Molecular mechanisms of chemoresistance induced by cisplatin in NSCLC cancer therapy

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Abstract:

Cancer cells utilize a number of mechanisms to increase their survival and progression as well as their resistance to anticancer therapy: deregulation of growth regulatory pathways by acquiring grow factor independence, immune system suppression, reducing the expression of antigens activating T lymphocyte cells (mimicry), induction of anti-apoptotic signals to counter the action of drugs, activation of several DNA repair mechanisms and driving the active efflux of drugs from the cell cytoplasm and epigenetic regulation by microRNAs (miRNAs). Due to the fact that it is commonly diagnosed late, lung cancer remains a major malignancy with a low five-year survival rate; when diagnosed, the cancer is often highly advanced and the cancer cells may have acquired drug resistance. This review summarizes the main mechanisms involved in cisplatin resistance and in interactions between cisplatin-resistant cancer cells and the tumor microenvironment. It also analyses changes in the gene expression profile of cisplatin sensitive *vs.* cisplatin resistant non–small cell lung cancer (NSCLC) cellular model using the GSE108214 Gene Expression Omnibus database. It describes a protein-protein interaction network that indicates highly-dysregulated *TP53, MDM2* and *CDKN1A* genes as they encodes the top networking proteins that may be involved in cisplatin tolerance, these all being upregulated in cisplatin-resistant cells. Furthermore, it illustrates the multifactorial nature of cisplatin resistance by examining the diversity of dysregulated pathways present in cisplatin-resistant NSCLC cells based on KEGG pathway analysis.

1. Lung cancer in global perspective

Globally, lung cancer continues to be the major cause of cancer deaths in both men and women [1,2], being, the most common cancer type for men, constituting 22% of total cancer incidence, and the third most common in women, in whom it represents 8.4% total cancer incidence after breast and

colorectal cancers [3]. About 2.1 million new cases of lung cancer were diagnosed worldwide in 2018, which accounts for 11.6% of the world's total cancer incidence. Overall lung cancer mortality amounted to 1.8 million in 2018, accounting for 18.4% of the total cancer deaths [3]. The 5-year survival index for early stage lung cancers exceeds 50% [4]. This high mortality is primarily due to the fact that only 15% of these cancers are discovered in the early stages; therefore, despite the presence of developed modalities for treatment, most cancers are diagnosed at an advanced stage [4] and the overall 5-year survival rate is only about 15% [5].

1.1. Histopathological type

Lung cancer may be classified into two major groups: small cell lung cancer (SCLC) and non–small cell lung cancer (NSCLC), according to histopathological diagnosis [6,7]. NSCLC accounts for the approximately 80-85% of all lung cancer cases [2,8,9]. It is comprises two predominant histological subtypes: adenocarcinoma (ADC, approximately 40-50% cases) squamous cell carcinoma (SCC, approximately 20-30% cases) [6,8,10].

1.2. Treatment of NSCLC

The choice of treatment of NSCLC depends on the histological subtype and genetic subtype of the tumor, but also on disease stage, comorbidity, and performance status [11]. In cases of early stage NSCLC with no contraindications, surgical resection of the tumor is indicated [4]; while unresectable tumors can be controlled to some degree with radiation therapy, only a small number of patients demonstrate positive outcomes [12]. Alternatively, patients with locally-advanced unresectable lung cancer, may achieve long-term survival by treatment with combination of radiation therapy and chemotherapy [12]. One of the "first choice" drugs used in treating various solid tumors, including lung cancer, is cisplatin, discovered in 1965 and approved by Food and Drug administration in 1978 [13]. Furthermore, in the case of advanced metastatic form of lung cancer, improved survival and palliation of symptoms may be achieved with chemotherapy, targeted agents, and other supportive measures [12].

1.3. The effect of cisplatin

NSCLC patients are less sensitive to chemotherapy based on doublet of cisplatin, cis-diamminedichloroplatinum (II), than SCC patients [12,11]. These compounds have a well-known mechanism of action [14]. Cisplatin generally enters cells by passive diffusion, where it is then activated [15,16]. In the cytosol, its chloride ligands are replaced by water molecules, generating positively-charged mono- and bi-aquated forms of cisplatin that react with various membrane and cytoplasmic components, as well as nuclear DNA and RNA [14,17]. Aquated species of cisplatin are able to form covalent bonds with endogenous nucleophilic such as methionine, cysteine-containing peptides and polypeptides including reduced glutathione (GSH) and metallothioneins (MT) [17,18]. These interactions increases oxidative stress

via depletinon cell of reducing equivalents, resulting in cytotoxic effects [17]; however, those molecules additionally function as cytoprotective buffer, as some of the chemically active cisplatin is inactivated by reacting with them, thus protecting more vital targets (DNA)[19,17].

Cisplatin also causes the formation of intrastrand and interstrand crosslinks in DNA [15,20]. Cross-links between guanine bases are induced by cisplatin, carboplatin and oxaliplatin [20,21]. While cisplatin and carboplatin form identical cross-links, those formed by oxaliplatin include the bulky 1,2-diaminocyclohexane group in the adduct [22]. Only a little DNA damage is needed to disrupt replication and transcription [23]. However, it may be an oversimplication that the cytotoxic properties of cisplatin are based on it binding to nuclear DNA, mainly via intrastand DNA crosslinks, leading to cell cycle arrest and subsequent apoptosis [16].

2. Mechanisms underlying cisplatin resistance

Compounds based on cisplatin are used in treatment of advanced disease of NSCLC and adjuvant chemotherapy [20]. However, this treatment entails a multipronged adaptive response in malignant cells, which renders them less susceptible to the antiproliferative and cytotoxic effects of the drugs [16,20,21], resulting in the resumption of proliferation [21]. These mechanisms allow the cancer cell to survive and progress in human organisms and thus develop resistance to therapy [24]. Such resistance is a major cause for therapeutic failure of NSCLC, leading to tumor recurrence and disease progression [25]. The mechanisms underlying cisplatin resistance are multifactorial [16]. A significant role is played by tolerance or repair of cisplatin-DNA adducts [16,26]; in addition, resistance has been associated with the induction of anti-apoptotic signals, the active efflux of drugs from the cell cytoplasm, epigenetic regulation by miRNA, deregulation of growth regulatory pathways by acquiring growth factor independence, suppression of the immune system, and low expression of antigens that activate T lymphocyte cells (mimicry) [24,27]. All these mechanisms appear to play crucial roles in cisplatin resistance. Greater knowledge of the extensive interactions of cisplatin taking place in the cytoplasm and nucleus, and of the multifactorial nature of resistance will enable a more complete understanding of cisplatin resistance in patients with NSCLC [16] (Fig.1).

