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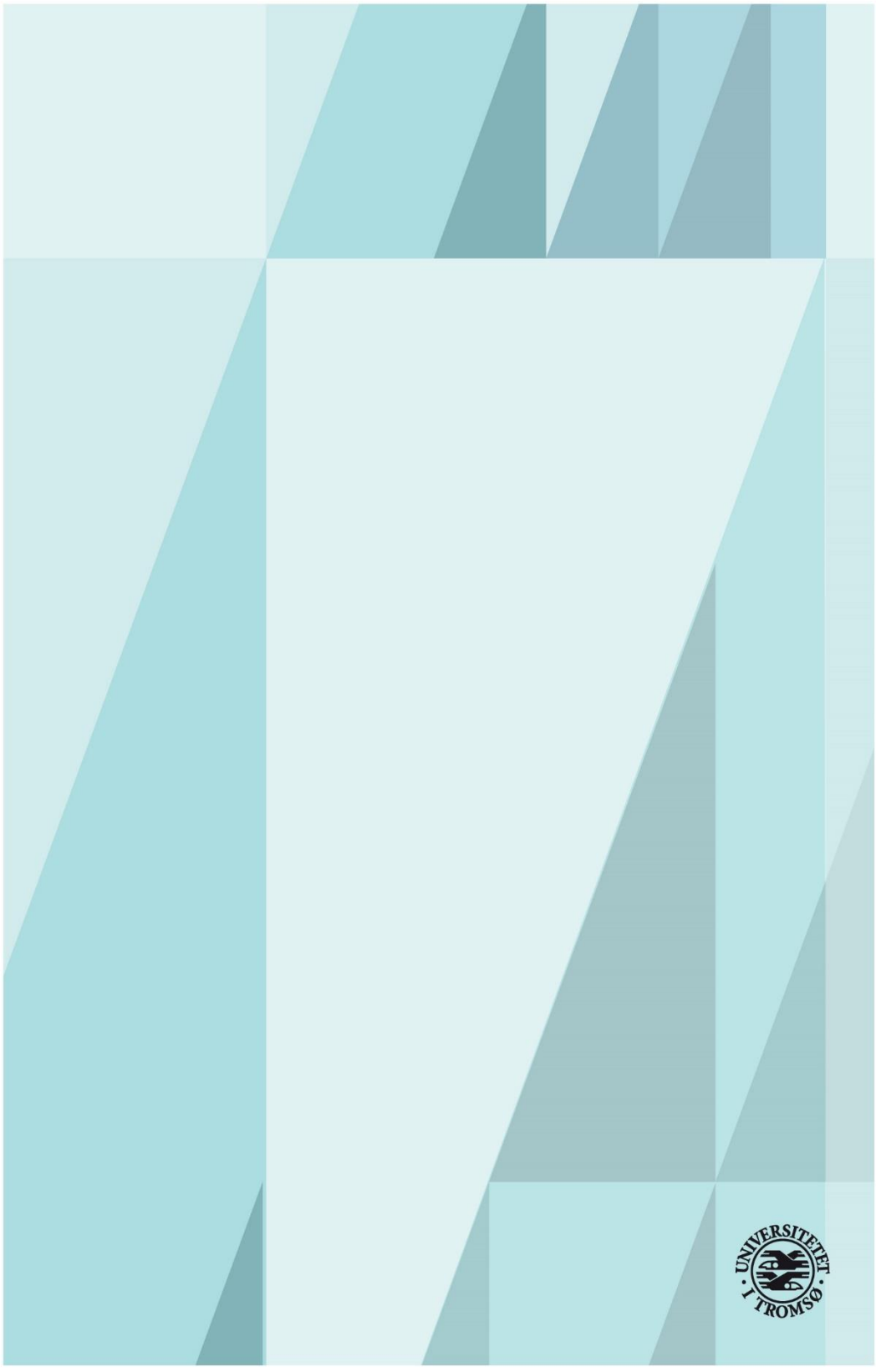
Swapnil Parashram Bhavsar

A dissertation for the degree of Philosophiae Doctor - *February 2019*



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A dissertation for the degree of Philosophiae Doctor

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UiT – The Arctic University of Norway

Faculty of Health Science

Department of Clinical Medicine

Pediatric Research Group

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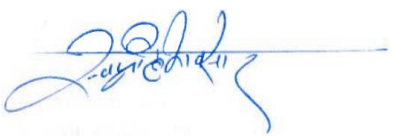

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Swapnil Parashram Bhavsar

Tromsø, Norway

List of abbreviations

ABC	ATP-Binding Cassette
ALK	Anaplastic Lymphoma Kinase
BCRP	Breast Cancer Resistance Protein
BCL-2	B-Cell Lymphoma 2
BCL2L1 (BCL-X_L)	BCL-2 Like 1
BAX	BCL-2 Associated X
BAK	BCL-2 Antagonist /Killer
CAR	Constitutive Androstane Receptor
CSCs	Cancer Stem Cells
CCND1	Cyclin D1
CDDP	Cisplatin
CDK	Cyclin-Dependent Kinase
c-MYC	v-Myc Avian Myelocytomatosis Viral Oncogene Homolog
dsRNA	Double-stranded RNA
DGCR8	DiGeorge Critical 8
DNMT	DNA Methyl Transferase
DXR	Doxorubicin
Exp5	Exportin 5
ETOP	Etoposide
EGFR	Epidermal Growth Factor Receptor
E2F3	E2F Transcription Factor 3
GSH	Glutathione
HDAC	Histone Deacetylase
HSRs	Homogeneously Staining Regions
IGH	Immunoglobulin Heavy Chain Locus
INSS	International Neuroblastoma Staging System
INRG	International Neuroblastoma Risk Group
INRGSS	International Neuroblastoma Risk Group Staging System
IDRF	Image-Defined Risk Factors
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
LOH	Loss of Heterozygosity

lncRNAs	Long noncoding RNAs
MYCN	v-Myc Avian Myelocytomatosis Viral Oncogene Neuroblastoma derived Homolog
miRNA	MicroRNA
miRISC	MicroRNA Inducing Silencing Complex
MNA	MYCN Amplified
mRNA	Messenger RNA
MDR1	Multidrug Resistance Protein-1
MRP-1	Multidrug Resistance associated Protein-1
MRE	MicroRNA Recognition Element
MMR	Mismatch Repair
MCL-1	Myeloid Cell Leukemia-1
MAP3K9	Mitogen-Activated Protein Kinase Kinase Kinase 9
NAIP	Neural Apoptosis Inhibitory Protein
NER	Nucleotide Excision Repair
NHEJ	Non-Homologous End Joining
NRAS	Neuroblastoma RAS Viral Oncogene Homolog
NSCLC	Non-Small Cell Lung Cancer
ORF	Open Reading Frame
Pre-miRNA	Precursor microRNA
Pri-mRNA	Primary microRNA
PTEN	Phosphatase and Tensin Homolog
PARP-1	Poly (ADP-Ribose)–Polymerase 1
RAS	Rat Sarcoma
RIIID	RNase III domain
SCID	Severe Combined Immunodeficient Mouse
siRNA	Small Interfering RNA
STAT3	Signal Transducer and Activator of Transcription 3
SLC	Solute Carrier
TERF1	Telomeric Repeat Binding Factor 1
TP53	Tumor Protein P53
UTR	Un-Translated Region

Summary

This thesis sheds light on the identification and functional role of microRNAs (miRNAs) in neuroblastoma chemoresistance. Neuroblastoma is an embryonal malignancy of childhood and the biology of neuroblastoma tumors is complex and dramatically heterogeneous (Brodeur, 2003). Most of the children diagnosed above one year of age, shows metastatic disease and poor prognosis (Brodeur, 2003). Chemotherapy is one of the principle mode of treatment for the metastatic disease. However, resistance to drug represents the major clinical obstacle for the effective treatment of cancer. MiRNAs are small, endogenous, non-coding RNAs that reduce the translation of target mRNAs (Croce and Calin, 2005). Recently, miRNAs have been shown to modulate drug resistance in multiple cancers. However, the role of miRNAs in neuroblastoma chemoresistance is limited and poorly understood. In this study, we set out to identify miRNAs (and their networks/pathways) along with their functional targets involved in mediating drug resistance in neuroblastoma.

In paper I, we performed miRNA profiling studies, by employing next generation deep sequencing technique to identify 34-downregulated and 8-upregulated miRNAs differentially expressed in neuroblastoma cell lines isolated from six patients at diagnosis (before therapy) and from the same patients at relapse (after therapy). Interestingly, 22 of the 34-downregulated miRNAs were located on chromosome 14q32 locus. MiRNAs from this locus are previously reported to be downregulated in multiple human cancers (Zehavi et al., 2012). Moreover, we also demonstrated that reduced expression of certain chromosome 14q32 miRNAs correlates with poor clinical outcome in a cohort consisting of 226-primary neuroblastomas. Furthermore, Ingenuity pathway analysis (IPA) of differentially expressed miRNAs revealed important biological pathways associated with the cancer progression and drug resistance. Hence, in this study, we identified a unique set of miRNAs which may be involved in the development of drug resistance in neuroblastoma.

In paper II and III, we have investigated the previously unknown functional role of chromosome 14q32 located miRNAs (identified in Paper I), *miR-376c-3p* and *miR-323a-3p* which are downregulated in resistant neuroblastoma cell lines. Using a set of molecular methods, like flow cytometry, reverse transcriptase-polymerase chain reaction (RT-qPCR), western blot and luciferase reporter assay we confirm and validate *CCND1* as a direct target of *miR-376c-3p* and *STAT3* as a direct target of *miR-323a-3p* in neuroblastoma. We show that, *miR-376c-3p* targets *CCND1* and induce G1-cell cycle arrest whereas *miR-323a-3p* targets *STAT3* and induce G1-cell cycle arrest and apoptosis in neuroblastoma cells.

List of papers

Paper-I

Next generation sequencing of microRNAs from isogenic neuroblastoma cell lines isolated before and after treatment

Roth S.A., Knutsen E., Fiskaa T., Utnes P., Bhavsar S.P., Hald O.H., Løkke C., Mestdagh P., Johansen S.D., Flægstad T., & Einvik C.

Cancer Letters (2016) 372(1):128-136

Paper-II

Hsa-miR-376c-3p targets Cyclin D1 and induces G1-cell cycle arrest in neuroblastoma cells

Bhavsar S.P., Løkke C., Flægstad T., & Einvik C.

Oncology Letters (2018) 16(5):6786-6794

Paper-III

Hsa-miR-323a-3p targets *STAT3* and induces G1-cell cycle arrest and apoptosis in neuroblastoma cells

Bhavsar S.P., Olsen L., Løkke C., Flægstad T., & Einvik C.

Manuscript

Introduction

Neuroblastoma

Origin and development

Neuroblastoma is an embryonal malignancy, which arises from the sympathoadrenal lineage of neural crest cells during development. Neural crest cells are the transient, migratory population of cells found in the embryo during development of the neural tube. These cells migrate and differentiates into diverse cell types including, peripheral neurons, enteric neurons, glia, melanocytes, cartilage, schwann cells, cells of the craniofacial skeleton and adrenal medulla (Knecht and Bronner-Fraser, 2002, Cheung and Dyer, 2013). The induction of neural crest cells and subsequent specification of the neural crest-derived cells is dependent upon the co-operative intrinsic and extrinsic functions of the regulatory gene network (Tsubota and Kadomatsu, 2018).

Since neuroblastoma arises from the developing tissues, it generally occurs in infants and young children and the median age of diagnosis is 17 months (Maris, 2010). In addition, most of the children diagnosed above one year of age, shows metastatic disease and poor prognosis (Brodeur, 2003). The incidence of neuroblastoma is 10.2 per million children under 15 years of age and it is necessary to improve outcome because 15% of all pediatric cancer related deaths are due to neuroblastoma (Maris, 2010, Cheung and Dyer, 2013)

The biology of neuroblastoma tumors is complex and dramatically heterogeneous (Brodeur, 2003). The tumors may contain different types of neuroblastoma cells, which include cells resembling immature sympathetic neurons (N-type cells), non-neuronal schwann cell-like (S-type cells) and stem-like cells (I-type) cells. Patients with S-type Schwann cells have better outcome (Ratner et al., 2016).

Biology and genomics

Amplification of the MYC-family proto-oncogene, v-Myc Avian Myelocytomatosis Viral Oncogene Neuroblastoma Derived Homolog (*MYCN)* is one of the most common genetic abnormalities in neuroblastoma. *MYCN* amplification is found in approximately 25% of cases and it is highly correlated with advanced stage of disease and poor prognosis (Brodeur et al., 1984, Huang and Weiss, 2013). The *MYCN* gene was originally discovered as amplified DNA sequences in the form of double minute chromosomes (DMs) and homogeneously staining regions (HSRs) in cells of neuroblastoma (Schwab et al., 1983, Kohl et al., 1983).

Because of the *MYCN* amplification, high levels of N-myc protein is generated; this protein is shown to act as a transcriptional regulator of certain genes involved in proliferation and differentiation processes, thus leading to tumorigenesis (Negroni et al., 1991, Wenzel et al., 1991, Wenzel and Schwab, 1995). In addition, *MYCN* has been shown to regulate oncogenic miRNAs in neuroblastoma (Schulte et al., 2008). Moreover, *MYCN* is also shown to sensitize the neuroblastoma cells for drug-induced apoptosis (Fulda et al., 1999). *MYCN* is therefore considered a promising target for therapeutic intervention. Although direct targeting of *MYCN* is not yet achieved, alternative indirect approaches of *MYCN* targeting will most certainly gain success in future (Chen et al., 2018b).

Chromosomal gains and losses are the means of frequently occurring genetic variations in human neuroblastoma. Caron and colleagues studied the allelic loss of certain chromosomes in 89-neuroblastomas and they found that loss of chromosome 1p is a strong prognostic factor in patients with neuroblastoma, independently of age and stage (Caron et al., 1996). Similarly, chromosome 11q is also frequently deleted in neuroblastoma and that unbalanced 11q and 1p36 loss of heterozygosity (LOH) are independently associated with a worse outcome in patients with neuroblastoma (Attiyeh et al., 2005). In contrast to deletions, gain of 17q is one of the most frequent cytogenetic abnormalities in neuroblastoma cells. The 17q gain serves as an important prognostic factor with negative outcome in children with neuroblastoma (Bown et al., 1999). Additionally, gains of 1q, 2p, 7q, 11p and losses of 3p, 4p, 9p and 14q have also been documented in neuroblastoma (Cheung and Dyer, 2013).

Mutations are observed in certain genes in neuroblastoma. Mutations in the paired like homeobox 2b (*PHOX2B*) which is the regulator of normal autonomic nervous system development was the first predisposing gene identified and linked to hereditary neuroblastoma (Trochet et al., 2004). In addition, heritable mutations in the anaplastic lymphoma kinase (*ALK*) gene, which encodes a receptor tyrosine kinase, lead to its constitutive activation and *in vivo* tumor formation in mouse models (George et al., 2008, Janoueix-Lerosey et al., 2008, Mosse et al., 2008, Chen et al., 2008). Moreover, somatic mutations were also observed in alpha thalassemia/mental retardation syndrome X-linked (*ATRX*) gene in a cohort of 104 patients with advanced stage neuroblastoma (Cheung et al., 2012). Albeit with low frequencies, mutations have also been identified in protein tyrosine phosphatase-non receptor type 11 (*PTPN11*), neuroblastoma RAS viral oncogene homolog (*NRAS*) and *MYCN* (Pugh et al., 2013).

Clinical aspects

Neuroblastoma may arise anywhere along the parasympathetic nervous system in the neck, chest, abdomen or pelvis. However, tumors are mostly found in the adrenal medulla or paraspinal ganglia (Maris, 2010) (Figure 1). The presence of tumor cells in the bone marrow or tissue biopsies and the higher levels of urine catecholamines can be used to diagnose neuroblastomas (Maris et al., 2007).

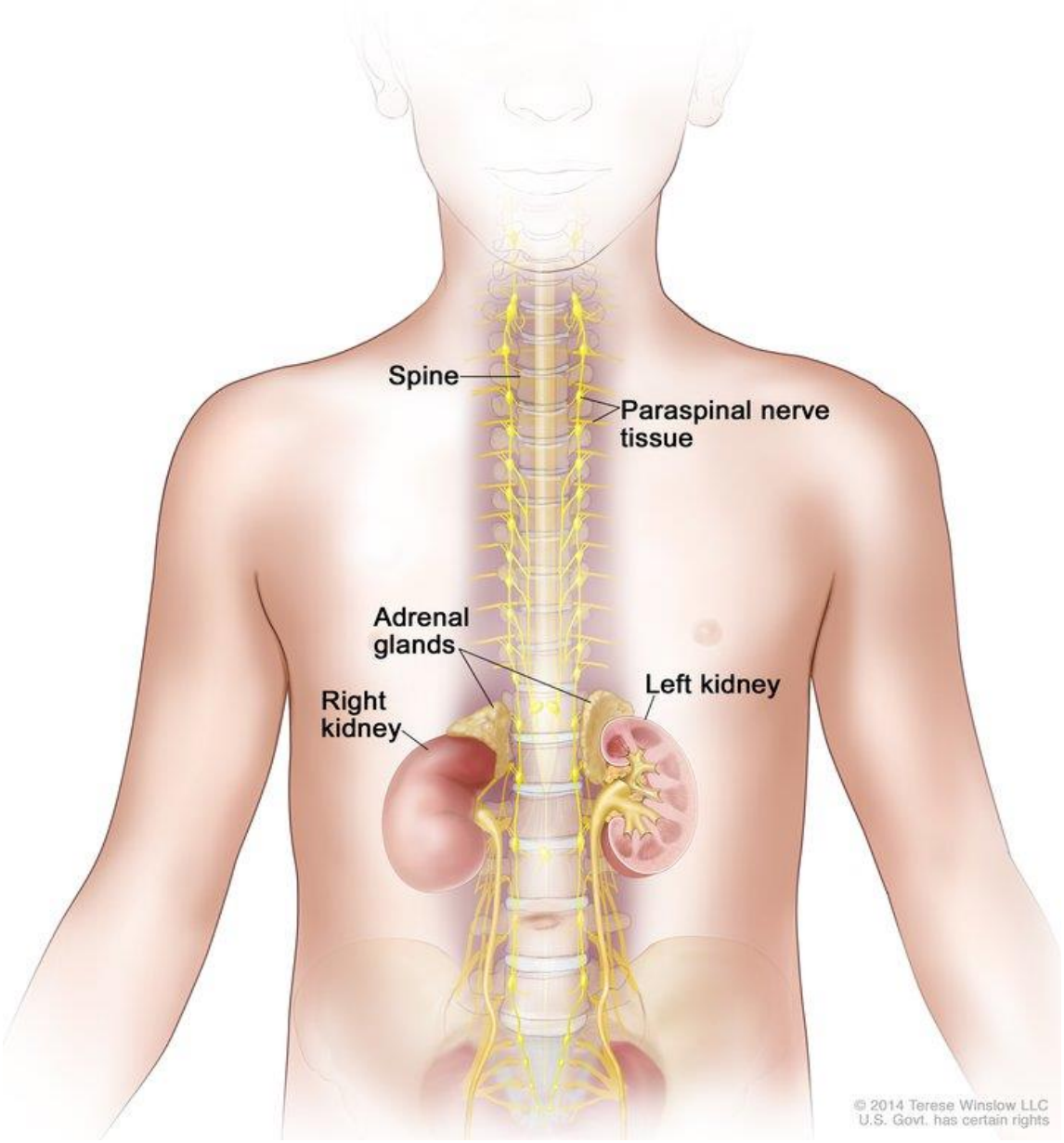


Figure 1. Clinical presentations in neuroblastoma. The primary distribution of neuroblastoma in children is mostly in the neck, chest, abdomen and pelvis. Tumors are mainly observed in paraspnial nerve tissue and adrenal glands. Illustration available from: https://ghr.nlm.nih.gov/condition/neuroblastoma with permission from the National Cancer Institute © (2014) Terese Winslow LLC, U.S. Govt.

Staging of cancer helps to plan treatment and predict patient's outcome. In 1988, an internationally accepted staging system based on clinical, radiographic and surgical evaluation was developed called International Neuroblastoma Staging System (INSS). However, modifications of INSS were proposed later for the incorporation of both clinical and biological features in the prediction of prognosis (Brodeur et al., 1993, Brodeur et al., 1988). Therefore, in 2009, a new pre-treatment risk-stratification classification system called International Neuroblastoma Risk Group (INRG) was established using 13 prognostic factors in a cohort of 8,800 children diagnosed with neuroblastoma to facilitate the comparison of risk based clinical trials conducted in different parts of the world. The INRG task force later proposed a new staging system based on clinical criteria and image-defined risk factors (IDRFs) called International Neuroblastoma Risk Group Staging System (INRGSS) (Monclair et al., 2009, Cohn et al., 2009). The currently followed INRG classification system broadly categorize neuroblastoma tumors into four risk groups (very low risk, low risk, intermediate risk and high risk) based on the analysis of age at diagnosis, INRG tumor stage, histologic category, grade of tumor differentiation, DNA ploidy, *MYCN* oncogene status and chromosome 11q status (Maris, 2010).

Due to the clinical and biological heterogeneity of neuroblastoma tumors, the treatment approaches are mainly based on INRG risk group system. Treatment of low and intermediate risk patients rely on surgery with or without chemotherapy. However, subsets of infants with localized tumors are cured with observation alone, without any cytotoxic treatment. Patients of low and intermediate risk group show excellent prognosis as opposed to high-risk patients. Treatment of high-risk neuroblastoma patients is mainly categorized into three phases: Induction of remission phase (multiple rounds of chemotherapy and surgery), consolidation of remission phase (high-dose chemotherapy with autologous stem-cell rescue and external-beam radiotherapy) and the post-consolidation phase (where efforts are made to treat the therapy resistant minimal residue disease by immunotherapy regimens, cytokines and isotretinoin) (Pinto et al., 2015). Despite the multimodal treatment regimens, 50 to 60% of patients with high-risk neuroblastoma will ultimately relapse with no curative treatment available for these patients. The treatment failure has been associated with acquired drug resistance or the selection of rare resistant clones from a heterogeneous tumor environment or both (Maris, 2010). Therefore, recent research is focused around developing biological based therapies which specifically target important genes, proteins or oncogenic pathways responsible for malignant transformation and progression in neuroblastoma (Brodeur, 2003).

