

Overexpression of the erythropoietin receptor is associated with upregulation of tubulin beta 6 and paclitaxel resistance in ovarian adenocarcinoma cells

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Abstract

The emergence of tumor cell resistance is one of the major issues in current oncology practice. It reduces the effectiveness of therapy and worsens cancer patients' prognoses. However, it confirms a wide range of molecular interactions as well as the complexity of the human organism. Our previous research confirmed the functionality of the erythropoietin receptor (EPOR) in ovarian and breast cancer cells, as well as its relationship to these cells' sensitivity to specific therapies. The current study demonstrates that EPOR overexpression in human ovarian adenocarcinoma cells A2780 is directly linked to paclitaxel resistance. Furthermore, EPOR overexpression results in morphological changes that vary according to the pattern of EPOR isotypes expressed. In this regard, the most interesting result appears to be the change in the shape of the T clone, which has a tendency to form spheroidal structures. In addition, functional enrichment analysis demonstrated that EPOR-associated differentially expressed genes are involved in several biological and cell processes. Indeed, a T clone with a single 68 kDa EPOR isotype demonstrates significant resistance to paclitaxel therapy and is associated with the upregulation of tubulin beta 6.

Keywords: ovarian adenocarcinoma, A2780, erythropoietin receptor, paclitaxel, resistance

1. Introduction

The emergence of resistance in cancer cells represents a significant problem in current oncology practice. It limits successful therapy and worsens the prognosis for cancer patients. However, it also confirms a wide range of interactions at the molecular level and the high complexity of the human body.¹ For this reason, deeper investigation aimed at understanding processes at the cellular level is essential for the successful treatment of various types of diseases, including cancer. The goal of scientific groups in this area is primarily to eliminate the symptoms of tumor resistance and subsequently improve therapeutic options and thus the prognosis for patients.^{2,3}

Several clinical studies have demonstrated a poor prognosis for cancer patients in the case of therapy with

recombinant erythropoietin (EPO).⁴ This protein is a growth hormone that has a wide range of uses, especially in the adjustment of anemic conditions, which are a common side effect of chemotherapy. Its effect is mediated by the EPO receptor (EPOR). As a result of EPOR stimulation, various signaling pathways are activated, leading to the activation of transcription factors involved in regulating various cellular processes.^{5,6} The activation of EPOR signaling pathways can thus induce mitogenic, anti-apoptotic, anti-inflammatory, and other protective effects.⁷ Although it was long assumed that EPO acts exclusively on hematopoietic cells,⁸ it is now proven to also have effects in non-hematopoietic environments, including nerve, retinal, myocardial, and other types of cells, where the presence of EPOR has also been detected.⁹ In addition to

growth stimulation, EPOR-expressing tumor cells can potentially be favored in relation to antitumor therapy. In this context, breast cancer RAMA 37-28 cells overexpressed by EPOR were more resistant to tamoxifen and proliferated more rapidly in the presence of tamoxifen. In addition, these cells showed prolonged activation of AKT signaling, which we suppose is a mechanism that can lead to EPOR-induced tamoxifen resistance in EPOR-overexpressed cells.¹⁰ Moreover, enhanced activation of signaling pathways mediated by pERK1/2 in RAMA 37-28 cells as detected by western blot analysis was demonstrated to be essential for paclitaxel resistance.¹¹ Interestingly, EPOR siRNA silencing sensitized and/or potentiated the anti-proliferative effect of tamoxifen on human ovarian adenocarcinoma - A2780 cells.¹²

Supportive treatment with EPO currently has its use in anti-cancer therapy with paclitaxel, which belongs to the group of unique mitotic inhibitors – taxanes.¹³ Taxanes represent a great hope in anti-cancer therapy due to their antiproliferative and anti-angiogenic properties, as well as anti-metastatic effects observed in cancer cells. Although taxanes are considered highly potent for the treatment of various types of cancer, they are not truly effective in all cases.¹⁴

The goal of our research was to confirm the role of EPOR in ovarian tumor cells. As a result, we generated a stably EPOR-overexpressed human ovarian adenocarcinoma cell line by transfecting parental A2780 cells with the expression vector containing human EPOR cDNA. In addition, in this work we point out a positive correlation between EPOR expression and tubulin beta 6 protein levels, which probably plays an important role in paclitaxel sensitivity of ovarian adenocarcinoma cells.

2. Materials and methods

2.1. Cell culture and transfection

The human ovarian adenocarcinoma cell line A2780 was obtained from the European Collection of Animal Culture (ECAC, Salisbury, UK). Cells were cultured in RPMI 1640 medium with L-glutamine (Gibco BRL, Paisley, UK) supplemented with 10% fetal calf serum (Gibco BRL) and antibiotic/antimycotic solution (100 U/mL of penicillin, 100 mg/mL of streptomycin, and 0.25 mg/mL of amphotericin B; Gibco BRL) and were maintained under standard tissue culture conditions at 37 °C and 5% humidified atmosphere of CO₂. A2780 cells (1×10⁶/well) were transfected by expression vector pUNO1 (100 ng) (Invivogen, Toulouse, France) containing fully sequenced open reading frame of EPOR using Lipofectamine LTX DNA transfection reagents (0.35 µL of Lipofectamine LTX reagent + 0.1 µL of PLUSTM reagent) (ThermoFisher, Waltham, MA, USA) in a 6-well plate. Cells transfected by the pUNO1-mcs vector (Invivogen) served as a negative control (NEG), and

the parental A2780 cells served as an intact control (INT). 25 µg of blasticidin was used in the combination with limiting dilution analysis (by diluting a cell suspension to the point, where statistically each well of a 96-well plate contains a single cell) for a selection of A2780 clones overexpressing EPOR.

