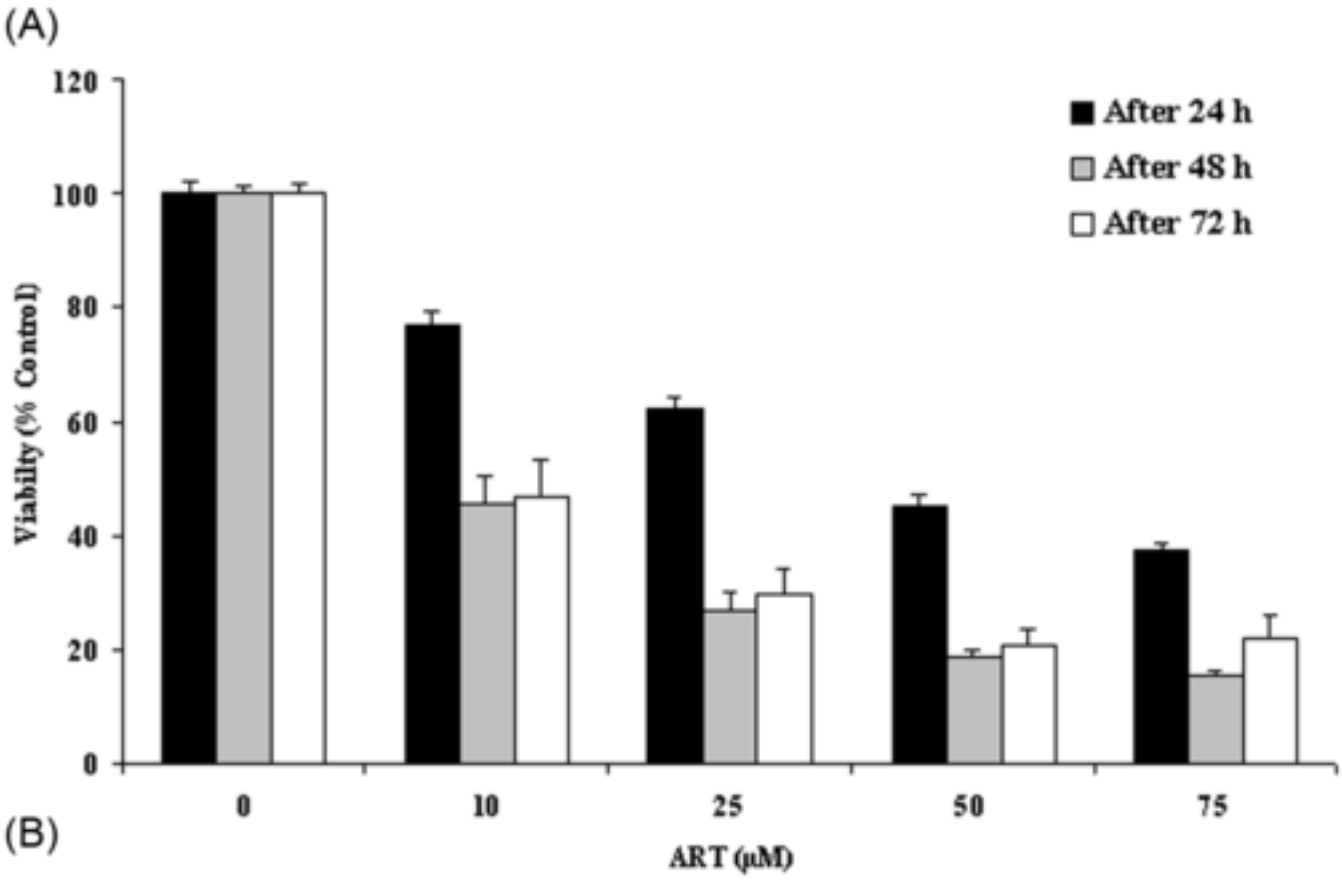
Fig.6. Biomarker analysis displaying common genes that were significantly regulated after treatment with 25μM ART for 48 h in Miapaca-2 (A) and BxPc-3 (B) cells.