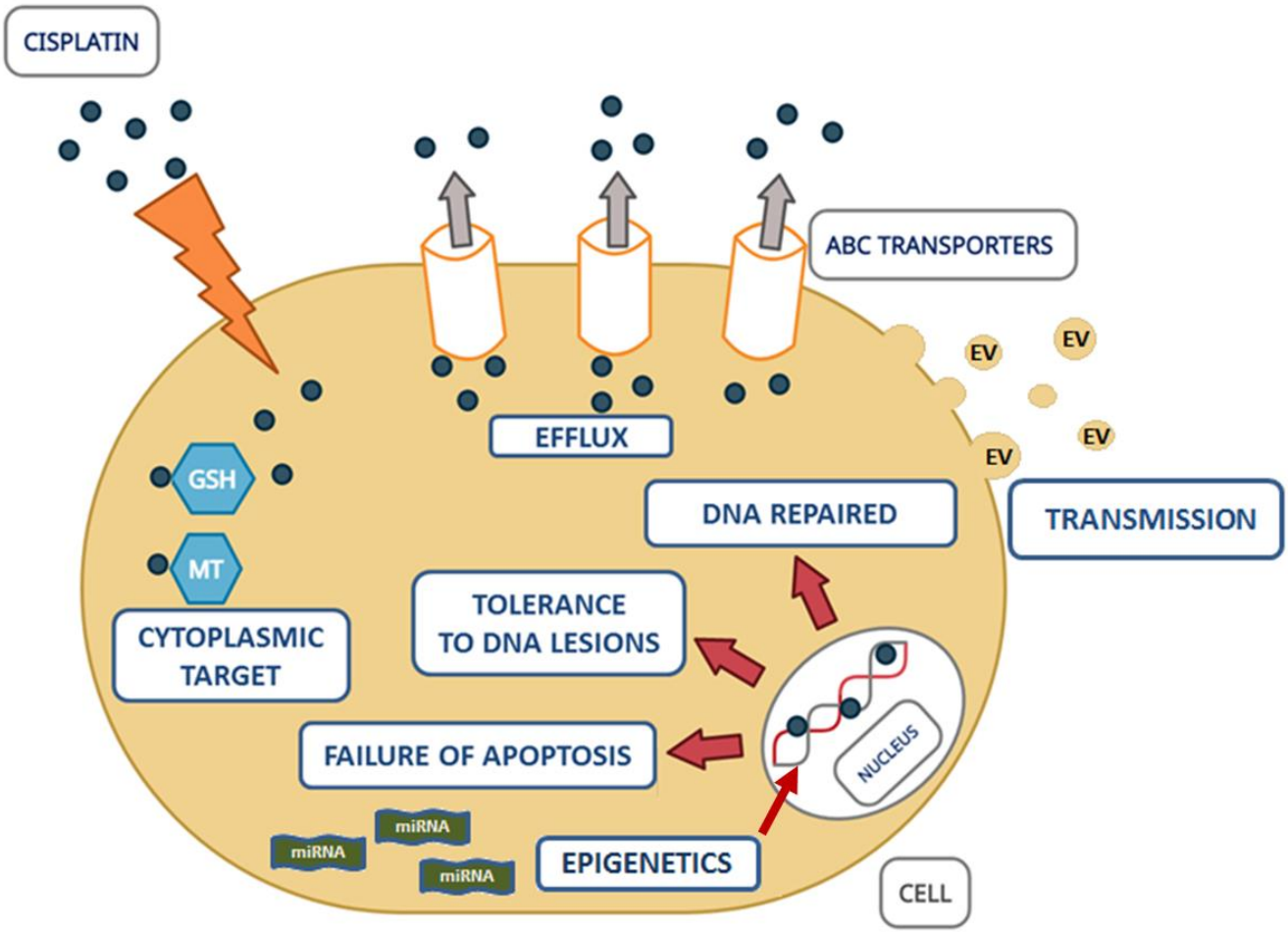

Fig.1 Molecular mechanisms of cisplatin resistance. *GSH - Reduced glutathione, MT – metallothioneins, EV – Extracellular vesicles.*

2.1. Repair of DNA damage

Platinum compounds are believed to be the most active anticancer agents currently used in clinical therapies of NSCLC. Their cytotoxic activity is based on their ability to form DNA adducts [28,29]. Cisplatin induces both intrastrand crosslinks, comprising around 90% of cases, and interstrand crosslinks (ICLs), comprising 5-8% of DNA adducts. Generally, there are two forms of intrastand crosslink: the 1,2-intrastrand crosslink between two adjacent purines, being the predominant form, and the 1,3-intrastrand adducts [28–30]. ICLs link two bases on the opposite strands of DNA[30].

The formation of platinum adducts is particularly deleterious, as distortion of the DNA double helix blocks DNA replication and transcription. In addition, if the damage is not repaired, they can lead to single-strand breaks (SSBs), double-strand breaks (DSBs), and chromosomal rearrangements. Processes such as DNA damage response (DDR) and DNA damage tolerance (DDT) upregulation are advantageous to cancer cells because they allow them to resist these damaging lesions. For example, many types of cancers exhibiting chemoresistance, including lung cancer, demonstrate upregulated DDR and DDT pathways [30]. In NSCLC many processes aimed at removing or repairing the DNA lesions

activate the cellular DDR [30,31]. Depending on the type and location of DNA damage, several repair pathways exist, such as nucleotide excision repair (NER), homologous recombination repair (HRR), nonhomologous end joining (NHEJ), and translesion synthesis (TLS) or post-replication repair (PRR) [28,30,32,33]. These repair mechanisms demonstrate different degrees of specificity and fidelity; however, they may be mutually complementary in some types of damage [30,33]. If the DNA lesions are not repaired before replication, the damaged DNA cannot be utilized as a template for replication by high fidelity DNA polymerases. Damage results in the replication fork stalling and the development of a replication gap. To enable completion of DNA replication across the lesion and in consequences cell survival, cells utilize error-free or error-prone lesion bypass mechanisms to synthesize DNA. To ensure error-free lesion bypass, and allow synthesis past the lesion, the template must be switched from the damaged to the undamaged DNA strand [30].

Mechanism of error-prone repair involves a concerted and coordinated interplay between different cell-cycle checkpoints and DDT pathways. The primary pathways are homologous recombination (HR), homologous recombination repair (HRR), Fanconi Anemia (FA), nucleotide excision repair (NER) and translesion synthesis (TLS) [28]. ICL repair begins by TLS using low fidelity DNA polymerases, which prepares the leading template strand for repair by the HR pathway [30]. Stalled replication forks activate the FA pathway, which detects and repairs the stalled replication forks with the common biochemical FA/BRCA HRR pathway [30].

