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Chapter IX

Perspectives of Proteomics Investigations of Neuroblastoma Chemoresistance

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Abstract

Neuroblastoma, the third most common paediatric solid tumors after leukaemiae and brain neoplasiae, with an incidence of approximately 1.3 child out of 100.000, is responsible of 15% of all childhood cancer death.

The acquisition of multidrug resistance upon treatment with anticancer drugs is a common feature of highly malignant Neuroblastoma. The identification of marker proteins involved in chemo-resistance might significantly help in the prognosis of this neoplasia by individualising the drug treatment .

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Proteomics investigation might represent a powerful holistic scientific approach in order to possibly characterised the molecular hallmarks of Neuroblastoma chemoresistance. Combining high-resolution protein separations with mass spectrometry protein identification, proteomics allows to explore the molecular mechanisms of cancer chemoresistance in a data driven experimental design, therefore enabling the construction of novel hypothesis not necessarily linked to a define researcher theory.

In the following we review the current state of the art in the proteomics investigations devoted to the characterisation of Neuroblastoma drug resistance.

Keywords: Proteomics, Neuroblastoma, Chemoresistance, Pediatry, Cancer.

Introduction

Neuroblastoma is the most common extracranial tumor of childhood and the most common cancer diagnosed during infancy, at a median age of 18 months (Landis S.H. et al., 1999). The incidence of this tumor is fairly uniform throughout the world; the aetiology of neuroblastoma is still not clear, but it seems unlikely that environmental exposure has a significant role (Kushner B.H. et al., 1986). This neoplasia is characterized by a heterogeneous clinical behaviour, which have been used for the classification and prognosis of the disease.

Mainly the classification divides Neuroblastoma class of tumors in unfavourable and favourable subtypes, depending on the genetic changes occurred. A subset of patient can inherit a genetic predisposition to neuroblastoma, but mostly somatic gene changes have been shown to be correlated to different tumor subtypes. Usually a favourable prognosis is associated to near triploid karyotypes with whole chromosome gains, rare structural chromosomes rearrangements and high expression of the TrkA neurotrophin receptor. On the contrary unfavourable tumors are characterized by chromosome structural changes (i.e. deletion, or umbalanced gain of a chromosome copy), amplification of the MYCN protooncogene and expression of the TrkB neurotrophin receptor and its ligand (Brodeur G.M., 2003).

A heterogeneous histology is also characteristic for this tumor which derived from developing neural crest, an organ rich of multipotent cells, that give rise multiple cell phenotypes. The International Neuroblastoma Pathological Classification (INPC) has also established histological favourable and unfavourable types that, in combination with the genetic markers can be prognostic for the tumor (Shimada H. et al., 1999).

Distinct Cell Type of Neuroblastoma

To proceed with research studies aimed to better understand aetiology progression and possible therapy for NB, the availability of a cellular model resembling tumor heterogeneity is of primary importance. In the last 30 -years course of studies on the growth, differentiation and malignant properties of Neuroblastoma more then 25 different parental cells lines have been examined and among all the cell lines and clones studied three distinct cellular

phenotypes have been identified:

- N type, neuroblastic cells that resembles a sympathoadrenal precursor cell in culture, express biochemical markers (enzymes and cell surface receptors) typical of developing neuroblasts, and are tumorigenic;
- S-type, non-neuronal substrate adherent cells representing the glial /melanoblastic precursor, non expressing neuronal markers proteins and non tumorigenic;
- I-type, first described as a cell with intermediate phenotype between N and S subtypes more recently defined as stem cell precursor of the first two types, expressing both N and S cell marker proteins (Ross R.A. et al., 2003).

These three cell types have been recently demonstrated to occur in human NB tumors, by bone marrow aspiration (Valent A. et al., 1999) using laser capture microdissection (Mora J. et al., 2001) and by immunocitochemistry (Ross R.A. et al., 2003) demonstrating that human neuroblastoma cells variants are not an in vitro artifact but are representative of the tumor in vivo.