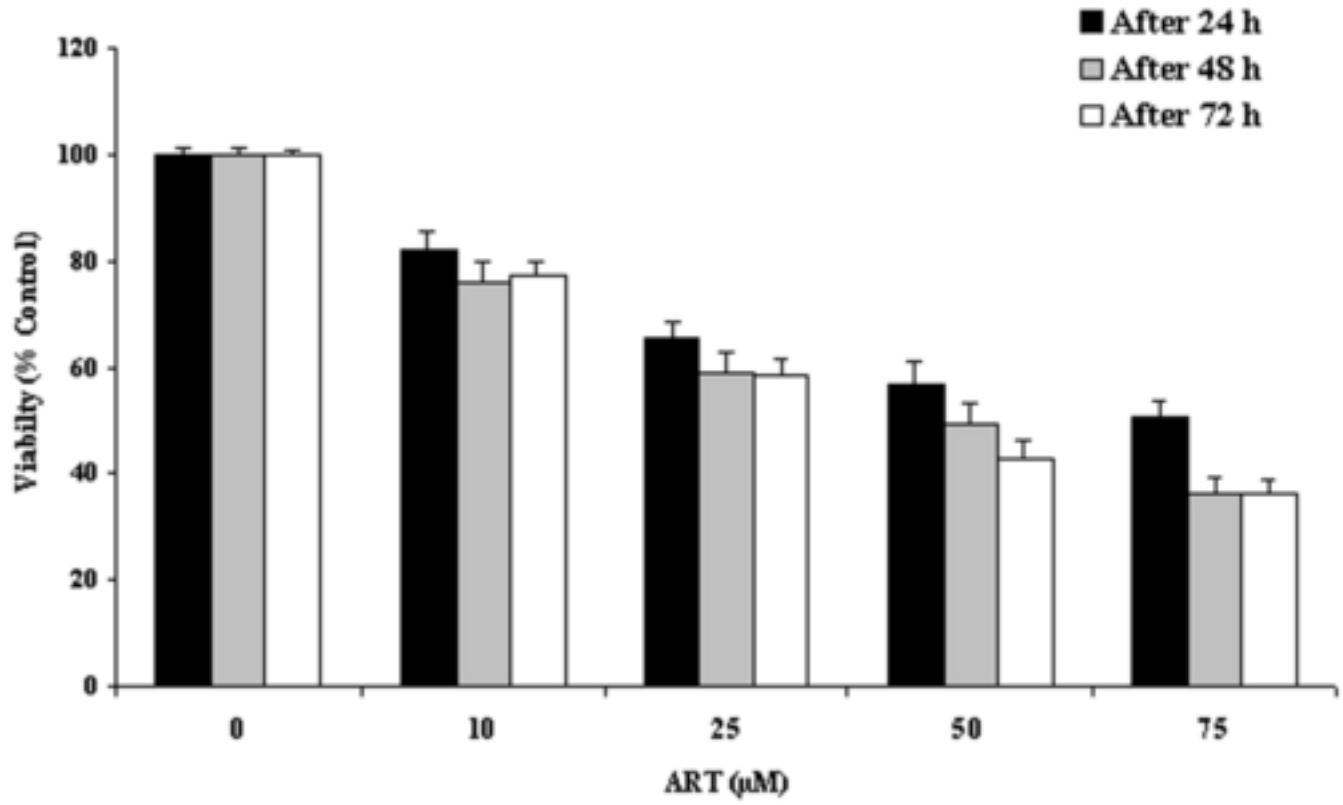
Fig.7. Specific biomarker genes significantly regulated after 48 h treatment with Artesunate (25μM) in Miapaca-2 (A) and BxPc-3 (B) cells.

Fig.8. Action of ART on topoisomerase IIα: (A) Supercoiled plasmid DNA (pRYG) was incubated with topoisomerase IIα and various concentrations of ART or etoposide. The reaction products were separated in 1% agarose gel; ethidium bromide staining was performed subsequent to electrophoresis. The supercoiled DNA was relaxed by the enzyme and separated according to its supercoils status. Relaxed DNA labels all molecules of zero to seven supercoils. All other molecules ran in one band (supercoiled DNA). Lane 1, pRYG; lane 2, pRYG and topoisomerase IIα (no solvent control); All other lanes show reactions done in the presence of DMSO, which was used as a drug solvent. Lane 6, pRYG and topoisomerase IIα plus DMSO (solvent control); Lanes 3 to 5, pRYG and topoisomerase IIa in the presence of ART 25, 50 and 100 μM; lanes 7 to 9, pRYG and topoisomerase IIα in the