2.1.1. Nucleotide Excision Repair (NER)

Intra-strand DNA cross-links are relatively straightforward. Only one strand is damaged, and the second strand remains available as a template for repair synthesis. These adducts are most commonly repaired by nucleotide excision repair (NER) [28,32]. NER involves several proteins used for damage recognition and damage excision, as well as a helicase [34]. Lesions in the DNA helix are recognized by the XPC-RAD23B damage recognition protein complex, which binds to the DNA strand [34]. An oligonucleotide of 24–32 nucleotides in length is excised on both sides of the lesion on the DNA strand [30,34], and the resulting gap is patched by repair synthesis and ligation [32].

Additionally, NER acts as an important mediator of responsiveness to cisplatin-based chemotherapy. Recent studies showed that lung cancer cell line, Calu-1, which is moderately resistant to cisplatin, exhibited elevated level of NER factors, participating in DNA repair including XPA, XPC-hHR23B, XPG, ERCC1-XPF, TFIIH, PCNA and DNA ligase [33].

2.1.2. Post-replication repair (PRR)

Studies on *Saccharomyces cerevisiae* yeast have provided an good understanding of the activity of PRR pathways [35]. Stalled DNA replication is typically restarted by PRR pathways such as translesion DNA synthesis (TLS) or template switching (TS). Both pathways are regulated by ubiquitination of the proliferating cell nuclear antigen (PCNA) at Lysine 164 (K164) or Lysine 63 (K63) [36].

The TLS pathway is initiated by a protein complex formed by RAD6 (an E2 ubiquitin-conjugating enzyme) and RAD18 (an E3 ubiquitin ligase) [30,36]. The RAD6-RAD18 complex (an E2–E3 complex) induces posttranslational monoubiquitination of PCNA at K164 (monoUb-PCNA); such monoubiquitination is the primary modification of PCNA in mammals. Following this, monoUb-PCNA recruits one of the four Y-family specialized polymerases TLS: Pol κ, Pol η, Pol τ, Rev1 [30]. Interactions between the ubiquitin (Ub) moiety of monoUb-PCNA and the Ub-binding domains allows the TLS polymerases bind to the stalled 3′-ends or to the damage sites, thus allowing replication over the DNA lesion [30,36]. Following the incorporation of the nucleotide opposite the damage site, the insertion of TLS polymerase is replaced by polymerase Pol ζ, an error-prone polymerase belonging to the B-family formed as a heterodimeric complex of Rec3/Rev7 [30]. In PCNA-dependent TLS, Pol ζ forms a complex with the Pol31 and Pol32 subunits of Pol β (Rev3-Rev7-Pol31-Pol32, referred to as Pol ζ4) [30,37(p31)]. Pol ζ contains active sites that can accommodate distorted DNA bases and base pair mismatches and extends the TLS patch by ~18 nucleotides [30]. This extension step allows the lesion to escape detection because TLS polymerases do not have intrinsic exonuclease activity for do not proofreading capability [30]; the incorporation of faulty nucleotides by low-fidelity TLS polymerases may r increase spontaneous mutagenesis, therefore resulting in platinum-chemotherapy tolerance and toxicity within normal cells [30,36,38]. When the strand is extended past the DNA lesion, Pol ζ is replayed by the high fidelity DNA polymerase [30].

The TS pathway is promoted by additional factors, such as MMS2-UBC13 (a UEV–E2 complex) and HLTF (an E3 ligase), which are functional homologues of yeast Rad5 [30,36,39]. This stable complex allows polyubiquitination of PCNA (polyUb-PCNA) [30,36]; however, PCNA polyubiquitination occurring in response to alkylating agents is ~20-fold slower than monoubiquitination [30]. PCNA polyubiquitination predominantly occurs via en bloc transfer of preformed ubiquitin chains, initiated by the MMS2–UBC13 complex, which initiates the formation of ubiquitin chains at the K63 linkages of PCNA [30,36]. Briefly, HTLF forms a thiol-linked ubiquitin chain on UBC13, which is then transferred to RAD6~ubiquitin to form RAD6~ubiquitinn+1 [30]; subsequently RAD18 transfers the resultant Ub chain to PCNA en bloc. In the TS pathway, PolyUb-PCNA stimulates the release of the stalled primer end from the damaged template [36], which then joins with the newly-synthesized daughter strand of the sister chromosome. The TS pathway is essentially error-free, as the repair is based on an undamaged template [36].

Recently studies suggested that PRR pathways, contribute to the chemoresistant phenotype in NSCLC. The TLS function of Pol ζ is believed to play a key role in its ability to enhance resistance to platinum-based chemotherapies. Doles *et al.* showed that reduction of the Pol ζ activity can make an intractable lung cancer model of NSCLC susceptible to cisplatin-based chemotherapy. Inhibition of Rev3L activity or expression may be particularly effective as cisplatin treatment, itself, increases Rev3L mRNA levels and elevated Rev3L was shown to promote cisplatin resistance [40]. Additionally, Ceppi *et al*. confirmed the association of platinum sensitivity with the endogenous Pol η mRNA levels in several

NSCLC cell lines. Their results indicate a linear relationship between basal Pol η levels and *in vitro* cisplatin sensitivity. Endogenous Pol η mRNA presented significantly higher level in the most cisplatin-sensitive NSCLC cell lines, while lower level was observed in resistant cell lines (with comparable degree of cisplatin resistance) [41].

2.1.3. Fanconi Anemia (FA) and ICL Repair

FA is an autosomal recessive genetic disease which is caused by mutations in the Fanconi anemia protein cluster. It is characterized by hypersensitivity to various agents that induce ICL and chromosomal instability, and can favor the development of various cancers [30]. FA pathways function mostly during the S phase and are involved in ICL repair [30,42]. Additionally, FA or FA-like proteins have been found to mediate cellular resistance NSCLC against agents which induce ICL [33,42].

In response to ICL, the FA pathway induces phosphorylation of FANCI by the FA core complex, which contains MHF1-2, FAAP24 and large multi-subunit ubiquitin E3 ligase [30,42]. FANCD2 is phosphorylated by the checkpoint kinase ATR at threonine 691 (T 691) and at serine 717 (S 717) [30,42,43]. Such phosphorylation of FANCD2 induces enhanced cellular resistance to ICL stimulating agents and is also required for establishment of the intra-S-phase checkpoint response [42]. This modification helps stabilize the replication forks but is not required for FA pathway activation [30].

MHF1-2 and FAAP24 recruit FANCL, a large multi-subunit ubiquitin E3 ligase (FA core complex). FANCL contains a plant homeodomain (PHD) which catalyzes the mono-ubiquitylation of FANCD2-FANCI [42]. The complex of FANCL with UBE2T and UBE2W (enzymes E2) induces monoubiquitination of FANCD2 at Lysine 561 (K561), additionally FANCL in complex with UBE2T promote monoubiquitination of FANCI at Lysine 521 (K521). FANCD2 monoubiquitination is an essential modification for the FA network and is also considered a surrogate marker of activation [30].