Drug resistance in human cancer

Mechanisms of drug resistance

Although there are significant advances in the cancer treatment approaches, no approach is yet 100% effective against this deadly disease (Gottesman, 2002). Chemotherapy is one of the principle mode of treatment for cancer. However, resistance to drug represents the major clinical obstacle for effective treatment of cancer. Drug resistance accounts for 90% of treatment failure in cancer patients (Longley and Johnston, 2005). The resistance can be developed against every effective anticancer drugs and resistance to anticancer treatment can be multifactorial. Owing to this complexity, resistance mechanisms are broadly categorized into intrinsic and extrinsic mechanisms. Whereas intrinsic resistance mechanisms are inherent or pre-existing in the tumor cells, the extrinsic or acquired resistance mechanisms are developed during the course of treatment. Acquired resistance can be due to mutations or adaptive responses which increases the expression of the therapeutic targets, the activation of alternative compensatory signaling pathway not targeted by the treatment, selection of the resistant clones from the subpopulation of tumor cells among others (Holohan et al., 2013, Buhagiar and Ayers, 2015, Kartal-Yandim et al., 2016).

A range of molecular mechanisms have been observed which lead to drug resistance including alterations in drug transport and drug metabolism, alterations in drug targets, DNA-damage repair mechanisms, downstream resistance mechanisms like dysfunctional apoptosis and autophagy, local tumor microenvironment, cancer stem cells and adaptive off-target responses (Figure 2) (Holohan et al., 2013, Kartal-Yandim et al., 2016). All these drug resistance mechanisms can act independently or in combination leading to multidrug resistance (MDR). MDR is a resistance mechanism in which malignant cells become resistant to structurally and functionally unrelated chemotherapeutic agents (Gillet and Gottesman, 2010).

Drug transport: Influx and efflux

In order for the drug to exert its effect on the tumor cells, it must be efficiently distributed along the body and reach the tumorous cells. However, pharmacokinetic effects such as absorption, distribution, metabolism and elimination are important factors, which can limit the amount of drug reaching the tumor cells (Holohan et al., 2013). After the drug reaches the tumor cells, it must be transported into the cells in sufficient dosage to exert its effect. The drug can enter the cells mainly through three different routes. Drug can get into cells through i)

passive diffusion across the plasma membrane (e.g. vinblastine, doxorubicin), ii) by energy dependent transport

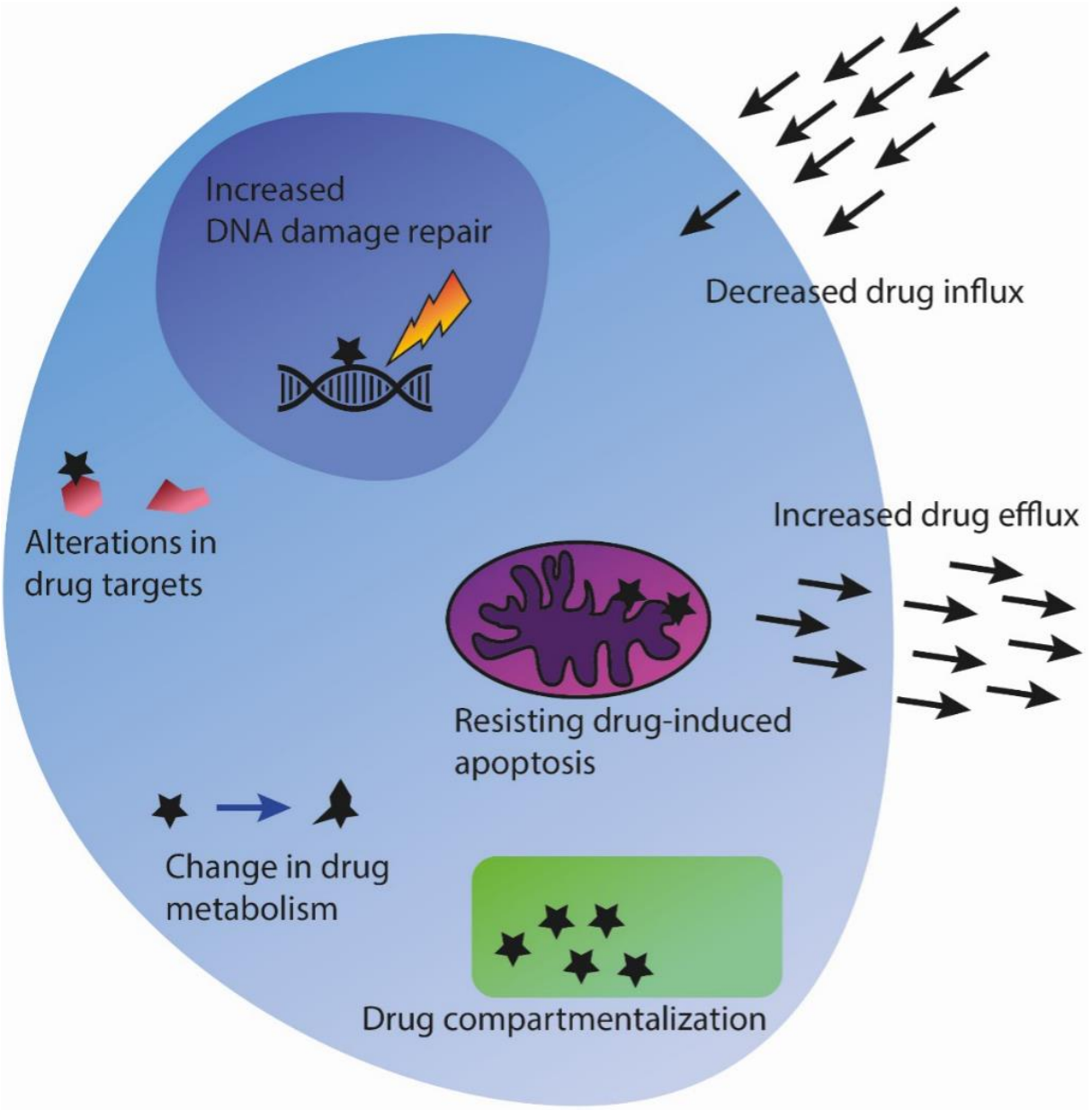


Figure 2. The drug resistance mechanisms in cancer cell. Resistance to drug can be acquired through various mechanisms including alterations in DNA damage repair processes, alterations in drug targets, decreased drug influx and increased drug efflux, resisting drug-induced apoptosis, drug compartmentalization and drug metabolism.

through transmembrane transporter proteins (e.g. nucleoside analogs) and iii) by endocytosis (e.g. immunotoxins) (Gottesman, 2002). The influx of drug into the cells is often altered leading to decreased drug uptake. Therefore, drug accumulation deficiencies is one of the major cause of drug resistance in cancer. The solute carrier (SLC) transporter superfamily is primarily involved in uptake of small molecules into cells including chemotherapeutic drugs. Mutations

and/or reduced expression have been observed in the SLC family transporter proteins, limiting the entry of drugs and causing cells resistant to drugs (Gillet and Gottesman, 2010). For example, reduced expression of *SLC22A4* was reported in a cohort of 251 primary neuroblastomas (Fletcher J.I., 2012).

Contrary to influx, cell membrane transporter proteins also mediate removal or efflux of drugs from the cells. Three efflux pumps have been extensively studied namely ABCB1, ABCC1 and ABCG2. These transporter proteins belong to a large family of ATP-dependent transporters known as ATP binding cassette (ABC) family (Higgins, 1992). ABC family members are involved in transport of multiple structurally and mechanistically unrelated chemotherapeutic agents like the removal of taxanes, topoisomerase inhibitors and antimetabolites like 5-fluorouracil, methotrexate and pemetrexed. They are widely expressed in tissues and human cancers and are not only involve in transport of drugs but also in the transport of nutrients and necessary biological molecules across plasma and intracellular membranes (Goldstein et al., 1989, Gottesman, 2002).

The multidrug transporter energy-dependent drug efflux pump ABCB1 (also called P-gp or MDR1) is a product of the *MDR1* gene (Chen et al., 1986). The MDR1 transporter was the first ABC transporter identified. It is involved in transport of a large variety of hydrophobic anticancer drugs like doxorubicin, etoposide, irinotecan, daunorubicin, vinblastine, vincristine and taxol (Fletcher J.I., 2012, Gottesman, 2002). MDR1 is over-expressed in many tumors thus causing intrinsic drug resistance. MDR1 is also shown induced by chemotherapy thus resulting in acquired drug resistance (Thomas and Coley, 2003). Therefore, the obvious therapeutic strategy would be to develop MDR1 inhibitors but the results from clinical trials for MDR1 inhibitors (e.g. *Tariquidar* in advanced breast carcinoma) are unsuccessful (Pusztai et al., 2005). In neuroblastoma, MDR1 was shown highly expressed in drug resistant cell lines and in post-treatment samples from relapsed patients, however, results from these studies were equivocal due to small sample size and had no prognostic significance (Goldstein et al., 1990, Flahaut et al., 2009).

Another ABC family transporter, ABCC1 (also called multidrug resistance associated protein1; MRP1) transports negatively charged anticancer drugs and neutral drugs that have been modified by conjugation with acidic ligands such as, sulfate, glutathione (GSH), and glucuronate (Borst et al., 2000). This member is also widely expressed across tissues and human cancers and have a broad spectrum of anticancer drug transport activity. MRP1 over-expression is associated with drug resistance and treatment failure across cancer types (Zalcberg et al.,

2000, Triller et al., 2006). In neuroblastoma, MRP1 has been shown to efflux etoposide, vincristine, doxorubicin and irinotecan. Interestingly, high expression of MRP1 is associated with poor clinical outcome in primary neuroblastomas (Fletcher J.I., 2012, Haber et al., 2006, Oberthuer et al., 2006). In addition, Manohar and colleagues demonstrated that MRP1 is a direct transcriptional target of N-myc and it is highly expressed in *MYCN*-amplified neuroblastoma (Manohar et al., 2004). Moreover, an inhibitor of MRP1, reversan was shown to mediate drug resistance in a mouse model of neuroblastoma (Burkhart et al., 2009).

The ABCG2 transporter (also called breast cancer resistance protein; BCRP) has a narrow range of drugs as compared to MDR1 and MRP1. BCRP is capable of transporting doxorubicin, topotecan, mitoxantrone, methotrezate and it is over-expressed in multiple drug resistant tumors (Alisi et al., 2013). Other members of ABC family are also involved in drug transport in human cancer but they are not well studied.

Drug sequestration is another major factor in facilitating multidrug resistance in cancer by limiting the amount of drug having access to intracellular targets. Whereas most commonly used anticancer drugs have their targets located in the nucleus such as DNA or topoisomerases, many new drugs have targets in the intracellular compartments like mitochondria, endosomes, lysosomes, golgi apparatus and endoplasmic reticulum (ER). Therefore, the ability of anticancer drugs to effectively concentrate in these cellular compartments will determine the drug's therapeutic efficiency. However, multiple resistance mechanisms can lead to altered intracellular distribution of drugs resulting in drug resistance (Duvvuri and Krise, 2005).

Drug metabolism: activation and inactivation

After the drug enters the body, it is absorbed and systemically distributed throughout the body. Most drugs undergo chemical transformation in the body. There are many biochemical factors involved in metabolism and pharmacological activity of drugs. Drugs are transformed in the body by a variety of drug metabolism enzymes (DMEs) to yield pharmacologically active or inactive metabolites (Axelrod, 1960). DMEs are thus the second line of cellular resistance. Different mechanisms exist for each different class of drugs as reviewed in detail by Sheweita SA (Sheweita, 2000).

Alterations in drug targets

Alterations in drug or its targets have profound effect on the anti-cancer treatment therapy. The genomic instability persistent in cancer cells can cause mutations or aberrant expression of drug targets. The under or over-expression of drug targets could thus result in

loss of therapeutic potential, leading to drug resistance (Kartal-Yandim et al., 2016). For example, topoisomerase inhibitors (e.g. anthracyclins) are widely used in anticancer treatment and are highly dependent on the target for its effect. However, reduced drug target expression reduces the effectiveness of inhibitors thus conferring resistance to the anticancer drugs (Beck et al., 1999). In another example, germline and somatic activating mutations were identified in *ALK* in neuroblastoma. These mutations led to autophosphorylation and constitutive activation of the receptor tyrosine kinase (RTK). Inhibition of *ALK* by crizotinib or ceritinib (ALK inhibitors) showed poor response rate and acquired secondary mutations were observed in the *ALK* kinase domain of the treated patients (Wang et al., 2017a). Similarly, acquired resistance was observed against the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (Gefinitib) in advanced non-small cell lung cancer (NSCLC) patient. The initially responsive patient ultimately suffered relapse after 2 years of complete remission. The development of secondary mutation was observed in the *EGFR*, which conferred resistance to gefitinib (Kobayashi et al., 2005).

DNA damage repair mechanisms

Most anticancer drugs (e.g. platinum drugs or alkylating agents) are designed to target actively dividing cells causing DNA damage and ultimately leading to cell cycle arrest and programmed cell death (apoptosis). Depending upon the type and extent of DNA damage, a complex of interacting pathways is activated which can detect the damage and activate a set of proteins that can induce either cell cycle arrest or apoptosis. The cell cycle arrest induced upon DNA damage allows cells to repair the damaged DNA (Bouwman and Jonkers, 2012, Gillet and Gottesman, 2009). In order to fix the damaged DNA, cells have developed multiple DNA repair processes including mismatch repair system (MMR), nucleotide excision repair (NER), base excision repair system (BER), homologous recombination (HR), inter-strand crosslink (ICL) repair and non-homologous end-joining (NHEJ). This ability of cells to repair the damaged DNA by a multitude of repair mechanisms can reduce the efficiency of anticancer drug treatments. But, mutations in the components involved in these DNA repair pathways can lead to deficiencies in DNA damage repair systems, which are mainly associated with drug resistance (Ciccia and Elledge, 2010, Bouwman and Jonkers, 2012). For example, the excision repair cross complementing 1 (ERCC1), an important component of NER machinery has been linked to drug resistance in gastric and non-small cell lung cancer (Lord et al., 2002, Kwon et al., 2007) and loss of human mutL homolog (hMLH1) expression, a MMR repair system component has been associated with drug resistance in ovarian cancer (Strathdee et al., 1999).

Therefore, alterations in DNA damage repair mechanisms will be crucial in determining the drug resistant phenotype in cancer cells. Importantly, the obvious therapeutic strategy will be to inhibit the DNA repair machinery in combination with increased cytotoxic therapy in cancer cells.

Downstream resistance mechanisms in cancer

The principle aim of any chemotherapeutic drug treatment is to induce cell death. However, several adaptive responses are initiated within cells, which help evade apoptosis and promote cancer cell survival. These adaptive responses include deregulation of apoptosis, activation of upstream pro-survival signaling pathways and stress induced autophagy (Holohan et al., 2013).

The deregulation or dysfunction of apoptosis is one of the classic hallmarks of cancer (Hanahan and Weinberg, 2000) and therefore resistance of cell to induce apoptosis can cause resistance to cancer drug treatment. Apoptosis can be induced either by the mitochondrial pathway (also called intrinsic pathway) or by the activation of death receptors (extrinsic pathway). Both pathways ultimately lead to activation of specific proteases, the caspases. The caspases mediate the biochemical and morphological activities in the cell responsible for the DNA fragmentation and membrane blebbing, which are the characteristic features of apoptosis. The BCL-2 family of genes mainly regulate apoptosis mediated by the mitochondrial pathway. The anti-apoptotic BCL-2 family proteins (BCL-X_L and MCL-1) and the pro-apoptotic family proteins (BAX, BAD, and BAK) are important mediators of therapy response. They can interplay to inhibit or facilitate cell death, which involves mitochondrial outer membrane permeabilization (Elmore, 2007). Therefore, amplification, translocation, mutations or other alterations in the BCL-2 family genes could ultimately lead to drug resistance.

Autophagy (here, macroautophagy) is the evolutionary conserved catabolic process wherein targeted cytoplasmic components are sequestered and engulfed by double membrane vesicles (called autophagosome) which eventually fuses with lysosome (called autolysosome) for bulk degradation (Sui et al., 2013, Yang and Klionsky, 2010, Bhujabal, 2017). Autophagy has a complex role in cellular biology in that it not only allows degradation of damaged organelles or excessive proteins, but also it is an adaptive response to metabolic stresses like nutrient deprivation, absence of growth factors, hypoxia, glucose deprivation, cytotoxic drugs, etc. Autophagy can be upregulated or downregulated in response to various signaling pathways and by chemotherapy. The induction of autophagy in response to chemotherapeutic drug treatment can have either pro-death or pro-survival role depending on the cell type. Autophagy

can be activated to protect cells from the anti-cancer drug induced metabolic or therapeutic stress thus allowing the resistant cells to survive, eventually promoting drug resistance. Here, autophagy can be therapeutically inhibited to re-sensitize the resistant tumor cells and enhance effectiveness of chemotherapeutic agents. Contrary to this, autophagy could also induce autophagic cell death which is physiologically different from type I programmed cell death or apoptosis. As mentioned earlier, dysfunctional apoptosis is a hallmark of cancer, thus autophagic cell death is an alternative death mechanism, which can be activated to circumvent drug resistance (Sui et al., 2013).

Role of tumor microenvironment in drug resistance

The malignant and non-transformed cells together create a tumor microenvironment (TME), which may consist of extra cellular matrix (ECM), fibroblasts, pericytes, adipocytes, immune cells, inflammatory cells and blood vessels. These TME cells communicate intracellularly by a complex network of integrin, cytokines, chemokines, and growth factors thus promoting the tumor growth and maturation (Balkwill et al., 2012). TME can thus protect malignant cells from the toxic effects of drugs thus allowing them to evade apoptosis and to develop cancer drug resistance (Holohan et al., 2013). For example, Challagundla and colleagues discovered a complicated molecular mechanism wherein *miR-155* is exchanged between neuroblastoma tumors (via exosomes) and tumor associated macrophages (TAMs) present in the tumor microenvironment. *MiR-155* targets telomeric repeat binding factor 1 (*TERF1*), which functions as an inhibitor of telomerase. High telomerase activity is one of the hallmarks of cancer. Interestingly, the TERF1-proteins levels were low in the cancer cells resistant to chemotherapy (Challagundla et al., 2015). In another example, the growth factor interlukin-6 (IL-6) produced by bone-marrow derived mesenchymal stem cells (BMMSC) and TAMs in the bone-marrow microenvironment promotes the growth and survival of neuroblastoma cells. IL-6 mediated drug resistance by activating the signal transducer and activator of transcription 3 (*STAT3*), which is necessary for the upregulation of multiple survival factors including survivin and anti-apoptotic BCL-2 family members (Ara et al., 2009, Ara et al., 2013)

Cancer stem cells and drug resistance

Cancer stem cells (CSCs) are the rare immortal cells found within tumors that have stem cell like capacity to self-renew. They exhibit the characteristics of both stem cells and cancer cells and have the ability to produce tumors in transplanted host animals (Yu et al., 2012). In 1994, Lapidot and colleagues first proposed the evidence of cancer stem cells. They identified

a population of cells from acute myeloid leukemia (AML) patients which, when transplanted, initiated AML in severe combined immune-deficient (SCID) mice (Lapidot et al., 1994). Subsequently, cancer stem cells were identified in various cancer types including breast, brain, colon, pancreas, lung, prostate, melanoma, glioblastoma (Yu et al., 2012) and neuroblastoma (Ross et al., 1995).

CSCs are inherently drug resistant. They show high levels of drug efflux proteins, amplified checkpoint activation, DNA damage repair, increased Wnt/β-catenin and Notch signaling (Shervington and Lu, 2008, Eyler and Rich, 2008) and therefore they are one of the major factors in cancer relapse and poor patient outcome. In one study, it was demonstrated that a subpopulation of neuroblastoma cells termed 'side population' express higher levels of ABCG2 transporter in mouse neuroblastoma and are enriched for neuroblastoma stem cells (Stepanova, 2015).

The CD133 cell surface marker expression is a characteristic of the stem cells. The treatment with variety of drugs have shown the enrichment of CD133 positive stem cells *in vivo* in different chemotherapy resistant cancers (Alisi et al., 2013). Vangipuram and colleagues demonstrated that CD133 positive neuroblastoma cells were more resistant to anticancer drugs than the CD133 negative cells (Vangipuram et al., 2010).

Small RNAs in human cancer

The 'small RNAs' are generally referred to as small non-coding RNA molecules which are less than 300 nucleotides in length (Hagemann-Jensen et al., 2018). There are different classes of small RNAs which includes transfer RNAs (tRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), miRNAs, PIWI-interacting RNAs (piRNAs), small interfering RNAs (siRNAs), transcription initiation RNAs (tiRNAs) and splice site RNAs (spliRNAs) (Morris and Mattick, 2014). These small RNAs participate in processes like RNA translation, RNA splicing, RNA modifications, mRNA destabilization or degradation, epigenetic processing, gene silencing, transcription initiation and splicing mechanisms (Hagemann-Jensen et al., 2018, Morris and Mattick, 2014). In short, they are involved in the regulation of the genome organization and gene expression. Therefore, the functional role of these small RNAs needs to be investigated further. In this thesis, we will discuss in details about the role of miRNAs in general and in relation to neuroblastoma.