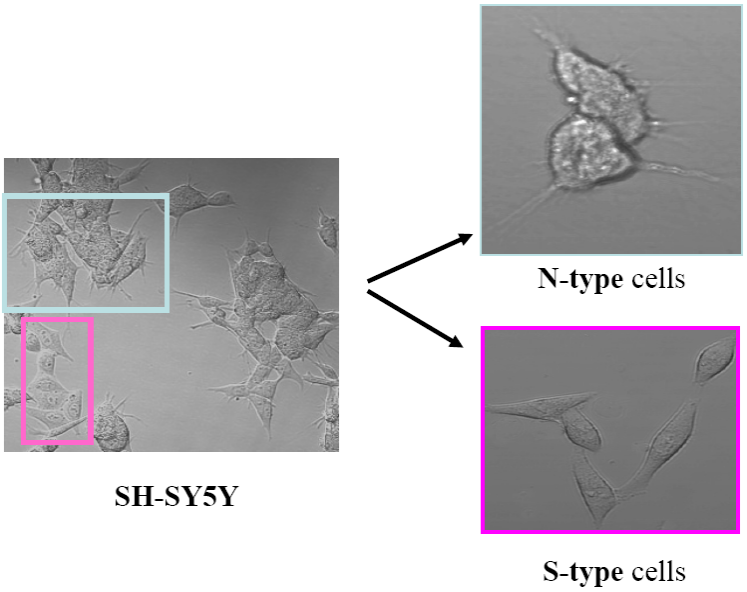

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Figure 1. Cytology of Neuroblastoma human cell line : Confocal images of heterogeneous Neuroblastoma human cell line (SH-SY5Y) and sorted subpopulations N-type and S-type.

Cancer Chemoresistance

A common feature of highly malignant neuroblastoma is the acquisition of multidrug resistance (Keshelava N. et al., 1998). Despite the intensive multimodal therapies and the recent advances in combined chemotherapy, the poor clinical outcome and low response to conventional therapy of Neuroblastoma, due to drug resistance in patients with advanced stage, limit efficacy of the effective chemotherapy (Lange B. et al., 2003).

Neoplastic cells, in fact, can developed an intrinsic (permanent resistance caused by genetic alterations) or an acquired resistance of the cells that, initially are highly responsive to anticancer therapy, but become resistant during the course of the disease.

Multiple cellular mechanism have been identified to contribute to the drug resistance phenotype of cells treated with compounds used in many chemotherapeutic protocols (Table 3). In addition, pharmacologic factors, such as inadequate dosing or route of delivery, may play a role in clinical resistance of tumours (Table 1), (Broker L.E. et al., 2004).

Table 1. Drug Resistance Mechanisms

Pharmacologic events:	Insufficient drug dose	References
	Improper infusion rate	(Castel V., 2001)
	Inadequate route of delivery	(Erdlenbruch E., 2001)
	Drug metabolism	(Donelli M.G., 1992)
		(Rivory L.P., 2002)
Cellular events:	Alteration in drug transport systems	(Juliano R.L., 1976)
	Modification in drug activation or	(Puchalski R.B., 1990)
	detoxification	(Kubo T., 1995)
	Alteration in drug targets	(Chaney S.G., 1996)
	Enhanced repair of drug-caused damage	(Wyllie A.H., 1997)
	Alteration in drug-induced apoptosis	(Yu D., 1998)
	Change in signaling pathways	

Alterations in drug transport system, that cause a reduced intracellular accumulation of drugs, is one of the most common mechanism of Multi Drug Resistance (MDR). It is caused by enhanced drug efflux or also result from a decreased uptake of the cytotoxic agent caused by defect in the import system (i.e. methotrexate resistance) (Gorlick R. et al., 1997). Many transporter proteins are involved in MDR, such as P-glycoprotein (P-gp) (Juliano R.L. et al., 1976), MDR-associated proteins (MRPs) (Cole S.P. et al., 1992), the transporter associated with antigen presentation (TAP) (Izquierdo M.A. et al., 1996) and others specific for different neoplasiae.

These proteins are able to remove cytotoxic drugs from the cells and can move across cellular membranes against a concentration gradient, by using energy derived from ATP hydrolysis. The best characterized drug exporter is the P-glycoprotein; the substrate list of P-gp contains a wide spectrum of chemotherapeutic drugs, including Vinca alkaloids, Taxanes, Anthracyclines and Epipodophyllotoxins (Litman T. et al., 2001).

