

Original Paper

New Insights in Gene Expression Alteration as Effect of Paclitaxel Drug Resistance in Triple Negative Breast Cancer Cells

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Key Words

Triple negative breast cancer • Paclitaxel • Drug resistance

Abstract

Background/Aims: Triple negative breast cancer (TNBC) is a highly aggressive form of cancer which lacks targeted therapy options. Thus, TNBC patients have poor outcomes and a decreased survival rate than patients with other types of breast cancers. Due to the lack of surface receptors, TNBC needs a comprehensive investigation to provide more information regarding patient's therapy, as well as to understand the way how to counteract drug resistance mechanisms. Nowadays, chemotherapy remains an unsolved issue which rise a lot of questions in oncology field. **Methods:** In this article, we investigated the implication of paclitaxel in TNBC cell lines after a prolong administration, after 12, respectively 24 passages followed by evaluation of morphological alteration, mutational pattern by next generation sequencing and the altered gene expression pattern by microarray technology and validation by qRT-PCR of the resistance to therapy relevant genes. **Results:** Using functional assays, we showed that paclitaxel exhibits antiproliferative activity on Hs578T/Pax and MDA-MB-231/Pax demonstrating the activation of cell death mechanisms. Confocal microscopy revealed significant modifications which occur in the morphological structure with a disruption of the actin-filaments and also mitotic catastrophe. The presence of these nuclear alterations is due to some modifications at the cellular and molecular levels. Important alterations at the tran-

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scriptomic and genomic levels were observed from this a common drug resistance signature (*IL-6*, *CXCL8*, *VEGFA*, *EGR1*, *PTGS2* and *TRIB1*) for both cell lines at 24 passages was discovered. Also, an important mutation (TP53) linked with drug response was identified. **Conclusion:** These results might be used to furnish novel biomarkers in TNBC, as well as to find a strategy to counteract the resistance to therapy in order to increase survival rate and to enhance the prognosis of patients with TNBC.

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Introduction

Breast cancer persists to be the most prevalent solid tumor and remains the second-leading source of cancer-related death in women worldwide [1]. Triple-negative breast cancer (TNBC), which represents approximately 10-15% of all diagnosed breast cancers, is defined by the lack of expression level of estrogen and progesterone receptors, as well as exhibits a negative expression of Her2 protein (human epidermal growth factor receptor 2). TNBC manifests a more aggressive clinical behavior among patients and is the tumor subtype with the poorest prognosis compared to other breast cancer subtypes [2-5]. TNBC is a heterogeneous pathology with different subtypes characterized by distinct pathologic and clinical features [4, 6]. Nowadays, chemotherapy and radiotherapy remain the standard clinical therapies for TNBC where tolerance to chemotherapy severely affects the prognosis of patients with rapid development of drug resistance mechanisms [5, 7, 8].

Chemoresistance (anthracyclines, taxanes, alone or in combination with biological drugs) [9] is a major cause of treatment failure in a wide range of carcinomas raising a terrible issue in the oncology field. This issue affects the patients' recovery and adds a tremendous decrease in the survival rate. Most of patients develop resistance during chemotherapy (acquired resistance) or de novo lack of therapeutic response (primary resistance). Through acquired resistance, the presence of processes such as drug inactivation, drug target alteration, cell death inhibition, drug efflux, DNA damage repair, epigenetic alterations and epithelial-mesenchymal transition (EMT) are observed [10].

Paclitaxel is one of the most widely used chemotherapeutic agents in different types of cancer, including breast cancer, bladder cancer, non-small cell lung cancer, endometrial cancer and cervical carcinoma. It's mechanism of action is to suppress the dynamics of microtubule spindle resulting in the blockage of metaphase-anaphase transitions, which ultimately inhibits mitosis and induces apoptosis [1, 11]. In the most cases, development of cancer resistance to chemotherapeutic agents involves primarily modifications to their cellular target, including tubulin isotope selection, post-translational alterations in tubulin and associated regulatory proteins [11].

In this study, we investigate the impact of multiple dose exposures of paclitaxel on TNBC cells at cellular and molecular level; therefore, cell proliferation using MTT test, cytoskeletal alteration using confocal microscopy, genomic alteration using next generation sequencing and those at transcriptomic level by microarray at 12 respectively 24 passages on two TNBC cell lines are evaluated. Though bioinformatics tools we created significant networks to provide important clues related to the response to paclitaxel on TNBC cell lines. Furthermore, we aimed to determine if paclitaxel therapy induces the activation of specific genes involved in drug resistance mechanisms.

Materials and Methods

Cell culture and induction of paclitaxel resistance into TNBC cells

The experiments were performed on TNBC cell lines, MDA-MB-231 and Hs578T, which were kept in humidified atmosphere at 37°C with 95% air, and 5% of carbon dioxide. The Hs578T cell line was maintained in D-MEM high glucose (Gibco) supplemented with 10% fetal bovine serum (Gibco), 2 mM L-glutamine (Gibco), 0.01 mg/ml insulin, 1% Non-Essential Amino Acids Solution (100X, Gibco) and 1% Penicillin-

Streptomycin (Gibco), meanwhile MDA-MB-231 cell line was cultured in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco), 2 mM L-glutamine (Gibco) and 1% Penicillin-Streptomycin (Gibco). The paclitaxel-resistance TNBC cells was established by multiple dose exposures of paclitaxel (0.1 nM) following by the evaluation of different biological processes at passage number 12 (P12) respectively 24 (P24) (Supplementary Fig. S1 – for all supplemental material see www.cellphysiolbiochem.com).

Assessment of paclitaxel sensitivity

The cellular viability and the sensibility induced by paclitaxel were assessed through MTT assay on both TNBC cell lines. According to MTT assay, IC_{50} (half maximal inhibitory concentration) of the parental cells (Hs578T and MDA-MB-231) and paclitaxel-induced resistance cells (Hs578T/Pax and MDA-MB-231/Pax) was determined. At a seeding density of 10^4 cells/mL, TNBC cells were cultured and treated with step-wise doses of paclitaxel. Forty-eight hours after incubation, the proliferative activity was evaluated through the incubation of the treated cells with 1mg/ml MTT solution for 2 hours. Furthermore, the metabolized formazan salt was resolubilized in DMSO and the absorbance was read at 570 nm with a multi-plate spectrophotometer Synergy H1 (BioTek). Resulted data were expressed as mean \pm SD (standard deviation). The differences between experimental conditions and controls were analyzed using t test (statistically significant was considered $p < 0.05$). Statistical analyses were carried out using GraphPad Prism.

Actin-filaments assessment through confocal microscopy

To evaluate the modifications of actin-filaments, a triple fluorescence protocol was performed. At a seeding density of 1.2×10^4 cells/ml, TNBC cells were cultured and treated with 0.1nM paclitaxel as described in Supplementary Fig. S1. Forty-eight hours after incubation, cells were stained with DAPI dye (blue for nucleus), MytoTracker dye (red for mitochondria) and Phalloidin dye (green for actin filaments). Stained cells were examined using confocal laser scanning with an Olympus FLUOVIEW FV1200 microscope. Images were taken using the PLAPON60xOSC2 (1.4 NA) objective and a channel mode (three channels: 405/488/543 nm excitation). Acquisition was performed using FV10-ASW software.

Mutation induction as effect of paclitaxel exposure using Next-Generation Sequencing Panel (NGS) and Ion Torrent PGM platform

DNA was extracted from control TNBC cells (Hs578T and MDA-MB-231, both cell lines at passage P0) as well as paclitaxel-resistance TNBC cells (Hs578T/Pax and MDA-MB-231/Pax, at passage P12 and P24) using Purelink Genomic DNA mini kit from Life Technologies according to manufacturer's instructions. Samples were sequenced using Ion Ampliseq Cancer Panel Pool v2, which consists of the most relevant hot spot mutations and Ion Torrent PGM Next Generation Sequencer (Thermo Fisher Scientific). 20 ng of DNA was used to prepare the amplicon libraries and the Ion Ampliseq™ Library Kit 2.0 (Life Technologies) following by a purification step using AMPure XP Beads (Beckman Coulter). Further, Qubit 2.0 was used for the quantification using Qubit HS DNA kit. Four bar-coded 100pM-diluted libraries were used for each Ion 316 Chip (Thermo Fischer Scientific) and using the Ion PGM HI-Q Sequencing 200 kit the sequencing was performed on Ion Torrent PGM Machine (Thermo Fischer Scientific). The software Torrent Suit 5.6 and Ion Reporter 5.6 executed the bioinformatic analysis for data trimming alignment and variant calling.