presence of 25, 50 and 100 μM etoposide (inhibition control). (B) Supercoiled plasmid DNA (pRYG) was incubated with topoisomerase IIα and various concentrations of ART or etoposide. The reaction products were separated in 1% agarose gel in presence of 0.5 μg/mL ethidium bromide. Lane 1, pRYG; lane 2 pRYG and topoisomerase IIα (no solvet control); lane 3, pRYG and topoisomerase IIα in the presence of DMSO (solvent control); Lane 4 to 6,

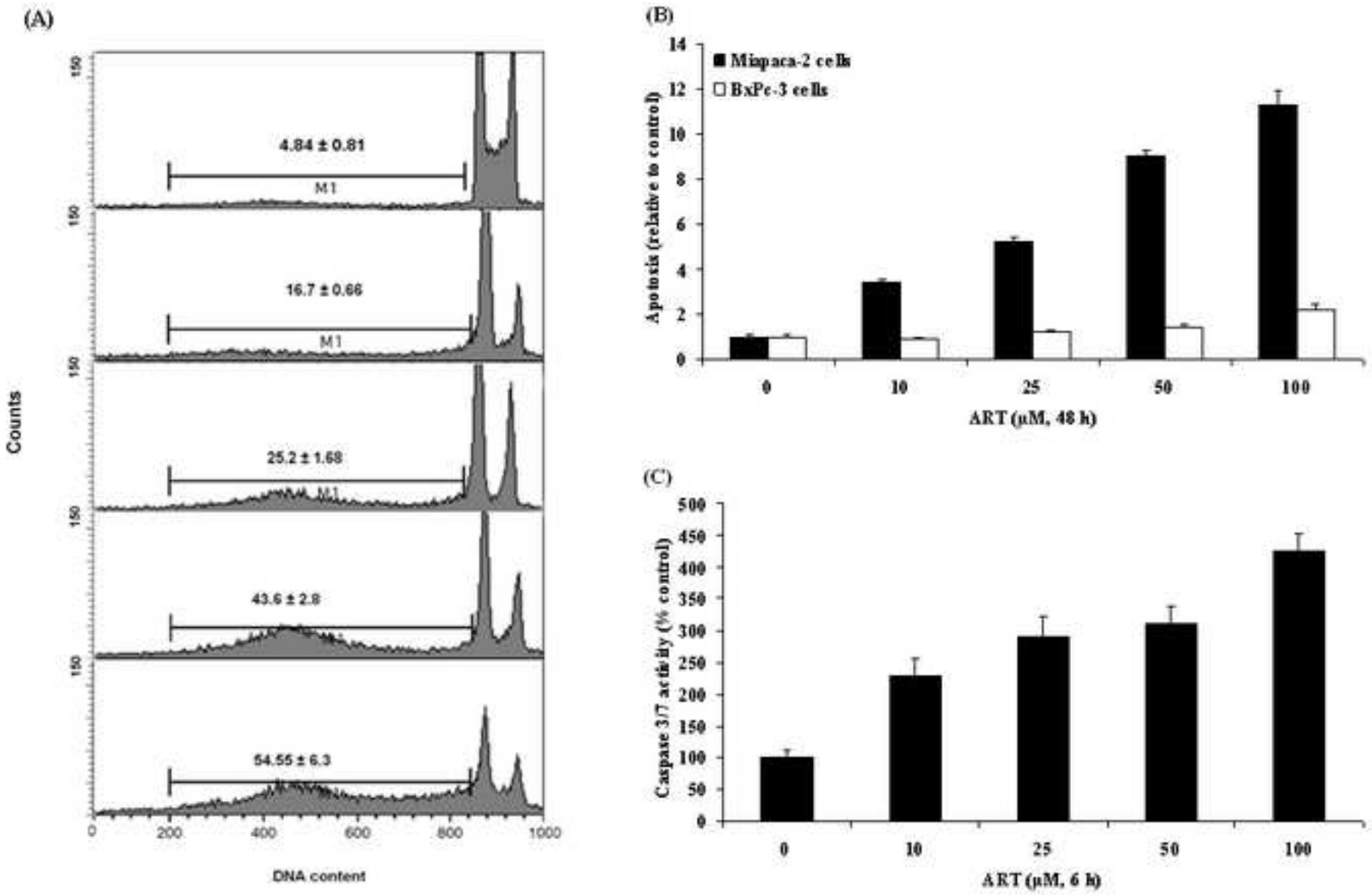
pRYG and topoisomerase IIα in the presence of 25, 50 and 100 μM ART; lanes 7 to 8, pRYG and topoisomerase IIα in the presence of 100, 150 μM etoposide; lanes 9 is linear pRYG DNA.

Fig.9. Potentiating effect of Artesunate on the growth inhibition of gemcitabine. The viability of MiaPaCa-2 (A) and BxPc-3 (B) cell lines after treatment with or without gemcitabine alone or in combination with Artesunate (10 μM) was assessed by MTS assay All results are represented as mean values ± SE of the percentage cell viability relative to the untreated control. * $P < 0.05$.