MicroRNAs: Biogenesis and mode of action

MiRNAs are a large family of short (~22-25 nucleotides), endogenous, non-coding RNAs, which binds the partial or perfect complementary sequences in the 3'-untranslated region (UTR) of target messenger RNAs (mRNAs) leading to translational repression or mRNA degradation (Croce and Calin, 2005). Mounting evidence have established the roles of miRNAs in regulation of important cellular processes like survival, proliferation, metastasis, development, apoptosis and stress response (Croce and Calin, 2005). In 1993, the first miRNA was discovered while studying the development timing of the nematode *Caenorhabditis elegans* (Lee et al., 1993). Since then thousands of miRNAs have been identified across different species and the number is still increasing (Table 1).

Table 1: List of microRNA databases

Database	Link	Reference
deepBase	http://deepbase.sysu.edu.cn/	(Yang et al., 2010)
miRGen2.0	http://www.microrna.gr/mirgen/	(Alexiou et al., 2010)
miRNAMap	http://miRNAMap.mbc.nctu.edu.tw/	(Hsu et al., 2006)
miRBASE	http://microrna.sanger.ac.uk/	(Griffiths-Jones, 2006)

The biogenesis of miRNAs takes place in a sequential manner which starts in the nucleus and ends in the cytoplasm (Figure 3). The miRNA-genes are mostly transcribed in the nucleus by RNA polymerase II (Pol II) enzyme into long primary miRNAs (pri-miRNAs) characterized by unique hairpin structure with 5'-cap and polyadenylated tail (Lee et al., 2004). The microprocessor complex (drosha ribonuclease III; DROSHA and its essential co-factor DiGeorge critical 8; DGCR8) further crops these pri-miRNAs into ~70-100 nucleotides long precursor miRNAs (pre-miRNAs) (Gregory et al., 2004, Denli et al., 2004). However, an alternative 'splicing machinery' has been reported for intronic miRNAs (called Mirtrons) which does not involve drosha-mediated cleavage (Ruby et al., 2007, Berezikov et al., 2007). Mirtrons have been discovered in several species including mammals, fruit-fly, *Drosophila melanogaster* and the nematode, *Caenorhabditis elegans* (Winter et al., 2009). After nuclear processing, the pre-miRNAs produced are then exported to the cytoplasm by exportin-5 (XPO5) in complex with GTP-binding nuclear protein, RAN (Ran-GTP) (Yi et al., 2003).

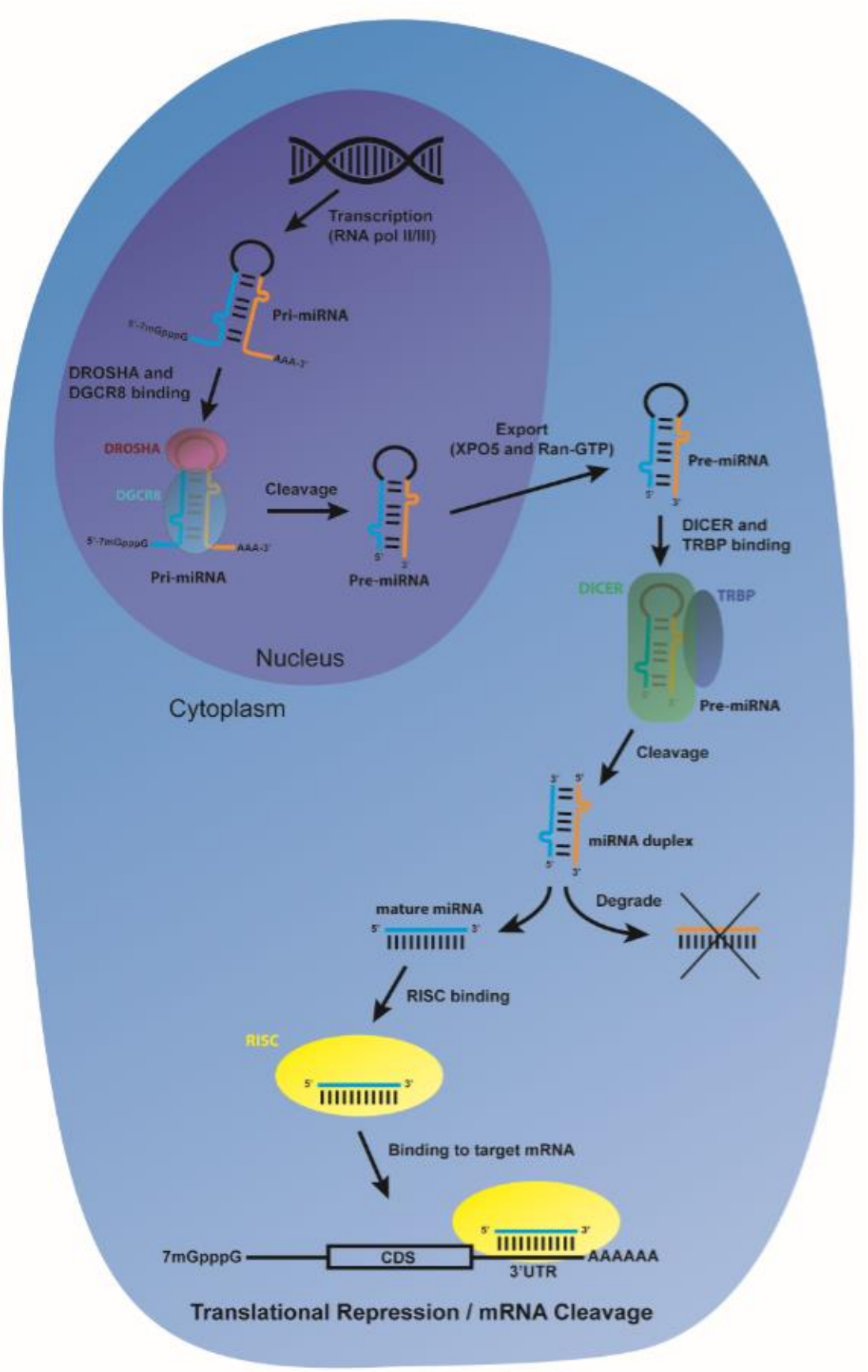


Figure 3: The microRNA biogenesis pathway

In the cytoplasm, an RNase III enzyme-DICER1 along with transactivation-responsive RNA-binding protein (TRBP) cleaves the pre-miRNA into an approximately 22 nucleotides long double-stranded (ds) miRNA with 2-nucleotide 3' overhangs (Chendrimada et al., 2005). For some miRNAs, an additional endonuclease step by argonaute protein 2 (AGO2) cleaves the pre-miRNA generating the nicked Ago2-cleaved-precursor-miRNA (ac-pre-miRNA), which may facilitate the strand dissociation of mature miRNA (Diederichs and Haber, 2007). After DICER1 mediated cleavage, the ds miRNA is unwinded by helicases (like p68, p72, RNA helicase A, RCK/p54, TNRC6B, Gemin3/4 and Mov10) into single stranded mature miRNA (the guide strand) and the complementary passenger strand is subsequently degraded (Winter et al., 2009). The mature miRNA is then incorporated into the miRNA-induced silencing complex (miRISC) containing the argonaute proteins (AGO1, AGO2, AGO3 or AGO4) together with the members of GW182 family proteins and accessory factors. The mature miRNA-miRISC complex recognizes the complementary sequences in the 3'-UTRs of target mRNAs leading to mRNA degradation, destabilization or translational repression (Gregory et al., 2005, Winter et al., 2009).

Studies have, however, shown that the mature miRNA can also bind the 5'-UTR or the open reading frame (ORF) of the mRNA (Lytle et al., 2007, Moretti et al., 2010). In addition, instead of its usual function of guiding argonaute protein complexes for target mRNA silencing, miRNAs have been shown to act independently of argonaute proteins by interacting directly with ribonucleoproteins (decoy activity) (Beitzinger and Meister, 2010). Moreover, miRNAs have also been shown to directly interact with DNA and regulate the gene expression at transcriptional level (Kim et al., 2008).

Given the nature of miRNA and its interaction with target mRNA, it is not surprising that a single miRNA can target multiple genes. This regulatory function of miRNAs can thus affect many cellular pathways controlling important developmental and oncogenic processes. Scientists have developed various different bioinformatic tools to predict miRNA targets (Table 2). Some of the predicted miRNA targets have been experimentally validated in various cancer types, which suggest a global role of miRNA regulation in cancer (Iorio and Croce, 2012).

Table 2: List of microRNA target prediction tools

Tool	Link	Reference
miRTarBase	http://miRTarBase.mbc.nctu.edu.tw/	(Chou et al., 2018)
miRDB	http://mirdb.org	(Wong and Wang, 2015)
DIANA-microT	http://www.microrna.gr/webServer	(Paraskevopoulou et al., 2013)
miRWalk	http://mirwalk.uni-hd.de/	(Dweep et al., 2011)
microRNA.org	http://www.microrna.org	(Betel et al., 2008)
Targetscan	http://www.targetscan.org	(Lewis et al., 2005)

MicroRNAs and their roles in human cancer

In 2002, Calin and colleagues reported the first study identifying the involvement of miRNAs in cancer. They observed a frequent deletion and downregulation of chromosomal region 13q14 in B-cell chronic lymphocytic leukemia (CLL). This chromosomal region, which harbored *miR-15* and *miR-16* miRNA genes, was deleted or downregulated in about 68% of patients with CLL (Calin et al., 2002). The same group reported in 2004 that about 50% of miRNA genes are mapped to cancer-associated genomic regions or in fragile sites. They also demonstrated that the miRNAs located in the deleted regions have low levels of expression in many cancers (Calin et al., 2004).

Numerous studies have demonstrated miRNAs acting as oncogenes and/or tumor suppressors and affecting the different hallmarks of cancer (Hanahan and Weinberg, 2000) like sustaining proliferative signaling (Si et al., 2007, Medina et al., 2010), activating invasion and metastasis (Gregory et al., 2008, Chen et al., 2011), inducing angiogenesis (Hua et al., 2006) and resisting cell death (Lima et al., 2011) (Figure 4). Thus, the deregulated expression of miRNAs can have significant impact on the normal functioning of the cellular processes leading to the diseased condition or cancer.

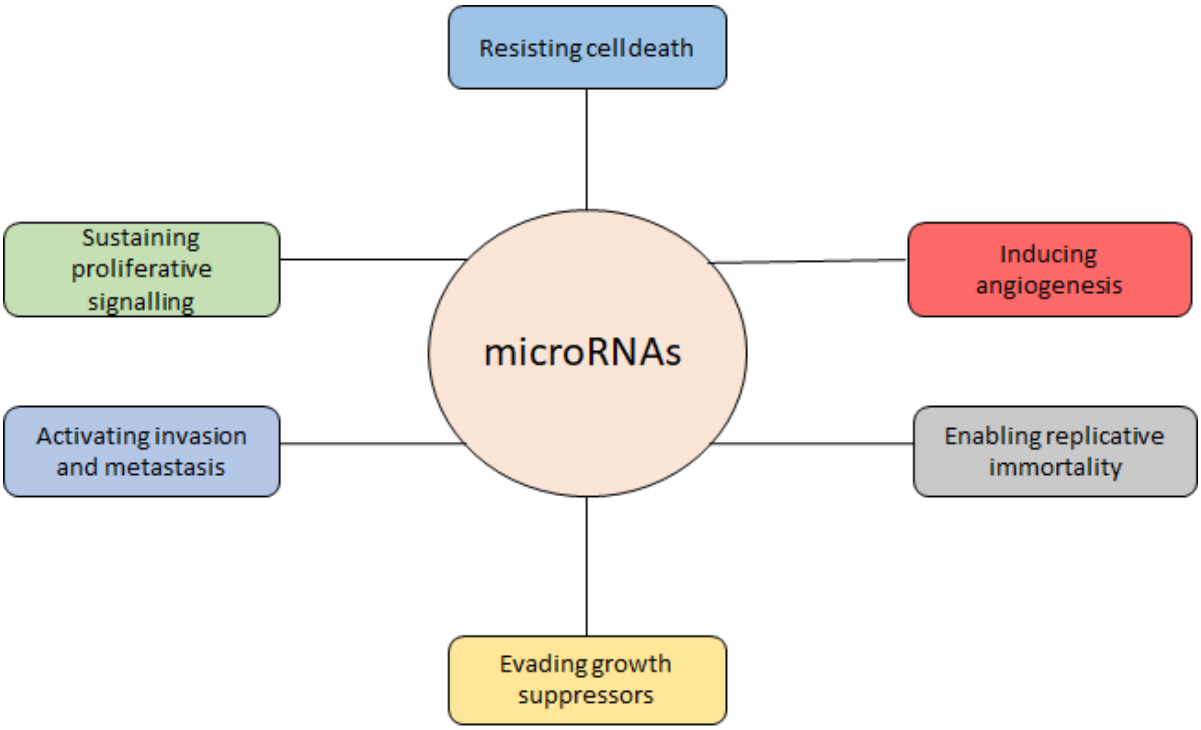


Figure 4: MicroRNAs targeting hallmarks of cancer

Dysregulation of miRNA expression is a common feature of cancer. MiRNAs are shown to be over-expressed or under-expressed, and this aberrant expression has been associated with cancer phenotype (Deng et al., 2008). Whole-genome miRNA expression profiling has been used to detect the global expression of miRNAs in tumor specimens relative to normal tissues (Lu et al., 2005). In addition, miRNA profiling not only distinguish between cancerous and normal tumors but also between parental and resistant tumors. For instance, in our study, we employed the next generation sequencing technique to identify differentially expressed miRNAs from six different parental and resistant neuroblastoma cell lines isolated before and after chemotherapy treatment. We observed a downregulated expression of several miRNAs in the resistant cell lines, which is in concordance with the earlier studies demonstrating a general downregulation of miRNAs in cancer (Roth et al., 2016, Williams et al., 2017)

Several methods are developed for detecting miRNAs like bead-based miRNA profiling, miRNA microarrays, RT-qPCR, *in situ* hybridization techniques and the recent high-throughput sequencing. Moreover, loss and gain-of-function studies have been established to study the biology of miRNAs by overexpressing or silencing of particular miRNAs with synthetic mimics or antagomirs, respectively (Iorio and Croce, 2012). Overall, these studies have demonstrated the potential of miRNAs to be used as diagnostic and prognostic markers in cancer.

Due to their small size, miRNAs are more stable and resistant to degradation. In addition, extra-cellular miRNAs can be easily detected and extracted from body fluids such as blood (total blood, plasma or serum), exosomes, and even from urine, saliva and sputum (Weber et al., 2010). These so called circulating miRNAs are associated with various pathophysiological conditions and can thus be used as prognostic biomarkers for early diagnosis. For example, Lawrie et al. (2008), were the first to detect increased levels of tumor associated miRNAs (*miR-155*, *miR-210* and *miR-21*) in serum of patients with diffuse large B-cell lymphoma and that increased levels of these miRNAs correlated with improved relapse-free survival (Lawrie et al., 2008).

MiRNAs can also response to specific therapies. In cholangiocarcinoma cell lines, targeted inhibition of *miR-21* and *miR-200b* led to increased sensitivity to gemcitabine. This was the first study demonstrating the involvement of miRNAs in modulating drug resistance in cancer cells (Meng et al., 2006).

MicroRNAs as oncogenes and tumor suppressors

As mentioned earlier, dysregulation of miRNAs can affect one or several cellular processes including survival, proliferation, invasion, migration, metastasis, differentiation and apoptosis by acting as oncogenes or tumor suppressor genes (Babashah and Soleimani, 2011). Cancer cells generally show the abundance of specific oncogenic miRNAs (also called oncomiRs) and the loss of tumor-suppressor miRNAs (Figure 5) (Table 3) (Esquela-Kerscher and Slack, 2006).

The oncomiRs repress the tumor suppressor genes, and/or genes that control cell differentiation or apoptosis (Esquela-Kerscher and Slack, 2006, Lu et al., 2005, Babashah and Soleimani, 2011). For instance, *miR-155* is over-expressed and acts as an oncomiR by targeting SH2 domain-containing inositol 5-phosphatase 1 (*SHIP1*) in acute myeloid leukemia (Xue et al., 2014). In breast cancer, *miR-21* was highly over-expressed compared to matched normal breast tissues. Thus, knockdown of *miR-21* by anti-miR-21 oligonucleotides, suppressed cell growth *in vitro* and tumor growth in xenograft mouse model probably by indirect regulation of *BCL2* expression (Si et al., 2007).

The tumor-suppressor miRNAs negatively regulate protein-coding oncogenes and or genes that inhibit cell differentiation or apoptosis (Esquela-Kerscher and Slack, 2006, Lu et al., 2005, Babashah and Soleimani, 2011). For example, the tumor suppressor, *miR-34a* have been shown to target *MYCN* (Wei et al., 2008) and E2F transcription factor 3 (*E2F3*) and induce

apoptosis in neuroblastoma (Welch et al., 2007). In chronic lymphocytic leukemia, *miR-15a* and *miR-16-1* are deleted or downregulated, however over-expression of these miRNAs in leukemic cell line model negatively regulated the expression of anti-apoptotic BCL2 protein (Cimmino et al., 2005).

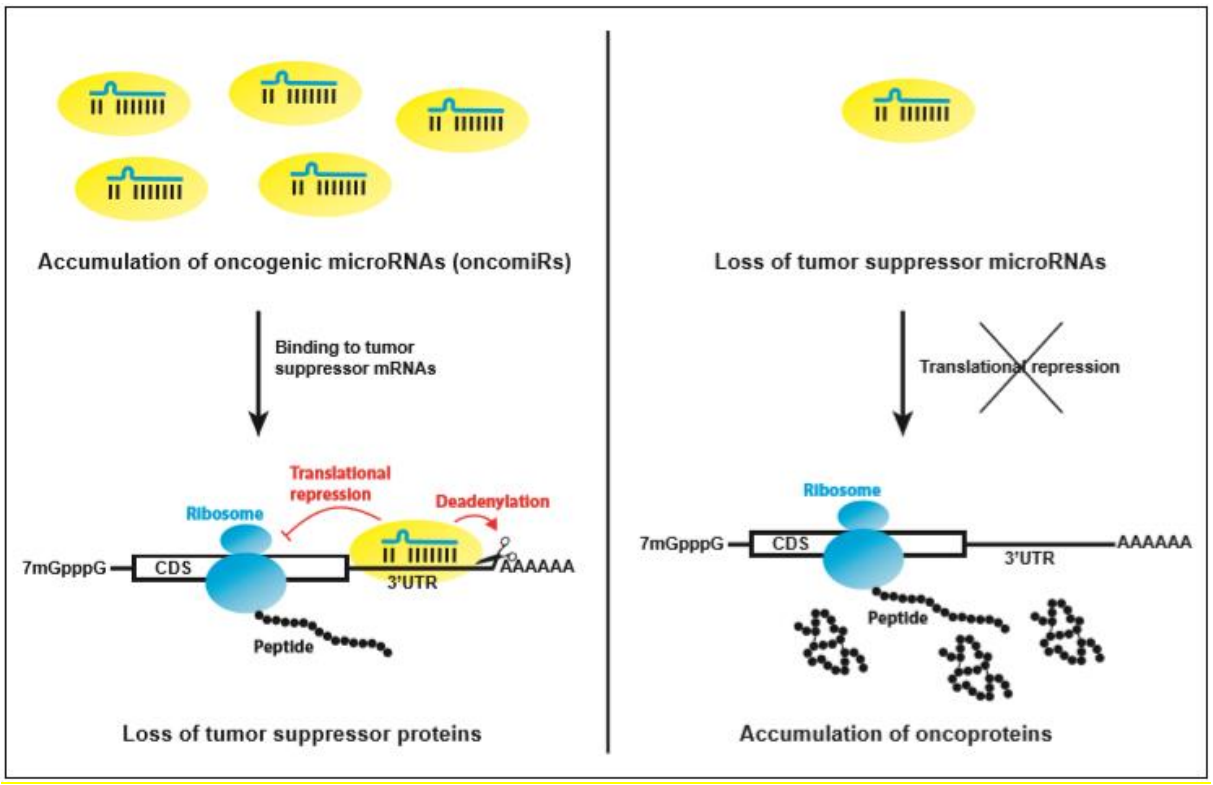


Figure 5: MicroRNA as oncogenes and tumor suppressors

Depending on the cellular context, miRNAs could function either as oncomiRs or tumor-suppressors. For instance, the polycistronic *miR-17-92* cluster (includes *miR-17-3p*, *miR-17-5p*, *miR-18a*, *miR-19a*, *miR-19b-1*, *miR-20a* and *miR-92a-1*) located at the genomic locus 13q31, was not only over-expressed in tumor-cell lines but also amplified in tumors in diffuse large B-cell lymphoma. In addition, *in vivo* studies of *miR-17-92* over-expression in transgenic mouse model of human B-cell lymphoma resulted in aggressive tumors, indicating the oncogenic role of *miR-17-92* miRNA in cancer progression (Ota et al., 2004, He et al., 2005). However, in another study, the c-Myc induced *miR-17-92* cluster targeted and decreased the expression of the *E2F1*, involved in transition of G1-S phase of cell cycle progression, suggesting a tumor suppressor activity of this miRNA cluster (O'Donnell et al., 2005).

MiRNAs can function in complex regulatory circuits and feedback mechanisms. They are shown to work together in groups and co-operate to regulate oncogenes necessary for tumor progression. In human burkitt lymphomas, a group of miRNAs targeting the *c-Myc* oncogene

were silenced, which led to the over-expression of c-Myc and its targets involved in proliferation and survival. Interestingly, over-expression of c-Myc led to repression of some *c-Myc* targeting miRNAs, indicating a feedback mechanism in regulation of *c-Myc* expression (Bueno et al., 2011).