Gene expression evaluation as effect of multiple paclitaxel dose exposure

Total RNA extraction from TNBC cells was performed using TriReagent (Invitrogen) according to the manufacturer's instruction. The RNA concentration and quality were assessed using Nanodrop-1000 spectrophotometer (Thermo Scientific) respectively 2100 Bioanalyzer (Agilent Technologies). 200 ng of total RNA ($RIN \geq 7$) was used to synthesize fluorescent cyanine-3-labeled cDNA following the Agilent one-color Quick-Amp labeling protocol (Agilent Technologies) and hybridized onto Agilent Whole Human Genome 8 \times 60K microarrays according to Agilent standard protocol. The microarray slides were scanned using the Agilent G2505C Microarray Scanner (Agilent Technologies). The scanned images were transformed into TXT files using the Agilent Feature Extraction Software (Version 10.7.3.1, Agilent Technologies), and integration of the data in the biological context were done using Venny (<https://bioinfogp.cnb.csic.es/tools/venny/>), Panther Gene Ontology (<http://pantherdb.org>) and String 11.0 (<https://string-db.org>).

qRT-PCR data validation

1000 ng of total RNA was reversed transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression evaluation was conducted using SYBR Select Master Mix (Applied Biosystems) and RT-qPCR was performed on ViiA7 System as a 10 μ l volume reaction using a 384-well plate. As an internal control we used B2M and GAPDH genes. Relative quantification was conducted using the $2^{-\Delta\Delta CT}$ method.

Results

Cellular viability and evaluation of paclitaxel sensibility on TNBC cell lines

Cellular viability was investigated on TNBC cells to evaluate the cytotoxicity effect of paclitaxel treatment. The activity was assessed on both cell lines, Hs578T and MDA-MB-231 (both at passage P0, considered as control group) as well as paclitaxel-induced resistance cells (Hs578T/Pax and MDA-MB-231/Pax, both at passage P12, respectively P24). Cells, at passage P0, P12 respectively, P24 were treated with stepwise doses of paclitaxel with concentration between 0-200 μ M. In Fig. 1, IC_{50} values are shown at 24h; data presented as % of control in relation with log (concentration, μ M). According to IC_{50} values, was shown that multiple dose exposures to paclitaxel increase the response to paclitaxel treatment. Only at passage P24 a slightly increased of IC_{50} values was observed, which is related to the activation of drug resistance mechanisms after a multiple dose exposure to paclitaxel.

Morphological traits assessment post-therapy with multiple paclitaxel doses

Confocal microscopy was used to investigate if the loss of viable cells was associated with morphological alteration, particular with apoptosis activation. Fig. 2 shows that Hs578T and MDA-MB-231 control cells exhibited no morphological alterations and no sights of apoptosis. At P0, a concentration of 0.1 nM paclitaxel was added, and some alterations were observed in morphology structure compared to the control group. In this regard, the presence of nuclear damage/fragmentation (indicated with orange arrow) was found. After a prolonged multiple dose exposure with paclitaxel (P12 and P24), significant alterations in cellular morphology, which highlighted the presence of micronucleus (magenta arrows), nuclear damage/fragmentation (orange arrows) and multinucleated/polynuclear cells (yellow arrow) were observed. Also, the effect of paclitaxel at passage P24 induced actin-filaments disruption and giant cells were observed, traits that are important sights involved in apoptosis stimulation. In the case of MDA-MB-231 cells, we shown that paclitaxel therapy induced morphological traits alterations characteristic of apoptosis stimulation. P0 exhibited the presence of cellular shrinkage (red arrows) and actin-filaments disruption. At passage, P12 and P24 the presence of cellular shrinkage (red arrows), nuclear damage/fragmentations

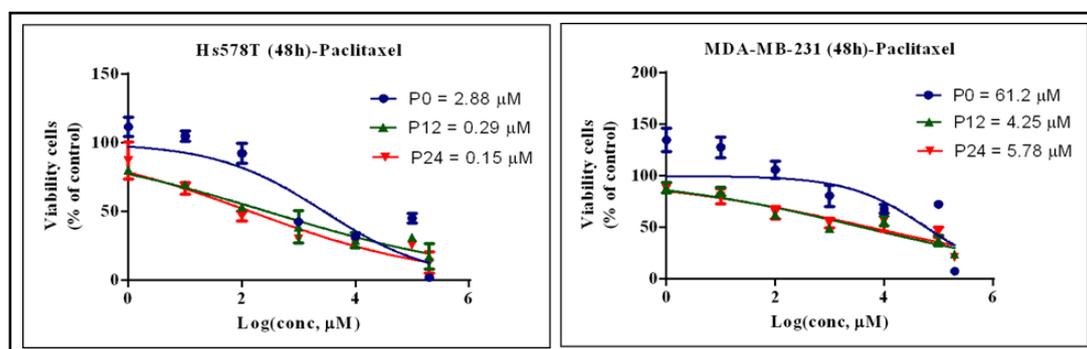


Fig. 1. The antiproliferative effect of paclitaxel evaluated at 48 hours on (A) Hs578T (parental cells and Hs578T/Pax at passage P12, respectively P24) and (b) MDA-MB-231 (P1 and MDA-MB-231/Pax at passage P12, respectively P24) cell lines using MTT assay. Log (concentration, μ M) = Log (concentration of bioactive compound, μ M) (mean \pm SD, n=3).

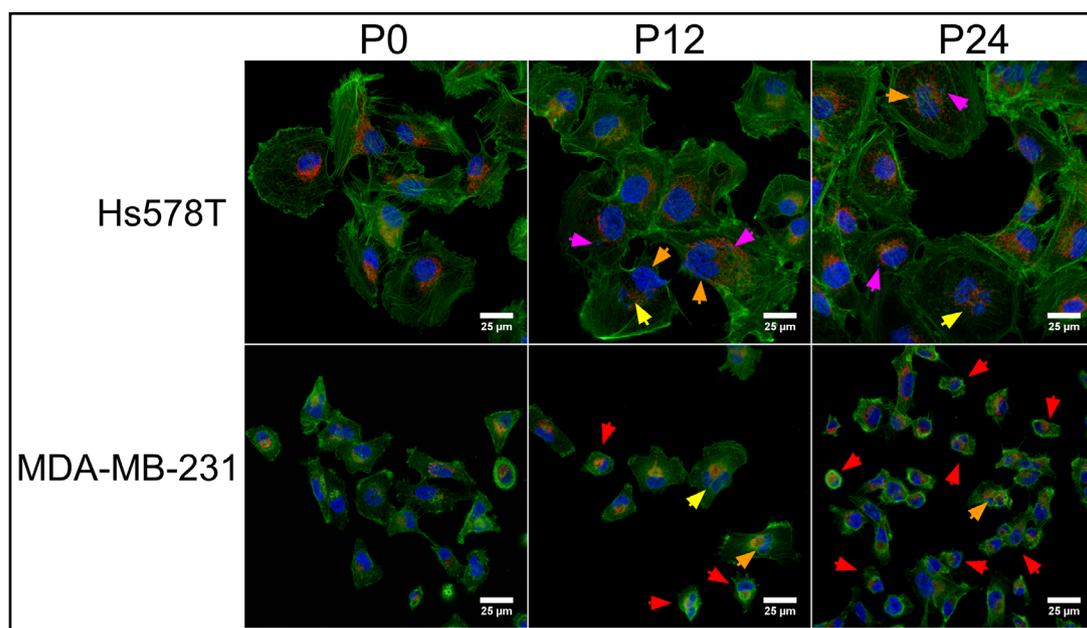


Fig. 2. Alterations of cellular morphology after a prolonged exposure with multiple doses of 0.1 nM paclitaxel. Morphological traits were visualized using confocal microscopy. The cellular components were stained with Phalloidin-FITC dye specific for actin filaments, DAPI dye for nucleus and MytoTracker for mitochondria. Scale represents 25 µm. Images were captured with 60x oil immersed objective. Magenta arrows represent the formation of the micronucleus, yellow arrows point multinucleate/polynuclear cells, red arrows denote cellular shrinkage and orange arrows point nuclear damage/fragmentation.