2.2. Isolation, protein purification, and western blot analysis

A2780 cells, including a negative control and selected C, T, and V EPOR-overexpressing clones cultivated in 6-well plates, were washed twice with PBS after reaching 80% confluence, scraped with a scraper, and lysed using lysis buffer (FNN0011, ThermoFisher) supplemented with 1× protease inhibitor cocktail (PIC P8340, Sigma-Aldrich). The cells were sonicated on ice (2 cycles; 30-second pulses at 100% amplitude). The lysates were then subjected to an additional sonication for 15 seconds at 30% power using a BANDELIN SONOPULS HD2070 (BANDELIN electronic, Berlin, Germany) on ice, followed by centrifugation at 13,000 g for 10 minutes at 4 °C.

Protein concentration was determined using a detergent-compatible protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein (30 µg), supplemented with 0.01% bromophenol blue, 1% 2-mercaptoethanol, 0.4% SDS, and 5% glycerol, were separated on a 10% SDS-polyacrylamide gel (acrylamide/bis solution, 37.5:1) and transferred onto a nitrocellulose (NC) membrane (Bio-Rad Laboratories) via dry transfer for 10 minutes using the iBlot 2 Gel Transfer Device (ThermoFisher, USA).

Membranes were blocked for 1 hour at room temperature in 5% non-fat milk prepared in 20 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.1% TWEEN 20 (pH 7.4). They were then incubated overnight at 4 °C with primary antibodies: rabbit polyclonal anti-EPOR (1:2000, A82, Amgen) and rabbit polyclonal anti-tubulin beta 6 (1:3000, ABIN7076054, Antibodies Online). After washing for 20 minutes with wash buffer, the membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature: goat anti-rabbit IgG (1:2000, 31461, ThermoFisher) and rabbit anti-mouse IgG (1:5000, Santa Cruz).

Detection was performed using a Pierce ECL Western Blotting Substrate (Pierce) and visualized with a bio-imaging system (DNR MF Chemibis 2.0, Israel). Equal sample loading was verified by immunodetection of β-actin using mouse monoclonal antibody (1:1000, #3700, Cell Signaling).

2.3. Metabolic activity of cells

Metabolic activity of cells was monitored via 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS; Sigma-

Aldrich, Saint Louis, MO, USA), and was analyzed 72 h after the incubation of cells (1×10^4 /well) in a 96-well plate with different concentrations of paclitaxel (3.91–1000 nM; Fresenius Kabi s.r.o., Praha, Czech Republic). Ethanol was used as a vehicle for the paclitaxel solution. In our case, the highest tested concentration of paclitaxel (1000 nM) contained 0.007% ethanol, which had no effect on the metabolic activity of the cell lines tested so far. Viable cells with active metabolism converted MTS into a soluble formazan product, the absorbance of which was measured at 570 nm using a spectrophotometer (Epoch; BioTek, Winooski, VT, USA). MTS data are presented as mean fold-changes in cell proliferation $\pm$ SD of three independent experiments.

2. 4. RNA isolation and library preparation

After incubation of A2780 cells and clones in 6-well plates, mRNA was isolated using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. DNase I treatment was incorporated during the RNA isolation process to remove potential genomic DNA contamination. The concentration and purity of total RNA (measured by OD 260/280 and 260/230 ratios) were assessed using a Nanodrop spectrophotometer (Thermo Fisher Scientific). The RNA samples were stored at -80°C .

For library preparation, 250 ng of total RNA was reverse transcribed into first-strand cDNA using oligo(dT) primers and the QuantSeq 3' mRNA-Seq Library Prep Kit (Lexogen, Austria). During the process, the RNA template was degraded using the RNA removal solution (RS buffer, Lexogen), and the second strand was synthesized using random hexamer primers containing Illumina-compatible linker sequences at their 5' ends.

The resulting double-stranded DNA (dsDNA) libraries were purified using magnetic beads, then amplified by PCR with specific single i7 indexing primers. This step added necessary adapter sequences for cluster generation and produced DNA suitable for quality control and sequencing. For A2780 cells, 20 PCR cycles were determined to be optimal using the PCR Add-on Kit for Illumina (Lexogen). The amplified libraries were purified again with magnetic beads, and their fragment lengths were assessed using a fragment analyzer.