Table 3: Key microRNAs with oncogenic and tumor suppressor roles in neuroblastoma

miRNA	Regulation	mRNA targets	Function	Reference
miR-17-92	Up-regulated	DKK3	Oncogenic	(De Brouwer et al., 2012)
miR-34a	Down-regulated	MAP3K9	Tumor suppressive	(Tivnan et al., 2011)
miR-21	Up-regulated	PTEN	Oncogenic	(Chen et al., 2012)
miR-376c	Down-regulated	CCND1	Tumor suppressive	(Bhavsar et al., 2018)
miR-380	Up-regulated	TP53	Oncogenic	(Swarbrick et al., 2010)
miR-193b	Down-regulated	MCL1,CCND1,MYCN	Tumor suppressive	(Roth et al., 2018)
miR-15a	Up-regulated	RECK	Oncogenic	(Xin et al., 2013)
miR-323a	Down-regulated	STAT3	Tumor suppressive	Manuscript I

The expression and function of oncomiRs can be increased or upregulated by multiple mechanisms including genomic amplifications, activating mutations, transcriptional activation and loss of epigenetic silencing. In contrast, loss of tumor-suppressor miRNAs can be due to genetic deletions, in-activating mutations, transcriptional repression and epigenetic silencing mechanisms (Lujambio and Lowe, 2012). Overall, the regulation of miRNA expression in cancer is very complex and therefore the mechanisms pertaining to the deregulation of miRNAs are discussed in the next section.

Mechanisms of microRNA deregulation in cancer

The deregulation or differential expression of miRNAs in cancer is undisputed. Not a single universal mechanism but a combination of several different mechanisms operate to modulate the expression profiles of individual or group of miRNAs in cancer setting (Deng et al., 2008). The mechanisms of miRNA deregulation can be broadly categorized into structural genetic variations, epigenetic modifications, transcriptional deregulation and defects in the miRNA biogenesis machinery (Figure 6) (Lin and Gregory, 2015, Deng et al., 2008).

The structural genetic variations include the DNA-copy number alterations (amplifications, deletions, and translocations) which are implicated in modulating the expression of miRNAs in cancers (Lujambio and Lowe, 2012, Deng et al., 2008). For instance, in chronic lymphocytic leukemia frequent deletions of chromosomal region 13q14 harboring the miRNAs *miR-15* and *miR-16* were observed in more than 50% of patients (Calin et al., 2002). In another study, an amplification of *C13orf25* locus at 13q31-32 containing seven miRNA polycistronic cluster was reported in lymphoma patients (Ota et al., 2004, Tagawa and Seto, 2005).

In addition to genomic alterations, transcriptional regulators also play an important role in modulating the expression of miRNAs in cancer. For example, the activation of tumor suppressor gene, tumor protein P53 *(TP53)* led to the significant upregulation of 34-miRNAs and downregulation of 16-miRNAs in a genome-wide screen for *TP53*-regulated miRNAs in cancer. Among the deregulated miRNAs, *miR-34* showed a marked upregulation, which is a well-known tumor suppressor shown to target genes, involved in promoting cell growth and proliferation. In the same study, other miRNAs like tumor suppressive, *let-7a* targeting the oncogenes rat sarcoma (*RAS*), high mobility group AT-Hook 2 (*HMGA2*) and *miR-15a/16* targeting the *BCL-2* were also identified (Tarasov et al., 2007). In another example, the transcriptional factor encoded by the proto-oncogene *c-Myc*, directly activates the expression of oncogenic *miR-17-92* cluster (O'Donnell et al., 2005). Interestingly, c-Myc has been shown to repress a broader set of miRNA expression in mouse models of B cell lymphoma (Chang et al., 2008).

Defects in the miRNA biogenesis machinery can also affect the miRNA expression. In the first step of miRNA biogenesis, RNA polymerase II transcribes pri-miRNAs from miRNA genes (Lee et al., 2004), which has been shown to be deregulated in several cancers. As mentioned earlier, different genetic abnormalities like deletions, amplifications, and translocations can alter the expression of miRNA genes (Lin and Gregory, 2015, Deng et al., 2008).

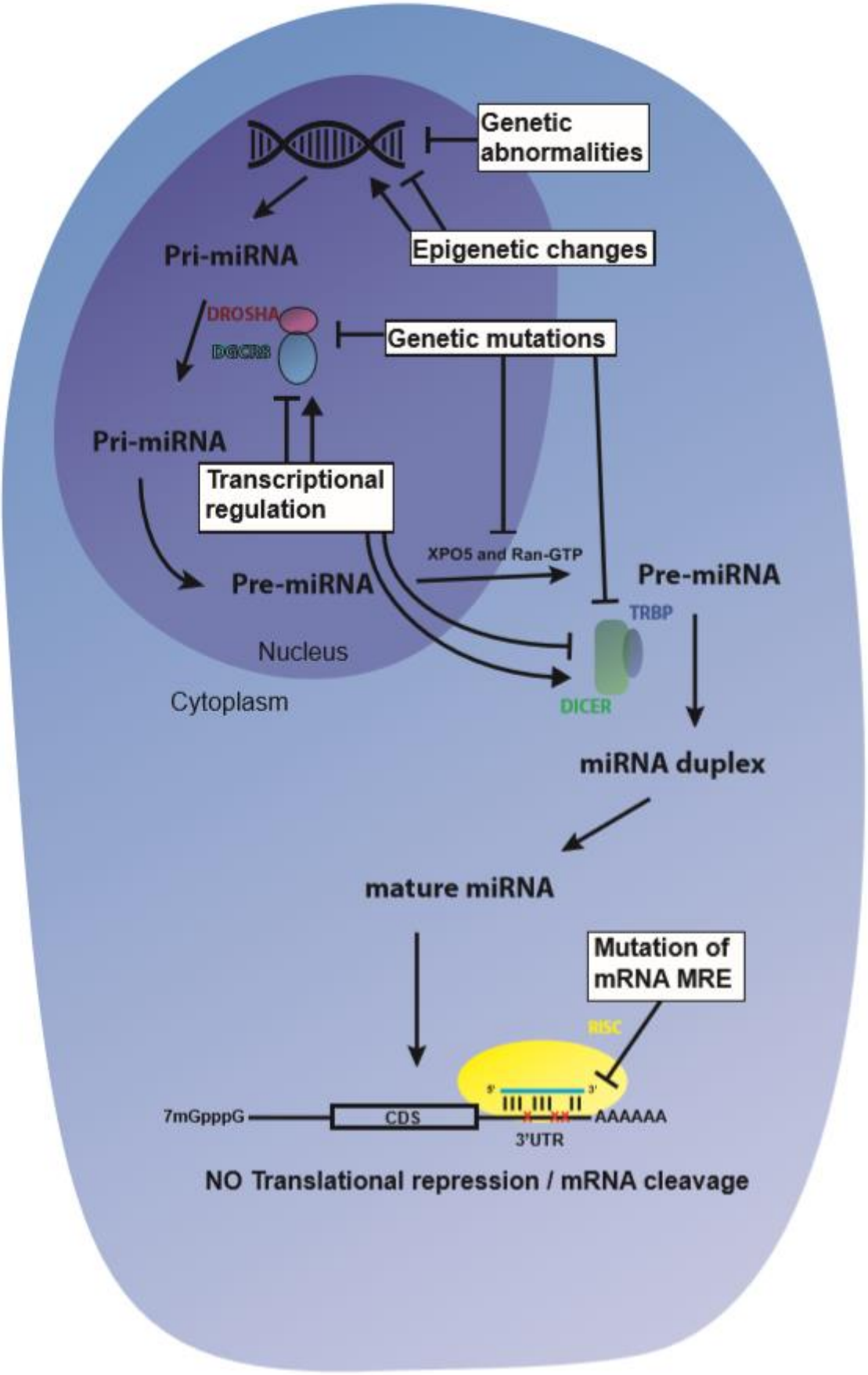


Figure 6: The mechanisms of microRNA deregulation

In the second step of miRNA processing pathway, microprocessor complex, which contains DROSHA and DGCR8 enzymes, cleaves the pri-miRNA to generate pre-miRNA (Gregory et al., 2004, Denli et al., 2004). The microprocessor components DROSHA and DGCR8 are often dysregulated in cancer. For instance, a study by Lin and colleagues identified the downregulation of DROSHA in advanced stage neuroblastoma tumors, which correlated with the global downregulation of the miRNAs and poor clinical outcome (Lin et al., 2010). However, in cervical squamous cell carcinoma, the upregulation of DROSHA was observed to link with altered miRNA expression (Muralidhar et al., 2011). In the third step, the pre-miRNAs are exported to cytoplasm by XPO5 via RAN-GTP (Yi et al., 2003). A study by Melo and colleagues demonstrated that, an inactivating mutation in XPO5 results in trapping of pre-miRNAs in the nucleus, impairing miRNA biogenesis machinery (Melo et al., 2010). Defects have also been observed in DICER and its essential co-factor TRBP, which are responsible for further processing of pre-miRNAs into ~22 nucleotides mature miRNAs (Chendrimada et al., 2005). For instance, DICER1 was shown downregulated in neuroblastoma and it correlated with global downregulation of miRNAs and poor clinical outcome (Lin et al., 2010). However, DICER was upregulated in metastatic prostate adenocarcinoma with global upregulation of miRNA expression and correlated with the increase in clinical stages (Chiosea et al., 2006). Frameshift mutations were identified in *TRBP2*, which caused decreased protein expression in sporadic and hereditary colorectal carcinomas (Melo et al., 2009).

Epigenetic modifications like DNA methylations (mostly occurs in CpG sequences) and histone covalent modifications (acetylation, methylation and phosphorylation) alter the chromatin structure and regulate the pattern of gene expression (Portela and Esteller, 2010). Weber and colleagues thoroughly analyzed miRNA genes and demonstrated that 50% of miRNA genes are associated with CpG islands (short DNA sequences located at the gene promoter). Moreover, the frequency of miRNA gene promotor methylation was higher as compared to protein coding genes (Weber et al., 2007). Studies have reported that CpG islands associated with miRNA gene promoter are frequently hypermethylated in cancer leading to epigenetic silencing of tumor suppressor miRNAs. These miRNAs can be re-activated by the treatment of chromatin-modifying drugs such as inhibitors of DNA methylation and/or histone deacetylase (Lujambio et al., 2008). For example, *miR-127*, which targets the proto-oncogene *BCL6*, is hypermethylated in human cancer cells. Upon treatment with chromatin modifying agents, a strong upregulation of *miR-127* was observed (Saito et al., 2006). In another study, Parodi et al., (2016) analyzed the methylation status of a set of miRNAs in neuroblastoma cell

lines and identified a subset of hypermethylated and downregulated miRNAs (*miR-34 and miR-124*) whose targets have important roles in fundamental cell processes like growth and apoptosis (Parodi et al., 2016). Similar to DNA hypermethylation, DNA hypomethylation have been shown to upregulate the expression of oncogenic miRNAs. The *let-7a-3* gene was hypomethylated in lung adenocarcinomas, leading to high expression of *let-7a-3* having oncogenic functions (Brueckner et al., 2007). In addition to DNA methylation, histone modifications could also modulate the miRNA expression profiles in cancer (Guil and Esteller, 2009).

MicroRNAs and drug resistance in human cancer

The role of miRNAs in mediating drug resistance in neuroblastoma is poorly understood. Very few studies have reported the direct involvement of miRNAs in modulating the drug resistance mechanisms in neuroblastoma (Table 4).

In one of our miRNA profiling studies, we employed deep sequencing technique to identify 34-downregulated and 8-upregulated miRNAs differentially expressed in neuroblastoma cell lines isolated from six patients at diagnosis and at relapse following intensive treatments (Roth et al., 2016). In another study, Ayers and colleagues identified differential expression of miRNAs in chemoresistant cell line models (SH-SY5Y and UKF-NB-3) of neuroblastoma (Ayers et al., 2015).

Chen and collaborators found *miR-21* as the first miRNA associated with drug resistance in neuroblastoma. They observed the increased expression of *miR-21* in cisplatin-resistant SH-SY5Y and BE(2)-M17 neuroblastoma cells as compared to parental cells. Therefore, by using antagomir against *miR-21,* they knocked down the expression of *miR-21*, which sensitized the cisplatin-resistant cells. Further, they ectopically expressed *pre-miR-21* in parental cells, which led to increased resistance to cisplatin treatment and enhanced proliferation by modulating the phosphatase and tensin homolog (PTEN) protein levels (Chen et al., 2012).

In another study, *miR-204* was shown to increase sensitivity of neuroblastoma cell lines to cisplatin and etoposide. Surprisingly, *miR-204* had no effect on neuroblastoma cell growth in the absence of chemotherapeutic agents. The *miR-204* has been shown to directly target the 3'UTR sequence of *BCL2* and an oncogene, neurotrophic receptor tyrosine kinase 2 (*NTRK2*) both of which are important mediators in facilitating resistance to several chemotherapeutic agents. Moreover, *BCL2* and *NTRK2* are significantly associated with poor patient survival in chemo-resistant neuroblastoma (Ryan et al., 2012).

Table 4: MiRNAs involved in modulating drug resistance in neuroblastoma

microRNA	Drug/s	Target/s	Reference
miR-17-5p-92	Not determined	p21 and BIM	(Fontana et al., 2008)
miR-21	Cisplatin	PTEN	(Chen et al., 2012)
miR-204	Cisplatin, Etoposide	BCL2 and NTRK2	(Ryan et al., 2012)
miR-137	Doxorubicin	CAR	(Takwi et al., 2014)
miR-520f	Cisplatin, Etoposide	NAIP	(Harvey et al., 2015)
miR-155	Cisplatin	TERF1	(Challagundla et al., 2015)
miR-497	Not determined	CHEK1, AKT and VEGFA	(Soriano et al., 2016)
miR-141	Cisplatin	FUS	(Wang et al., 2016)
miR-137	Doxorubicin	CAR	(Zhao et al., 2017)

Two independent reports highlighted the importance of *miR-137* in modulating the doxorubicin sensitivity of neuroblastoma cells. Takwi and colleagues observed downregulated expression of *miR-137* and an inverse high expression of constitutive androstane receptor (CAR) and MDR1 in doxorubicin-resistant neuroblastoma cells as compared to parental cells. Furthermore, *miR-137* was shown to directly target *CAR* and that over-expression of *miR-137* led to sensitization of resistant cells to doxorubicin and reduction of the resistant tumor growth *in vivo* (Takwi et al., 2014). Zhao et al., (2017) proposed yet another mechanism to increase the sensitivity of neuroblastoma cells to doxorubicin. In this study, short-interfering RNA (siRNA) knockdown of histone deacetylase 8 (*HDAC8*) which is often upregulated and correlated with advance stage disease, increased sensitivity of neuroblastoma cells to doxorubicin via upregulation of *miR-137* and inhibition of the *MDR1* (Zhao et al., 2017).

Tumor microenvironment has been shown to play important role in mediating drug resistance in cancers via release of exosomic miRNAs. The study was carried out to show how exchange of exosomic miRNAs (*miR-21* and *miR-155*) takes place between neuroblastoma cells and neighboring human monocytes and how these exchanged miRNAs affect drug resistance (Challagundla et al., 2015).

Harvey and colleagues showed that the expression of *miR-520f* was significantly reduced in post-chemotherapy tumors as compared to matched pre-chemotherapy samples. In the same study, a cisplatin and etoposide resistant SK-N-AS cell line was developed which acquired resistance via increase in expression of neural apoptosis inhibitor protein (*NAIP*) and the downregulation of *miR-520f* targeting 3'UTR of *NAIP* (Harvey et al., 2015).

In another study, the over-expression of *miR-497* reduced proliferation of multiple chemo-resistant neuroblastoma cell lines and induced apoptosis in *MYCN*-amplified cell lines. In addition, increased *miR-497* expression also reduced tumor growth and inhibited vascular permeabilization in preclinical neuroblastoma mouse model. Moreover, low *miR-497* expression correlated with poor patient outcome in a cohort of human neuroblastoma samples (Soriano et al., 2016).

Recently, Wang and colleagues, found that *miR-141* was downregulated in both *MYCN*-amplified and non-amplified neuroblastoma cell lines. Over-expression of *miR-141* inhibited proliferation, migration and increased cisplatin chemo-sensitivity in neuroblastoma cells by targeting fused in sarcoma (*FUS*) (Wang et al., 2016).

MicroRNA as therapeutics

Since the discovery of miRNAs in 1993, there have been significant advances in deciphering the molecular mechanisms of cancer progression. Mounting studies have documented the prominent role of miRNAs as the post-transcriptional regulators of developmental and cellular processes (Croce and Calin, 2005). Indeed, they are shown to function as oncogenes or tumor suppressors which make them promising targets for the development of cancer therapeutics (Babashah and Soleimani, 2011). The most important advantage of miRNA is that, single miRNAs can target multiple mRNAs, affecting different oncogenic or tumor suppressive pathways (Croce and Calin, 2005). Moreover, miRNAs are shown to differentially express not only across normal and cancerous tissues but also across parental and drug resistant cells (Roth et al., 2016). Thus effective modulation of cancer associated-miRNAs could be an interesting approach in the treatment of cancer.

Broadly, there are two main strategies to modulate the expression of miRNAs in cancer. Direct strategy involves the use of either the chemically-modified synthetic ds oligonucleotides (called mimics) or the viral based vector-constructs to re-express tumor suppressive miRNAs; and/or chemically-modified single-stranded (ss) anti-miR oligonucleotides (called antagomirs) to inhibit oncogenic miRNAs in cancer. Indirect strategy employs drugs to target the

components of miRNA biogenesis machinery or the epigenetic machinery, thus modulating miRNA expression profile in cancerous cells (Iorio and Croce, 2012, Thorsen et al., 2012).

The stability and effective delivery of miRNAs into the target tissues are the challenges mainly faced in the miRNA-based therapy. Hence, scientists have come up with different designs and formulations for the efficient and robust delivery of miRNAs *in vivo* (Thorsen et al., 2012).

For the success of miRNA-based therapy, efficient and safe delivery of miRNA mimics or antagomirs *in vivo* is essential. MiRNA mimics or antagomirs can be administered via intranasal, local and systemic routes through viruses (adeno associated viruses, AAVs or lentivirus constructs), lipids (neutral lipid emulsion, polyethyleneimine, atelocollagen), nanoparticles (iNOPs) or antimiR oligonucleotides (AMOs) (Iorio and Croce, 2012).

Table 5: List of microRNAs with therapeutic potential in human cancers

Micro-RNA	Model	AntimiRs or mimics	Route of admin.	Therapeutic effect	Cancer type	Reference
miR-380-5p	Orthotopic mouse model	LNA, 2'-F/MOE	Intra-peritoneal	Reduced tumor growth	Neuroblastoma	(Swarbrick et al., 2010)
miR-17-5p	Orthotopic LAN-5 neuroblastoma xenografts	Cholesterol-conjugated 2'-O-Me antagomir	Intra-tumoral	Reduced tumor growth	Neuroblastoma	(Fontana et al., 2008)
miR-34	Mouse K-Ras G12D model	Lipid-formulated miR-34a mimics	Intra-vessicle	Reduced tumor growth	Lung cancer	(Trang et al., 2011)
let-7	Mouse K-Ras G12D model	Lenti-viral construct expressing let-7a	Intra-nasal	Reduced tumor growth	Lung cancer	(Trang et al., 2010)

Several types of chemical modifications are done in order to confer nuclear resistance, enhance binding affinity and facilitate cellular uptake of antagomirs so that relevant miRNAs are exactly targeted. These antimiR chemistries of antagomirs include the use of high-affinity 2' sugar modifications such as locked nucleic acid (LNA), 2'-O-methyl (2'-O-Me); 2'-O-methoxyethyl (2'-MOE) and 2'-fluoro (2'-F) as reviewed by Eva van Rooij (van Rooij and Olson, 2012).

MRX34 is the first miRNA mimic to enter phase I clinical trial (ClinicalTrials.gov Identifier: NCT01829971) in cancer. This ds synthetic miRNA mimic is delivered by liposome technology. The tumor suppressor miRNA, *miR-34* (MRX34) has been shown to target 24 known oncogenes having role in diverse cell processes including survival, proliferation, metastasis, cell cycle and chemoresistance (Bouchie, 2013). Miravirsen (from Santris Pharma), an antagomir targeting *miR-122* is another therapeutic microRNA to enter clinical trial for the treatment of hepatitis C virus (HCV). In addition, the follow up phase IIa clinical trial study (ClinicalTrials.gov Identifier: NCT01200420) reported miravirsen to be safe, well tolerated and provided prolonged antiviral activity without evidence of viral resistance (Janssen et al., 2013). Recently, phase II clinical trial of a synthetic *miR-29* miRNA mimic, MRG-201 (ClinicalTrials.gov Identifier: NCT02603224) from miRagen therapeutics was initiated for the treatment of patients with fibrotic diseases (Rupaimoole and Slack, 2017) (Retrieved from: http://www.miragen.com/press-release/miragen-therapeutics-announces-initiation-phase-2-clinical-trial-mrg-201/)

All the above strategies mentioned take into account the modulation of single miRNA or single miRNA family. However, the cancer phenotype may be as a result of aberrant expression of multiple miRNAs in co-ordination with various mRNA targets. Therefore, an indirect approach which employs drugs to target the epigenetic machinery is considered. Epigenetic modifications like methylation and acetylation of genomic DNA affects global miRNA expression levels. Thus, epigenetic drugs or modifiers like DNA methyltransferase (DNMT) inhibitors and histone deacetylase (HDAC) inhibitors which modulate the methylation and acetylation status, respectively, are used to revert the aberrant miRNA expression levels with corresponding change in relevant genes. In other words, epigenetic treatment can be used for the induction of specific miRNAs to serve as novel anticancer therapy (Monroig and Calin, 2013). For example, the *miR-127* which is located within a CpG island and silenced in human cancer cells was upregulated by the treatment of 5-aza-2'-deoxycytidine (inhibits DNA methylation) and 4-phenylbutyric acid (inhibits histone deacetylase). In addition, this

upregulation of *miR-127* lead to corresponding downregulation of its potential target, *BCL6*, in human cancer cells (Saito et al., 2006).