(orange arrows) as well as multinucleated/polynuclear cells (yellow arrow) were observed. Moreover, treated cells exhibited intracellular vacuolization, chromatin condensation and actin-filaments disruption, being important morphological traits specific of apoptosis.

Identification of mutation signatures in TNBC cell lines post-therapy with paclitaxel

In order to highlight the presence of mutation in all three time-points, P0 (control group), P12 respectively P24, we analyzed the mutation patterns for both TNBC cell lines, Hs578T/Pax and MDA-MB-231/Pax. Fig. 3 showed the number of mutations identified in each treatment scenario. Thus, all passages for Hs578T/Pax cell line retained the same mutations, but it can be observed in Fig. 3A that P0 do not present any mutations in *TP53* (c.215 C>G) and *NOTCH1* (c.4732_4734 delGTG) genes. We observed that at P12 and P24, a mutation identified in *TP53* gene exhibit implication in drug response according to Clinvar data base, mechanisms which altered the response to paclitaxel therapy. Based to NGS data, it was found that the mutation in *NOTCH1* gene in P24, is variant of unknown significance. The alterations that occur in *HRAS* (c.35G>A) and *HNF1A* (c.817 A>G) genes are associated with pathogenic mutations. Meanwhile, the second TNBC cell line, MDA-MB-231/Pax, exhibited different mutations compared to Hs578T/Pax cell line (Fig. 3A-B). Also, P0 revealed the absence of mutations in several genes, such as *PIK3CA* (c.352+40 A>G), *KDR* (c.798+54 G>A), *CSF1A* (c.*37delT), *CSF1A* (c.35instA), *TP53* (c.215 C>G) and *SMAD4* (c.955+58 C>T). Also, the mutation observed in *TP53* gene is similar between both TNBC cell lines, which is associated to drug response. Moreover, the alteration in *KRAS* (G>A) gene is a pathogenic mutation. The mutated genes in TNBC have both intronic and exonic locations. The exact locations of the mutations identified in this study are presented in Fig. 3 (Fig. 3D for Hs578T cell line, respectively Fig. 3E for MDA-MB-231 cell line).

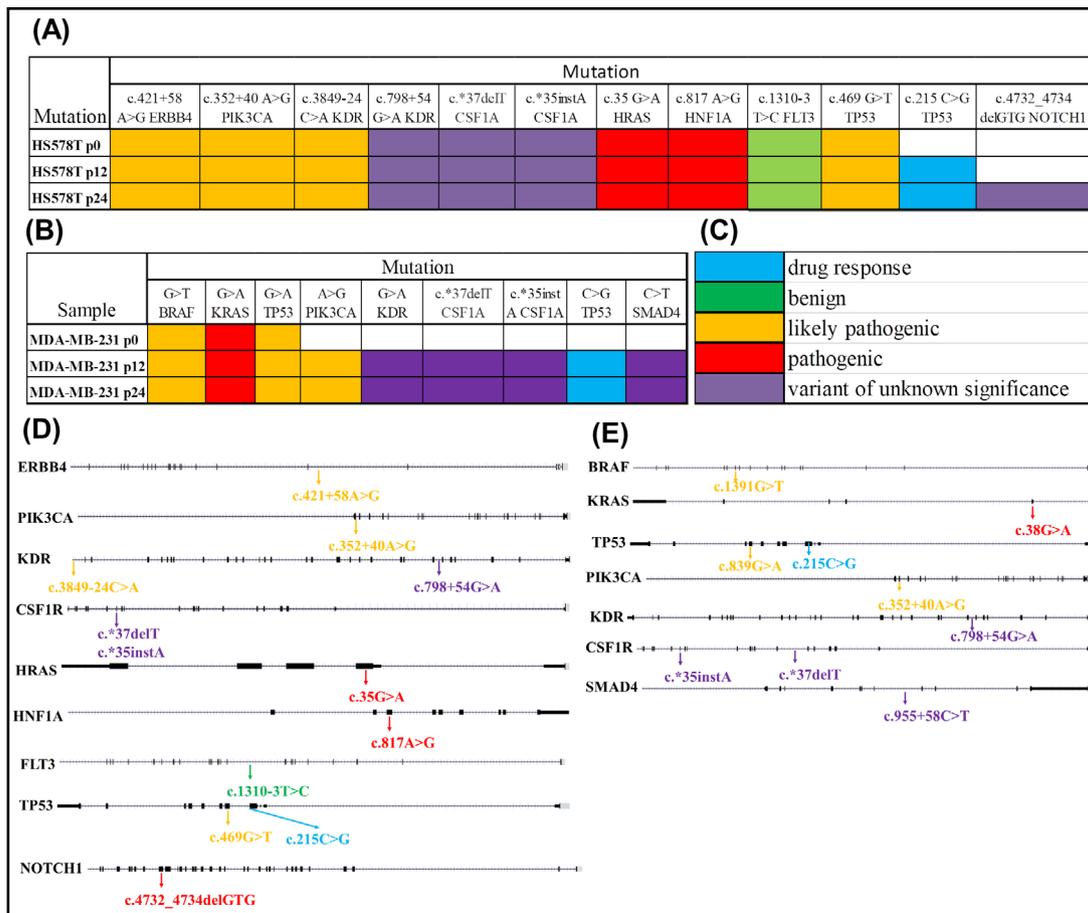


Fig. 3. Evaluation of mutation patterns in TNBC cell lines, Hs578T/Pax and MDA-MB-231/Pax, post-therapy with paclitaxel using Ion Torrent PGM Machine and Ion AmpliSeq Cancer Hotspot Panel Pool v2. (A) Mutation patterns detected in the HS578T/Pax samples (B) Mutation patterns detected in the MDA-MB-231/Pax samples. (C) mutation legend: blue - drug response, green - benign, orange - likely pathogenic, red - pathogenic, purple - unknown significance (according to Clinvar). Localization of mutations in each of the gene sequences for the most frequent mutated genes in TNBC cells for (D) Hs578T samples and (E) MDA-MB-231 samples.

Alteration of the mRNA profiles of the TNBC cells as effect of multiple doses exposure of paclitaxel

We performed microarray profiling of mRNAs in order to identify the most relevant altered mRNAs based on the analysis of P12 versus P0 (control group), respectively P24 versus P0. A cut-off value for FC (fold change) of ± 2 and $p \leq 0.05$ was selected to determine the modifications occur in gene expression patterns, in Table 1 are presented top 20 altered transcripts. The heatmap for this data is shown in Fig. 4A and 4B. The bioinformatics analysis showed significant alteration on gene-expression profile at P12 indicating 2731 differentially expressed mRNAs between Hs578T/Pax and MDA-MB-231/Pax, including 1384 down-regulated and 1347 upregulated mRNAs. In addition, 2406 mRNAs were differentially expressed between Hs578T/Pax and MDA-MB-231/Pax at P24, including 1317 down-regulated and 1089 upregulated mRNAs. We overlapped the altered mRNAs profiling data obtained from the datasets mentioned above and though Venn diagram are represented the common mRNAs altered in both TNBC cell lines in all passages, P12 respectively P24 (Fig. 4C, D). An overlapping of the common signature among at P24 on the two cell lines is being presented in Fig. 5A for the genes and Fig. 5B for lncRNAs.