An important pathway that leads to inactivation of anticancer drugs is the glutathione/glutathione-S-transferase (GSH/GST) system that conjugates electrophilic metabolites, such as alkylating agents, cisplatin and doxorubicin, with the intracellular

antioxidant GSH (Puchalski R.B. et al., 1990). A lot of drugs, such as Methotrexate, to perform their cytotoxic function, needs to be modified, by polyglutamylation, essential mechanism for the retention of the drug in the cells. Polyglutamylated methotrexate is not recognized by export proteins such as MRP (Zeng H. et al., 2001) and can accumulate inside the cell (Gorlick R. et al., 1999).

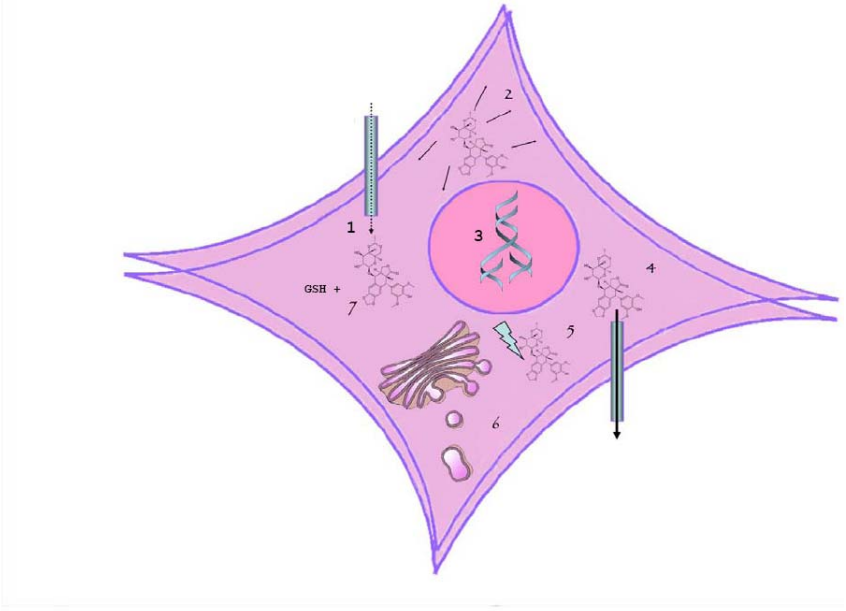

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Figure 2. Schematic representation of resistance mechanism : 1) Reduced entry of the drug (i.e. reduced permeability of membrane); 2) Reduced activation of the drug; 3) Promotion of repairing processes of DNA (i.e. ↑ DNA polymerase); 4) Increased ejection of drugs (altered expression of MRPs and P-gp); 5) Increased inactivation of the drug (i.e. ↑ aldehyde dehydrogenase); 6) Altered intracellular distribution of the drug (i.e. toward lysosome); 7) Increase of drug's intracellular link (i.e. GSH).

Alteration in the cellular targets of chemotherapeutic drugs may disturb effective drug-target interaction and thus lead to impaired drug response; defect in topoisomerases, thymidylate synthase, β-tubulin and dihydrofolate reductase, may render tumour cells resistant to drugs that target these proteins. Topoisomerase I and II, enzymes involved in the DNA replication, transcription, chromosome segregation and DNA recombination, are specific targets for the topoisomerase inhibitors (irinotecan, topotecan and etoposide) and alterations of these enzymes lead to resistance against topoisomerase inhibitors in vitro (Kubo T. et al., 1995). In addiction binding of Paclitaxel to β-tubulin (a microtubule-disrupting agent) induces polymerization and bundling of microtubules, which leads to cell-cycle arrest and subsequent cell death (Schiff P.B. et al., 1979).

To preserve genome integrity, cells use a complex machinery to repair the accidental lesions that occur in DNA; these repair damage processes are induced by the action of anticancer drugs and result increased in cells that shown resistance (Chaney S.G. et al., 1996). Four pathways, summarized in Table 2, are involved in the repair of DNA damage induced by anticancer drugs.