Yet another alternative to chemically modified antisense oligonucleotides and demethylating agents is the use of competitive inhibitors called miRNA sponges. These are transcripts expressed from strong promoters containing multiple, tandem binding sites to a miRNA of interest. Therefore, miRNA sponges can be used as an antagomir to decrease the level of oncogenic miRNAs in the cultured cells. The miRNA sponge plasmids together with luciferase constructs can be used for assessing transfection efficiency (by inclusion of GFP reporter in sponge mRNA), validation of predicted miRNA targets and to assay miRNA loss of function phenotype (Ebert et al., 2007).

Choi and colleagues developed another strategy in contrast to miRNA sponges. They developed target protector morpholinos (ss chemically-modified oligonucleotides) to mask the miRNA recognition element (MRE) or the complementary miRNA binding sites in the 3'UTR of the target mRNA (Choi et al., 2007).

The *CCND1* and *STAT3* oncogenes in human cancer

CCND1

The *CCND1* (cyclin D1) gene was first characterized in 1991 (Xiong et al., 1991). More than two decades of research have established *CCND1* as an important regulator of cell cycle progression and cancer. It is one of the most extensively documented oncogenes in human cancers. Given its prominent role in cell cycle regulation, alterations in levels of *CCND1* have had oncogenic effects. Multiple converging studies point out to *CCND1* as the biomarker of cancer phenotype and disease progression (Musgrove et al., 2011).

During the process of cell cycle, the regulatory catalytic subunits called "cyclins" (especially D-type cyclins which includes cyclin D1, D2 and D3) are produced in a periodic, cell cycle dependent manner. Biologically, in response to growth factors or mitogenic stimuli, cyclin D1 gets associated with the serine-threonine protein kinases called cyclin dependent kinases (CDKs) - CDK4 and CDK6. The cyclin D1-CDK4/6 complex thus phosphorylate the tumor suppressor retinoblastoma (RB) protein which in turn releases the RB from E2F transcription factors. The E2F therefore transactivate genes necessary for G1-S phase of cell cycle progression. Consequently, the cell cycle enters DNA replication and cell division phase leading to cellular proliferation (Motokura et al., 1991, Baldin et al., 1993, Bartek et al., 1996, Musgrove et al., 2011). Moreover, other than its catalytic function in cell cycle regulation,

CCND1 plays a non-catalytic role in DNA repair mechanisms. Jirawatnotai and colleagues reported that *RAD51*, a component of DNA-repair machinery, interacts directly with *CCND1*. The RAD51 is an enzyme necessary for homologous recombination process, to seal DNA breaks using the intact copy of the DNA sequence as templates (Jirawatnotai et al., 2011). Furthermore, cyclin D-CDK4 also targets genes including *SMAD*s (Matsuura et al., 2004), *RUNX* family (Shen et al., 2006) and *MEF2* (Lazaro et al., 2002) associated with proliferation, degradation and differentiation respectively.

Knockdown of *CCND1* and its partner *CDK4* leads to inactivation of RB pathway, E2F transcription and thus G1-cell cycle arrest in neuroblastoma (Molenaar et al., 2008) and apoptosis in cutaneous melanoma (Sauter et al., 1999) and squamous carcinomas (Sauter et al., 2002). Given the role of cyclin D/CDK4/CDK6/RB pathway members in numerous aspects of cellular biology, the deregulation of these proteins is therefore associated with unlimited growth or proliferation, hence tumorigenesis.

Extracellular signals like growth factors or mitogenic stimuli influence transcription, translation, degradation and thus the abundance of cyclin D1 protein in the cell system. Therefore, oncogenic activation of mitogenic signaling pathways may lead to abnormal increase in cyclin D1 protein levels. Cyclin D1 is shown to interact with numerous proteins involved in cell cycle regulation, transcriptional regulation and DNA repair mechanisms. Therefore, deregulation of cyclin D1 will significantly affect different cellular processes directly or indirectly and therefore have oncogenic effects (Fu et al., 2004).

CCND1 amplification and its subsequent over-expression is one of the most common genetic aberrations in human cancers. *CCND1* was shown to be amplified and over-expressed in multiple cancers including breast cancer, lung cancer, oral squamous cell carcinoma and malignant melanoma (Santarius et al., 2010). In mantle cell lymphoma and multiple myeloma, the translocation event juxtaposes *CCND1* with the immunoglobulin heavy chain locus (IGH), leading to *CCND1* over-expression (Li et al., 1999, Pratt, 2002). In addition, mutations that result in deregulation of *CCND1* expression have been shown in mantle cell lymphoma (Wiestner et al., 2007), esophageal (Benzeno et al., 2006) and endometrial (Moreno-Bueno et al., 2003) cancers. Furthermore, altered expression of miRNAs targeting *CCND1* is another mechanism deregulating *CCND1* in human cancers. Both *miR-15a* and *miR-16* are deleted or downregulated and inversely associated with *CCND1* expression in non-small cell lung cancer (Bandi et al., 2009) and prostate cancer (Bonci et al., 2008).

In case of neuroblastoma, genetic aberrations and over-expression of *CCND1* is observed, similar to other cancers. Amplification of *CCND1*, *CDK4* and mutation of *CDK6* have been shown in a subset of neuroblastoma cell lines and tumors (Molenaar et al., 2008). In addition, a rearrangement in the 3'UTR sequence of *CCND1* has also been shown in neuroblastoma tumor (Molenaar et al., 2003). Taken together, these findings suggest a crucial role of cyclins and CDKs in promoting cellular proliferation in neuroblastoma and demonstrate their potential for therapeutic intervention.

STAT3

The *STAT3* gene was first described as a DNA binding protein activated in response to growth factors and having a dual function of signal transduction and activation of transcription (Zhong et al., 1994). *STAT3* encodes one of the seven members (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6) of the STAT protein family (Copeland et al., 1995, Darnell, 1997). This protein is activated in response to binding of cytokines and growth factors to cellular receptors, which activates membrane-associated janus kinases (JAKs). JAK in turn phosphorylates STAT3 at specific residues to form homo/heterodimers and translocate to cell nucleus. In the nucleus, STAT3 acts as a transcription factor, which regulates the expression of a wide range of genes involved in survival, proliferation, invasion, metastasis, angiogenesis, and immunosuppression (Yu et al., 2014).

STAT3 has been shown to be activated by numerous activators (e.g. cytokines, growth factors, toll-like receptors, etc.) and therefore it has a diverse range of biological functions (Aggarwal et al., 2006). Hence, deregulation of *STAT3* can lead to oncogenesis through various different mechanisms. Yu et al., (2014) have reviewed the role of JAK-STAT3 signaling pathway in multiple biological processes including obesity and metabolism, pre-metastatic niche formation, cancer stem cells, and role in inflammation-mediated cancer (Yu et al., 2014).

STAT3 is constitutively activated in diverse human cancers and hence it functions as an oncogene (Levy and Lee, 2002, Aggarwal et al., 2006). In a study by Ling and Arlinghaus, mammary tumors were induced in mice by the 4T1 breast cancer cell line followed by effective knockdown of *STAT3* using a lentivirus small hairpin (shRNA) delivery system. This led to efficient blockage of tumor formation. However, mice without shRNA for *STAT3* efficiently formed tumors. Thus, this study concluded that constitutively activated STAT3 plays an important role in the induction of breast tumors by regulating the expression of c-Myc and Twist (Ling and Arlinghaus, 2005). Contrary to this, a tumor suppressive role of *STAT3* has also been identified in some cancers (Zhang et al., 2016).

In addition, multiple studies have addressed the regulation of JAK-STAT3 signaling by miRNAs in different cancers. MiRNAs like *miR-135a* (Navarro et al., 2009), *miR-26a* (Yang et al., 2013), *miR-337* (Du et al., 2012), *miR-9* (Zhuang et al., 2012) and *let-7* (Sugimura et al., 2012) have shown reciprocal interactions with JAK-STAT3 signaling pathway, which have a crucial role in regulating oncogenesis. Recent studies from Odate et al., (2017) have demonstrated the specific targeting and inhibition of *STAT3* by generation 2.5 antisense oligonucleotide, AZD9150, which decreases neuroblastoma tumorigenicity and increases chemosensitivity (Odate et al., 2017). Also, from our studies, we have demonstrated that *miR-323a-3p* has been reduced in resistant neuroblastoma cell lines and advance stage primary neuroblastoma tumors. Thus, over-expression of *miR-323a-3p* leads to reduced cell viability, G1-cell cycle arrest and apoptosis by targeting *STAT3* in neuroblastoma (Manuscript I).

Given the importance of JAK-STAT3 signaling in cancer, it is one of the most promising targets for cancer therapy. *STAT3* inhibitors are being developed. For instance, several tyrosine kinase inhibitors are available in clinic, which could target JAK-STAT3 signaling indirectly, ultimately leading to reduction of tumor growth and survival by promoting apoptosis (Yu et al., 2009).

Aims of the study

The overall aim of this study is to identify and elucidate the functional role of miRNAs involved in the drug resistance mechanisms in neuroblastoma.

The specific aims of the study are as follows:

i) To identify miRNAs and their pathways that are involved in the underlying drug resistance mechanisms in neuroblastoma (Paper I)

ii) To evaluate the functional role of downregulated 14q32 locus miRNAs, *miR-376c-3p* and *miR-323a-3p* in neuroblastoma (Paper II & III)

Summary of papers

Paper-I

Next generation sequencing of microRNAs from isogenic neuroblastoma cell lines isolated before and after treatment

Roth S.A., Knutsen E., Fiskaa T., Utnes P., Bhavsar S.P., Hald O.H., Løkke C., Mestdagh P., Johansen S.D., Flægstad T., & Einvik C.
Cancer Letters (2016) 372(1):128-136

Recently, microRNAs (miRNAs) have been shown to modulate drug resistance in multiple cancers. However, the role of miRNAs in neuroblastoma chemoresistance is limited and poorly understood. In this study, we set out to identify miRNAs and their pathways involved in mediating drug resistance in neuroblastoma. We hypothesize that differential expression of miRNAs among parental and resistant cells could lead to development of drug resistance in neuroblastoma. We used next generation deep sequencing technology to determine the miRNA expression profiles from matched parental cell lines (isolated at diagnosis) and resistant cell lines (isolated at relapse after intensive treatments) from six neuroblastoma patients.

We observed a downregulation of 34 miRNAs and upregulation of 8 miRNAs in the cell lines isolated post-treatment. RT-qPCR technique was used to confirm and validate the deregulated miRNA expressions. Our results were in line with previous reports, where a general downregulation of miRNAs is more common than upregulation of miRNAs in cancers. Interestingly, 22 of the 34 downregulated miRNAs were located on chromosome 14q32 locus, which have earlier been reported to be downregulated in multiple human cancers. Moreover, we also demonstrated that the reduced expression of certain chromosome 14q32 miRNAs correlates with poor clinical outcome in a cohort consisting of 226 primary neuroblastomas. Furthermore, Ingenuity pathway analysis (IPA) of differentially expressed miRNAs revealed important biological pathways associated with cancer progression and drug resistance. Hence, in this study, we identified a unique set of miRNAs which may be involved in the development of drug resistance in neuroblastoma. In addition, we showed that reduced expression of certain miRNAs is correlated with poor prognostic factors in neuroblastoma.

Paper-II

Hsa-miR-376c-3p targets Cyclin D1 and induces G1-cell cycle arrest in neuroblastoma cells

Bhavsar S.P., Løkke C., Flægstad T., & Einvik C.

Oncology Letters (2018) 16(5):6786-6794

In paper I, we showed that downregulation of chromosome 14q32 miRNAs is associated with drug resistance and poor clinical outcome in neuroblastoma. In this paper, we investigated the previously unknown functional role of chromosome 14q32 locus miRNA, *miR-376c-3p* that is downregulated in post-therapy neuroblastoma cell line pairs. We hypothesize that restoration of the downregulated *miR-376c-3p* could reverse the resistant phenotype caused due to reduced expression of *miR-376c-3p.*

Therefore, we first over-expressed *miR-376c-3p* in multiple neuroblastoma cell lines and demonstrated that upregulation of *miR-376c-3p* results in significant reduction of cell growth and G1-cell cycle arrest in most of these cell lines. We then used miRNA target-prediction software (TargetScan) to identify *miR-376c-3p* targets mainly related to cell cycle. Dual luciferase reporter assay was performed to confirm and validate *miR-376c-3p* target. Finally, RT-qPCR and western blot assays were performed to quantitate *miR-376c-3p* mediated suppression of target mRNA and protein levels, respectively.

Interestingly, we observed cell cycle related *CCND1* as one of the several genes predicted to target by *miR-376c-3p.* Luciferase reporter assay further confirmed and validated *CCND1* as a direct target of *miR-376c-3p*. As expected, the mRNA and protein levels of *CCND1* were significantly reduced in *miR-376c-3p* transfected cells as compared to negative control (NC) transfected cells. Hence, in this paper we proposed that *miR-376c-3p* could affect cell growth and induce G1-cell cycle arrest by targeting *CCND1* in neuroblastoma cells.

Paper-III

Hsa-miR-323a-3p targets *STAT3* and induces G1-cell cycle arrest and apoptosis in neuroblastoma cells

Bhavsar S.P., Olsen L., Løkke C., Flægstad T., & Einvik C.

Manuscript

Recently, the role of microRNAs (miRNAs) in modulating drug resistance in multiple cancers has emerged as a promising therapeutic approach in the treatment of therapy-resistant tumors. However, the role of miRNAs in neuroblastoma drug resistance is poorly understood.

In our previous research, we have demonstrated that *miR-323a-3p* is downregulated in post-chemotherapy neuroblastoma cells as compared to matched pre-chemotherapy cells. Moreover, *miR-323a-3p* is downregulated in stage 4 vs stage 1-2 in a cohort of 226 primary neuroblastoma tumors. Therefore, in this study, we set out to investigate the functional role of *miR-323a-3p* in neuroblastoma.

We used RT-qPCR to quantitate the basic expression of *miR-323a-3p* in BE(2)-C, Kelly and SHSY-5Y neuroblastoma cell lines. The functional role of *miR-323a-3p* in these cell lines was evaluated by alamar blue-cell viability, flow cytometry and western blot assays. Luciferase reporter assay, RT-qPCR and western blotting techniques were used for *miR-323a-3p* target identification and quantification in neuroblastoma cell lines.

Over-expression of *miR-323a-3p* resulted in significant reduction of cell viability in BE(2)-C, Kelly and SHSY-5Y cell lines. By flow cytometric cell cycle analysis we found G1-cell cycle arrest in Kelly and SHSY-5Y but not in BE(2)-C cells. By western blot analysis, we found that *miR-323a-3p* induces apoptosis in BE(2)-C, Kelly and SHSY-5Y cell lines.

Bioinformatic miRNA target prediction software (miRDB) predicted 793 potential target genes for *miR-323a-3p*. Subsequent literature search, target analysis and luciferase reporter assay identified and confirmed *STAT3* as a direct target of *miR-323a-3p*. Furthermore, RT-qPCR and western blot analysis revealed that the mRNA and protein levels of *STAT3* were significantly reduced in *miR-323a-3p* transfected cells as compared to negative control (NC) transfected cells.

Thus, the study showed that over-expression of *miR-323a-3p* led to significant inhibition of cell viability, G1 cell cycle arrest and apoptosis by targeting *STAT3* in neuroblastoma cells.

Discussion (Paper I, II & III)

Chemotherapy is the primary choice of treatment for patients with high-risk neuroblastoma. However, resistance to chemotherapy has emerged as an important clinical obstacle. These high-risk patients usually relapse after prolonged treatment and eventually die (Habib et al., 2012). Therefore, instead of the conventional chemotherapy approaches, alternative strategies need to be employed which may target specific genes to combat this lethal disease. Mounting studies have demonstrated the ability of miRNAs to regulate the expression of multiple genes affecting the growth and survival of multiple cancer types (Gurtan and Sharp, 2013). Thus, the known functionality of miRNAs to act as oncogenes or tumor suppressors establish that aberrant expression of miRNAs may represent a general feature of human cancer and provides an opportunity for therapeutic intervention.

In this study, by deep sequencing analysis, we observed reduced expression of *miR-376c-3p* and *miR-323a-3p* in resistant neuroblastoma cell lines as compared to matched parental neuroblastoma cell lines. In addition, these miRNAs were also downregulated in a cohort consisting of 226 primary neuroblastoma tumors. We thus propose that downregulation of *miR-376c-3p* and *miR-323a-3p* miRNAs may be associated with underlying resistance mechanisms observed in resistant neuroblastoma cell lines. Over-expression of these downregulated miRNAs correlated with decreased survival in neuroblastoma cells by promoting G1-cell cycle arrest and apoptosis. Therefore, this study has implications for understanding the functional roles of miRNAs in neuroblastoma tumorigenesis and drug resistance.

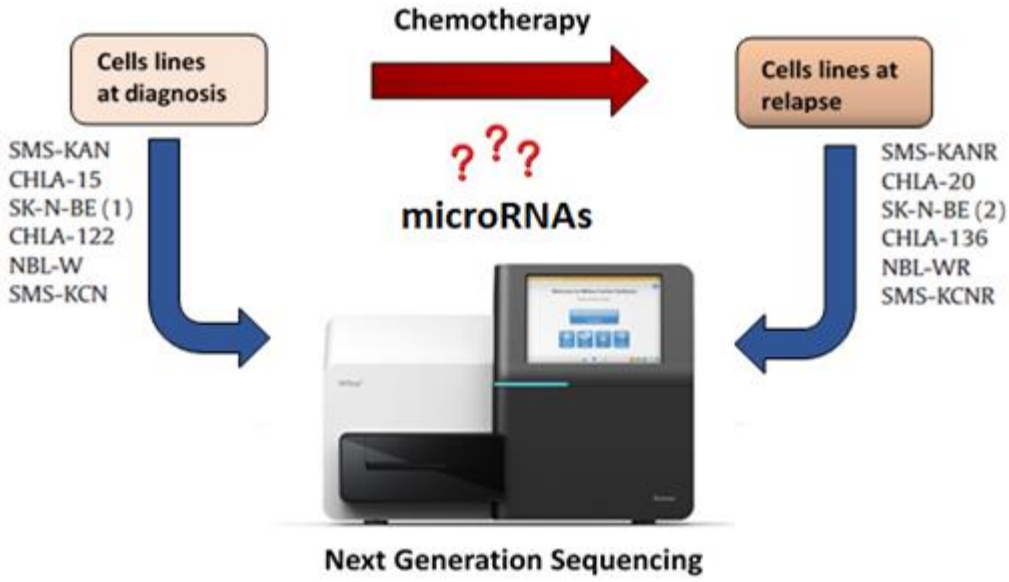
MicroRNAs are differentially expressed in parental and resistant neuroblastoma cell lines

Drug resistance is one of the most important clinical obstacles in treatment of cancer. Multiple factors are involved in mediating cells resistant to drugs, which are described in detail in the introduction section. Growing evidence have established the roles of miRNAs in mediating drug resistance in human cancers (Ayers et al., 2015, Chen et al., 2012). In this study, we have characterized the roles of certain miRNAs in drug resistant neuroblastoma.

Generally, researchers create resistant cell lines by exposing cancer cells to high concentrations of drugs. These *in vitro*-selected resistant cell lines are then subjected for the analysis of underlying drug resistance mechanisms. However, this process of making cells resistant to drug may create a significant impact on the cellular system as a whole causing high expression of a myriad of genes and proteins. In short, the cell system might be restructured drastically due to high concentration of drugs. This scenario is far from what we can imagine

about resistant cancerous cells present in the tumor microenvironment in the body. Owing to the numerous possibilities or complexities offered by the tumor microenvironment as described in the introduction section and the drugs pharmacokinetic and pharmacodynamic properties. It is very difficult to correlate the results obtained from such resistant cell lines with the naturally acquired resistance of the cancerous cells present in the patient's body. Therefore, we opt for another strategy, which included cells isolated from neuroblastoma patients at diagnosis and from the same patients at relapse following chemotherapy. This strategy provided us with the parental cells at diagnosis which were not treated with any kind of drugs and we also got the resistant cells from the same patient at relapse which were treated with chemotherapeutic drugs. Therefore, these resistant cells obtained via naturally acquired resistance in the body at relapse would mimic the underlying drug resistance mechanisms more closely than the cells, which are created by exposure to high concentrations of drugs.

Very few studies have demonstrated the deregulation of miRNAs and their association with neuroblastoma pathogenesis and drug resistance. Bray et al., (2009) have shown the dysregulation of miRNAs in neuroblastoma tumors caused by large-scale chromosomal imbalances and the over-expression of *MYCN*. In addition, this study concluded that patterns of miRNA expression might be predictive of clinical outcome (Bray et al., 2009). In another study by Chen and Stallings, expression profiling of 157 miRNA loci in 35 primary neuroblastoma tumors resulted in differential expression of 32 loci in favorable and unfavorable tumor subtypes. Interestingly, several miRNAs were downregulated in tumors with *MYCN* amplification. They further assessed the functional role of single locus (*miR-184*), and found that *miR-184* had significant role in apoptosis. Therefore, this study concludes that differential patterns of miRNA expression are correlated with clinical and biological factors (Chen and Stallings, 2007). Similarly, Schulte and colleagues, used next-generation sequencing technology to analyze five favorable and five unfavorable neuroblastoma tumor samples. This study also revealed the differential pattern of miRNA expression in favorable versus unfavorable neuroblastoma (Schulte et al., 2010). Taken together all these studies, points towards the clinical and functional relevance of differentially expressed miRNAs in the pathogenesis of neuroblastoma.