Table 1. Top 20 most abundant altered coding and non-coding transcripts as effect on paclitaxel exposure after 12 (p12), respectively 24 serial doses (p24) on MDA-MB-231 and Hs678T cell lines

Cell line	Analysis	RNA species	Upregulated	Downregulated
Hs578T	P12 versus P0	Gene	FOS, MX1, FOSB, OAS1, IFITM1, IFI44L, IFI27, MX2, RCSD1, EGR1, BST2, CXCL2, RSAD2, OAS2, BST2, IFI44, CHI3L1, ATF3, PKNOX2, PCSK2	LCP1, NEFL, LMO7DN, JAKMIP3, NR0B1, NCAM1, LGI4, ARHGDIB, HLA-DPB1, EPB41L4A, KRT83, NAP1L3, SULT6B1, EMILIN2, GABBR2, SH3GL3, NEFM, CPEB1, SCN3A, TSPYL5
		lncRNA	LOC100133669, RNA5-8S5, LINC01016, lnc-ARRDC3-1, SNORD3B-1, LOC100506474, LOC101929174, LOC152286, lnc-ACACA-1, lnc-RNF13-2, lnc-PRDM9-3, LOC102723456, XLOC_I2_012847, C1orf167, LINC01127, lnc-CD44-1, CATIP-AS1, LOC100506098, lnc-IL1R2-1, XLOC_I2_012847	TMEM200C, LINC01197, LOC101928942, CAMK1D, RNA18S5, LOC100507377, XLOC_I2_015885, LOC284009, C16orf97, MKL2, lnc-FGF20-2, MSR1, ZNF790-AS1, lnc-SPATA9-1, SP3P, ZNF385D-AS2, XLOC_I2_008783, lnc-C11orf44-2, LOC100132495, STAM-AS1
	P24 versus P0	Gene	FOS, PCSK2, RCSD1, SLC12A7, CXCL2, EGR1, CXCL3, PKNOX2, DRD1, IFITM1, KIR2DS4, MX1, IFI27, OAS1, CXCL8, IL-6, SLC2A5, SMIM1, HS3ST2, BST2	LCP1, HLA-DPB1, NCAM1, GALNT14, KRT83, LPP, NEFM, RHOJ, ZBTB32, DCLK1, C3AR1, SH3GL3, SIRPB2, TAS2R30, CRISPLD2, CILP, CNN1, MSR1, TMEM108, SOX6
		lncRNA	CYP2S1, lnc-KLF3-2, lnc-ARRDC3-1, C8orf4, lnc-CTD-2054N24.2.1-3, LOC100128001, LOC101927041, C1orf167, LOC152286, lnc-MYBP2-2, LOC100133669, C1orf100, lnc-KIF1C-2, LOC100506098, GLYCAM1, LOC100506474, lnc-SDK2-5, FER1L4, FCGR2C, MRV1-AS1	XLOC_I2_015885, TMEM200C, RNA28S5, RNA18S5, lnc-ANKRD53-1, lnc-CDKAL1-1, ZNF385D-AS2, lnc-CELA3A-2, LOC101930333, lnc-PLEKHA5-1, lnc-ARSL-1, lnc-AC078802.1-1, HAAO, LOC101929910, lnc-CTBP1-1, ASAP1-IT1, lnc-SYT16-1, LOC100128563, STAG2, LOC100507377
MDA-MB-231	P12 versus P0	Gene	OR2T34, HSPA6, TMEM95, APOL6, CCDC177, ARSH, OR5B21, RAPSN, NTSR2, TRPV4, ADRA1A, SELP, AXIN2, ANKRD20A2, ADAMTSL1, LAMB4, MGAT5, SUGCT, CCDC181	ZP1, CCL3, DOCK8, HS6ST2, CCDC85A, RAP1GAP2, KCNK7, DEFB124, C4A, ADAM11, PTPN6, FHAD1, FAM104B, SULT6B1, GAB3, AKR1B10, OLR1, HBD, CNH2, FGFR4
		lncRNA	LOC100505784, LINC01349, lnc-C14orf23-6, lnc-AP000790.1-2, LOC100996249, IGANRP, lnc-UEVLD-2, lnc-PER2-1, lnc-TBC1D12-1, lnc-TPCN2-5, CRHR1, lnc-CCDC68-1, LOC102724050, lnc-FAM22B-1, LOC_I2_009159, LOC100507073, lnc-RNF13-2, LOC100128563, lnc-PRR7-2, lnc-SERPINB3-3	lnc-POTEB-5, EPB41L4A-AS2, LINC01038, lnc-MCCC2-2, KLK13, lnc-DNAJC24-1, C16orf97, lnc-SYNPR-1, lnc-LRRTM1-1, TBC1D22A, lnc-ARID1B-1, ADAMTS17, LINC00969, lnc-UTP23-1, lnc-ZFHX4-2, FAM95B1, PLCD4, lnc-DHX34-2, lnc-TBC1D22A-2, LINC01545
	P24 versus P0	Gene	FOSB, FOS, SCN8A, HES1, NR4A2, LILRA5, FBLN2, ARHGEF18, ATF3, ABL2, CXCL2, IGANRP, DUSP1, KRT14, IL-6, NRCAM, PTGS2, ANTXRL, NKX2-1, ZFP36	KLHL10, SPARC, LGALS12, DOCK8, KCNMB3, PCBP3, ALOX5, KLRC1, GPR87, TEPP, DLGAP1, GPR65, PLG, LIPG, N4BP2L1, CRTAM, C1QTNF3, NAT16, NMNAT3, BEX5
		lncRNA	RNA5-8S5, ZNF385D-AS2, lnc-MRPL32-1, C21orf91-OT1, OR12D2, HCG2040054, FGF10-AS1, lnc-DYNLRB1-1, lnc-ROPN1L-2, XLOC_I2_005438, lnc-ENPP2-1, lnc-LPPR5.1-1, lnc-MTX2-1, lnc-CGN-1, lnc-TRAPPC8-2, SNORA80B, XLOC_I2_012748, LOC100128644, SNORD21, FAM230B	MIR146A, lnc-IL1R2-1, lnc-POTEB-5, LOC344887, lnc-RP11-712L6.5.1-2, XLOC_I2_002945, STAM-AS1, SAP30L-AS1, LOC101927751, LOC101928710, lnc-PRKRIR-1, EPB41L4A-AS2, lnc-RALGDS-2, LOC100131099, IGFBP7-AS1, lnc-CHSY1-5, lnc-GNLY-1, lnc-TCF19-1, DPY19L2P3, LOC100289230

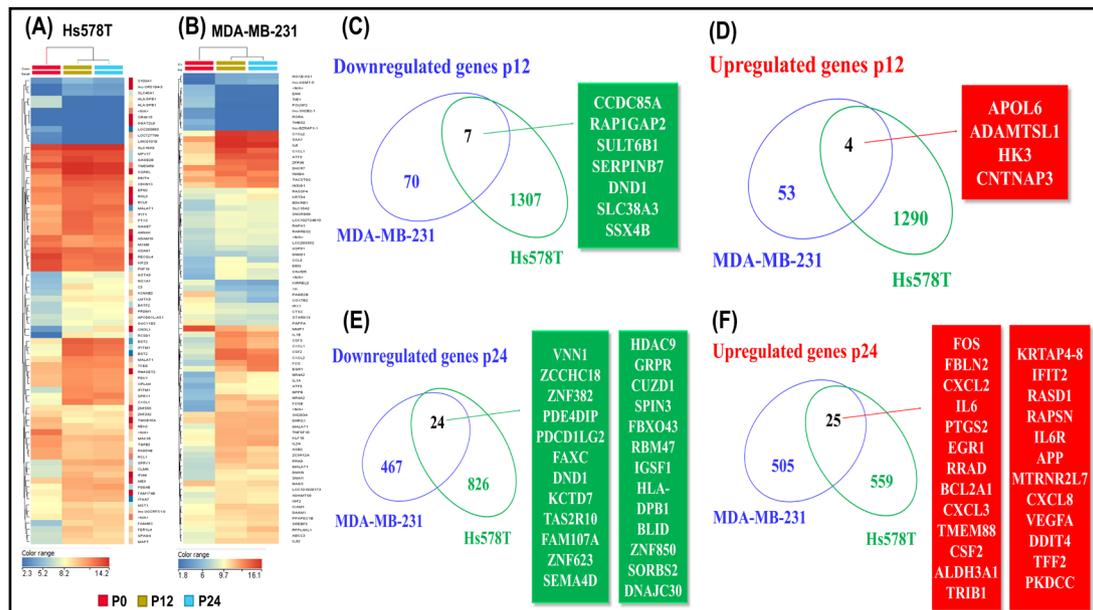


Fig. 4. Gene expression profiling of TNBC cell lines as effect of multiple dose exposure to paclitaxel. Heatmap emphasizing the altered genes signature at passage P12 and P24 in (A) Hs578T cell line and (B) MDA-MB-231 cell line; Venn diagram of the statistically significant ($FC \pm 2$ and $p\text{-value} \leq 0.05$) upregulated and downregulated gene expression by overlapping both TNBC cell lines; (C, D) common signature at p12, (C) downregulated and (D) upregulated genes selected cell line; (E, F) common signature at p24, (E) downregulated and (F) upregulated genes selected cell line.