Table 2. DNA Repair Mechanisms in Drug Resistance

Repair Mechanism	Drugs involved
Base excision repair	Alkylating agents (Hansen W.K., 2000)
Nucleotide excision repair	Platinum compounds (Furuta T., 2002)
Mismatch repair	/
O^6-alkylguanine DNA alkyltransferase	Nitrosurea-derivatives (Ishiguro K., 2005)

Deregulation of apoptotic pathway favors carcinogenesis by providing tumours cells with a survival advantage (Wyllie A.H., 1997). In addiction, because many anticancer agents exert their effect at least through activation of the apoptotic cascade (Fisher D.E., 1994), alteration in apoptosis can lead to a wide-spectrum of drug resistance intracellular target such as p53 (Smith M.L. et al., 2002), Bcl-2 family members (Reed J.C. et al., 1996), c-myc (Nasi S. et al., 2001) and inhibitors of apoptosis proteins (LaCasse E.C. et al., 1998).

Cellular processes such as cell proliferation and differentiation are controlled by various signal transduction pathways; the development of biologic response modifiers, which target abnormal signalling pathway in tumours, has led to the recognition of alternative resistance mechanism that stem from alterations in signalling pathways. For example Her-2,a member of the erbB receptor can cause enhanced DNA repair, defective cell-cycle checkpoints and altered apoptoic responses, result in resistance against DNA-damaging agents and antimitotic drugs in vitro (Pietras R.J. et al., 1994).

New therapeutic strategies are therefore needed, including the promising approach represented by specific targeting of drugs, enlucidated by a deep investigation on the neuroblastoma cells' proteome.

Table 3. Cellular Mechanisms of Resistance against Chemotherapeutic Drugs

Cisplatin	MRPs, Inactivation by Glutathione, Enhanced DNA Repair, Altered Apoptotic Response
Irinotecan	P-gp, Mutation in Topoisomerase I, Degradation of Topoisomerase I-DNA complexes
Paclitaxel	P-gp, β-tubulin Mutations, Altered Apoptotic Response
Methotrexate	MRPs, Decreased Polyglutamylation, Increased Levels of Dihydrofolate Reductase
Doxorubicin	P-gp, MRPs, Mutation in Topoisomerase II, Enhanced DNA Repair, Altered Apoptotic Response

Cell Line Variants as in Vitro Model

As it will be discussed later on in this review, one of the major problem to treat Neuroblastoma at the moment is its ability to develop a resistance to most of chemotherapeutic agent used so far. Therefore, in order to extend the potential results to what occur in the patient, the investigations of drug resistance response and interaction should be pursued with all the cultured neuroblastoma cell variants.

Among all the cell lines already characterized to investigate Neuroblastoma one of the most commonly used is the SH-SY5Y. Those cells are derived from a human neuroblastoma metastasis in bone marrow and are a thrice cloned subline of the neuroblastoma cell line SK-N-SH which was established in 1970 from a metastatic bone tumor. The cells grow as clusters of neuroblastic cells with multiple, short, fine cell processes (neuritis).

The identification of marker proteins involved in chemoresistance mechanisms following an un-bias data driven molecular approach combined differential analysis and protein characterisation by mass spectrometry (MS) will be the focus of this review.

For this kind of studies several cell lines can be employed (i.e. SK-N-SH, IMR32, BE(2)C, N1E-115, etc.) nevertheless the protein content of SH-SY5Y is the most widely characherized so far :

High resolution map of the mitochondrial proteome has been generated by Scheffler N.K. et al. (2001), which produced in those cells a cybrid model for neurodegenerative disorders (Scheffler N.K. et al., 2001);

A profiling of the cell surface proteome of different tumor cell lines including SH-SY5Y , useful to identify novel targets for diagnostic and therapeutic for lots of disease, is available since 2003 (Shin B.K. et al., 2003);

Changes of protein and phosphoroteins profile in SH-SY5Y under oxidative stress (Nakamura M. et al., 2006);

A first proteomic investigation in the field of Neuroblastoma developing drug resistance was pursued to characterize an etoposide chemo-resistant SH-SY5Y derived cloned (Urbani A. et al., 2005).