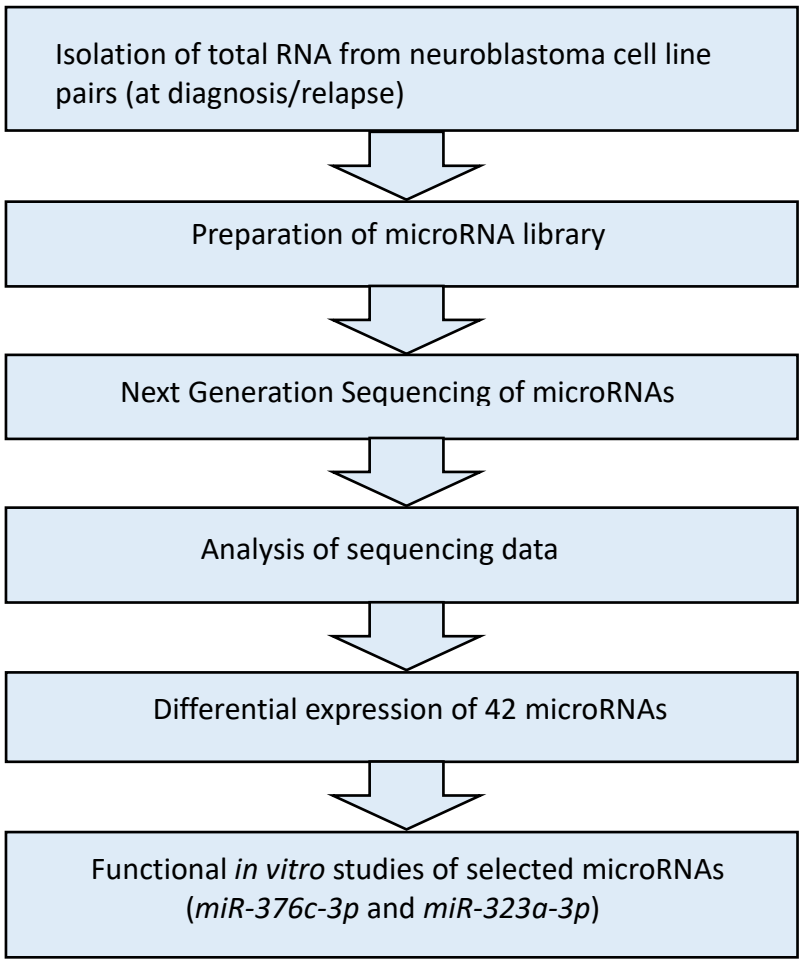


Figure 7. Schematic representation summarizing the workflow of the study. Total RNA extracted from neuroblastoma cell lines at diagnosis and after chemotherapeutic treatments at relapse are reverse-transcribed to produce microRNA-cDNA-libraries, which are further subjected to next generation sequencing. The data obtained after sequencing is systematically analyzed to study the functional role of miRNAs in neuroblastoma pathogenesis and drug resistance.

The roles of miRNAs in mediating drug resistance is poorly understood. Few studies have reported the involvement of miRNAs in mediating drug resistance in neuroblastoma. These studies have employed cell lines, which were created by exposing them to high concentration of drugs (Chen et al., 2012, Ayers et al., 2015). We therefore hypothesize that miRNAs may be differentially expressed across parental and naturally acquired resistant cells and that functional analyzes of these aberrantly miRNAs could be important in understanding the drug resistance mechanisms in neuroblastoma.

Table 6. The neuroblastoma cell line pairs used in the study.

Cell lines isolated at diagnosis	Cell lines isolated at relapse	Chemotherapy given to the patients
CHLA-15	CHLA-20	DOX ,CTX, CDDP, VM-26
CHLA-122	CHLA-136	DOX, ETOP, autoSCT with TBI
NBL-W	NBL-WR	DOX, CTX, DAU, VCR, CDDP, DTIC, ETOP
SK-N-BE(1)	SK-N-BE(2)-C	DOX, CTX, VCR, RAD
SMS-KAN	SMS-KANR	DOX, CTX, RAD
SMS-KCN	SMS-KCNR	DOX, CTX

DOX: Doxorubicin; CTX: Cyclophosphamide; CDDP: Cisplatin; VM-26: Teniposide; ETOP: Etoposide; autoSCT: Autologous stem cell transplantation; TBI: Total body irradiation; DAU: Daunorubicin; VCR: Vincristine; DTIC: Dacarbazine; RAD: Local radiation.

We used next-generation deep sequencing technology to sequence small RNA transcriptome from the neuroblastoma cell lines isolated from six patients at diagnosis and at relapse after intensive treatment regimens (Figure 7) (Table 6) (Paper I). As expected, this led us to identification of several hundred miRNAs differentially expressed across parental and resistant cell lines. Systematic analysis of the miRNA expression profiles revealed a panel of 42 differentially expressed miRNAs, 34 of which were downregulated and eight of which were upregulated significantly. Interestingly, 22 out of 34 downregulated miRNAs belonged to the chromosome 14q32 miRNA clusters (Figure 8). The results obtained from deep sequencing were further confirmed and validated by RT-qPCR. The miRNA expression profiles obtained using deep sequencing technology correlated well with the data obtained from RT-qPCR.

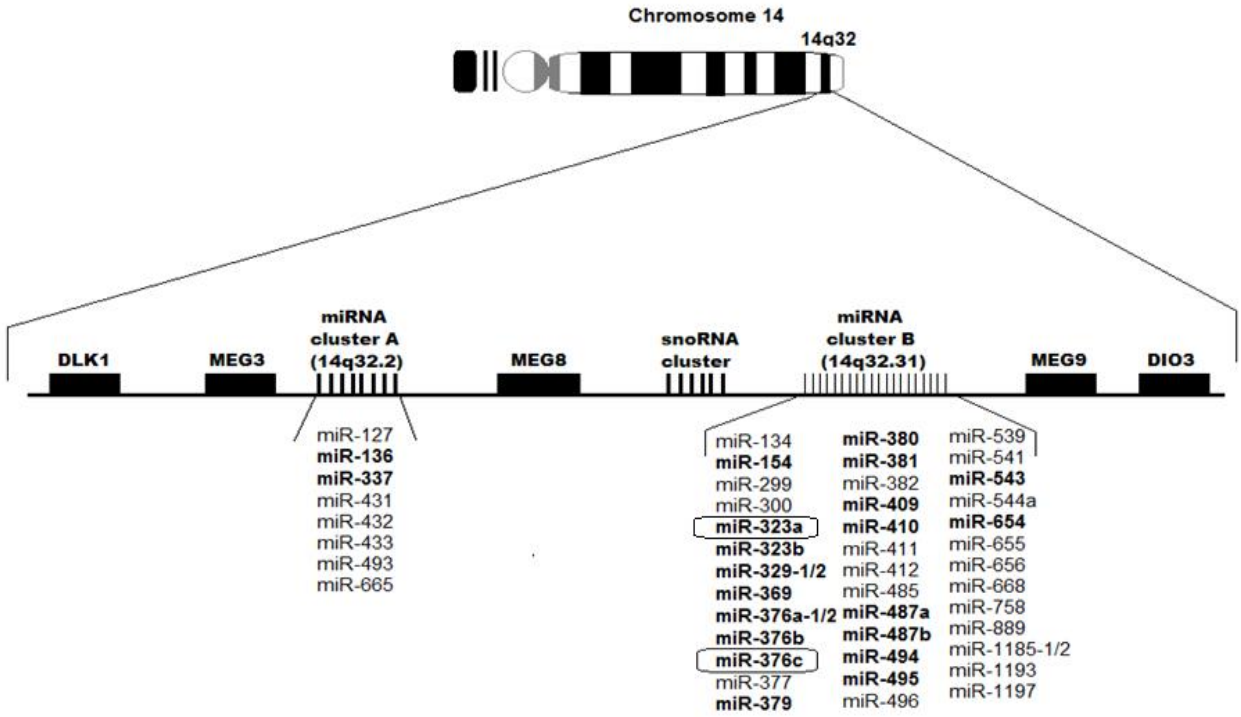


Figure 8. Schematic representation of the miRNA-clusters at 14q32 chromosomal region. Multiple miRNAs located at 14q32 chromosomal region are downregulated (bold type) and associated with poor prognosis factors in neuroblastoma. The miRNAs of interest, *miR-376c* and *miR-323a* are highlighted in bold and rectangular box. DLK1, delta like non-canonical notch ligand 1; MEG, maternally expressed; snoRNA, small nucleolar RNA; DIO3, iodothyronine deiodinase 3; miRNA, microRNA.

The reduced expression of chromosome 14q32 miRNAs in tumors associated with poor prognosis factors was confirmed in a cohort consisting of 226 primary neuroblastomas. Moreover, we also applied Ingenuity Pathway Analysis (IPA), which identifies new targets, pathways and candidate biomarkers within the context of biological systems to gain insights into the nature of genes and pathways affected by the deregulated miRNAs in neuroblastoma. The results of this analysis revealed several biological pathways associated with cancer and drug resistance (Paper I).

MiR-376c-3p and *miR-323a-3p* are novel microRNAs with tumor suppressive activity in neuroblastoma

Since the chromosome 14q32 miRNA clusters are shown downregulated in multiple cancers (Zehavi et al., 2012), we focused on miRNAs from this region to study their functional roles in neuroblastoma. We selected *miR-376c-3p* and *miR-323a-3p*, to assess their role in the pathogenesis of neuroblastoma. Both miRNAs were downregulated in most of the cell lines isolated from patients after chemotherapeutic treatments relative to matched cell lines isolated

at diagnosis. In addition, the expression of *miR-376c-3p* and *miR-323a-3p,* was reduced in INSS stage 4 compared to stage 1-2 in a cohort of neuroblastoma tumors. Interestingly, literature search on *miR-376c-3p* and *miR-323a-3p* showed a dual nature of these miRNAs to act as either oncogenic or the tumor suppressive miRNA depending on cellular context. Taken together, all these important properties of *miR-376c-3p* and *miR-323a-3p,* prompted us to evaluate the unknown functional role of these miRNAs in neuroblastoma pathogenesis.

The *miR-376c-3p* is found downregulated in multiple human cancers, including prostate cancer (Formosa et al., 2014), cervical cancer (Deng et al., 2016), oral squamous cell carcinoma (Wang et al., 2017b), intrahepatic cholangiocarcinoma (Iwaki et al., 2013), melanoma (Zehavi et al., 2012), osteosarcoma (Jin et al., 2013) and gliomas (Huang et al., 2017). However, it was upregulated in acute myeloid leukemia (Dixon-McIver et al., 2008) and gastric cancer (Shiotani et al., 2013). From these studies, *miR-376c-3p* was shown to target a set of genes including B-cell-specific moloney murine leukemia virus insertion site 1 (*BMI1*), homeobox B7 (*HOXB7*), growth factor receptor-bound protein 2 (*GRB2*), transforming growth factor-alpha (*TGFA*), liver receptor homolog-1 (*LRH-1*), insulin growth factor 1 receptor (*IGF1R*) and activin receptor-like kinase 7 (*ALK7*) involved in cell growth, cell invasion (Deng et al., 2016, Wang et al., 2017b, Jin et al., 2013, Jiang et al., 2016), migration (Formosa et al., 2014, Wang et al., 2017b, Iwaki et al., 2013, Zehavi et al., 2012), cell cycle arrest (Deng et al., 2016), apoptosis (Wang et al., 2017b) and drug resistance (Ye et al., 2011).

We therefore performed functional studies to evaluate the effect of *miR-376c-3p* over-expression on the neuroblastoma cells (Paper II). We demonstrated that over-expression of *miR-376c-3p* led to suppression of cell viability and G1-cell cycle arrest in neuroblastoma cell lines. These results suggested that the cell cycle genes might be affected and could serve as *miR-376c-3p* targets. To test this hypothesis, we used bioinformatics algorithms to predict *miR-376c-3p* targets related to cell cycle regulation. TargetScan algorithm predicted *CCNDl* as a target of *miR-376c-3p,* which was further confirmed and validated by dual-luciferase reporter assay. Our experimental data showed that the expression of *CCND1* mRNA and protein levels were significantly reduced after transfection of *miR-376c-3p* as compared to *NC* transfected cells. In addition, we performed a rescue experiment to see if *CCND1* counteracts the effect of *miR-376c-3p* induced G1-cell cycle arrest upon *CCND1* over-expression. However, a modest and statistically insignificant effect of *CCND1* over-expression on reducing the effect of *miR-376c-3p* over-expression (i.e. G1-cell cycle arrest) was observed in neuroblsatoma cells. This is because *CCND1* might be targeted not only by *miR-376c-3p* but also by some other miRNAs

simultaneously. Many studies have demonstrated that several miRNAs can target the same mRNA to effectively repress the target gene expression (Shukla et al., 2011). This result thus point out towards the role of additional miRNAs in the regulation of *CCND1* expression in neuroblastoma cell lines. The *CCND1* has been proposed as an important oncogene in a variety of cancers (Musgrove et al., 2011). Multiple studies have demonstrated increased expression of *CCND1* and that knockdown of *CCND1* induced G1-cell cycle arrest and inhibited Cyclin D-Rb pathway in neuroblastoma cells (Molenaar et al., 2008, Molenaar et al., 2003, Rihani et al., 2015). Moreover, *CCND1* was also shown to be targeted by *miR-34* and induce G1-cell cycle arrest in NSCLC (Sun et al., 2008). Taken together, our findings suggest that *miR-376c-3p* has the ability of arresting growth in neuroblastoma cells by targeting *CCND1* oncogene. Further studies are necessitated to understand the role of additional miRNAs in the regulation of *CCND1* in neuroblastoma.

A similar study was performed to evaluate the functional role of *miR-323a-3p* in neuroblastoma (Manuscript I). Previous studies have demonstrated that *miR-323a-3p* is downregulated in multiple cancers including glioblastoma (Shahar et al., 2016), osteosarcoma (Chen et al., 2018a), bladder cancer (Li et al., 2017) and pancreatic ductal adenocarcinoma (PDAC) (Wang et al., 2016) whereas upregulated in prostate cancer (Gao et al., 2015, Gao and Zheng, 2018). Thus, *miR-323a-3p* functions as both tumor suppressive or oncogenic miRNA by regulating growth, apoptosis, invasion and migration depending on the cellular context.

We demonstrated that over-expression of *miR-323a-3p* led to suppression of cell viability, G1-cell cycle arrest and apoptosis in neuroblastoma cell lines. Given the tumor suppressive nature of this miRNA, we searched the literature for probable targets of *miR-323a-3p* validated in other cancer types. RT-qPCR was performed on such selected targets. In addition, we used online bioinformatic tool (miRDB) to predict *miR-323a-3p* targets. We thus identified *STAT3* as a target of *miR-323a-3p,* which was further confirmed and validated by dual-luciferase reporter assay. Next, we over-expressed *miR-323a-3p* in neuroblastoma cell lines and quantitated the expression of *STAT3* mRNA and protein levels. Interestingly, the expression of *STAT3* mRNA and protein levels was significantly reduced after transfection with *miR-323a-3p* as compared to *NC* transfected cells. Thus, we propose that *miR-323a-3p* decreases cell growth and induces G1-cell cycle arrest and apoptosis by targeting *STAT3* in neuroblastoma.

Multiple studies have demonstrated the oncogenic role of *STAT3* in diverse cancers. And, given the importance of JAK-STAT3 signaling in cancer, it is one of the most promising targets for cancer therapy (Yu et al., 2014). *STAT3* inhibitors are being developed, which show decreased neuroblastoma tumorigenicity and increases chemosensitivity (Odate et al., 2017). Furthermore, several tyrosine kinase inhibitors are also developed and now available in clinic, which could target JAK-STAT3 signaling indirectly, ultimately leading to reduction of tumor growth and survival by promoting apoptosis (Yu et al., 2009).

In a recent study, Ara et al., (2013) identified an IL-6/sIL-6R/STAT3 interactive pathway functioning between neuroblastoma cells and its microenvironment, which led to drug resistance. This study showed that STAT3 is not constitutively active in neuroblastoma cells and that treatment of IL-6 alone or in combination with soluble IL-6 receptor (sIL-6R) results in high activation of STAT3. The study demonstrated that drug-induced apoptosis of neuroblastoma cells was rescued with the treatment of IL-6 alone and/or in combination with sIL-6R. The rescue effect is observed due to the high activation of STAT3 in the cells since STAT3 inhibition (with STAT3 inhibitor, *Stattic*) was not able to protect cells from drug-induced apoptosis (Ara et al., 2013).

Given the important role of IL-6/sIL-6R activated STAT3 in drug resistance and our recent results showing that *STAT3* is a direct target of *miR-323a-3p* in neuroblastoma. We hypothesize that *miR-323a-3p* could target *STAT3* and rescue the drug-induced apoptosis in neuroblastoma cells. In addition, it would be interesting to find the expression of STAT3 downstream factors such as survivin, BCL-2, etc. affected by STAT3 repression.

To summarize, in this thesis, we were not able to address the direct role of miRNA in modulating drug resistance in neuroblastoma. However, we propose that *miR-323a-3p* could play a potential role in drug resistance by targeting *STAT3* in neuroblastoma cells. Further experimental studies with chemotherapeutic drugs are necessitated to address the role of IL-6/sIL-6R/STAT3/miR-323a pathway in modulating drug resistance in neuroblastoma.

Lastly, our findings suggest that the reduced expression of miRNAs may contribute to a resistant phenotype in neuroblastoma cell lines, and therefore ectopic over-expression of these miRNAs should be considered for overcoming resistance. The reduced expression of multiple miRNAs in post-chemotherapy neuroblastoma cell lines and primary tumors indicate a strong functional connection between miRNAs and underlying drug resistance mechanisms in the worst prognostic class of neuroblastoma. In addition, our study establishes that the restoration of certain miRNAs could be key therapeutic goal in neuroblastoma. According to our

knowledge, there are very few studies demonstrating the importance of chromosome 14q32 miRNA downregulation in neuroblastoma. Hence, additional studies are warranted to establish the role of all other chromosome 14q32 miRNAs in neuroblastoma pathogenesis and drug resistance.

References

AGGARWAL, B. B., SETHI, G., AHN, K. S., SANDUR, S. K., PANDEY, M. K., KUNNUMAKKARA, A. B., SUNG, B. & ICHIKAWA, H. 2006. Targeting signal-transducer-and-activator-of-transcription-3 for prevention and therapy of cancer: modern target but ancient solution. *Ann N Y Acad Sci,* 1091**,** 151-69.

ALEXIOU, P., VERGOULIS, T., GLEDITZSCH, M., PREKAS, G., DALAMAGAS, T., MEGRAW, M., GROSSE, I., SELLIS, T. & HATZIGEORGIOU, A. G. 2010. miRGen 2.0: a database of microRNA genomic information and regulation. *Nucleic Acids Res,* 38**,** D137-41.

ALISI, A., CHO, W. C., LOCATELLI, F. & FRUCI, D. 2013. Multidrug resistance and cancer stem cells in neuroblastoma and hepatoblastoma. *Int J Mol Sci,* 14**,** 24706-25.

ARA, T., NAKATA, R., SHEARD, M. A., SHIMADA, H., BUETTNER, R., GROSHEN, S. G., JI, L., YU, H., JOVE, R., SEEGER, R. C. & DECLERCK, Y. A. 2013. Critical role of STAT3 in IL-6-mediated drug resistance in human neuroblastoma. *Cancer Res,* 73**,** 3852-64.

ARA, T., SONG, L., SHIMADA, H., KESHELAVA, N., RUSSELL, H. V., METELITSA, L. S., GROSHEN, S. G., SEEGER, R. C. & DECLERCK, Y. A. 2009. Interleukin-6 in the bone marrow microenvironment promotes the growth and survival of neuroblastoma cells. *Cancer Res,* 69**,** 329-37.

ATTIYEH, E. F., LONDON, W. B., MOSSE, Y. P., WANG, Q., WINTER, C., KHAZI, D., MCGRADY, P. W., SEEGER, R. C., LOOK, A. T., SHIMADA, H., BRODEUR, G. M., COHN, S. L., MATTHAY, K. K., MARIS, J. M. & CHILDREN'S ONCOLOGY, G. 2005. Chromosome 1p and 11q deletions and outcome in neuroblastoma. *N Engl J Med,* 353**,** 2243-53.

AXELROD, J. 1960. Biochemical factors in the activation and inactivation of drugs. *Naunyn Schmiedebergs Arch Exp Pathol Pharmakol,* 238**,** 24-35.

AYERS, D., MESTDAGH, P., VAN MAERKEN, T. & VANDESOMPELE, J. 2015. Identification of miRNAs contributing to neuroblastoma chemoresistance. *Comput Struct Biotechnol J,* 13**,** 307-19.

BABASHAH, S. & SOLEIMANI, M. 2011. The oncogenic and tumour suppressive roles of microRNAs in cancer and apoptosis. *Eur J Cancer,* 47**,** 1127-37.

BALDIN, V., LUKAS, J., MARCOTE, M. J., PAGANO, M. & DRAETTA, G. 1993. Cyclin D1 is a nuclear protein required for cell cycle progression in G1. *Genes Dev,* 7**,** 812-21.

BALKWILL, F. R., CAPASSO, M. & HAGEMANN, T. 2012. The tumor microenvironment at a glance. *J Cell Sci,* 125**,** 5591-6.

BANDI, N., ZBINDEN, S., GUGGER, M., ARNOLD, M., KOCHER, V., HASAN, L., KAPPELER, A., BRUNNER, T. & VASSELLA, E. 2009. miR-15a and miR-16 are implicated in cell cycle regulation in a Rb-dependent manner and are frequently deleted or down-regulated in non-small cell lung cancer. *Cancer Res,* 69**,** 5553-9.

BARTEK, J., BARTKOVA, J. & LUKAS, J. 1996. The retinoblastoma protein pathway and the restriction point. *Curr Opin Cell Biol,* 8**,** 805-14.

BECK, W. T., MORGAN, S. E., MO, Y. Y. & BHAT, U. G. 1999. Tumor cell resistance to DNA topoisomerase II inhibitors: new developments. *Drug Resist Updat,* 2**,** 382-389.