Protein ubiquitylation regulates various biological processes, including DNA damage checkpoints and DNA repair pathways. The monoubiquitination of FANCD2 and FANCI results in the production of the FANCI/FANCD2 complex; this is which is translocated and assembled into DNA repair sites. The complex also recruits FAN1 endonucleases which colocalize FA proteins (PALB2, BRCA2, FANCJ, RAD51C and SLX4) for the removal of the ICL through NER [30,42]. It is believed that TLS polymerases are then recruited to repair the damage (the polymerases are promoted by RAD6/RAD18 following induction by monoUb-PCNA); however, the exact sequences of repair pathways concerning NER/TLS remain unclear. It is possible that FANCI/FANCD2 complex creates the incision at the site of the ICL, and the gap at the lesion is filled by TLS. Alternatively, TLS may promote the incision at the site of DNA damage, and the FANCI/FANCD2 complex then induces TLS activity nearby[30].

Given that the FA pathway plays an essential role in response to therapy-induced DNA interstrand cross-links, it is highly possible that cancers with defective FA pathway are more sensitive to cisplatin based therapy [44]. Ping *et al.* found that the cisplatin-resistant NSCLC cell line A549/DR exhibits significantly elevated expression level of the FA factors compared to its parent cell line A549

[33]. Additionally, previous studies, that used specific small molecule inhibitors or RNA targeting FA pathway-associated genes, showed a variable sensitization of tumor cells to cisplatin [33,44–46].

2.1.4. Homologous recombination repair (HRR)

In NSCLC, in response to DSBs, HRR pathway is activated during S and G2 phases of the cell cycle [30,33,47]. HRR uses the undamaged sister chromatid as a template. The procedure consists of three main steps: end resection, strand invasion and resolution [30]. In the initial step involving nucleolytic resection, MRE11-RAD50-NBS1 (MRN) complex and the 5′ to 3′ exonuclease Exo1 are activated to resect nucleotides and extend the annealed 3'-single-stranded DNA (3'-ssDNA) overhangs; MRN and Exo1 are activated singly or in combination with Bloom's syndrome RecQ helicase like protein (BLM) and helicase/endonuclease DNA2 [30,48,49]. Following activation, the 3′-ssDNA tails generated via DNA end resection are stabilized by replication protein A (RPA) [30,49]. RPA is then removed and exchanged for the Rad51 recombinase in a process aided by the mediator proteins Rad52 or BRCA2 with localizer PALB2. These proteins are involved in Rad51-ssDNA filament formation and protect Rad51 from removal [30,48,49]. The Rad51-ssDNA complex is a right-handed helical polymer, with the DNA being held in an extended conformation [49]. The complex performs a "presynaptic" search for a homologous sequence in double-stranded DNA [30,48,49], leading to the production of heteroduplex DNA (hDNA) and the formation of a transient structure known as the displacement loop or D-loop structure [30,48–50]. During homologous paring, the activity of Rad51 is stimulated by Rad 54, member of the Swi2/Snf2 family of chromatin remodeling proteins/ATPases [48].

When the 3′ overhang of the invading strand is free from RAD51, it serves as a primer for initiation of DNA synthesis [30,48,49], thus allowing the extension of the D-loop structure [49]. Currently, it is unclear which replication machinery is used for this elongation but it has been shown that Pol η of TLS polymerases, in particular, demonstrate affinity for D-loop elongation [30].

After elongation, two pathways can be utilized to resolve the D-loop: the DSB repair (DSBR) and synthesis-dependent strand annealing (SDSA) models [30,49,50]. In the SDSA pathway [30] D-loop extension continues for a short distance. The D-loop is disassembled by dissociation of the newly-synthesized strand with the ssDNA associated with the other DSB end; this step is performed by regulator of telomere elongation helicase 1 (RTEL1) [30,49,50]. This pathway is preferred for mitotically-dividing cells, and it always provides a noncrossover gene conversion product [50]. In contrast, in the DSBR pathway, the gap is filled by capturing and ligating the second end to create a double Holliday Junction (dHJ) [30,50]. This mechanism involves BLM helicase, an ATP-dependent 3′-5′ DNA helicase used to unwind D-loops [30]. Resolution of the dHJ can lead to the formation of a crossover or non-crossover product [50].

Emerging data on the role of HR in the repair of cisplatin adducts is becoming increasingly significant and highlights the importance of this DNA repair process in NSCLC chemoresistance. Previous studies revealed a variable sensitization of tumor cells to cisplatin depending on activity of HR pathway-associated genes [47]. Moreover, in other study, Ping et al. found that the cisplatin-resistant

NSCLC-derived cell line A549/DR exhibits dramatically elevated expression levels of the HR factors compared to both its parent cell line A549 and moderately resistant to cisplatin Calu-1 cell line. Additionally, they proved that the depletion of HR associated factors, correlates with the increased ICL damage and decreased HR repair, thus leading to that the NSCLC hypersensitization to cisplatin [33].

2.2. Apoptosis

The major goal of cancer chemotherapy is to force tumor cells to execute apoptosis following exposure to anticancer agents, such as cisplatin used in treatment of NSCLC. However, cellular damage caused by chemotherapeutics has to pass a certain threshold level to trigger programmed cell death [51]. The effector phase of apoptosis involves several pro- and anti- apoptotic proteins, including pro-apoptotic Bax (Bcl-2-associated X protein), Bak (Bcl-2 homologous antagonist/killer), Bad (BCL2 associated agonist of cell death) and anti-apoptotic Bcl-2 (B-cell lymphoma 2), Bcl-XL (B-cell lymphoma-extra-large) and Bcl-w (Bcl-2-like protein 2). However, cancer cells commonly demonstrate mutations in the genes involved in various signaling pathways, including apoptotic ones, thus often leading to their dysfunction. This may result in the formation of resistance to cisplatin, as any interference which mediates the induction of anti-apoptotic signal transduction or inhibition of pro-apoptotic pathways, including transcriptional and translational responses, is a potential mechanism of drug resistance. Furthermore, apoptosis induced by cisplatin in both cisplatin-sensitive and cisplatin-resistant cancer cells leads to increased levels of Bax mRNA and Bak protein, and decreased expression of Bcl-2. While cisplatin activates a robust apoptotic response based on activation of the JNK pathway in cisplatin-sensitive cancer cells, no such response is observed in resistant cells.