Human Neuroblastoma Characterization by Proteomic Approach

The aim of a proteomics investigation is to investigate the protein repertoire system biology rather than the role of a single protein. Such a vision considers distinct proteins in their roles as part of a larger system or network. (Liebler D.C., 2002). Different analytical strategies can be followed to achieve the direct analysis and a comprehensive characterization of thousands of proteins (changes in expression, de novo synthesis, new protein isoforms, post-translational modifications, etc) (Figeys D., 2003; Link A.J. et al., 1999; Mann M. et al., 2003): among these the combination of high-resolution two-dimensional electrophoresis (2-DE) and mass spectrometry are the most frequently used.

In the recent years proteome analysis is becoming a key tool in possible new biomarkers discovery, in studying the several cascades involved in the different biological responces, in describing patient serum profiling or in understanding multidrug resistence (MDR) upon treatment with anticancer drugs. An increasing number of studies have been conducted using neuroblastoma as model.

Sitek B. et al. (2005) tried to gain deeper insights into TrkA and TrkB signaling pathways, two biologically active receptors for the neurotrophins involved in growth, survival, and differentiation of normal sympathetic neurons, using the human neuroblastoma SH-SY5Y cell line stably transfected with the TrkA or TrkB cDNA as model system. They used the difference gel electrophoresis (DIGE) system together with MALDI-peptide mass fingerprint (PMF)-MS analysis to identify differences in protein expression. Functional assignment revealed that the majority of these proteins are involved in organization and maintenance of cellular structures. A systematic study for differential expression of signaling

proteins (SP) in undifferentiated vs. differentiated cell lineages were performed by Oh J.E. et al. (2005). The N1E-115 cell line was cultivated and an aliquot was differentiated with dimethylsulfoxide (DMSO). Cell lysates were prepared, run on two-dimensional gel electrophoresis (2-DE) followed by MALDI-TOF-TOF identification of proteins and maps of identified SPs were generated. Switching-on/off of several individual SPs from different signaling cascades have been detected during the differentiation. However futher investigations are necessary to understand these process.

Even if the resolving power of 2DE has been improved by the use of more sensitive techniques of protein detection, to increase the likelihood of visualize the low-abundance gene products, complex biological mixtures need to be divided into simpler fractions prior to the proteomic analysis by separating the total protein content into cytosolic, mitochondrial, nuclei and membrane fractions. Scheffler N.K. et al. (2001) obtained mitochondrial fractions by multiple-step percoll/metrizamide gradient . The absence of many membrane-associated proteins known to be associated with these organelles and the limited number of total protein observed in the 2DE gel colloidal coomassie blue maps suggest that the majority of mitochondrial proteins are not being detected under these separation and staining conditions. Fountoulakis M. et al. (2003) obtained one of the larghest organelle databases starting from mitochondrial fraction of the neuroblastoma cell line IMR-32. Protein were resolved by 2DE and stained by coomassie blue. The database comprises 185 different gene products, resulting from the MALDI-MS analysis of approximately 600 spots. The most frequently detected species are heat shock proteins and house-keeping enzymes.

In order to identify novel biomarkers Escobar M.A. et al. (2005) focused their attention on nuclear extracts from three different human NB cell lines SK-N-AS, SK-N-DZ, and SK-N-FI. Proteins were analyzed for differential expression by 2-DE and polypeptides of interest were subsequently identified by liquid chromatography–linked tandem mass spectrometry (LC-MS/MS). They described 20 different proteins, in prelevance oncoproteins, many of which have prior associations with NB and cancer in general. In particular they chose a panel of 3 proteins (SET, grp94, and stathmin) as a potential test for NB detection for future work to validate these proteins as markers in NB by testing other NB cell lines and tissues as well as nonmalignant cells and tissues.

Another cellular compartment of substantial interest is the surface membrane. Comprehensive profiling of proteins expressed on the cell surface could provide a better understanding of the manner in which the cell surface proteome is regulated and how it responds to a variety of intracellular and extracellular signals. Shin B.K. et al. (2003) implemented a biotinylation-based proteome strategy in order to obtain membrane proteins enrichment. Membrane proteins were derivatized with biotin on the surface of intact SH-SY5Y cells. Solubilized biotinylated membrane proteins were purified by avidin column, separated by 2-DE, detected by silver staining and identified by MALDI-TOF-MS or by nanoLC-MS/MS. They identified both glucose-regulated proteins and heat-shock proteins as relatively highly abundant proteins on the cell surface.