Alteration of lncRNA profiles in TNBC cells as effect of multiple doses exposure of paclitaxel

The microarray data reveal important alteration on Hs578T/Pax and MDA-MB-231/Pax cell lines versus parental cell lines at the level of lncRNA. A total of 4342 lncRNAs were analyzed using the Agilent microarray technology and differences in lncRNAs-expression levels according to FC cut-off of ± 2 and $p \leq 0.05$ were considered significant (Table 2). In this regard, we identified the most relevant altered lncRNAs between both cell lines at P12 and P24, with the results indicating 1955 differentially expressed lncRNAs between Hs578T/Pax and MDA-MB-231/Pax at P12, of which 1027 were downregulated and 928 were upregulated. A total of 2102 differentially expressed lncRNAs were identified in the Hs578T/Pax and MDA-MB-231/Pax at P24, of which 1117 were downregulated and 985 were upregulated. We overlapped the altered lncRNAs profiling data obtained from the datasets mentioned above and though Venn diagram are represented the common lncRNAs altered in both TNBC cell lines at P12 respectively P24 (Fig. 6A-D), emphasis a common downregulated signature of 10 lncRNA at P12, respectively 28 lncRNA at P24, meanwhile in the case of overexpressed lncRNAs 8 common at P12, respectively 14 at P24 were identified.

Biological analysis

In our survey of gene ontology (GO) biological processes, the function of the upregulated mRNAs in Hs578T/Pax cells at P12 were primarily involved in molecular function related to catalytic and binding activity. Otherwise, the main altered processes observed for both cell lines involved cellular or biological regulation processes (Supplementary Fig. S2). Circos representation for the cellular processes, biological processes and molecular function is presented in Fig. 7, which emphasizes the common processes among the two passages in the two cell lines.

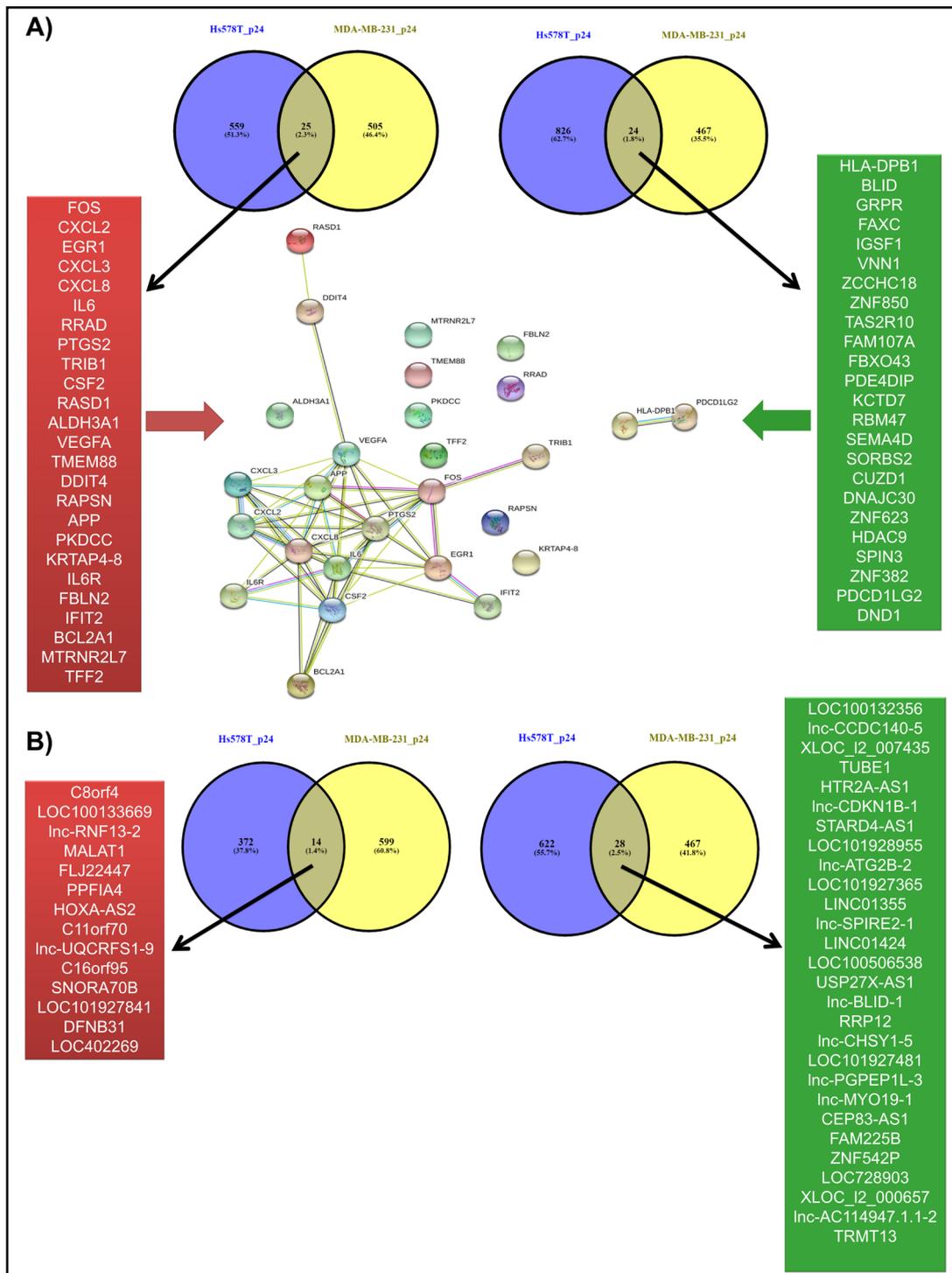


Fig. 5. Venn diagram in order to highlight the common. (A) mRNAs and (B) lncRNAs between Hs578T/Pax and MDA-MB-231/Pax at P24, both for up- and downregulated.

Table 2. Altered drug resistance genes as effect on paclitaxel exposure after 12 (P12), respectively 24 serial doses (P24) on MDA-MB-231 and Hs678T cell lines

Cell line	Analysis	RNA species	Upregulated	Downregulated
Hs578T	P12 versus P0	Gene	56: ABCB4, EGFR, HIF1A, TNF, VEGFA, IL-6, PTGS2, MYC, CD44, HLA-A, SOD2, LGALS3, TNFSF10, ABCC2, STAT1, CCL5, APOBEC3G, NFKBIA, ABCC6, KLF4, EGR1, SOCS3, SNAI1, CAT, ABCC3, MVP, FASN, S100A8, BMP7, ITGA6, CASP1, CLDN1, APOL1, ITGB4, CXCL1, LPIN1, ANGPTL4, AKR1C3, OCLN, IL24, ASS1, JUNB, ABCA3, AKR1C1, MSMB, SLC16A4, TFEB, KCNJ10, UGCG, TRIB1, SLC2A1, CISH, GSTA4, RNASET2, AKR1C4, SLC22A18, CCR10, MKNK2, LY6E, DTX3L	56: TP53, BCL2, BIRC5, BRCA1, CTNNB1, TERT, BRCA2, KIT, CXCL12, ATM, EZH2, ITGB3, MAPK8, MKI67, HSP90AA1, CDK1, EGF, PTPN11, RAD51, E2F1, DNMT1, TOP2A, THBS1, PRKDC, WNT5A, TPMT, POSTN, CDH2, SPHK1, NOX4, TGF2, BLM, FANCD2, RELN, ATF2, DIABLO, ATP7A, SALL4, MECOM, NEK2, KIF11, BRIP1, RRM2, SFPO, ST6GAL1, DAB2IP, FKBP4, ANXA3, SKIL, ASF1, KIF4A, WASF1, UBE2S, DAPK2, CCNF, E2F7, VASH2, E2F8, COBLL1, ADAL
	P24 versus P0	Gene	34: TNF, VEGFA, IL-6, PTGS2, CD44, SOD2, LGALS3, TNFSF10, ABCC2, PDGFRA, APOBEC3G, ABCC6, EGR1, SOCS3, CAT, ABCC3, FASN, ITGA6, ITGB4, CXCL1, LPIN1, IL24, ASS1, SLC16A4, TFEB, TRIB1, SLC2A1, GSTA4, RNASET2, SLC22A18, CCR10, PARP4, LY6E, TP53111	36: TP53, BCL2, CTNNB1, TERT, BRCA2, CXCL12, ATM, CD274, MAPK8, MKI67, HSP90AA1, CDK1, EGF, PTPN11, THBS1, GHR, EIF4E, CDH2, ITGA4, TGF2, FANCD2, RELN, DIABLO, ATP7A, BRIP1, RRM2, RALBP1, ST6GAL1, RAD18, ANXA3, ATP1B1, CLCN3, WASF1, MCTS1, CCNF, ADAL
MDA-MB-231	P12 versus P0	Gene	1: ATM	36: FGFR4, NRP2, MSX2
	P24 versus P0	Gene	20: VEGFA, IL-6, PTGS2, NR3C1, ABL1, PTPN11, RUNX1, CSNK2A1, EGR1, SNAI1, CDX2, PTPRC, CD24, MECOM, HIPK2, HOXA9, KCNJ10, TRIB1, WASF1, TSPAN8	13: TLR4, ADAM12, ZEB2, AKR1C3, CD19, AKR1C1, MSX2, HOXB4, BACH2, IL9R, SIRT5, RPS6KA2, APK2