2. 5. Sequencing and data analysis

cDNA libraries were sequenced on the Illumina NextSeq platform, generating single-end reads of 75 bp, with approximately 8 million reads per sample. The resulting FASTQ files were processed and aligned to the reference genome (*Rattus norvegicus*, mRatBN7.2) using the STAR aligner (version 2.5.2b; <https://github.com/alex-dobin/STAR/releases/tag/2.5.2b>). Pre-processing included adapter trimming and removal of the initial 10 bases, as recommended. Reads were counted using STAR (version

2.5.2b). Differential gene expression analysis was performed using the open-source R package edgeR (version 3.12; <https://bioconductor.org/packages/release/bioc/html/edgeR.html>). Genes with low read counts (< 3 CPM, counts per million) were filtered out using the `filterByExp` function of edgeR. Differentially expressed genes (DEGs) were identified using the `glmTreat` and `glmQLFit` (quasi-likelihood, QL) functions within edgeR, considering genes with a log fold change (logFC) greater than ± 1.2 and a false discovery rate (FDR) less than 0.05. Gene Ontology (GO) biological process enrichment analysis was performed using the Reactome Pathway Database (<https://reactome.org/>).

2. 6. Silencing of EPOR gene expression by siRNA

To silence EPOR expression in EPOR-overexpressed T clone of A2780 cells, we used siRNAs (SR301438A - EPOR (Human) – 3 unique 27mer siRNA duplexes – 2 nM each, OriGene Technologies, Rockville, MD, USA), ntRNAs (SR30004 – universal scrambled negative control siRNA duplex – 2 nM, OriGene Technologies), and the transfection reagent siTran 2.0 siRNA (OriGene Technologies), following the manufacturer's instructions. Briefly, cells of the T clone were seeded into 6-well plates at a density of 5×10^5 cells per well in RPMI medium, supplemented with 10% FBS, and incubated for 24 hours at 37°C in the presence of 5% CO_2 to reach 70–90% confluence at the time of siRNA addition. Both the target and control siRNAs were dissolved in RNase-free siRNA duplex re-suspension buffer (OriGene Technologies, Inc.; #SR30005) according to the manufacturer's instructions. The dissolved siRNA (20 μM) was diluted in transfection buffer (1:99), mixed with siTran reagent at a 1:43 ratio, incubated for 15 minutes at room temperature, and then added to the cells at a 1:10 ratio. The siRNA was left in contact with the cells for 24 hours, after which the medium was replaced with fresh culture medium. To verify the efficiency of EPOR silencing by siRNA, the cells were analyzed by western blot 48 hours after the medium change according to the protocol described above. The transfection reagents were stable, and none of the tested conditions significantly affected cell viability.

2. 7. Statistical analysis

All experiments under various conditions were performed in at least three independent replicates. The mean and standard deviation were calculated using descriptive statistics. Data analysis was conducted using RTCA software Pro 1.2.1 (ACEA Bioscience). Statistical significance was assessed with a non-parametric method, one-way ANOVA using SigmaPlot (Ver. 12.0). Differences were considered statistically significant at $p < 0.05$ as *; $p < 0.01$ as **; $p < 0.001$ as ***.

3. Results

Following transfection of A2780 cells (INT) with EPOR expression or control (NEG) vector, we generated twenty-two different clones using the limiting dilution

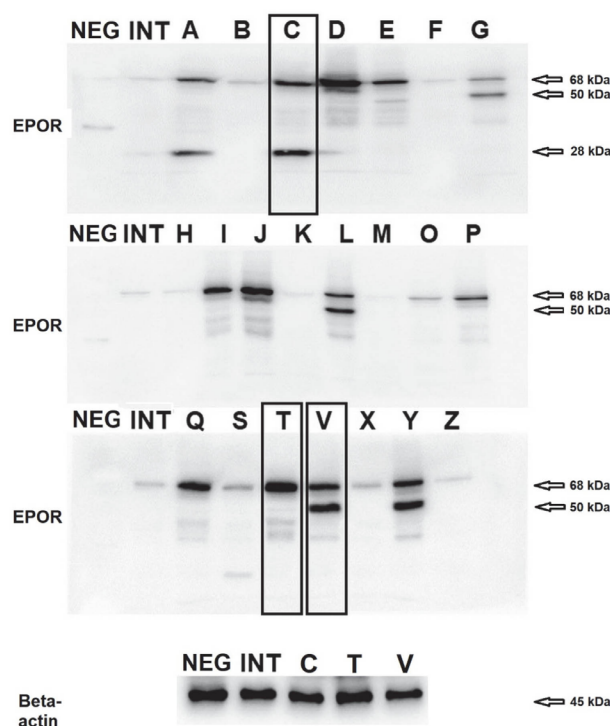


Figure 1. Overexpression of EPOR in A2780 cells results in a different pattern of expressed EPOR isotypes. Clones C, T, and V were selected as representative clones for further testing. As controls, we used intact A2780 cells (INT) and cells transfected with an empty expression vector (NEG). Beta actin protein (mouse monoclonal antibody #3700, Cell Signaling) was used as a loading control for both NEG and INT controls and C, T, and V clones.

protocol and the blasticidin resistance selection marker. We observed three distinct patterns of EPOR expression when we tested each individual clone using western blot analysis and the specific anti-EPOR antibody A82, and therefore we selected representatives from each type, namely clones C, T, and V, for further analysis (Figure 1). The first pattern (clone C) was characterized by the presence of two 68 and 28 kDa EPOR isotypes; the second contained only the 68 kDa EPOR fragment (clone T); and the third (clone V) a combination of 68 and 50 kDa EPOR (Figure 1).