Additionally, three important mediators of chemoresistance in cancer cells are X-linked inhibitor of apoptosis protein (Xiap), Akt, and p53 [51]. Of the three, p53 is the main tumor suppressor. Cisplatin treatment of cancer cells leads to p53 activation and its stabilization by phosphorylation at the sites of Ser15 and/or Ser20 inhibit the association of p53 with E3 ubiquitin ligase mouse double minute 2 (Mdm2); this blocks the degradation of p53, which is regulated by Mdm2 in normal cells. In contrast, the cell survival factor Akt inhibits apoptosis. Akt participates directly in the suppression of pro-apoptotic proteins and indirectly induces growth factor-mediated and cytokine-mediated expression of anti-apoptotic protein [16,52].

Additionally, 34% of patients with NSCLC have a mutation of the tumor suppressor gene *TP53* that encodes p53 protein (including nonsense mutation and pro-oncogenic “gain-of-function mutation”) which has been associated with frequent smoking [52]. Such mutations in *TP53* , including “gain of function mutation” cause the dysregulation of multiple signaling cascades, such as apoptotic pathways [52,53]. For example, p53 status strongly influences the action of cyclin-dependent kinase inhibitor 1A (CDKN1A), which regulates G1/S and G2/M checkpoints, and is transiently recruited to facilitate cisplatin-induced DNA damage. Upregulation of CDKN1A allows cells to acquire a highly-aggressive phenotype and to escape cell cycle blockage and apoptosis [54]. Cisplatin also accumulates in

mitochondria, forming adducts with mitochondrial DNA. This process leads to the impaired synthesis of proteins involved in the electron transport chain and an increase in the intracellular ROS level [52].
Interestingly, p53 also demonstrates an antioxidant function by regulating a wide range of antioxidant genes. Furthermore, ROS impair the function of tumor suppressors such as p53 by inflicting DNA damage. They also activate the PI3K/Akt pathway involved in cell survival and proliferation by the activity of Epidermal Growth Factor Receptor (EGFR), thus further enhancing resistance to chemotherapy among cancer cells. Moreover, Akt is involved in the activation of EGFR and down-regulation of ROS. The PI3K/Akt pathway inhibits ROS production by regulating the expression of Forkhead Box Protein O1 (Foxo1) transcription factor and Caspase-3, which are involved in the intrinsic apoptosis. Thus EGFR promotes Akt activation, and Akt promotes EGFR signaling in return, forming a positive feedback circle within the EGFR-Akt axis[52].

Another mechanism by which cells may resist apoptosis is related with the overexpression of the inhibitor of apoptosis protein (IAP) family of proteins [55]. XIAP (X-linked IAP) directly inhibits the apoptotic activity of caspases, including caspase-3 and caspase-7 through its BIR2 domain, and caspase-9 through its BIR3 domain [55,56]. During apoptosis, cells prevent the binding of XIAP to caspases and trigger its redistribution from the cytosol to the nucleus using endogenous antagonists of XIAP, such as SMAC (second mitochondria-derived activator of caspases), HtrA2/Omi (high-temperature-requirement A2), ARTS (endoplasmic reticulum aminopeptidase) and XAF1 (XIAP associated factor 1) [55]. Furthermore IRES (internal ribosome entry segment) can initiate XIAP mRNA translation and enhance it using various IRES transacting factors e.g. La autoantigen, hnRNP C1/C2 (heterogeneous nuclear ribonucleoproteins C1/C2) and MDM2 protein. Additionally, in cancer cells, the level of XIAP can be upregulated through phosphorylation by Akt kinase or by interaction with survivin, Notch receptor or p34SEI-1 protein, which protects proteins by promoting degradation by ubiquitination [56]. In contrast, the upregulation in XIAP expression observed in cancer cells in response to DNA damage is associated with two proteins: Che-1 protein mediates activation of XIAP NF-κB-dependent transcription, while Mdm2 mediates XIAP by IRES-dependent translation[56]. In turn, XIAP over-expression provides resistance to apoptosis through the stimulation of both the intrinsic (mitochondrial directed) and extrinsic (death receptor directed) pathways [55].
The numerous genes involved in apoptosis indicate a highly-complex interwoven network of checks and balances. In lung cancers, in addition to inhibition of proapoptotic proteins, chemotherapy resistance can be induced by activation or overexpression of antiapoptotic molecules [57].

2.3. ABC transporters

Over expression of ATP-binding cassette (ABC) transporters play an important role in the development of multiple drug resistance in NSCLC. ABC proteins have the ability to efflux a variety of small molecules, including toxic chemicals, from the cytosol by using energy from ATP hydrolysis [58,59]. The ABC protein family consists of 49 membrane proteins [60] divided into seven subfamilies (ABCA -

ABCG) based on their gene structure, amino acid sequence, domain organization and phylogenetic properties [24,58–60]. ABC transporters are expressed in various tissues, such as the liver, intestine, kidney, and brain, where they play important roles in the distribution and excretion of various drugs [60].

Members of three subfamilies, *viz.* ABCB, ABCC and ABCG (comprising at least 11 ABC superfamily transporters), are involved in the active efflux of anticancer drugs from cytoplasm of cell; these are named Multidrug Resistant Proteins (ABCB) or Multidrug Resistant-associated Proteins (ABCC) [24,60]. Their overexpression can confer resistance to drugs such as cisplatin by lowering intracellular accumulation of chemotherapeutics [61]. Interestingly, in cancer cells, exposure to one drug often elicits resistance to various structurally-unrelated others. This phenomena is related to the broad substrate specificity of ABC transporters [62]. In lung cancer cells, several ABC proteins are involved in the reduction of intracellular drug concentrations: ABCA1, ABCA2 (ABC transports not classified as multidrug resistant proteins), ABCB1 (P-glycoprotein/multidrug resistance protein 1; MDR1), the multidrug resistance-associated proteins (MRPs) ABCB4, ABCB11 and ABCC1-6; as well as ABCC10, ABCC11, and ABCG2 (BCRP/MXR) [58]. However, only ABCA1, ABCC2 and ABCC6 enable cisplatin resistance by its direct efflux from the cell [63,64].

ABC transporters are membrane integral proteins typically consisting of evolutionarily-conserved structures named nucleotide binding domains (NBDs), which transfer the energy to transport the substrate across the membrane, and six α-helical transmembrane domains (TMDs), which provide the specificity for the substrate. Full transporters (TMD1-NBD1-TMD2-NBD2 or NBD1-TMD1-NBD2-TMD2) are composed of four domains: 2 × TMD (TMD1 and TMD2) and 2 × NBD (NBD1 and NBD2), while half transporters include only two, *viz.* TMD-NBD or NBD-TMD, and act as functional transporters by homo- or hetero-dimerization [24,60].