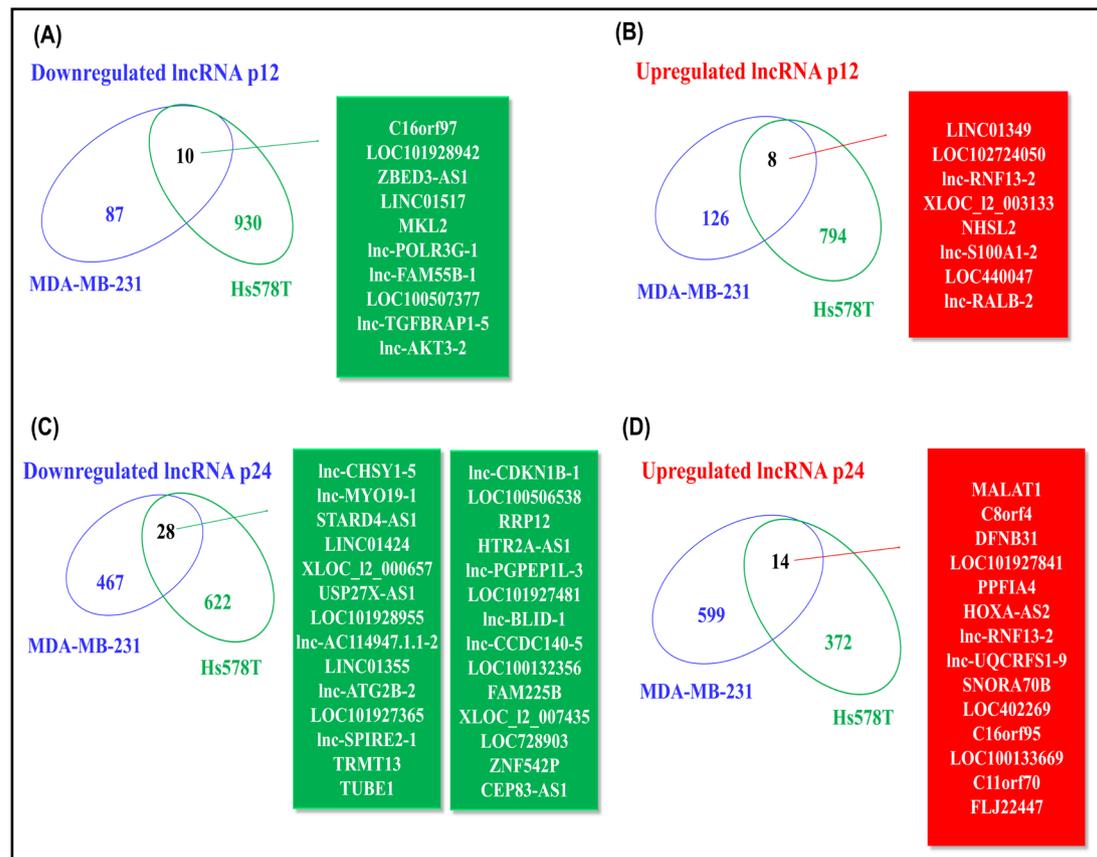


Fig. 6. Alteration of lncRNAs in TNBC cell lines as effect of multiple dose exposure to paclitaxel therapy on selected TNBC cell lines. Venn diagram of the statistically significant ($FC \pm 2$ and $p\text{-value} \leq 0.05$) upregulated and downregulated lncRNAs by overlapping both TNBC cell lines; (A, C) Common downregulated lncRNAs signature at P12, respectively P24; (B, D) Common overexpressed lncRNAs signature at P12, respectively P24.

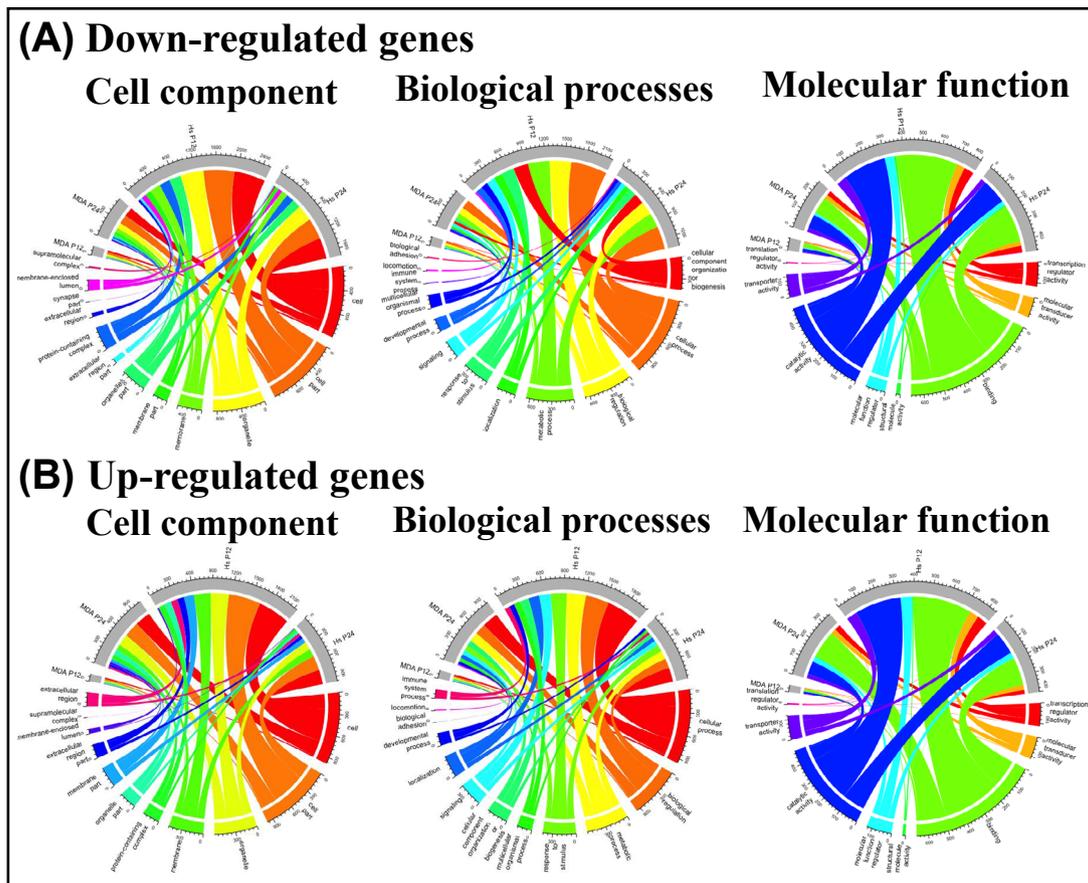


Fig. 7. Circos plot of the Gene Ontology enrichment analysis. Graphical representation of the cell component, biological processes and molecular function for (A) up-regulated genes for Hs578T/Pax and MDA-MB-231/Pax at P12 and P24 and (B) down-regulated genes for Hs578T/Pax and MDA-MB-231/Pax at P12 and P24.