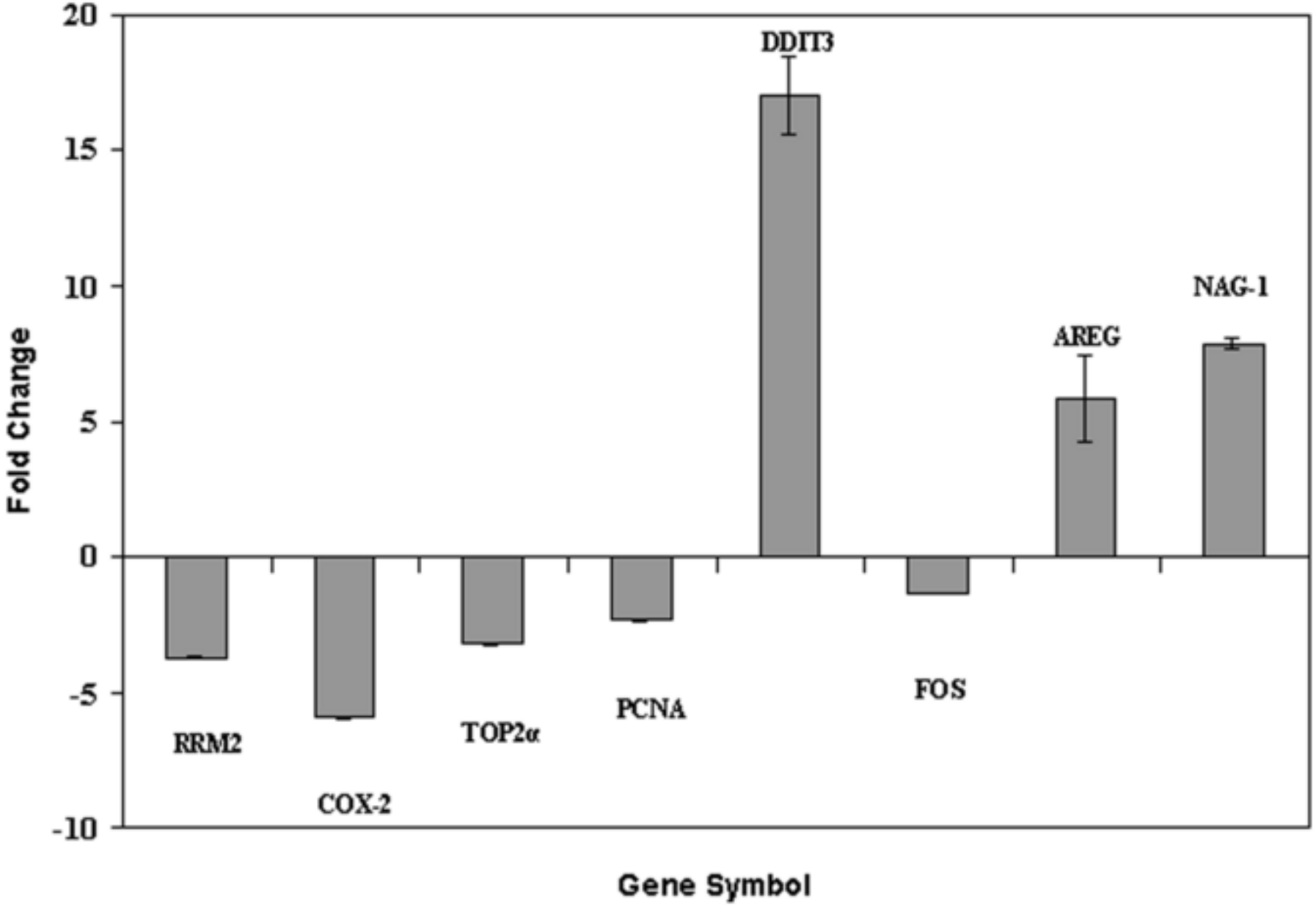




Figure


(A)
4.84 ± 0.81
M1
16.7 ± 0.66
M1
25.2 ± 1.68
M1
43.6 ± 2.8
54.55 ± 6.3
Counts