The NBD domains are typically located in the cytoplasm; they comprise 200–220 aa with an α-helical domain and a catalytic core domain. The latter includes most of the conserved regions, organized within the Walker A motif (or phosphate-binding P-loop) and Walker B motif (for the binding and hydrolysis of ATP), as well as the LSGGQ signature motif (involved in the binding of the nucleotide) and the A, D, H and Q loops [24,60]. One ATP molecule can be bound and hydrolysed by the Walker A and Walker B motifs of one NBD subunit and the C-loop and D-loop of the second subunit, one homodimer/heterodimer can bind and hydrolyze two ATP molecules [24,60]. This ATP hydrolysis indices conformational changes in the TMD domain, which lead on alternating access from inside and outside of the cell, resulting in unidirectional transport across the cell membrane; it is also likely that ATP binding is sufficient to trigger NBD dimerization and the transport of substrates [24].

While NBDs present an open conformation and are separated from one another in the absence of nucleotides [24], in the presence of ATP, they form a complete interface by approaching each other and "sandwiching" any bound ATP molecules [60]. The NBDs of ABC transporters that actively transport substrates against a gradient function as ATPases. Whereas some of these proteins form channels, in which passive anions flow occurs, that require energy input [24]. ATP hydrolysis disrupts the dimer

interface and releases the ADP and inorganic phosphate. Additionally, effective coupling of substrates transport, utilize by binding of ATP molecules requires the transmission of the molecular motion from the NBD to the TMD domains [60]. The interaction between TMDs and NBDs takes place on a coupling helix, which is located in the cytoplasmic loops of the TMD [60]. Furthermore, several ABCC family transporters (ABCC1, -2, -3, -4 and -8) use glutathione (GSH) to enable the transport of several substrates. GSH conjugates present higher affinity to transporters or act as stimulators of active transport [24]. In addition, the WNT/β-catenin pathway is an important signal transduction pathway that regulates tumor cell cisplatin resistance [63]. Activation of the WNT signaling pathway draws non-phosphorylated (activated) β-catenin into the nucleus, thus promoting the expression of downstream signaling molecules, including ABCB1, ABCC1 and ABCG2, and promoting the occurrence of cisplatin resistance in NSCLC [63,65].

2.4. Epigenetic regulation by miRNAs

Cisplatin resistance is also regulated by microRNA (miRNA), small, endogenous, noncoding RNA molecules that consist of about 18–23 nucleotides that have influence on posttranscriptional regulation of gene expression [27]. Their expression and wide range of targeted genes influences almost every genetic pathway from cell cycle checkpoint, cell proliferation to apoptosis. Even though, miRNAs expression correlates with various cancers they may act as tumor suppressors and oncogenes [66]. Furthermore, one particular miRNA being tumor suppressor for one type of cancer may act as oncogene in another. One of such miRNA is miR-630, it inhibits tumor growth and metastasis in esophageal squamous cell carcinoma, hepatocellular carcinoma and breast cancer, whereas it plays an oncogenic role in renal cell carcinoma, colorectal cancer and gastric cancer [67]. However role of miR-630 in cisplatin resistance of NSCLC remains unclear. MiR-630 targets and inhibits activation of p-53, the master regulator of cisplatin-induced cell death, and blocks the early DNA damage response in lung cancer cells, it also reduces pro-apoptotic pathways regulated by p-53 [27], and targets several other apoptotic modulators such as PARP3, DDIT4, EP300 and EP300 downstream effector p-53 thus shifting apoptotic balance towards cell survival [68]. On the other hand miR-630 inhibits cell proliferation by targeting cell-cycle kinase 7 (CDC7), [68]. In NSCLC cell models miR-630 may confer cisplatin resistance in A549 cells while playing opposite role in other lung adenocarcinoma cell lines: CL1-0 and H358[69,70]. This Janus face mechanism of action may be attributed to the fact, that cancer cells usually mutate the TP53 gene in favor of their survival and propagation. Some of the mutant p-53 proteins not only lose the wild-type activity, but also acquire oncogenic function, namely “gain-of-function”, to promote cancer development [53]. TP53 mutations are especially common in stage I through III of NSCLC [71]. Interestingly, 34% of NSCLC patients have mutation in TP53 gene as an aftermath of frequent smoking [52]. Additionally, expression of p-53 protein and its pro-apoptotic activity (in response to cisplatin treatment) in NSCLC was shown to be upregulated and enhanced after inhibition of miRNA-98-5p, thus proving this miRNA involvement in cisplatin resistance [72].

Interestingly, the most upregulated miRNA found in cisplatin resistant variant of NSCLC cell line - A549 compared to parental A549 is miR-224 [73]. It targets potent cyclin-dependent kinase inhibitor p21WAF1/CIP, which is critical for p-53 - induced cell cycle arrest, dysregulating G1/S cell cycle transition and apoptosis, thus promoting tolerance to cisplatin [68,73].

Furthermore, one of the first discovered miRNA – miRNA-196a, upregulated in vast majority of cancer types including NSCLC was shown to be involved in mediation of cisplatin resistance, however, its mechanism is not clear [74]. MiRNA-196a targets Annexin-A1 (ANXA1) gene that regulates physiological mechanisms such as hormone secretion, apoptosis, exocytosis and signal transduction [75]. Anexin-A1 is also involved in the acquisition and maintenance of a cancer stem cell-like phenotype that is characterized by upregulation of several chemo-resistant mechanisms including activity of ABC proteins [76,77] . Furthermore, downregulation and/or silencing of microRNA-196a enhances the sensitivity of NSCLC cells to cisplatin treatment [74]. Moreover, miRNA-196a targets 3′-UTR region of HOXA5 gene that encodes the transcription factor Hox-A5 (homeobox protein), resulting in increased NSCLC cell proliferation and metastasis [78].

One of the most important tumor suppressor in lung cancer is phosphatase and tensin homolog deleted in chromosome 10 (PTEN) that inhibits NSCLC cell growth by promoting G0/G1 arrest and cell apoptosis [79,80]. In many types of cancer (including NSCLC) aggressive phenotype correlates with downregulation of PTEN [81,82]. Furthermore targeting PTEN by several miRNA such as: miRNA-21, miRNA-92b and miRNA‑328 confers cisplatin resistance in NSCLC [82–84].

However, some miRNA enhance cisplatin sensitivity or reduces cisplatin resistance by targeting: ABCC2 that mediates cisplatin efflux, or anti-apoptotic Bcl-xl by let7c [85], TGFβR2 by miRNA 17 family [86] or MET by miRNA-206 [87] and in turn inhibiting or reversing EMT phenotype, thus usually they are substantially downregulated in NSCLC [27].