The most interesting aspect appears to be the change in the shape of T clone, which had a tendency to form spheroidal structures (Figure 2). The C clone with an elongated spindle shape is also morphologically different from the controls and other spherical shape clones like V clone (Figure 2).

Assay of metabolic activity and/or EPOR clone response to paclitaxel therapy showed that the T clone was, in addition more resistant ($IC_{50} = 545$ nM) compared to the parental cell line ($IC_{50} = 20.3$ nM), negative vector ($IC_{50} = 35.5$ nM), as well as the C ($IC_{50} = 20.8$ nM) and V clones ($IC_{50} = 12.2$ nM) (Figure 3). The C and V clones did not differ significantly from INT and NEG controls in response to paclitaxel (Figure 3).

To understand the molecular changes that occurred after transfection and subsequent overexpression of human EPOR in the ovarian adenocarcinoma cell line A2780, we used RNA-seq analysis. RNA was isolated from the C, T, and V clones of A2780 cells with EPOR overexpression, as well as from INT and NEG controls, and evaluated for quality. cDNA libraries were prepared from biological replicates with an optimal fragment size between 150 and 300 nt. The sequencing itself produced approximately 10 million raw reads per sample from clones and controls. More

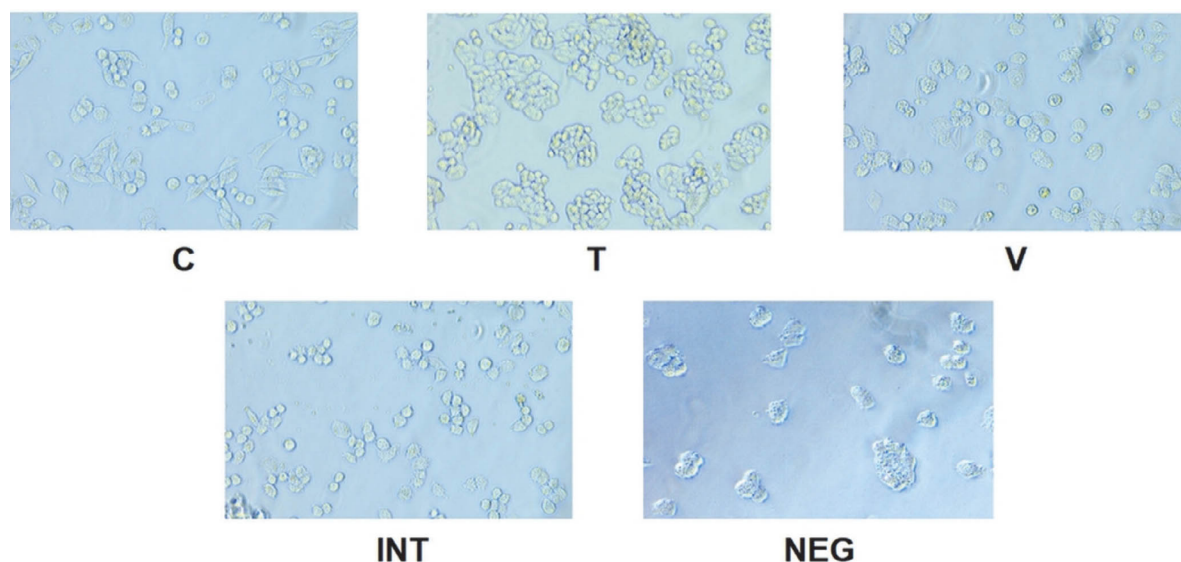


Figure 2. Overexpression of EPOR in A2780 cells results in changed cell morphology. The morphology of EPOR overexpressed A2780 clone C (C), clone T (T), and clone V (V) together with the morphology of intact (INT - untransfected A2780 cells) and negative control (NEG - A2780 cell transfected with non-EPOR vector) are shown (Olympus Plan Apo, 20x objective).