DNA content
(B)
Mispaca-2 cells
BxPc-3 cells
Apotosis (relative to control)
ART (μM, 48 h)
(C)
Caspase 3/7 activity (% control)
ART (μM, 6 h)

Figure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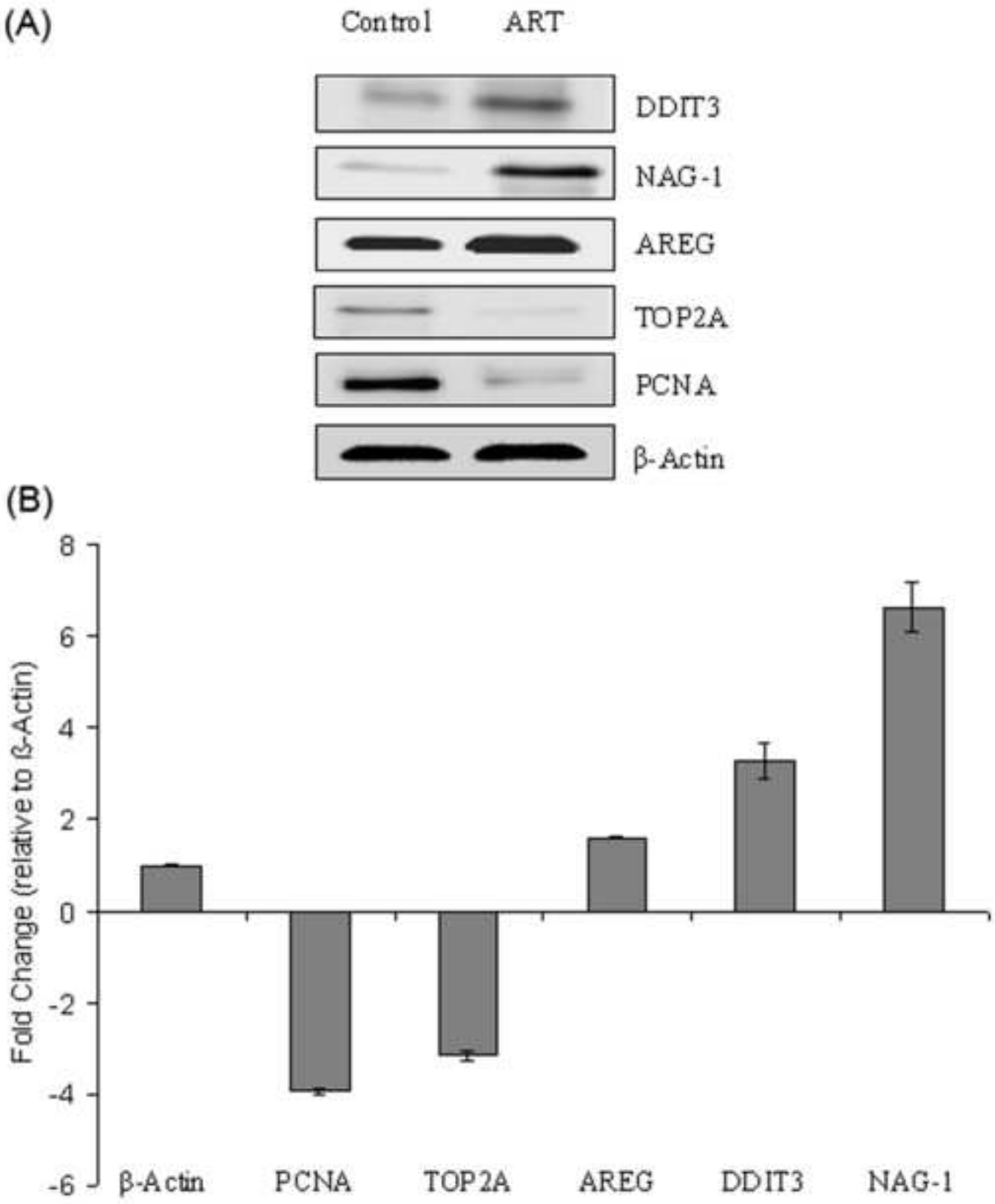
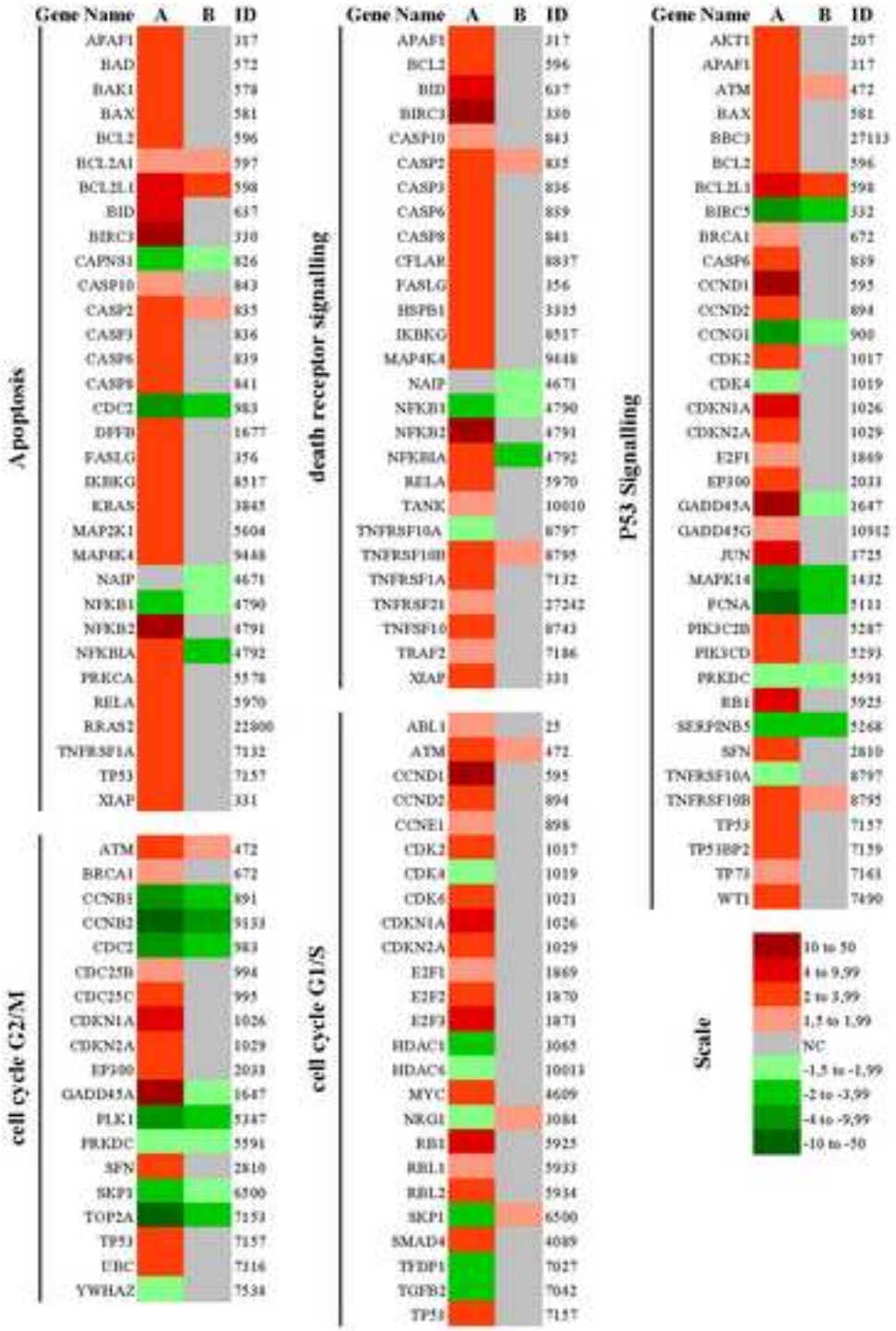