The most enigmatic miRNA involved in cisplatin resistance in NSCLC is miRNA-31 that targets 3′-UTR region of DICER1 gene [88]. Helicase with RNase motif, better known as Dicer is a critical regulator of the biogenesis of miRNA and small interfering RNA (siRNA) [89]. Thus, downregulation of Dicer by miRNA-31 leads to overall downregulation of miRNA production, both oncogenic (involved in acquisition of cisplatin resistance) and tumor suppressor (that renders NSCLC sensitization to cisplatin treatment) [88].

Interestingly, recent data suggests that acquired chemoresistance may be transferred to sensitive cells by extracellular vesicle as their cargo contains multiple particles including proteins, mRNA and microRNA [90]. Exosomes present in tumor microenvironments can be internalized by adjacent cells and modify the phenotype of the recipient cell to reflect the regulatory functions of the exosome cargo. This phenomena may be observed within the same tumor or at other anatomical sites [91]. Our recent research proved that exosome derived miRNA poses diagnostic value in early NSCLC diagnosis [2], however possible prognostic value for cisplatin based therapy outcome based on miRNA panel are yet to be determined.

2.5. Cisplatin resistance and the tumor microenvironment (TME)

The tumor microenvironment (TME) consists of both normal, non-malignant tissue cells and immune cells with diverse phenotypes and functions that can strongly modulate the response to chemotherapy and increase metastatic potential [92]. The least complicated TME activity leading to cisplatin resistance is the formation of a physiological barrier composed of a dense extracellular matrix (ECM) and closely-packed cells around the tumor, which substantially restricts the diffusion rate of anticancer drugs into the cancer cells [93]. The region comprising the tumor and the TME is often named the "wound that does not heal" as both states are characterized by similar molecular mechanisms, including inflammation [24]. One of the key components of the TME are tumor-associated macrophages (TAMs), which are responsible for promoting epithelial-to-mesenchymal transition (EMT), migration, tissue infiltration, dissemination and thus, distant metastasis [94]. Generally monocytes undergo differentiation towards one of two subpopulations: M1 (classical) or M2 (alternative) macrophages. Alternative activation, leads to the formation of regulatory macrophages and wound-healing macrophages. The activation of the M2 form results in the release of TGF-β, thus triggering EMT and increasing the metastatic potential of cancer cells [95]. EMT is considered to be a significant factor in chemoresistance, converting stationary epithelial cells into mobile, less proliferative mesenchymal cells [96]. In NSCLC, TAMs increase the population of CD133+ expressing cancer stem cells (CSCs), they also enhance the expression of genes associated with the inflammation proteins Sox2 and NF-κB [92]. Furthermore, cisplatin-resistant NSCLC cells present elevated expression of other oncogenic and stemness markers, such as Src, Notch1, macrophage inhibitory factor (MIF) and CD155, which promotes alternative activation of TAMs into pro-tumorigenic M2 (-like) macrophages [94]. Furthermore, cisplatin-stimulated classically-activated macrophages (CAMs) enhance ovarian cancer cell migration, triggering EMT via the CCL20/CCR6 axis [97]. Interestingly, the CCL20/CCR6 axis promotes NSCLC disease progression, and high expression of CCR6 has been associated with shorter disease-free survival [98]. The relationships between cisplatin resistance and the TME are summarized on Fig.2.

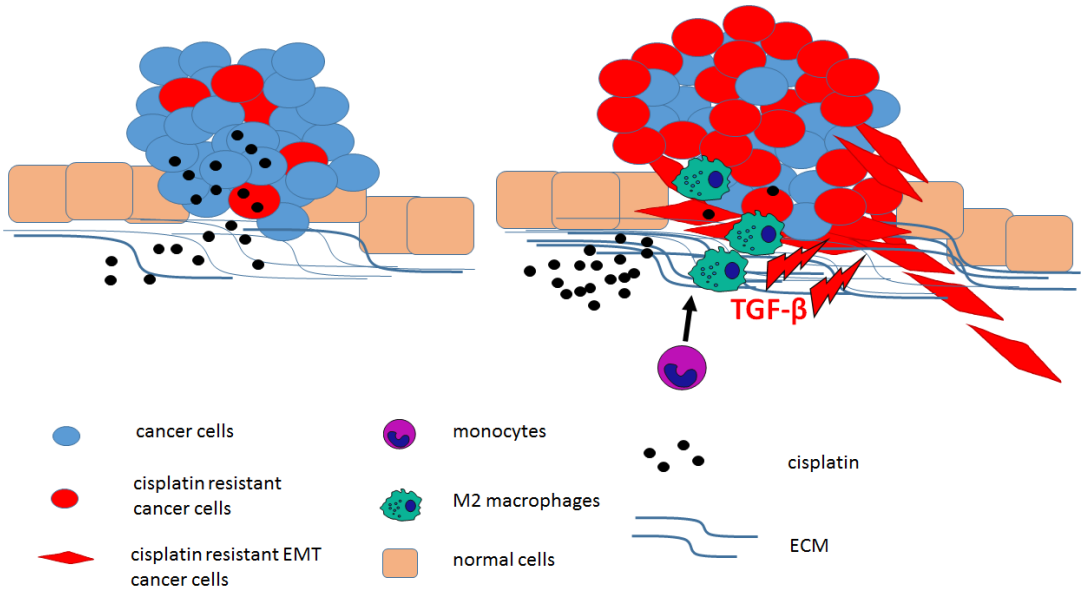

Fig.2 TME impact on cisplatin resistance. *Cisplatin administration leads to selection and acquisition of cisplatin-resistant cancer cells. The TME restricts the diffusion rate of cisplatin. Cisplatin-resistant cancer cells support the activation of M2 macrophages, which in turn induce EMT via TGF-β secretion resulting in enhanced chemo-resistance and metastatic potential.*

3. Protein-protein interaction changes in NSCLC caused by acquisition of cisplatin resistance

Resistance to cisplatin is acquired through many ostensibly unrelated mechanisms. To demonstrate this multifactorial nature, studies have analyzed changes in mRNA expression caused by cisplatin resistance in a NSCLC cellular model using the Gene Expression Omnibus data base [99] GSE108214, listing the mRNA expression profiles of the parental and cisplatin-resistant NSCLC cancer cell line A549. The findings were processed using GEO2R online analytical tool [100]. It was found that over 29000 genes were differently expressed between resistant and sensitive A549 cells. A protein-protein interaction (PPI) network was created using the top 250 differently-expressed genes (DEGs) using STRING version 11.0 online software [101] and the Cytoscape open source software platform for visualizing complex networks [102]. KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways analysis was performed using DAVID online tool [103] and KEGG PATHWAY Database [104]. The PPI network composed of 250 top differently-expressed mRNAs, enriched by known signaling proteins is shown on Fig.3.