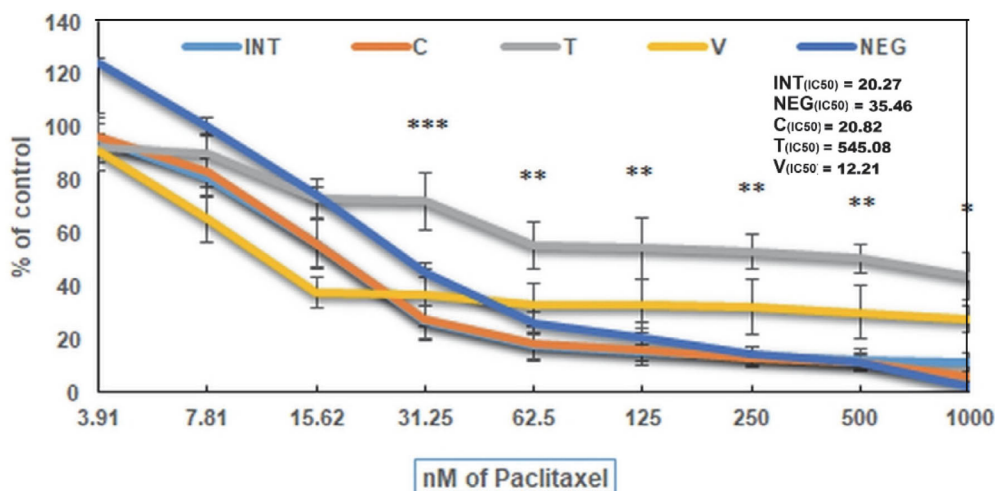


Figure 3. Overexpression of EPOR in A2780 cells results in paclitaxel resistance. The response of EPOR overexpressed A2780 clone C (C), clone T (T), and clone V (V) together with the response of intact (INT - untransfected A2780 cells) and negative control (NEG - A2780 cell transfected with non-EPOR vector) to paclitaxel treatment (3.91–1000 nM of paclitaxel) are shown. Cell growth/survival was measured using MTS after 72 h treatment. The results are expressed as the means $\pm$ SEM of triplicate experiments, and the significance of the T clone compared to the negative vector is shown as *** ($p < 0.001$), ** ($p < 0.01$), and * ($p < 0.05$).

than 15,000 genes were mapped in each sample. Genes with a minimum average logCPM (counts per million) of 3 were considered differentially expressed and included in the differential expression analysis. Genes with a logFC (fold change) greater than 1.2 were included in the final list of differentially expressed genes (DEGs). Additionally, we checked the p-value for selected differentially expressed genes and removed all genes with a $p > 0.01$. Overall, there were 1,258 DEGs in clone C compared to both controls, with 652 downregulated and 606 upregulated genes. In clone T, 1,411 DEGs were identified, of which 912 were downregulated and 499 upregulated. For clone V, 1,633 DEGs were recorded, with 894 downregulated and 739 upregulated (Figure 4).

The top 20 DEGs included the PRRX1 and FOXC1 genes, which were found to be downregulated in all C, T, and V clones. On the other hand, the genes TES and TSC22D3 were downregulated, along with the upregulated gene INSIG2, but only in clones T and V. In addition, genes SLIT2, ZMAT3, and FIBIN were found to be DEGs in clones C and V, while ATP2B1, CRABP, and RSU1 as DEGs in clones T, and V and SCN3A and NTS were DEGs in clones C and T. In this regard, some genes were identified as DEGs only in a single clone, such as SPOCK2 and NAV3 in clone C; FAM198B, CD47, and PCDH9 in clone T; and LINC00461 and BTG1 in clone V (Figure 4). Interestingly, our DEGs gene set shows enrichment (KEGG) typical of axon guidance-like (SLIT2, NAV3, PCDH9,

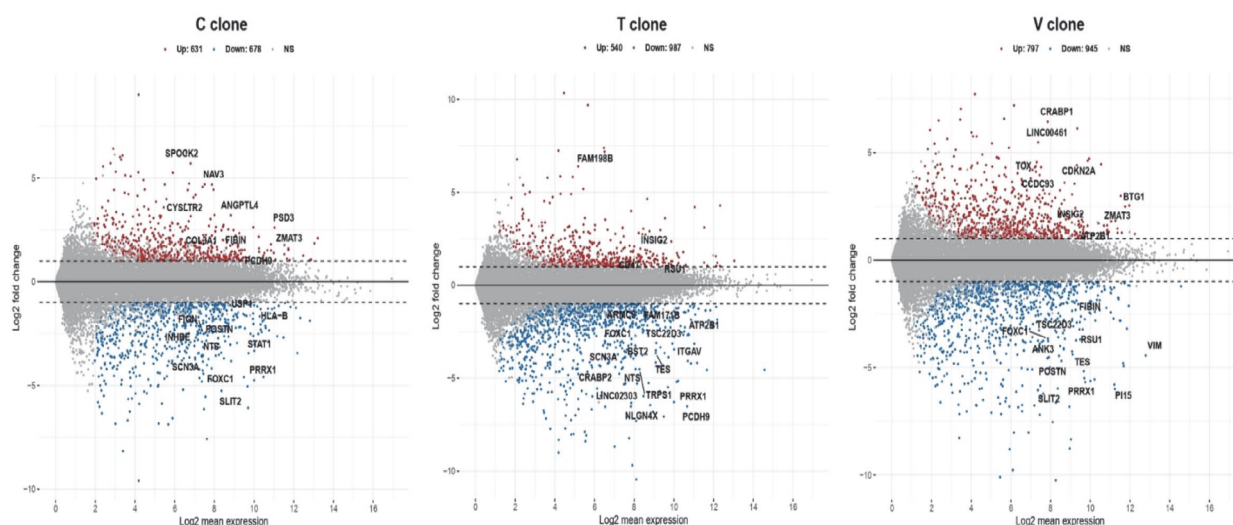


Figure 4. Overexpression of EPOR in A2780 cells results in differentially expressed genes (DEGs). Top 20 DEGs are shown in clone C (C clone), clone T (T clone) and clone V (V clone).

Table 1. The most relevant pathways involved in cellular signal transduction resulting from EPOR overexpression in A2780 cells, sorted by p-value (* False Discovery Rate).