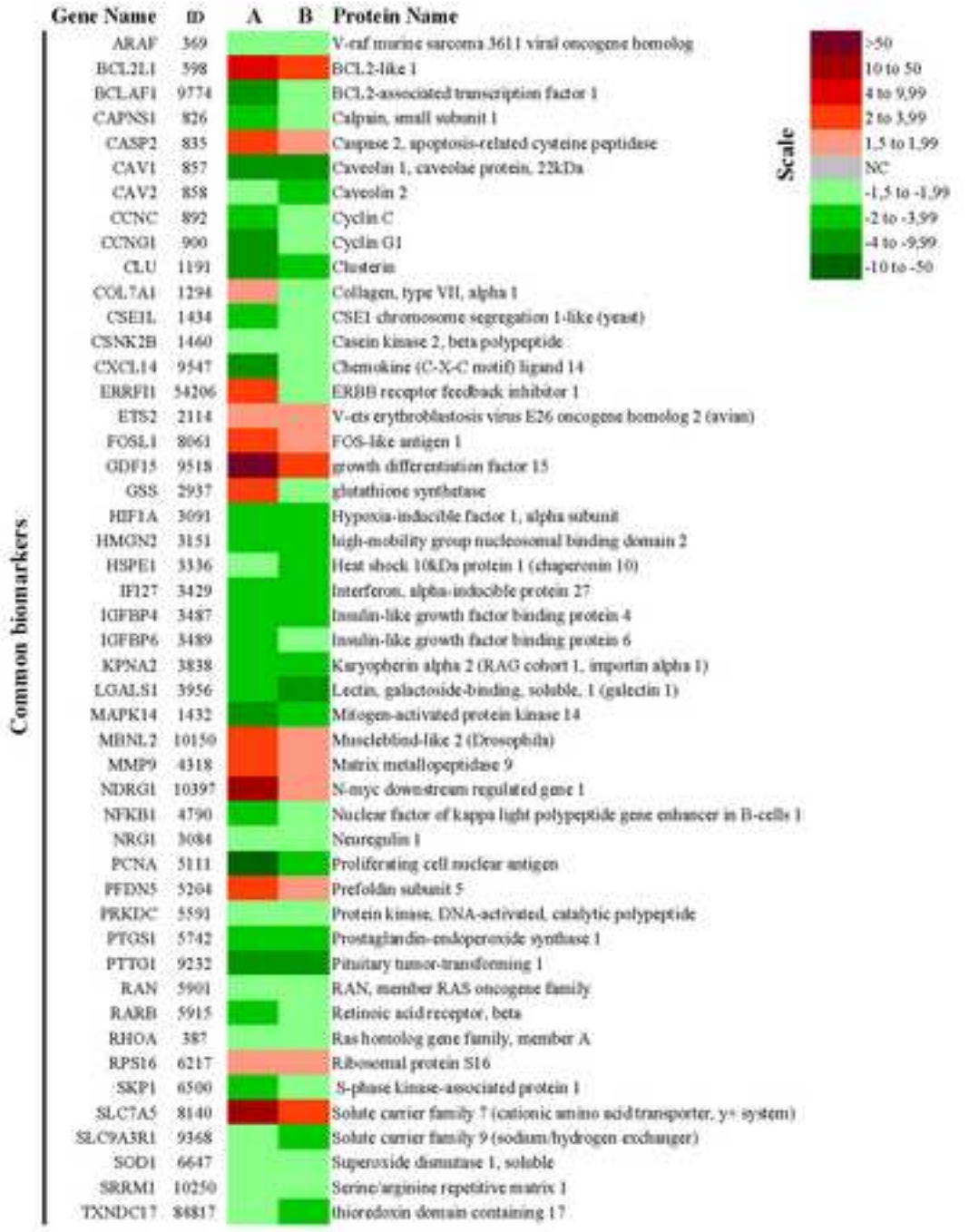
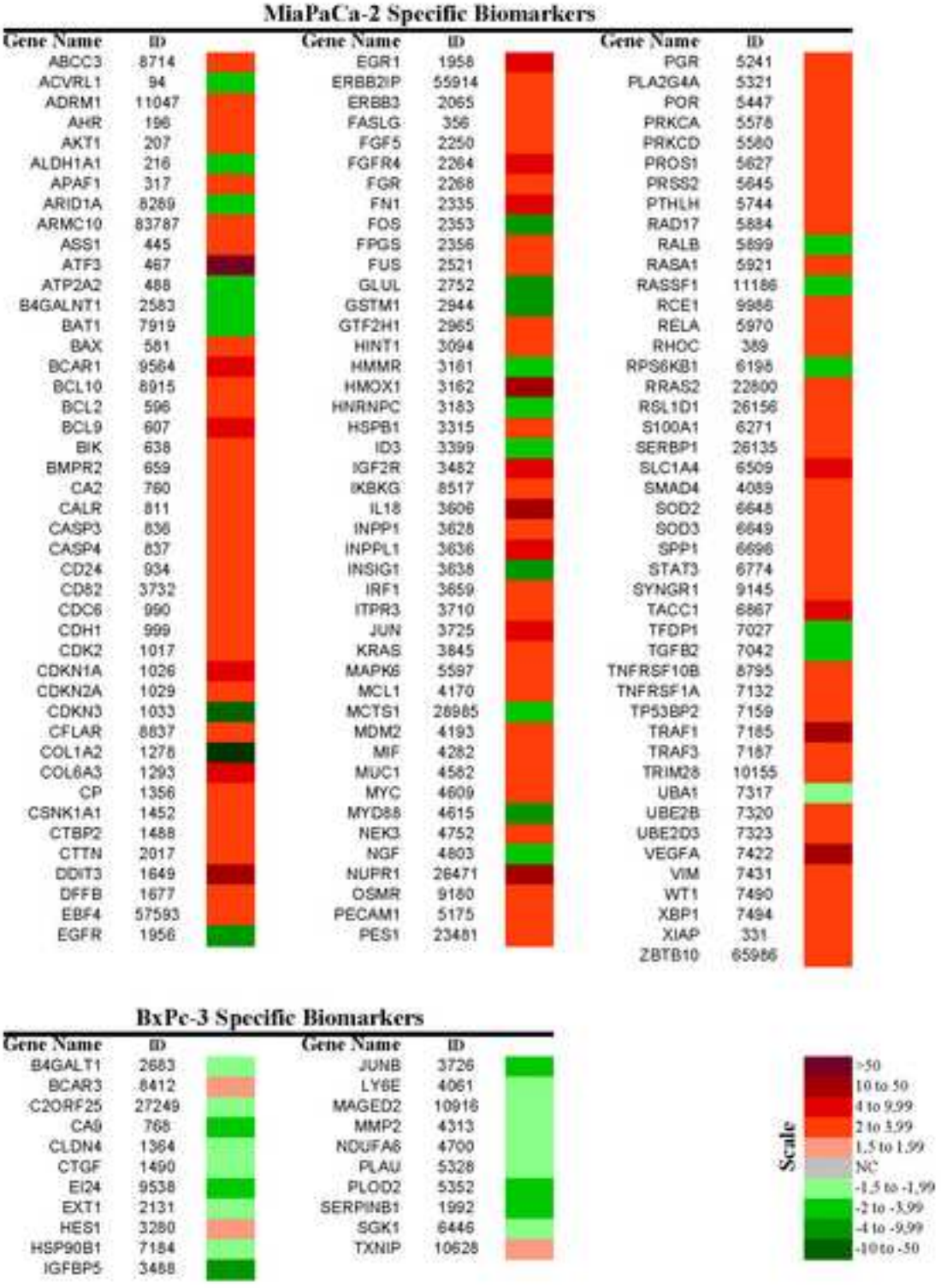
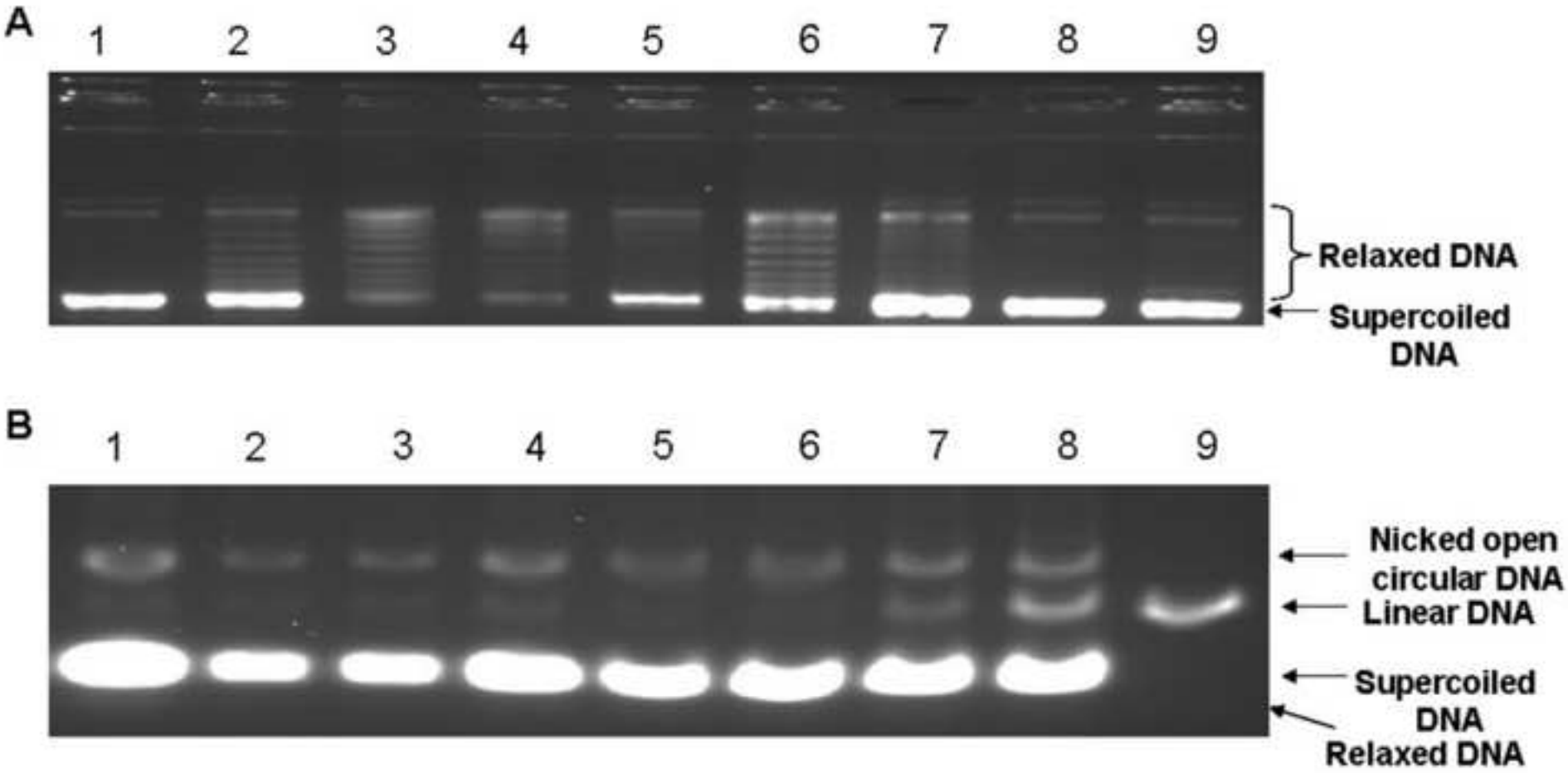








Figure


A
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Nicked open circular DNA
Linear DNA
Supercoiled DNA
Relaxed DNA