BEITZINGER, M. & MEISTER, G. 2010. Preview. MicroRNAs: from decay to decoy. *Cell,* 140**,** 612-4.

BENZENO, S., LU, F., GUO, M., BARBASH, O., ZHANG, F., HERMAN, J. G., KLEIN, P. S., RUSTGI, A. & DIEHL, J. A. 2006. Identification of mutations that disrupt phosphorylation-dependent nuclear export of cyclin D1. *Oncogene,* 25**,** 6291-303.

BEREZIKOV, E., CHUNG, W. J., WILLIS, J., CUPPEN, E. & LAI, E. C. 2007. Mammalian mirtron genes. *Mol Cell,* 28**,** 328-36.

BETEL, D., WILSON, M., GABOW, A., MARKS, D. S. & SANDER, C. 2008. The microRNA.org resource: targets and expression. *Nucleic Acids Res,* 36**,** D149-53.

BHAVSAR, S. P., LOKKE, C., FLAEGSTAD, T. & EINVIK, C. 2018. Hsa-miR-376c-3p targets Cyclin D1 and induces G1-cell cycle arrest in neuroblastoma cells. *Oncol Lett,* 16**,** 6786-6794.

BHUJABAL, Z. B. 2017. *FKBP8 and the autophagy inducing Class-III PI3K Complex.* PhD PhD thesis, University of Tromsø.

BONCI, D., COPPOLA, V., MUSUMECI, M., ADDARIO, A., GIUFFRIDA, R., MEMEO, L., D'URSO, L., PAGLIUCA, A., BIFFONI, M., LABBAYE, C., BARTUCCI, M., MUTO, G., PESCHLE, C. & DE MARIA, R. 2008. The miR-15a-miR-16-1 cluster controls prostate cancer by targeting multiple oncogenic activities. *Nat Med,* 14**,** 1271-7.

BORST, P., EVERS, R., KOOL, M. & WIJNHOLDS, J. 2000. A family of drug transporters: the multidrug resistance-associated proteins. *J Natl Cancer Inst,* 92**,** 1295-302.

BOUCHIE, A. 2013. First microRNA mimic enters clinic. *Nat Biotechnol,* 31**,** 577.

BOUWMAN, P. & JONKERS, J. 2012. The effects of deregulated DNA damage signalling on cancer chemotherapy response and resistance. *Nat Rev Cancer,* 12**,** 587-98.

BOWN, N., COTTERILL, S., LASTOWSKA, M., O'NEILL, S., PEARSON, A. D., PLANTAZ, D., MEDDEB, M., DANGLOT, G., BRINKSCHMIDT, C., CHRISTIANSEN, H., LAUREYS, G., SPELEMAN, F., NICHOLSON, J., BERNHEIM, A., BETTS, D. R., VANDESOMPELE, J. & VAN ROY, N. 1999. Gain of chromosome arm 17q and adverse outcome in patients with neuroblastoma. *N Engl J Med,* 340**,** 1954-61.

BRAY, I., BRYAN, K., PRENTER, S., BUCKLEY, P. G., FOLEY, N. H., MURPHY, D. M., ALCOCK, L., MESTDAGH, P., VANDESOMPELE, J., SPELEMAN, F., LONDON, W. B., MCGRADY, P. W., HIGGINS, D. G., O'MEARA, A., O'SULLIVAN, M. & STALLINGS, R. L. 2009. Widespread dysregulation of MiRNAs by MYCN amplification and chromosomal imbalances in neuroblastoma: association of miRNA expression with survival. *PLoS One,* 4**,** e7850.

BRODEUR, G. M. 2003. Neuroblastoma: biological insights into a clinical enigma. *Nat Rev Cancer,* 3**,** 203-16.

BRODEUR, G. M., PRITCHARD, J., BERTHOLD, F., CARLSEN, N. L., CASTEL, V., CASTELBERRY, R. P., DE BERNARDI, B., EVANS, A. E., FAVROT, M., HEDBORG, F. & ET AL. 1993. Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. *J Clin Oncol,* 11**,** 1466-77.

BRODEUR, G. M., SEEGER, R. C., BARRETT, A., BERTHOLD, F., CASTLEBERRY, R. P., D'ANGIO, G., DE BERNARDI, B., EVANS, A. E., FAVROT, M., FREEMAN, A. I. & ET AL. 1988. International criteria for diagnosis, staging, and response to treatment in patients with neuroblastoma. *J Clin Oncol,* 6**,** 1874-81.

BRODEUR, G. M., SEEGER, R. C., SCHWAB, M., VARMUS, H. E. & BISHOP, J. M. 1984. Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. *Science,* 224**,** 1121-4.

BRUECKNER, B., STRESEMANN, C., KUNER, R., MUND, C., MUSCH, T., MEISTER, M., SULTMANN, H. & LYKO, F. 2007. The human let-7a-3 locus contains an epigenetically regulated microRNA gene with oncogenic function. *Cancer Res,* 67**,** 1419-23.

BUENO, M. J., GOMEZ DE CEDRON, M., GOMEZ-LOPEZ, G., PEREZ DE CASTRO, I., DI LISIO, L., MONTES-MORENO, S., MARTINEZ, N., GUERRERO, M., SANCHEZ-MARTINEZ, R., SANTOS, J., PISANO, D. G., PIRIS, M. A., FERNANDEZ-PIQUERAS, J. & MALUMBRES, M. 2011. Combinatorial effects of microRNAs to suppress the Myc oncogenic pathway. *Blood,* 117**,** 6255-66.

BUHAGIAR, A. & AYERS, D. 2015. Chemoresistance, cancer stem cells, and miRNA influences: the case for neuroblastoma. *Anal Cell Pathol (Amst),* 2015**,** 150634.

BURKHART, C. A., WATT, F., MURRAY, J., PAJIC, M., PROKVOLIT, A., XUE, C., FLEMMING, C., SMITH, J., PURMAL, A., ISACHENKO, N., KOMAROV, P. G., GUROVA, K. V., SARTORELLI, A. C., MARSHALL, G. M., NORRIS, M. D., GUDKOV, A. V. & HABER, M. 2009. Small-molecule multidrug resistance-associated protein 1 inhibitor reversan increases the therapeutic index of chemotherapy in mouse models of neuroblastoma. *Cancer Res,* 69**,** 6573-80.

CALIN, G. A., DUMITRU, C. D., SHIMIZU, M., BICHI, R., ZUPO, S., NOCH, E., ALDLER, H., RATTAN, S., KEATING, M., RAI, K., RASSENTI, L., KIPPS, T., NEGRINI, M., BULLRICH, F. & CROCE, C. M. 2002. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci U S A,* 99**,** 15524-9.

CALIN, G. A., SEVIGNANI, C., DUMITRU, C. D., HYSLOP, T., NOCH, E., YENDAMURI, S., SHIMIZU, M., RATTAN, S., BULLRICH, F., NEGRINI, M. & CROCE, C. M. 2004. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci U S A,* 101**,** 2999-3004.

CARON, H., VAN SLUIS, P., DE KRAKER, J., BOKKERINK, J., EGELER, M., LAUREYS, G., SLATER, R., WESTERVELD, A., VOUTE, P. A. & VERSTEEG, R. 1996. Allelic loss of chromosome 1p as a predictor of unfavorable outcome in patients with neuroblastoma. *N Engl J Med,* 334**,** 225-30.

CHALLAGUNDLA, K. B., WISE, P. M., NEVIANI, P., CHAVA, H., MURTADHA, M., XU, T., KENNEDY, R., IVAN, C., ZHANG, X., VANNINI, I., FANINI, F., AMADORI, D., CALIN, G. A., HADJIDANIEL, M., SHIMADA, H., JONG, A., SEEGER, R. C., ASGHARZADEH, S., GOLDKORN, A. & FABBRI, M. 2015. Exosome-mediated transfer of microRNAs within the tumor microenvironment and neuroblastoma resistance to chemotherapy. *J Natl Cancer Inst,* 107.

CHANG, T. C., YU, D., LEE, Y. S., WENTZEL, E. A., ARKING, D. E., WEST, K. M., DANG, C. V., THOMAS-TIKHONENKO, A. & MENDELL, J. T. 2008. Widespread microRNA repression by Myc contributes to tumorigenesis. *Nat Genet,* 40**,** 43-50.

CHEN, C. J., CHIN, J. E., UEDA, K., CLARK, D. P., PASTAN, I., GOTTESMAN, M. M. & RONINSON, I. B. 1986. Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells. *Cell,* 47**,** 381-9.

CHEN, H., GAO, S. & CHENG, C. 2018a. MiR-323a-3p suppressed the glycolysis of osteosarcoma via targeting LDHA. *Hum Cell,* 31**,** 300-309.

CHEN, H., LIU, H. & QING, G. 2018b. Targeting oncogenic Myc as a strategy for cancer treatment. *Signal Transduct Target Ther,* 3**,** 5.

CHEN, J., WANG, L., MATYUNINA, L. V., HILL, C. G. & MCDONALD, J. F. 2011. Overexpression of miR-429 induces mesenchymal-to-epithelial transition (MET) in metastatic ovarian cancer cells. *Gynecol Oncol,* 121**,** 200-5.

CHEN, Y. & STALLINGS, R. L. 2007. Differential patterns of microRNA expression in neuroblastoma are correlated with prognosis, differentiation, and apoptosis. *Cancer Res,* 67**,** 976-83.

CHEN, Y., TAKITA, J., CHOI, Y. L., KATO, M., OHIRA, M., SANADA, M., WANG, L., SODA, M., KIKUCHI, A., IGARASHI, T., NAKAGAWARA, A., HAYASHI, Y., MANO, H. & OGAWA, S. 2008. Oncogenic mutations of ALK kinase in neuroblastoma. *Nature,* 455**,** 971-4.

CHEN, Y., TSAI, Y. H., FANG, Y. & TSENG, S. H. 2012. Micro-RNA-21 regulates the sensitivity to cisplatin in human neuroblastoma cells. *J Pediatr Surg,* 47**,** 1797-805.

CHENDRIMADA, T. P., GREGORY, R. I., KUMARASWAMY, E., NORMAN, J., COOCH, N., NISHIKURA, K. & SHIEKHATTAR, R. 2005. TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature,* 436**,** 740-4.

CHEUNG, N. K. & DYER, M. A. 2013. Neuroblastoma: developmental biology, cancer genomics and immunotherapy. *Nat Rev Cancer,* 13**,** 397-411.

CHEUNG, N. K., ZHANG, J., LU, C., PARKER, M., BAHRAMI, A., TICKOO, S. K., HEGUY, A., PAPPO, A. S., FEDERICO, S., DALTON, J., CHEUNG, I. Y., DING, L., FULTON, R., WANG, J., CHEN, X., BECKSFORT, J., WU, J., BILLUPS, C. A., ELLISON, D., MARDIS, E. R., WILSON, R. K., DOWNING, J. R., DYER, M. A. & ST JUDE CHILDREN'S RESEARCH HOSPITAL-WASHINGTON UNIVERSITY PEDIATRIC CANCER GENOME, P. 2012. Association of age at diagnosis and genetic mutations in patients with neuroblastoma. *JAMA,* 307**,** 1062-71.

CHIOSEA, S., JELEZCOVA, E., CHANDRAN, U., ACQUAFONDATA, M., MCHALE, T., SOBOL, R. W. & DHIR, R. 2006. Up-regulation of dicer, a component of the MicroRNA machinery, in prostate adenocarcinoma. *Am J Pathol,* 169**,** 1812-20.

CHOI, W. Y., GIRALDEZ, A. J. & SCHIER, A. F. 2007. Target protectors reveal dampening and balancing of Nodal agonist and antagonist by miR-430. *Science,* 318**,** 271-4.

CHOU, C. H., SHRESTHA, S., YANG, C. D., CHANG, N. W., LIN, Y. L., LIAO, K. W., HUANG, W. C., SUN, T. H., TU, S. J., LEE, W. H., CHIEW, M. Y., TAI, C. S., WEI, T. Y., TSAI, T. R., HUANG, H. T., WANG, C. Y., WU, H. Y., HO, S. Y., CHEN, P. R., CHUANG, C. H., HSIEH, P. J., WU, Y. S., CHEN, W. L., LI,

M. J., WU, Y. C., HUANG, X. Y., NG, F. L., BUDDHAKOSAI, W., HUANG, P. C., LAN, K. C., HUANG, C. Y., WENG, S. L., CHENG, Y. N., LIANG, C., HSU, W. L. & HUANG, H. D. 2018. miRTarBase update 2018: a resource for experimentally validated microRNA-target interactions. *Nucleic Acids Res,* 46**,** D296-D302.

CICCIA, A. & ELLEDGE, S. J. 2010. The DNA damage response: making it safe to play with knives. *Mol Cell,* 40**,** 179-204.

CIMMINO, A., CALIN, G. A., FABBRI, M., IORIO, M. V., FERRACIN, M., SHIMIZU, M., WOJCIK, S. E., AQEILAN, R. I., ZUPO, S., DONO, M., RASSENTI, L., ALDER, H., VOLINIA, S., LIU, C. G., KIPPS, T. J., NEGRINI, M. & CROCE, C. M. 2005. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci U S A,* 102**,** 13944-9.

COHN, S. L., PEARSON, A. D., LONDON, W. B., MONCLAIR, T., AMBROS, P. F., BRODEUR, G. M., FALDUM, A., HERO, B., IEHARA, T., MACHIN, D., MOSSERI, V., SIMON, T., GARAVENTA, A., CASTEL, V., MATTHAY, K. K. & FORCE, I. T. 2009. The International Neuroblastoma Risk Group (INRG) classification system: an INRG Task Force report. *J Clin Oncol,* 27**,** 289-97.

COPELAND, N. G., GILBERT, D. J., SCHINDLER, C., ZHONG, Z., WEN, Z., DARNELL, J. E., JR., MUI, A. L., MIYAJIMA, A., QUELLE, F. W., IHLE, J. N. & ET AL. 1995. Distribution of the mammalian Stat gene family in mouse chromosomes. *Genomics,* 29**,** 225-8.

CROCE, C. M. & CALIN, G. A. 2005. miRNAs, cancer, and stem cell division. *Cell,* 122**,** 6-7.

DARNELL, J. E., JR. 1997. STATs and gene regulation. *Science,* 277**,** 1630-5.

DE BROUWER, S., MESTDAGH, P., LAMBERTZ, I., PATTYN, F., DE PAEPE, A., WESTERMANN, F., SCHROEDER, C., SCHULTE, J. H., SCHRAMM, A., DE PRETER, K., VANDESOMPELE, J. & SPELEMAN, F. 2012. Dickkopf-3 is regulated by the MYCN-induced miR-17-92 cluster in neuroblastoma. *Int J Cancer,* 130**,** 2591-8.

DENG, S., CALIN, G. A., CROCE, C. M., COUKOS, G. & ZHANG, L. 2008. Mechanisms of microRNA deregulation in human cancer. *Cell Cycle,* 7**,** 2643-6.

DENG, Y., XIONG, Y. & LIU, Y. 2016. miR-376c inhibits cervical cancer cell proliferation and invasion by targeting BMI1. *Int J Exp Pathol,* 97**,** 257-65.

DENLI, A. M., TOPS, B. B., PLASTERK, R. H., KETTING, R. F. & HANNON, G. J. 2004. Processing of primary microRNAs by the Microprocessor complex. *Nature,* 432**,** 231-5.

DIEDERICHS, S. & HABER, D. A. 2007. Dual role for argonautes in microRNA processing and posttranscriptional regulation of microRNA expression. *Cell,* 131**,** 1097-108.

DIXON-MCIVER, A., EAST, P., MEIN, C. A., CAZIER, J. B., MOLLOY, G., CHAPLIN, T., ANDREW LISTER, T., YOUNG, B. D. & DEBERNARDI, S. 2008. Distinctive patterns of microRNA expression associated with karyotype in acute myeloid leukaemia. *PLoS One,* 3**,** e2141.

DU, L., SUBAUSTE, M. C., DESEVO, C., ZHAO, Z., BAKER, M., BORKOWSKI, R., SCHAGEMAN, J. J., GREER, R., YANG, C. R., SURAOKAR, M., WISTUBA, II, GAZDAR, A. F., MINNA, J. D. & PERTSEMLIDIS, A. 2012. miR-337-3p and its targets STAT3 and RAP1A modulate taxane sensitivity in non-small cell lung cancers. *PLoS One,* 7**,** e39167.

DUVVURI, M. & KRISE, J. P. 2005. Intracellular drug sequestration events associated with the emergence of multidrug resistance: a mechanistic review. *Front Biosci,* 10**,** 1499-509.

DWEEP, H., STICHT, C., PANDEY, P. & GRETZ, N. 2011. miRWalk--database: prediction of possible miRNA binding sites by "walking" the genes of three genomes. *J Biomed Inform,* 44**,** 839-47.

EBERT, M. S., NEILSON, J. R. & SHARP, P. A. 2007. MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells. *Nat Methods,* 4**,** 721-6.

ELMORE, S. 2007. Apoptosis: a review of programmed cell death. *Toxicol Pathol,* 35**,** 495-516.

ESQUELA-KERSCHER, A. & SLACK, F. J. 2006. Oncomirs - microRNAs with a role in cancer. *Nat Rev Cancer,* 6**,** 259-69.

EYLER, C. E. & RICH, J. N. 2008. Survival of the fittest: cancer stem cells in therapeutic resistance and angiogenesis. *J Clin Oncol,* 26**,** 2839-45.

FLAHAUT, M., MEIER, R., COULON, A., NARDOU, K. A., NIGGLI, F. K., MARTINET, D., BECKMANN, J. S., JOSEPH, J. M., MUHLETHALER-MOTTET, A. & GROSS, N. 2009. The Wnt receptor FZD1

mediates chemoresistance in neuroblastoma through activation of the Wnt/beta-catenin pathway. *Oncogene,* 28**,** 2245-56.
FLETCHER J.I., H. M., HENDERSON M.J. AND NORRIS M.D. 2012. *Targeting Multidrug Resistance in Neuroblastoma,* Netherlands, Springer.
FONTANA, L., FIORI, M. E., ALBINI, S., CIFALDI, L., GIOVINAZZI, S., FORLONI, M., BOLDRINI, R., DONFRANCESCO, A., FEDERICI, V., GIACOMINI, P., PESCHLE, C. & FRUCI, D. 2008. Antagomir-17-5p abolishes the growth of therapy-resistant neuroblastoma through p21 and BIM. *PLoS One,* 3**,** e2236.
FORMOSA, A., MARKERT, E. K., LENA, A. M., ITALIANO, D., FINAZZI-AGRO, E., LEVINE, A. J., BERNARDINI, S., GARABADGIU, A. V., MELINO, G. & CANDI, E. 2014. MicroRNAs, miR-154, miR-299-5p, miR-376a, miR-376c, miR-377, miR-381, miR-487b, miR-485-3p, miR-495 and miR-654-3p, mapped to the 14q32.31 locus, regulate proliferation, apoptosis, migration and invasion in metastatic prostate cancer cells. *Oncogene,* 33**,** 5173-82.
FU, M., WANG, C., LI, Z., SAKAMAKI, T. & PESTELL, R. G. 2004. Minireview: Cyclin D1: normal and abnormal functions. *Endocrinology,* 145**,** 5439-47.
FULDA, S., LUTZ, W., SCHWAB, M. & DEBATIN, K. M. 1999. MycN sensitizes neuroblastoma cells for drug-induced apoptosis. *Oncogene,* 18**,** 1479-86.
GAO, Q., YAO, X. & ZHENG, J. 2015. MiR-323 Inhibits Prostate Cancer Vascularization Through Adiponectin Receptor. *Cell Physiol Biochem,* 36**,** 1491-8.
GAO, Q. & ZHENG, J. 2018. microRNA-323 upregulation promotes prostate cancer growth and docetaxel resistance by repressing p73. *Biomed Pharmacother,* 97**,** 528-534.
GEORGE, R. E., SANDA, T., HANNA, M., FROHLING, S., LUTHER, W., 2ND, ZHANG, J., AHN, Y., ZHOU, W., LONDON, W. B., MCGRADY, P., XUE, L., ZOZULYA, S., GREGOR, V. E., WEBB, T. R., GRAY, N. S., GILLILAND, D. G., DILLER, L., GREULICH, H., MORRIS, S. W., MEYERSON, M. & LOOK, A. T. 2008. Activating mutations in ALK provide a therapeutic target in neuroblastoma. *Nature,* 455**,** 975-8.
GILLET, J. P. & GOTTESMAN, M. M. 2009. Identification of gene signatures involved in the mechanisms of multidrug resistance. *Per Med,* 6**,** 133-134.
GILLET, J. P. & GOTTESMAN, M. M. 2010. Mechanisms of multidrug resistance in cancer. *Methods Mol Biol,* 596**,** 47-76.
GOLDSTEIN, L. J., FOJO, A. T., UEDA, K., CRIST, W., GREEN, A., BRODEUR, G., PASTAN, I. & GOTTESMAN, M. M. 1990. Expression of the multidrug resistance, MDR1, gene in neuroblastomas. *J Clin Oncol,* 8**,** 128-36.
GOLDSTEIN, L. J., GALSKI, H., FOJO, A., WILLINGHAM, M., LAI, S. L., GAZDAR, A., PIRKER, R., GREEN, A., CRIST, W., BRODEUR, G. M. & ET AL. 1989. Expression of a multidrug resistance gene in human cancers. *J Natl Cancer Inst,* 81**,** 116-24.
GOTTESMAN, M. M. 2002. Mechanisms of cancer drug resistance. *Annu Rev Med,* 53**,** 615-27.
GREGORY, P. A., BRACKEN, C. P., BERT, A. G. & GOODALL, G. J. 2008. MicroRNAs as regulators of epithelial-mesenchymal transition. *Cell Cycle,* 7**,** 3112-8.
GREGORY, R. I., CHENDRIMADA, T. P., COOCH, N. & SHIEKHATTAR, R. 2005. Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. *Cell,* 123**,** 631-40.
GREGORY, R. I., YAN, K. P., AMUTHAN, G., CHENDRIMADA, T., DORATOTAJ, B., COOCH, N. & SHIEKHATTAR, R. 2004. The Microprocessor complex mediates the genesis of microRNAs. *Nature,* 432**,** 235-40.
GRIFFITHS-JONES, S. 2006. miRBase: the microRNA sequence database. *Methods Mol Biol,* 342**,** 129-38.
GUIL, S. & ESTELLER, M. 2009. DNA methylomes, histone codes and miRNAs: tying it all together. *Int J Biochem Cell Biol,* 41**,** 87-95.
GURTAN, A. M. & SHARP, P. A. 2013. The role of miRNAs in regulating gene expression networks. *J Mol Biol,* 425**,** 3582-600.