Pathway name	Entities				Reactions	
	found	ratio	p-value	FDR*	found	ratio
Serotonin receptors	7/13	8.35E-04	0.022	0.936	6/6	4.03E-04
Breakdown of the nuclear lamina	3/3	1.93E-04	0.027	0.936	3/3	2.02E-04
Regulation of gene expression by Hypoxia-inducible Factor	7/15	9.63E-04	0.043	0.936	7/7	4.70E-04
Activation, myristoylation of BID and translocation to mitochondria	3/4	2.57E-04	0.054	0.936	4/4	2.69E-04
NFE2L2 regulating inflammation associated genes	4/7	4.50E-04	0.063	0.936	3/3	2.02E-04
Drug-mediated inhibition of MET activation	2/2	1.28E-04	0.068	0.936	2/2	1.34E-04
Formation of annular gap junctions	5/11	7.06E-04	0.087	0.936	2/2	1.34E-04
Formation of a pool of free 40S subunits	29/106	0.007	0.102	0.936	2/2	1.34E-04
NOTCH4 Intracellular Domain Regulates Transcription	9/26	0.002	0.105	0.936	4/9	6.05E-04
Cell-extracellular matrix interactions	7/19	0.001	0.112	0.936	7/10	6.72E-04
Gap junction degradation	5/12	7.71E-04	0.114	0.936	4/4	2.69E-04
TRAF3 deficiency - HSE	2/3	1.93E-04	0.133	0.936	1/1	6.72E-05
Interleukin-38 signalling	3/6	3.85E-04	0.136	0.936	4/5	3.36E-04
Eukaryotic Translation Elongation	27/102	0.007	0.144	0.936	7/9	6.05E-04
Response of EIF2AK4 (GCN2) to amino acid deficiency	30/115	0.007	0.146	0.936	12/16	0.001
GTP hydrolysis and joining of the 60S ribosomal	31/120	0.008	0.153	0.936	3/3	2.02E-04
Role of ABL in ROBO-SLIT signalling	4/10	6.42E-04	0.164	0.936	4/4	2.69E-04
Cohesin Loading onto Chromatin	4/10	6.42E-04	0.164	0.936	2/2	1.34E-04
Formation of the ternary complex, and subsequently, the 43 complex	15/54	0.003	0.178	0.936	3/3	2.02E-04
Nonsense Mediated Decay (NMD) independent of the Exon Junction Complex (EJC)	26/101	0.006	0.182	0.936	1/1	6.72E-05
Peptide chain elongation	25/97	0.006	0.186	0.936	4/5	3.36E-04
LRR FLII-interacting protein 1 (LRRFIP1) activates type I IFN production	3/7	4.50E-04	0.186	0.936	4/5	3.36E-04
NrCAM interactions	3/7	4.50E-04	0.186	0.936	2/4	2.69E-04
Drug resistance of PDGFR mutants	1/1	6.42E-05	0.191	0.936	4/4	2.69E-04
PDGFR mutants bind TKIs	1/1	6.42E-05	0.191	0.936	2/2	1.34E-04

SPOCK2, SCN3A, NTS), focal adhesion (RSU1, TES, SPOCK2, CD47, PCDH9, NAV3), and cytoskeletal pathways (SLIT2, RSU1, NAV3, PCDH9, SPOCK2), with overlap into EMT, migration, neural plasticity (NTS, SCN3A), and p53-regulated stress responses (ZMAT3, BTG1, TSC22D3), which is characteristic of developmental and tumor expression programs (PRRX1, FOXC1, SLIT2, CD47, BTG1, RSU1).

The DEGs were subsequently analyzed based on the common cellular signaling, resulting in a table with the most relevant pathways ranked by p-value (Table 1).

Among the signaling pathways with the highest number of involved DEGs, surprisingly, some were related to protein synthesis, such as the “response of EIF2AK4 (GCN2) to amino acid deficiency”, “formation of a pool of free 40S subunits”, “peptide chain elongation”, “eukaryotic translation elongation”, “GTP hydrolysis and joining of the 60S ribosomal subunit.” Additionally, pathways related to hypoxia, such as “regulation of gene expression by hypoxia-inducible factor,” the extracellular matrix, such as “cell-extracellular matrix interactions,” gene transcription, such as “NOTCH4 intracellular domain regulates transcription,” and apoptosis, such as “activation, myristoyla-

tion of BID,” and “translocation to mitochondria,” were also identified (Table 1).

An association was even observed between the top 20 DEGs and the signaling pathways with the highest number of DEGs involved. In this regard, the strongest connections exist between SLIT2/NAV3/PCDH9 → ROBO-SLIT, ECM, neuronal migration; FOXC1/PRRX1 → EMT, hypoxia, RTK resistance; BTG1/ZMAT3/TSC22D3 → stress, translation, inflammation; and CD47/RSU1/SPOCK2 → ECM and cell communication.

In relation to the possible association of the upregulated tubulin beta 6 gene in the T clone, we used specific siRNA to silence the expression of EPOR and subsequently analyzed both the metabolic activity of the T clone under paclitaxel conditions and the expression of tubulin beta 6 itself. Indeed, EPOR siRNA reduced the sensitivity of the T clone to paclitaxel, both in comparison to the T clone alone and in comparison, to both control groups, INT and NEG (Figure 5). The increase in sensitivity (decrease in resistance) of the T clone correlated with the level of tubulin beta 6 protein, which, after EPOR siRNA treatment, decreased to the level of both controls (Figure 6).