Affinity cromatography coupled to mass spectrometry analysis could be a useful tool in investigating the role of proteins like receptors. For example Colabufo N.A. et al. (2006) synthesized a compound with high affinity for the σ_2 receptor to purify by chromatography possible receptor interactors.

Sigma (σ) receptors are classified in σ_1 and σ_2 subtypes and are localized in different tissues, including the central (CNS) and peripheral nervous systems. In the CNS, these receptors are involved in the modulation of neurotransmitter release, in memory and cognitive processes, and in locomotor activity, whereas their role in the peripheral nervous system and their signal transduction have to be clarified. Moreover, σ_1 and σ_2 receptor protein expression in normal tissues is lower than that in the corresponding tumor tissues. In human SK-N-SH neuroblastoma cell line the σ_2 receptors were overexpressed, whereas the σ_1 receptors were found in low affinity state so that they used the human SK-N-SH neuroblastoma as specific in vitro model to perform their experiment.

They characterized the SDS-PAGE gel electrophoresis stained bands by MALDI-MS and LC-MS/MS analysis. The six eluted proteins were identified as human histone proteins. These results disclosed a dual hypothesis about the σ_2 receptor, that it is formed by histones or that the σ_2 ligands also bind histone proteins.

A first attempt to characterize polypeptides repertoire secreted in the media by neuroblastoma cells has been done by Sandoval J.A., Hoelz D.J. et al. (2006). They resolved the secreted proteins by 2-DE gel electrophoresis and LC-MS/MS they identified 5 polypeptides that were secreted or shed by NB. Ubiquitin, b2-microglobulin, insulin-like growth factor binding protein–2, superoxide dismutase (copper and zinc), and heat shock cognate 70-kd proteins were secreted from NB cells, as compared with control media. Elevated levels of these proteins have been described in serum/tissues under intracellular stress and malignancies, including NB. The proteins may reveal additional tumor markers and possibly allow the employment in the diagnosis and treatment of NB. Detection of these proteins in serum of children with NB vs controls using the same approach is currently in progress by the same outhors.

Drug Response Investigations

Resistance to anti-neoplastic drugs is a major clinical problem and the proteomic approach could provide a useful tool in order to discover the mechanism underlying and overcome this trouble.

The aim of our initial study of (Urbani A. et al., 2005) was to determine potential markers of etoposide (a topoisomerase inhibitors extensively used in the treatment of many types of cancer and the most common drug adopted in neuroblastoma chemotherapeutic protocols) resistance in human neuroblastoma cell lines SH-SY5Y. The authors report on a proteomic investigation carried out to map the differential protein expression levels during the exposure of neuroblastoma cell line SH-SY5Y to etoposide. A comparison among parental chemosensitive cell line, parental cell line treated with 1μM etoposide for 10 hours and etoposide-resistant clone cultured with the same concentration of the topoisomerase inhibitor were analyzed by 2-DE and the differentially expressed proteins were identified by MALDI-TOF analysis. In the etoposide exposed SH-SY5Y cell line three protein altered their expression levels: FK506-binding protein 4, cyclophilin A and keratin 9. The first two polypeptides (immunophilines) might protect the cell either acting directly as chaperones towards protein damaged by etoposide or indirectly inducing the over-expression of Pgp.

Keratin 9, as all the intermediate filaments, plays a role in dynamic remodelling of cell during development of neoplastic phenotype, execution of apoptosis and maintaining cell integrity. The proteins involved in the establishment of the etoposide resistance are peroxiredoxin1, β-galactoside soluble lectin binding protein, vimentin, Hsp27, hnRNP K, dUTP pyrophosphatase. In particular Hsp27 has the function to augment the cellular survival in stress condition via its chaperone-activity and to modulate the redox state of the cell via the increase of the intracellular abundance of glutathione. This ability of Hsp27 to determine higher levels of glutathione may represent a key point for the study of chemoresistance; in fact it has been established that glutathione is involved in the onset of etoposide resistance in SH-SY5Y cells (Bernardini S. et al., 2002).