Therefore, we overlapped both cell lines, Hs578T/Pax and MDA-MB-231/Pax at P24 and we identified several common upregulated and downregulated genes. We identified 25 altered genes between both cell lines involved in pathways like pathways in cancer, PI3K-Akt signaling pathway, cytokine-cytokine receptor interaction, microRNAs in cancer, chemokine signaling pathway. Also, these genes are involved in different biological processes such as signaling, cell communication, signal transduction, positive regulation of biological process, cellular response to stimulus, positive regulation of cellular process, response to chemical, response to stimulus, negative regulation of biological process, multicellular organism development, regulation of biological process, regulation of cellular process, localization, cell differentiation. Meanwhile, Venn diagram showed a list of 24 altered genes with down-regulated expression which do not exhibit any connection between altered genes (Fig. 6A). Otherwise, common altered lncRNAs are also presented in both cell lines, Hs578T/Pax and MDA-MB-231/Pax at P24 (Fig. 6B).

Activation of relevant transcripts involved in drug resistance mechanisms

In this section, we intended to analyze if the obtained results are involved in the activation of drug resistance mechanisms. We downloaded from PubMed Central the list with all drug resistance genes in order to highlight the common genes involved in this process. Thus, we tried to provide a comprehensive understanding regarding the results obtained through microarray technique. Through the activation of drug resistance mechanism, cancer cells become resistance to therapy though the alterations which occur in specific genes. After a prolong therapy, it is known that tumor cells do not response to therapy, cell death is inhib-

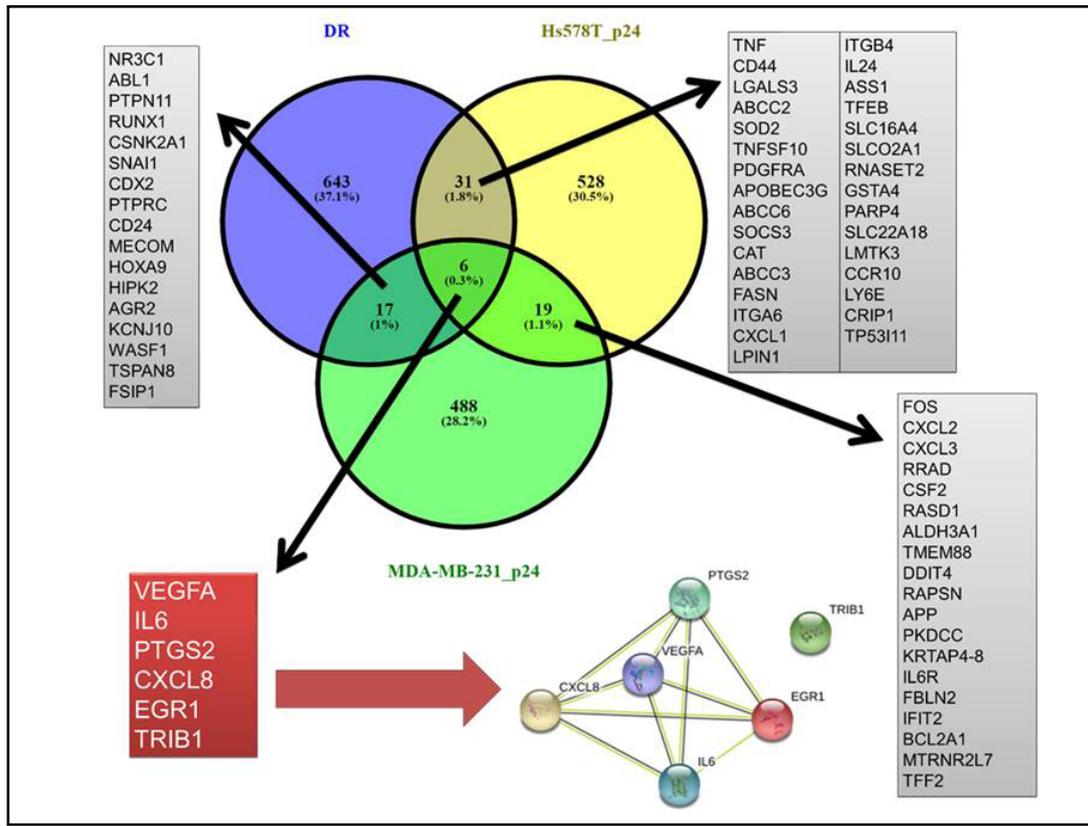


Fig. 8. Common drug resistance gene expression signature. (A) Venn diagram used to emphasize the common signature among the drug resistance gene list (downloaded from NCBI) and the overexpressed gene list at P24 on Hs578T/Pax and MBA-MB-231/Pax cell lines. (B) Interaction network using String software for common genes signature involved in drug resistance mechanisms.

ited and tumor growth is stimulated. In order to counteract this mechanism, a deep look and new therapeutic strategies are needed. In Fig. 8 are highlighted the common altered genes between drug resistance list and both cell lines. We observed the presence of six genes involved in drug resistance mechanisms, genes which exhibited an elevated expression profile, *VEGFA*, *IL-6*, *PTGS2*, *CXCL8*, *EGR1* and *TRIB1*. These genes are involved in important pathways, including pathways in cancer, PI3K-Akt signaling pathway, cytokine-cytokine receptor interaction, Human papillomavirus infection, microRNAs in cancer, transcriptional misregulation in cancer. As biological processes, the implication in regulation of molecular function, regulation of signal transduction, negative regulation of cellular process, negative regulation of nitrogen compound metabolic process, positive regulation of cellular process, regulation of catalytic activity and cell differentiation were observed.

RT-PCR data validation

Gene expression profiling for *IL-6* and *PTSG2* genes was evaluated using qRT-PCR technique to validate the results obtained by microarray technique and to reveal the gene expression level of some key transcripts involved in drug resistance mechanisms. The results showed that the relative gene expression level for both genes is statistically increased at P12 and P24 compared to the control group, meanwhile in MDA-MB-231/Pax cell line at P12 the *IL-6* expression level is slightly decreased compared to the control group (Fig. 9). Thus, these results confirmed the microarray data on both cell lines.

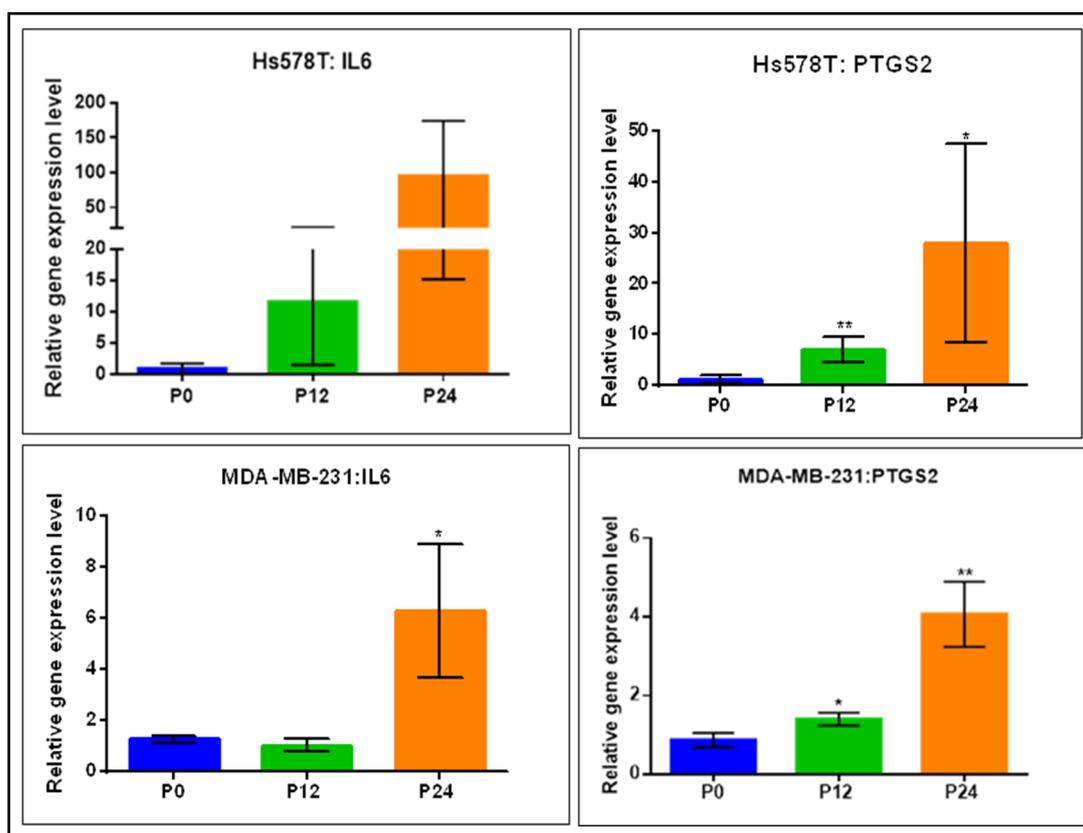


Fig. 9. Validation of microarray data through qRT-PCR technique. Gene expression profiling was investigated for IL-6 and PTGS2 at P0, P12 respectively P24 on both cell lines, Hs578T/Pax and MDA-MB-231/Pax; qRT-PCR results are normalised using $2\Delta\Delta CT$ method using B2M and GAPDH (* $P \leq 0.05$, ** $P \leq 0.01$).