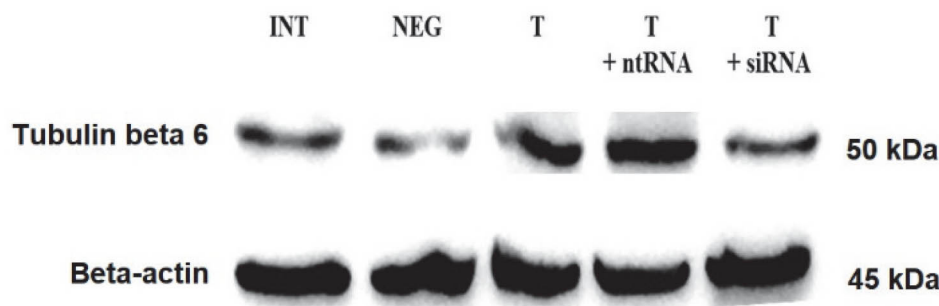


Figure 5. Overexpression of EPOR in the T clone of A2780 cells results in upregulation of tubulin beta 6. The effects of both EPOR siRNA and ntRNA on tubulin beta 6 protein levels are shown. Beta actin protein was used as a loading control.

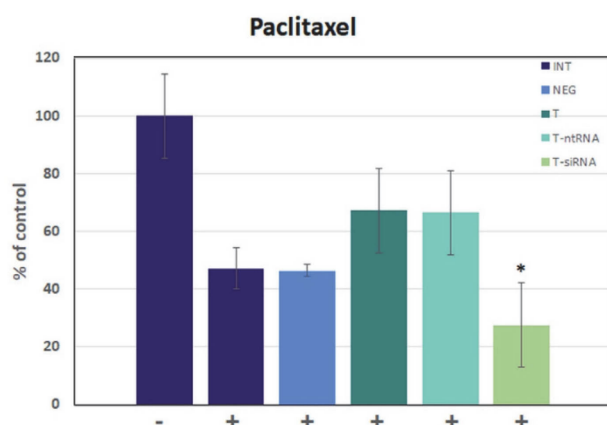


Figure 6. EPOR siRNA sensitizes EPOR-overexpressed A2780 cells to paclitaxel. The response of the EPOR-overexpressed A2780 clone T (T), treated with EPOR siRNA or ntRNA, is shown alongside the response of intact (INT - untransfected A2780 cells) and negative control (NEG - A2780 cells transfected with non-EPOR vector) cells to paclitaxel treatment (200 nM). Cell response was measured using MTS after 72 h of treatment. The results are expressed as the means $\pm$ SEM of triplicate experiments, and the significance of T clone + siRNA compared to T clone is shown as * ($p < 0.05$).

4. Discussion

Our previous study found that long-term incubation of parental A2780 cells with EPO altered the cell phenotype, as evidenced by increased levels of phospho-Erk1/2 proteins and resistance to paclitaxel. The phenotypic manifestation was unique to paclitaxel, as no change in sensitivity to cisplatin or carboplatin was observed. Moreover, the altered phenotype of A2780 cells remained stable even after EPO removal.¹⁵ Conversely, rat mammary adenocarcinoma human EPOR overexpression in RAMA 37-28 cells led to increased resistance to tamoxifen and faster cell proliferation compared to parental RAMA 37 cells.¹⁰ In addition, our recent study, similar to the current one, found that EPOR plays a role in the resistance of EPOR-overexpressed RAMA 37-28 cells to paclitaxel chemotherapy. In this regard, RAMA 37-28 cells showed a lower rate of paclitaxel-induced apoptosis and an increased activation of phospho-Erk1/2 signaling.¹¹

Transcriptomic analysis of the T clone revealed changes in the expression of several intriguing genes, some of which are directly involved in tumor progression. Several tumor suppressor genes, including FOXC1, TES, and RSU1, are among the top 25 genes. In the T clone, FOXC1 and TES show less expression, while RSU1 shows more. The latter, RSU1, may play an important role in tumor development and progression, although it was originally identified as a suppressor of Ras oncogenic transformation. Its expression and function in tumor cells are complex and situation-dependent. RSU1 can act as a tumor suppressor in some cases, promoting apoptosis and inhibiting cell invasion, while in other cases, it may promote metastasis, especially in aggressive cancer cells.¹⁶ Similarly, the tumor suppressor TES, which as a skeletal protein can also play a role in adhesion, migration, and reorganization of the actin cytoskeleton in tumor cells, is considered a tumor suppressor in humans. Decreased TES expression increases cell motility and reduces cell-cell contact, which contributes to tumor development and progression.¹⁷ The altered phenotype of our T clone may also be related to the increased expression of INSIG2 (observed in both T and V clones), which plays a role in regulating cholesterol synthesis. Its altered expression has been observed in various types of cancer. INSIG2 acts as a negative regulator of cholesterol biosynthesis and is associated with tumorigenesis and metastatic progression in several tumor types.¹⁸