Nakamura M. et al. (2006) examined overall protein alteration, including phosphorylation, in the SH-SY5Y cell line under oxidative stress induced by the dopaminergic neurotoxin 6-hydroxydopamine (6-OHDA). The experiments were performed by 2-DE and sequential gel staining with SYPRO Ruby and a novel fluorescent phosphosensor, Pro-Q Diamond Phosphoprotein stain for the detection of phosphorylated forms of protein. For mass spectrometric identification, the authors used a MALDI-TOF MS and a MALDI-QIT-TOF MS/MS instruments. After exposure to 6-OHDA several protein were identified as oxidative stress-responsive elements: elongation factor 2, heat shock cognate 71 kDa protein, lamin A/C, hnRNP H3 and TCP-1, glutathione S-transferase pi. Moreover the phosphorylation state of EF2, lamin A/C, hnRNP H3 and TCP-1 were altered in SH-SY5Y after the treatment. This change in the quantity and status of phosporylation of the identified protein may be an adaptive stress response in order to protects neuronal cells from oxidative stress.

To elucidate the molecular mechanism underlying the oxidative stress-mediated cell-degeneration Ishii T. et al (2005) analyzed the protein carbonylation on SH-SY5Y cells after treatment with an endogenous inducer of ROS production, the 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2). After treatment with this compound, biotin-LC-hydrazide was employed to detect protein-bound carbonyls. One-dimensional and two-dimensional electrophoresis were performed and the analysis with MALDI-TOF MS allowed the identification of glutathione-S-transferase P1 and the 19S regulatory cap, S6 ATPase, of the 26S Proteasome as a molecular target of protein oxidation under conditions of electrophile-induced oxidative stress.

All this proteomic studies agree with literature data asserting that some classes of the glutathione-S-transferase might be involved in anticancer-drug-resistance.

Heat shock protein 90 is an interesting anticancer drug target because of its function which is to protect various cellular protein involved in signaling, growth control and survival. Zhang M.H. et al. (2006) identify novel client proteins of Hsp90 and elucidate Hsp90 function through its inhibition by geldanamycin (an agent that exhibits potent antitumor activity). This drug inhibits ATPase activity of Hsp90 by specifically binding to its ATP-binding site and promoting proteolytic degradation of client proteins of Hsp90. Extracts from control and geldanamycin-treated SK-N-SH cells were analyzed by 2-DE and the five polypeptides down-regulated in the treated cells were identified by MALDI-TOF MS. Among these five proteins the authors choose vimentin to test the possibility that this intermediate filament could be a novel Hsp90 target and this result implies that geldanamycin can act as

anticancer drug and as an effective chemotherapeutic agent against human neuroblastoma cells promoting the release of vimentin from the Hsp90 complex. This mechanism led to the caspase-dependent vimentin cleavage, which increased sensitivity of the cell to apoptosis-inducing stimuli.

The involvement of one intermediate filament (vimentin) in the foregoing studies points to the importance of the cytoskeletal proteins and their alterated expressions in the onset of cellular resistance to chemotherapeutic agents. Among the several antineoplastic drugs used in neuroblastoma chemotherapeutic protocols, antimicrotubule agents such as paclitaxel, vincristine and vinblastine are extensively used. However resistance to these agents represents the major limit of the antimitotic therapies and the development of drug resistance has been associated with alterations in the drug target or differential expression of tubulin isotypes that confers altered sensitivity to antimicrotubule agents. In a recent study Verrillis N.M. et al. (2006) identified a different molecular mechanism of resistance to anticancer agents that target tubulin: the loss of wild-type γ-actin mediates the failure of the therapy. Proteins of the cytoskeleton from CCRF-CEM cells (a human T-cell acute lymphoblastic leukaemia cell line) and sublines that are resistant to vinblastine or desoxyepothilone B were analyzed for differential expression by 2-DE and the different γ-actin isoforms of the resistant cell lines were identified with MALDI-TOF mass spectrometry. The subsequent ESI-TOF MS/MS revealed the presence of amino acid substitution in the mutant polypeptides of interest that causes the loss of sensitivity toward the anticancer treatment. Trasfecting human neuroblastoma SH-EP cells with siRNA in order to eliminate wt- γ-actin expression suggested the possibility that the drug resistance phenotype was due to the loss of wt- γ-actin function and indicated that drug-resistance phenotype is mediated via altered cross-talk between microtubules and actin.