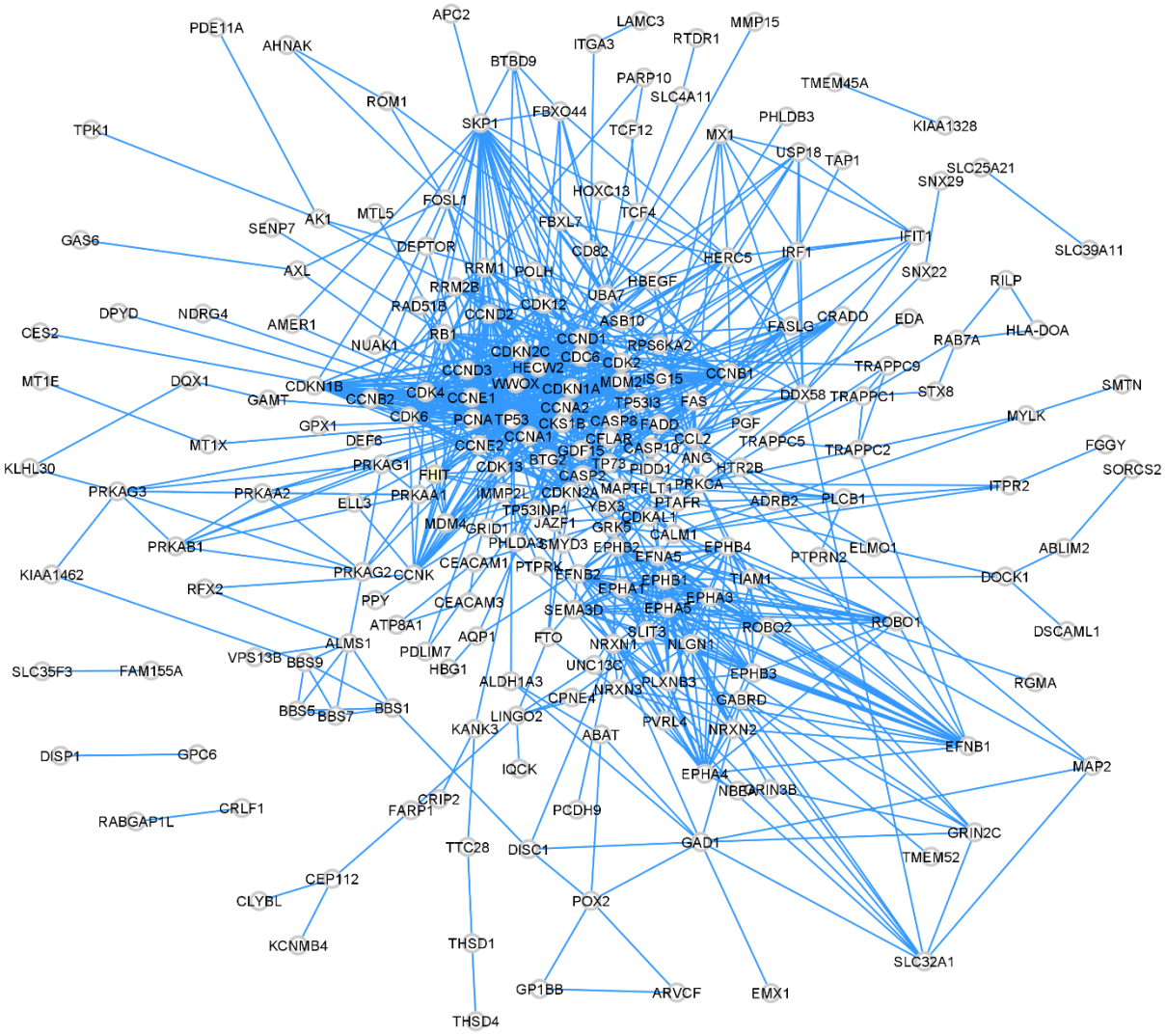

Fig.3 PPI network of 250 top differently-expressed genes between cisplatin-resistant vs. parental A549 cells. *The PPI pairs were imported and visualized by Cytoscape software.*

PPI network indicated the most efficiently-networking DEGs that are strongly related to cisplatin resistance. Among them, the top 3 (TP53, MDM2 and CDKN1A; Table 1) are reviewed in the *apoptosis* section as important anti-apoptotic factors involved in repairs of platinum-derived DNA damage. An analysis of the most dysregulated KEGG pathways (Table 2) demonstrated the complexity of mechanisms of cisplatin resistance, and to highlighted how mutually complementary they are. The KEGG pathways hsa01524, i.e. *platinum drug resistance*, and hsa04210, *Apoptosis*, are composed of a number of sub-pathways including PI3K-Akt and p53; these are also dysregulated, resulting in increased cell survivability. Interestingly, the observed dysregulation of focal adhesion and tight junctions may also suggest phenotypical changes toward a more aggressive and motile mesenchymal phenotype following epithelial to mesenchymal transition (EMT).

Table.1 Top networking DEGs *– upregulated in cisplatin resistant A549 cells. Fold change – A549 cisplatin resistant vs A549 parental, degree – presents the number of undirected edges.*

Gene	Betweenness	Degree	adj.P.Val	P.Value	Fold change
TP53	0.499325647	86	0.52935846	2.04e-01	1.3603568
MDM2	0.04074928	41	0.00026943	1.32e-06	10.4840794
CDKN1A	0.02606798	40	0.00032013	1.83e-06	10.1108217

Table.2 Major dysregulated KEEG pathways. *FDR – falls detection rate <0.05; p-value of <0.05*

KEGG	Pathway Name	Count In Network	FDR
hsa01524	Platinum drug resistance	9 of 70	2.58e-05
hsa04210	Apoptosis	27 of 135	7.38e-23
hsa04310	Wnt signaling pathway	11 of 143	0.00016
hsa04115	p53 signaling pathway	22 of 68	9.40e-19
hsa04151	PI3K-Akt signaling pathway	21 of 348	2.28e-06
hsa04510	Focal adhesion	10 of 197	0.0049
hsa04530	Tight junction	11 of 167	0.00048

Conclusion

Cisplatin resistance occurs as a result of multiple complex mechanisms operating at different cellular levels that either inhibit apoptosis, promote cell survival, or act simultaneously. Resistance can be enhanced by reducing cellular cisplatin levels, increasing inactivation by endogenous nucleophiles, altering the expression of regulatory genes, increasing repair of adducts and increased adduct tolerance. Resistance to cisplatin is a major impediment in NSCLC chemotherapy. An improved understanding of cisplatin resistance will better identify therapeutic targets and allow more accurate prediction of clinical response. Additionally, it will allow therapy to be better tailored to the needs of individual patients.

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