PRRX1, a transcription factor involved in tumor progression, also participates by promoting epithelial-mesenchymal transition (EMT), stemness, invasiveness, and angiogenesis, contributing to tumor aggressiveness. In high-grade serous ovarian carcinoma (HGSOC), PRRX1 is highly expressed and plays a key role in regulating tumor cell proliferation and migration.¹⁹ Paradoxically, in all our clones, PRRX1 expression was decreased. Similarly, reduced expression was observed for the SCN3A gene (in C and T clones), which encodes the alpha subunit of the voltage-gated sodium channel (also known as NaV1.3). While not directly linked to cancer, voltage-gated sodium channels, including NaV1.3, have been found to be aberrantly expressed

in various tumor types and play a role in cancer cell behavior. These channels can influence proliferation, migration, invasion, and potentially drug resistance of cancer cells.²⁰

A gene that did not make it into our top 25 DEGs was beta-tubulin class VI (TUBB6), which encodes a tubulin beta 6 isotype and plays a key role in the assembly and function of microtubules.²¹ The tubulin beta 6 protein, as a key microtubule protein, contributes to their dynamic behavior, where polymerization and depolymerization are critical not only for intracellular transport but also for the process of mitosis, during which they are responsible for the precise segregation of chromosomes via the mitotic spindle. Although tubulin beta 6 is expressed in various tissues, its levels can vary significantly,²² and abnormalities in its expression are associated with the progression of various types of cancer cells.^{23,24} In this context, increased or decreased expression of tubulin beta 6 in tumors is linked to heightened tumor aggressiveness and poor prognosis. Although changes in the expression of multiple genes may contribute to the resistance of the T clone, one such gene is certainly TUBB6. Its upregulation, as a result of EPOR overexpression, is associated with resistance to paclitaxel. Confirmation of tubulin beta 6's role in the T clone's resistance to paclitaxel is also evidenced by the silencing of EPOR expression, which increased sensitivity to paclitaxel and led to a decrease in the levels of the tubulin beta 6 protein itself.

The observed putative link between EPOR expression and the responses of human adenocarcinoma cells A2780 to paclitaxel might have clinical relevance. The detailed mechanisms that alter morphology and paclitaxel resistance need to be investigated further. The role of TUBB6 is also worth mentioning, as it could potentially serve in clinical practice in cancer patients as a marker of sensitivity to paclitaxel.

5. Conclusions

Finally, we would like to emphasize that we are the first to transfect human ovarian adenocarcinoma cell line A2780 with human EPOR gene and demonstrate some altered properties of the resulting clones. Indeed, the A2780-EPOR clone's increased resistance to paclitaxel chemotherapy, as well as significantly altered morphology of such cells, were closely related to the EPOR protein isotype pattern. In addition, functional enrichment analysis demonstrated that EPOR-associated differentially expressed genes are involved in several biological and cell processes. Our studies also found that EPOR knockdown inhibited metabolic activity of T clone and affected the tubulin beta 6 expression. Based on these findings, tubulin beta 6 may serve as a key protein and a contributor to paclitaxel resistance of ovarian adenocarcinoma cells induced by EPOR overexpression.

Conflict of Interest

The authors declare that they have no conflict of interest.

Authorship contribution statement

Pavol Harvaník: Writing – review & editing, Writing – original draft, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. Kristína Danková: Methodology, Formal analysis. Zuzana Solárová: Validation, Methodology, Investigation, Formal analysis, Conceptualization. Martin Šemeláková: Validation, Methodology, Formal analysis. Mangesh Bhide: Resources, Conceptualization. Peter Solár: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Availability of data and materials

The datasets used during the present study which are not provided in the manuscript are available from the corresponding author upon reasonable request.

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Povzetek

Pojav odpornosti tumorskih celic je eden od glavnih izzivov v sodobni onkološki praksi. Zmanjšuje učinkovitost terapije in poslabšuje prognoze za bolnike z rakom. Vendar potrjuje širok spekter molekularnih interakcij ter kompleksnost človeškega organizma. Naše prejšnje raziskave so potrdile funkcionalnost receptorja eritropoetina (EPOR) v jajčnikih in dojkah, kot tudi njegovo povezavo z občutljivostjo teh celic na določena zdravljenja. Trenutna študija dokazuje, da je prekomerna ekspresija EPOR v človeških jajčnikih adenokarcinomskih celicah A2780 neposredno povezana z odpornostjo na paklitaksel. Poleg tega prekomerna ekspresija EPOR povzroča morfološke spremembe, ki se razlikujejo glede na vzorec izraženih izotipov EPOR. V zvezi s tem se zdi najbolj zanimiv rezultat sprememba oblike klona T, ki ima tendenco k oblikovanju sferoidnih struktur. Poleg tega je funkcionalna analiza obogatitve pokazala, da so geni, povezani z EPOR, ki se izražajo diferencialno, vključeni v več bioloških in celičnih procesov. Klon T z enim izotipom EPOR 68 kDa dejansko kaže znatno odpornost na terapijo s paklitakselom in je povezan z zvišano regulacijo tubulina beta 6.



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