Altered expression of microtubule-associated protein is also linked to antimicrotubule resistance. Hailat N. et al. (1990) undertaken a quantitative analysis of the major tubulin regulatory protein stathmin by means of two-dimensional gel electrophoresis and revealed that more aggressive neuroblastoma (with high copies of the N-myc gene and less responsive to therapy) have reduced phosphorylation of stathmin.

Further research of cellular structural components by identification of protein isoforms should not only provide valuable insight into the nature of drug resistance mechanisms but also help to develop more successful therapy.

The induction of apoptosis is becoming a popular approach to the treatment of many human cancers, in fact evasion of apoptosis is a key determinant of therapy resistance and neoplastic progression. Over-expression of the protoncogene Bcl-2, found in most neuroblastoma cell lines and in primary neuroblastoma and correlated with a poor prognosis, is reported to be able to block apoptosis induced by some chemotherapeutic agents (cisplatin, doxorubicin and betulinic acid). In the study of Li Y. et al. (2005) the apoptosis and the protein profiles of antisense bcl-2 transfected human neuroblastoma SK-N-MC cells were compared to those of the control cells in order to evaluate the impact of an antisense bcl-2 therapy. Although flow cytometric data revealed that antisense bcl-2 transfection did not cause more extensive apoptosis, the proteomic approach based on 2-DE showed that this treatment induced changes in the expression of various proteins of which seven were identified by N-terminal sequencing. All these proteins were metabolic enzymes except one

matched in SWISS-PROT database to the anti-oxidant and anti-apoptosis protein thioredoxin. Based on the authors proposed pathway, up-regulation of thioredoxin may be a result of feedback mechanism induced by Bcl-2 suppression.

Although several mechanisms are responsible for the neuroblastoma multidrug resistance, heterogeneous cell population constituting these solid tumors has been shown to play a great role in the emergence of drug resistance. Sandoval J.A., Eppstein A.C. et al. (2006) investigated proteomic changes associated with resistance or sensitivity to MAPK kinase inhibition in three different neuroblastoma cell phenotypes: SH-SY5Y (N-type), BE (2)-C (I-type) and SK-N-AS (S-type).

Current therapies for neuroblastoma do not use MAPK-directed treatments, but the mitogen activated protein kinase (MAPK) signal transduction pathway is a well-characterized biochemical cascade mediating cell survival and death and is deregulated in a significant proportion of tumors. Several components of this pathway present strategic targets for cancer therapeutic development and the authors investigated whether inhibition of one of the key kinases involved in this pathway (MEK) represents a viable treatment option for neuroblastoma. The three neuroblastoma subtypes were treated with the MEK inhibitor U0126 (10μM) for 1 and 24 hours and analyzed for differential proteins expression by 2-DE. Spots that were down-regulated >2,5-fold after 1h and subsequently up-regulated >5,0-fold after 24h of Mek inhibition were identified by LC-MS/MS. N-type (Mek-resistant) showed the least altered proteomic profile whereas the I-type (MEK-sensitive) and S-type (MEK-intermediate) generated significant protein changes. Identified polypeptides all have roles in mediating an intracellular stress response suggesting that stress related protein expression may be targeted in response to ERK/MAPK therapeutics.

The work of Izbicka E. et al. (2006) reported the presence of differential sensitivity to docetaxel and paclitaxel in the human pediatric tumor xenograft models SK-N-MC and IMR32 (neuroblastoma), RHI and RH30 (rhabdomyosarcoma) and KHOS/NP (osteosarcoma). Six protein species were found by proteomic profiling (four ProteinChip arrays used) to be differentially regulated by docetaxel and paclitaxel in all KHOS/NP xenografts and five proteins in SK-N-MC xenografts. This mass spectrometry analysis could be the first step for the discovery of proteomic biomarkers for drug sensitivity.

Conclusions

The described studies are all useful approaches that will help towards a better understanding of the drug resistance problem by highlighting drug targets and helping in the comprehension of the cellular metabolic pathways involved. Nevertheless we still lack a metanalysis of the overall protein changes, such a data reconstruction will be an absolute requirement to possibly interpret the large amount of the data produced in the proteomics investigations. Moreover the use of cell fractionations and molecules specific tagging would allow to elicit a better view on the drug resistance of Neuroblastoma.

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