Discussion

Paclitaxel is an antimetabolic chemotherapeutic agent that suppresses the polymerization and stabilization of microtubules in living cells, which induce mitotic arrests as a consequence of the activation of the mitotic checkpoint. Paclitaxel has demonstrated antitumor activity against different types of cancers. Although, resistance to paclitaxel is often induced and is a multifactorial process that may originate through several types of modifications, including overexpression of the multidrug transporter P-glycoprotein, alterations in microtubule dynamics, altered metabolism of the drug, decreased sensibility to death-inducing stimuli and altered binding of paclitaxel to its target cell [12].

Paclitaxel has long been used to treat patients with breast cancer, but its use is limited due to several side effects which induce bone marrow suppression, cardiac disturbances and peripheral neuropathy. Since a tumor consists from a heterogenous population of cancer cells, in many cases patients developed resistance to paclitaxel which reduces the survival rate [13]. In our study, both cell lines, Hs578T/Pax and MDA-MB-231/Pax present a significant decreased in antiproliferative activity and cellular viability. In the case of MDA-MB-231/Pax cell line was showed that the value of IC₅₀ at passage 24 is increased compared to passage 12 which means that treated cells become resistance to paclitaxel treatment via activation of drug resistance mechanisms. The majority of the cells survived and had different morphologies compared to the parental cells. Among these, the majority of the cells were round and appeared highly proliferative (Fig. 2). Some of the treated cells were large, mostly after prolonged therapy, with an increased proliferation rate. It is believed that these cellular aspects primarily induced by paclitaxel therapy, may occur more frequently *in vivo* where

a tumor is formed with a complex structure of different types of cells and a sophisticated microenvironment, components which provides cancer cells with a protective microenvironment.

NGS is a reliable technique that provides essential clues regarding any mutations occurring in a certain gene profile. The main gene found in all samples is *TP53* highly mutated where its critical role is involved in driving cancer formation/progression, presenting an increased frequency in post-chemotherapy administration [14]. Therefore, it represents a tremendous way to highlight the activation of drug resistance mechanisms as effect of paclitaxel therapy on TNBC cells.

A specific pattern in breast cancer, especially in TNBC cell lines exposure for 12 respectively 24 passages to paclitaxel treatment was identified. This study represents the basis for developing mRNAs and lncRNAs expression signature as diagnosis tools for TNBC and also to provide a comprehensive portrait about mRNAs and lncRNAs alteration. The integrated mRNAs and lncRNAs profiling of TNBC reported here provides critical information on molecular and cellular mechanisms, especially on drug resistance mechanisms which reduced patient's lifespan and overall survival. In this regard, there is a tremendous demand to provide new insights regarding TNBC treatments and to understand how to counteract drug resistance mechanisms.

The mechanism of paclitaxel is strongly associated with the inhibition of proliferation and tumor growth. In this regard, was showed that paclitaxel initiated a cascade of signaling pathways which are involved in biological processes such as apoptosis [15]. Also, paclitaxel is involved in the modulation of immune response through regulation of immune cells, cytokine and chemokine [16, 17]. Moreover, the resistance induces in breast cancer as a results of paclitaxel therapy represents a disequilibrium in various signaling pathways, mutations occurring in different types of genes and epigenetic deregulation are the main functions which determine a worse clinical outcome for breast cancer patients [18]. Using microarray technique were identified several genes with an altered expression profile involved in drug resistance mechanisms. Thus, *IL-6*, *CXCL8*, *VEGFA*, *EGR1*, *PTGS2* and *TRIB1* are the common altered genes found in both cell lines, Hs578T/Pax and MDA-MB-231/Pax at passage 24. A prolonged exposure to paclitaxel therapy induce modifications at cellular and molecular levels by altering mRNAs and lncRNAs expression profiling, with impact on critical biological processes.

IL-6 is a pro-inflammatory cytokine which plays a critical role in the expansion and differentiation of tumor cells [19]. Also, *IL-6* can affect the tumorigenesis process by regulating proliferation, cell death, survival, angiogenesis, metabolism and metastasis, as well as modulate tumor therapeutic resistance such as multidrug resistance (MDR) [19]. Moreover, was showed that in the serum and tumor site, increased level of *IL-6* is usually associated with poor prognosis and lower survival in patients with breast cancer [20], meanwhile down-regulated level of *IL-6* is associated with better response to therapy [21].

An important chemokine is represented by *CXCL8* which enhance the immunoregulatory ability to defend against cancer, but also have the ability to modify the microenvironment to facilitate tumorigenesis. In breast cancer, *CXCL8* cooperates with *VEGF* to establish and expand tumor neovasculature [22]. In this regard, tumor neovascularization not only contribute to the initiation and tumor growth but also offers blood supply for distant metastasis. An increased profiling of *CXCL8* can stimulate angiogenesis and attract neutrophils to release enzymes involved in processes including tissues remodeling and tumor establishment [23]. According to its implication in the initiation, progression, angiogenesis and metastasis of breast cancer, *CXCL8* is considered an unfavorable prognostic factor. In patients with lymph node-TNBC, an elevated *CXCL8* level induces a poor prognosis being link with shorter survival time and distant metastasis [24, 25].

VEGFA a key modulator of angiogenesis, which is usually highly expressed in cancer tissue and is correlated with more aggressive features [26]. Also, this tumor secretory factor is highly correlated with tumor progression, angiogenesis and invasion in TNBC [27].

EGR1 is a member of the immediate early response gene family which can be activated by a wide range of cytokines, growth factors, DNA-damaging agents and hormones. In addition, *EGR1* can control *MDR1* expression profiling at the transcriptional level [28]. It was demonstrated in previous studies that the Erk1/2 pathway was constitutively active in the resistant cell line, indicating the possible involvement of this pathway in long-term drug resistance. Alongside, the activation of this pathway leads to multiple biological processes and diseases such as cell death, cell cycle arrest and carcinogenesis [29]. In this regard, *EGR1* expression can be activated by different stimuli through the Erk1/2 pathway and through the pathway activation which induce the development of MDR [28]. Through the presence of elevated expression profiling of genes involved in drug resistance mechanisms, we observed the activation of several biological processes including tumor progression, cell cycle, angiogenesis, apoptosis, processes which exhibit a poor prognosis for TNBC patients.

Conclusion

In conclusion, multiple dose exposure to paclitaxel therapy stimulates alterations in nuclear morphology, as well as overexpression of important transcripts involved in biological processes including cell proliferation and tumor growth, apoptosis, angiogenesis, metastasis and drug resistance. The transcriptomic alteration is also sustained by additional mutation that might affect the response to therapy. In order to provide a better understanding regarding drug resistance, deep investigation into cellular and molecular processes is needed.

Therefore, the activation of *IL-6*, *CXCL8*, *VEGFA*, *EGR1*, *PTGS2* and *TRIB1* genes can be considered an important and potential target for the diagnosis and treatment of paclitaxel resistant TNBC. Also, should not underestimate the role of drug response mutation (as we can observe for *TP53*) that might change the molecular portrait and affect the response to therapy. Though these obtained results we intended to enhance TNBC patients prognosis and survival rate, and to offer an overview against different signaling pathways activated in this pathology.

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Author Contributions

A.J. and L-A.P. manuscript preparation, A.J., O.Z., L.R. and C-A.C-C. cell culture multiple dose exposure, toxicological test, C.M. microscopical evaluation; R.C. bioinformatics microarray data analysis; L-A.P. and C.P-B evaluation and data analysis for mutational pattern; C-A.C-C., L.R. and A.J. data validation, C.B and O.Z. microarray evaluation; A.I, I.B.-N. and C.B. study design, writing-review and editing.

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Disclosure Statement

The authors declare that no conflicts of interest exist.

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