HABER, M., SMITH, J., BORDOW, S. B., FLEMMING, C., COHN, S. L., LONDON, W. B., MARSHALL, G. M. & NORRIS, M. D. 2006. Association of high-level MRP1 expression with poor clinical outcome in a large prospective study of primary neuroblastoma. *J Clin Oncol,* 24**,** 1546-53.

HABIB, E. E., EL-KASHEF, A. T. & FAHMY, E. S. 2012. Management of neuroblastoma: a study of first- and second-line chemotherapy responses, a single institution experience. *Oncol Rev,* 6**,** e3.

HAGEMANN-JENSEN, M., ABDULLAYEV, I., SANDBERG, R. & FARIDANI, O. R. 2018. Small-seq for single-cell small-RNA sequencing. *Nat Protoc,* 13**,** 2407-2424.

HANAHAN, D. & WEINBERG, R. A. 2000. The hallmarks of cancer. *Cell,* 100**,** 57-70.

HARVEY, H., PISKAREVA, O., CREEVEY, L., ALCOCK, L. C., BUCKLEY, P. G., O'SULLIVAN, M. J., SEGURA, M. F., GALLEGO, S., STALLINGS, R. L. & BRAY, I. M. 2015. Modulation of chemotherapeutic drug resistance in neuroblastoma SK-N-AS cells by the neural apoptosis inhibitory protein and miR-520f. *Int J Cancer,* 136**,** 1579-88.

HE, L., THOMSON, J. M., HEMANN, M. T., HERNANDO-MONGE, E., MU, D., GOODSON, S., POWERS, S., CORDON-CARDO, C., LOWE, S. W., HANNON, G. J. & HAMMOND, S. M. 2005. A microRNA polycistron as a potential human oncogene. *Nature,* 435**,** 828-33.

HIGGINS, C. F. 1992. ABC transporters: from microorganisms to man. *Annu Rev Cell Biol,* 8**,** 67-113.

HOLOHAN, C., VAN SCHAEYBROECK, S., LONGLEY, D. B. & JOHNSTON, P. G. 2013. Cancer drug resistance: an evolving paradigm. *Nat Rev Cancer,* 13**,** 714-26.

HSU, P. W., HUANG, H. D., HSU, S. D., LIN, L. Z., TSOU, A. P., TSENG, C. P., STADLER, P. F., WASHIETL, S. & HOFACKER, I. L. 2006. miRNAMap: genomic maps of microRNA genes and their target genes in mammalian genomes. *Nucleic Acids Res,* 34**,** D135-9.

HUA, Z., LV, Q., YE, W., WONG, C. K., CAI, G., GU, D., JI, Y., ZHAO, C., WANG, J., YANG, B. B. & ZHANG, Y. 2006. MiRNA-directed regulation of VEGF and other angiogenic factors under hypoxia. *PLoS One,* 1**,** e116.

HUANG, M. & WEISS, W. A. 2013. Neuroblastoma and MYCN. *Cold Spring Harb Perspect Med,* 3**,** a014415.

HUANG, Q., WANG, C., HOU, Z., WANG, G., LV, J., WANG, H., YANG, J., ZHANG, Z. & ZHANG, H. 2017. Serum microRNA-376 family as diagnostic and prognostic markers in human gliomas. *Cancer Biomark,* 19**,** 137-144.

IORIO, M. V. & CROCE, C. M. 2012. MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. *EMBO Mol Med,* 4**,** 143-59.

IWAKI, J., KIKUCHI, K., MIZUGUCHI, Y., KAWAHIGASHI, Y., YOSHIDA, H., UCHIDA, E. & TAKIZAWA, T. 2013. MiR-376c down-regulation accelerates EGF-dependent migration by targeting GRB2 in the HuCCT1 human intrahepatic cholangiocarcinoma cell line. *PLoS One,* 8**,** e69496.

JANOUEIX-LEROSEY, I., LEQUIN, D., BRUGIERES, L., RIBEIRO, A., DE PONTUAL, L., COMBARET, V., RAYNAL, V., PUISIEUX, A., SCHLEIERMACHER, G., PIERRON, G., VALTEAU-COUANET, D., FREBOURG, T., MICHON, J., LYONNET, S., AMIEL, J. & DELATTRE, O. 2008. Somatic and germline activating mutations of the ALK kinase receptor in neuroblastoma. *Nature,* 455**,** 967-70.

JANSSEN, H. L., REESINK, H. W., LAWITZ, E. J., ZEUZEM, S., RODRIGUEZ-TORRES, M., PATEL, K., VAN DER MEER, A. J., PATICK, A. K., CHEN, A., ZHOU, Y., PERSSON, R., KING, B. D., KAUPPINEN, S., LEVIN, A. A. & HODGES, M. R. 2013. Treatment of HCV infection by targeting microRNA. *N Engl J Med,* 368**,** 1685-94.

JIANG, W., TIAN, Y., JIANG, S., LIU, S., ZHAO, X. & TIAN, D. 2016. MicroRNA-376c suppresses non-small-cell lung cancer cell growth and invasion by targeting LRH-1-mediated Wnt signaling pathway. *Biochem Biophys Res Commun,* 473**,** 980-986.

JIN, Y., PENG, D., SHEN, Y., XU, M., LIANG, Y., XIAO, B. & LU, J. 2013. MicroRNA-376c inhibits cell proliferation and invasion in osteosarcoma by targeting to transforming growth factor-alpha. *DNA Cell Biol,* 32**,** 302-9.

JIRAWATNOTAI, S., HU, Y., MICHOWSKI, W., ELIAS, J. E., BECKS, L., BIENVENU, F., ZAGOZDZON, A., GOSWAMI, T., WANG, Y. E., CLARK, A. B., KUNKEL, T. A., VAN HARN, T., XIA, B., CORRELL, M., QUACKENBUSH, J., LIVINGSTON, D. M., GYGI, S. P. & SICINSKI, P. 2011. A function for cyclin

D1 in DNA repair uncovered by protein interactome analyses in human cancers. *Nature,* 474**,** 230-4.
KARTAL-YANDIM, M., ADAN-GOKBULUT, A. & BARAN, Y. 2016. Molecular mechanisms of drug resistance and its reversal in cancer. *Crit Rev Biotechnol,* 36**,** 716-26.
KIM, D. H., SAETROM, P., SNOVE, O., JR. & ROSSI, J. J. 2008. MicroRNA-directed transcriptional gene silencing in mammalian cells. *Proc Natl Acad Sci U S A,* 105**,** 16230-5.
KNECHT, A. K. & BRONNER-FRASER, M. 2002. Induction of the neural crest: a multigene process. *Nat Rev Genet,* 3**,** 453-61.
KOBAYASHI, S., BOGGON, T. J., DAYARAM, T., JANNE, P. A., KOCHER, O., MEYERSON, M., JOHNSON, B. E., ECK, M. J., TENEN, D. G. & HALMOS, B. 2005. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med,* 352**,** 786-92.
KOHL, N. E., KANDA, N., SCHRECK, R. R., BRUNS, G., LATT, S. A., GILBERT, F. & ALT, F. W. 1983. Transposition and amplification of oncogene-related sequences in human neuroblastomas. *Cell,* 35**,** 359-67.
KWON, H. C., ROH, M. S., OH, S. Y., KIM, S. H., KIM, M. C., KIM, J. S. & KIM, H. J. 2007. Prognostic value of expression of ERCC1, thymidylate synthase, and glutathione S-transferase P1 for 5-fluorouracil/oxaliplatin chemotherapy in advanced gastric cancer. *Ann Oncol,* 18**,** 504-9.
LAPIDOT, T., SIRARD, C., VORMOOR, J., MURDOCH, B., HOANG, T., CACERES-CORTES, J., MINDEN, M., PATERSON, B., CALIGIURI, M. A. & DICK, J. E. 1994. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature,* 367**,** 645-8.
LAWRIE, C. H., GAL, S., DUNLOP, H. M., PUSHKARAN, B., LIGGINS, A. P., PULFORD, K., BANHAM, A. H., PEZZELLA, F., BOULTWOOD, J., WAINSCOAT, J. S., HATTON, C. S. & HARRIS, A. L. 2008. Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. *Br J Haematol,* 141**,** 672-5.
LAZARO, J. B., BAILEY, P. J. & LASSAR, A. B. 2002. Cyclin D-cdk4 activity modulates the subnuclear localization and interaction of MEF2 with SRC-family coactivators during skeletal muscle differentiation. *Genes Dev,* 16**,** 1792-805.
LEE, R. C., FEINBAUM, R. L. & AMBROS, V. 1993. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell,* 75**,** 843-54.
LEE, Y., KIM, M., HAN, J., YEOM, K. H., LEE, S., BAEK, S. H. & KIM, V. N. 2004. MicroRNA genes are transcribed by RNA polymerase II. *EMBO J,* 23**,** 4051-60.
LEVY, D. E. & LEE, C. K. 2002. What does Stat3 do? *J Clin Invest,* 109**,** 1143-8.
LEWIS, B. P., BURGE, C. B. & BARTEL, D. P. 2005. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell,* 120**,** 15-20.
LI, J., XU, X., MENG, S., LIANG, Z., WANG, X., XU, M., WANG, S., LI, S., ZHU, Y., XIE, B., LIN, Y., ZHENG, X., LIU, B. & XIE, L. 2017. MET/SMAD3/SNAIL circuit mediated by miR-323a-3p is involved in regulating epithelial-mesenchymal transition progression in bladder cancer. *Cell Death Dis,* 8**,** e3010.
LI, J. Y., GAILLARD, F., MOREAU, A., HAROUSSEAU, J. L., LABOISSE, C., MILPIED, N., BATAILLE, R. & AVET-LOISEAU, H. 1999. Detection of translocation t(11;14)(q13;q32) in mantle cell lymphoma by fluorescence in situ hybridization. *Am J Pathol,* 154**,** 1449-52.
LIMA, R. T., BUSACCA, S., ALMEIDA, G. M., GAUDINO, G., FENNELL, D. A. & VASCONCELOS, M. H. 2011. MicroRNA regulation of core apoptosis pathways in cancer. *Eur J Cancer,* 47**,** 163-74.
LIN, R. J., LIN, Y. C., CHEN, J., KUO, H. H., CHEN, Y. Y., DICCIANNI, M. B., LONDON, W. B., CHANG, C. H. & YU, A. L. 2010. microRNA signature and expression of Dicer and Drosha can predict prognosis and delineate risk groups in neuroblastoma. *Cancer Res,* 70**,** 7841-50.
LIN, S. & GREGORY, R. I. 2015. MicroRNA biogenesis pathways in cancer. *Nat Rev Cancer,* 15**,** 321-33.
LING, X. & ARLINGHAUS, R. B. 2005. Knockdown of STAT3 expression by RNA interference inhibits the induction of breast tumors in immunocompetent mice. *Cancer Res,* 65**,** 2532-6.
LONGLEY, D. B. & JOHNSTON, P. G. 2005. Molecular mechanisms of drug resistance. *J Pathol,* 205**,** 275-92.

LORD, R. V., BRABENDER, J., GANDARA, D., ALBEROLA, V., CAMPS, C., DOMINE, M., CARDENAL, F., SANCHEZ, J. M., GUMERLOCK, P. H., TARON, M., SANCHEZ, J. J., DANENBERG, K. D., DANENBERG, P. V. & ROSELL, R. 2002. Low ERCC1 expression correlates with prolonged survival after cisplatin plus gemcitabine chemotherapy in non-small cell lung cancer. *Clin Cancer Res,* 8**,** 2286-91.

LU, J., GETZ, G., MISKA, E. A., ALVAREZ-SAAVEDRA, E., LAMB, J., PECK, D., SWEET-CORDERO, A., EBERT, B. L., MAK, R. H., FERRANDO, A. A., DOWNING, J. R., JACKS, T., HORVITZ, H. R. & GOLUB, T. R. 2005. MicroRNA expression profiles classify human cancers. *Nature,* 435**,** 834-8.

LUJAMBIO, A., CALIN, G. A., VILLANUEVA, A., ROPERO, S., SANCHEZ-CESPEDES, M., BLANCO, D., MONTUENGA, L. M., ROSSI, S., NICOLOSO, M. S., FALLER, W. J., GALLAGHER, W. M., ECCLES, S. A., CROCE, C. M. & ESTELLER, M. 2008. A microRNA DNA methylation signature for human cancer metastasis. *Proc Natl Acad Sci U S A,* 105**,** 13556-61.

LUJAMBIO, A. & LOWE, S. W. 2012. The microcosmos of cancer. *Nature,* 482**,** 347-55.

LYTLE, J. R., YARIO, T. A. & STEITZ, J. A. 2007. Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR. *Proc Natl Acad Sci U S A,* 104**,** 9667-72.

MANOHAR, C. F., BRAY, J. A., SALWEN, H. R., MADAFIGLIO, J., CHENG, A., FLEMMING, C., MARSHALL, G. M., NORRIS, M. D., HABER, M. & COHN, S. L. 2004. MYCN-mediated regulation of the MRP1 promoter in human neuroblastoma. *Oncogene,* 23**,** 753-62.

MARIS, J. M. 2010. Recent advances in neuroblastoma. *N Engl J Med,* 362**,** 2202-11.

MARIS, J. M., HOGARTY, M. D., BAGATELL, R. & COHN, S. L. 2007. Neuroblastoma. *Lancet,* 369**,** 2106-20.

MATSUURA, I., DENISSOVA, N. G., WANG, G., HE, D., LONG, J. & LIU, F. 2004. Cyclin-dependent kinases regulate the antiproliferative function of Smads. *Nature,* 430**,** 226-31.

MEDINA, P. P., NOLDE, M. & SLACK, F. J. 2010. OncomiR addiction in an in vivo model of microRNA-21-induced pre-B-cell lymphoma. *Nature,* 467**,** 86-90.

MELO, S. A., MOUTINHO, C., ROPERO, S., CALIN, G. A., ROSSI, S., SPIZZO, R., FERNANDEZ, A. F., DAVALOS, V., VILLANUEVA, A., MONTOYA, G., YAMAMOTO, H., SCHWARTZ, S., JR. & ESTELLER, M. 2010. A genetic defect in exportin-5 traps precursor microRNAs in the nucleus of cancer cells. *Cancer Cell,* 18**,** 303-15.

MELO, S. A., ROPERO, S., MOUTINHO, C., AALTONEN, L. A., YAMAMOTO, H., CALIN, G. A., ROSSI, S., FERNANDEZ, A. F., CARNEIRO, F., OLIVEIRA, C., FERREIRA, B., LIU, C. G., VILLANUEVA, A., CAPELLA, G., SCHWARTZ, S., JR., SHIEKHATTAR, R. & ESTELLER, M. 2009. A TARBP2 mutation in human cancer impairs microRNA processing and DICER1 function. *Nat Genet,* 41**,** 365-70.

MENG, F., HENSON, R., LANG, M., WEHBE, H., MAHESHWARI, S., MENDELL, J. T., JIANG, J., SCHMITTGEN, T. D. & PATEL, T. 2006. Involvement of human micro-RNA in growth and response to chemotherapy in human cholangiocarcinoma cell lines. *Gastroenterology,* 130**,** 2113-29.

MOLENAAR, J. J., EBUS, M. E., KOSTER, J., VAN SLUIS, P., VAN NOESEL, C. J., VERSTEEG, R. & CARON, H. N. 2008. Cyclin D1 and CDK4 activity contribute to the undifferentiated phenotype in neuroblastoma. *Cancer Res,* 68**,** 2599-609.

MOLENAAR, J. J., VAN SLUIS, P., BOON, K., VERSTEEG, R. & CARON, H. N. 2003. Rearrangements and increased expression of cyclin D1 (CCND1) in neuroblastoma. *Genes Chromosomes Cancer,* 36**,** 242-9.

MONCLAIR, T., BRODEUR, G. M., AMBROS, P. F., BRISSE, H. J., CECCHETTO, G., HOLMES, K., KANEKO, M., LONDON, W. B., MATTHAY, K. K., NUCHTERN, J. G., VON SCHWEINITZ, D., SIMON, T., COHN, S. L., PEARSON, A. D. & FORCE, I. T. 2009. The International Neuroblastoma Risk Group (INRG) staging system: an INRG Task Force report. *J Clin Oncol,* 27**,** 298-303.

MONROIG, P. D. & CALIN, G. A. 2013. MicroRNA and Epigenetics: Diagnostic and Therapeutic Opportunities. *Curr Pathobiol Rep,* 1**,** 43-52.

MORENO-BUENO, G., RODRIGUEZ-PERALES, S., SANCHEZ-ESTEVEZ, C., HARDISSON, D., SARRIO, D., PRAT, J., CIGUDOSA, J. C., MATIAS-GUIU, X. & PALACIOS, J. 2003. Cyclin D1 gene (CCND1) mutations in endometrial cancer. *Oncogene,* 22**,** 6115-8.

MORETTI, F., THERMANN, R. & HENTZE, M. W. 2010. Mechanism of translational regulation by miR-2 from sites in the 5' untranslated region or the open reading frame. *RNA,* 16**,** 2493-502.
MORRIS, K. V. & MATTICK, J. S. 2014. The rise of regulatory RNA. *Nat Rev Genet,* 15**,** 423-37.
MOSSE, Y. P., LAUDENSLAGER, M., LONGO, L., COLE, K. A., WOOD, A., ATTIYEH, E. F., LAQUAGLIA, M. J., SENNETT, R., LYNCH, J. E., PERRI, P., LAUREYS, G., SPELEMAN, F., KIM, C., HOU, C., HAKONARSON, H., TORKAMANI, A., SCHORK, N. J., BRODEUR, G. M., TONINI, G. P., RAPPAPORT, E., DEVOTO, M. & MARIS, J. M. 2008. Identification of ALK as a major familial neuroblastoma predisposition gene. *Nature,* 455**,** 930-5.
MOTOKURA, T., BLOOM, T., KIM, H. G., JUPPNER, H., RUDERMAN, J. V., KRONENBERG, H. M. & ARNOLD, A. 1991. A novel cyclin encoded by a bcl1-linked candidate oncogene. *Nature,* 350**,** 512-5.
MURALIDHAR, B., WINDER, D., MURRAY, M., PALMER, R., BARBOSA-MORAIS, N., SAINI, H., ROBERTS, I., PETT, M. & COLEMAN, N. 2011. Functional evidence that Drosha overexpression in cervical squamous cell carcinoma affects cell phenotype and microRNA profiles. *J Pathol,* 224**,** 496-507.
MUSGROVE, E. A., CALDON, C. E., BARRACLOUGH, J., STONE, A. & SUTHERLAND, R. L. 2011. Cyclin D as a therapeutic target in cancer. *Nat Rev Cancer,* 11**,** 558-72.
NAVARRO, A., DIAZ, T., MARTINEZ, A., GAYA, A., PONS, A., GEL, B., CODONY, C., FERRER, G., MARTINEZ, C., MONTSERRAT, E. & MONZO, M. 2009. Regulation of JAK2 by miR-135a: prognostic impact in classic Hodgkin lymphoma. *Blood,* 114**,** 2945-51.
NEGRONI, A., SCARPA, S., ROMEO, A., FERRARI, S., MODESTI, A. & RASCHELLA, G. 1991. Decrease of proliferation rate and induction of differentiation by a MYCN antisense DNA oligomer in a human neuroblastoma cell line. *Cell Growth Differ,* 2**,** 511-8.
O'DONNELL, K. A., WENTZEL, E. A., ZELLER, K. I., DANG, C. V. & MENDELL, J. T. 2005. c-Myc-regulated microRNAs modulate E2F1 expression. *Nature,* 435**,** 839-43.
OBERTHUER, A., BERTHOLD, F., WARNAT, P., HERO, B., KAHLERT, Y., SPITZ, R., ERNESTUS, K., KONIG, R., HAAS, S., EILS, R., SCHWAB, M., BRORS, B., WESTERMANN, F. & FISCHER, M. 2006. Customized oligonucleotide microarray gene expression-based classification of neuroblastoma patients outperforms current clinical risk stratification. *J Clin Oncol,* 24**,** 5070-8.
ODATE, S., VESCHI, V., YAN, S., LAM, N., WOESSNER, R. & THIELE, C. J. 2017. Inhibition of STAT3 with the Generation 2.5 Antisense Oligonucleotide, AZD9150, Decreases Neuroblastoma Tumorigenicity and Increases Chemosensitivity. *Clin Cancer Res,* 23**,** 1771-1784.
OTA, A., TAGAWA, H., KARNAN, S., TSUZUKI, S., KARPAS, A., KIRA, S., YOSHIDA, Y. & SETO, M. 2004. Identification and characterization of a novel gene, C13orf25, as a target for 13q31-q32 amplification in malignant lymphoma. *Cancer Res,* 64**,** 3087-95.
PARASKEVOPOULOU, M. D., GEORGAKILAS, G., KOSTOULAS, N., VLACHOS, I. S., VERGOULIS, T., RECZKO, M., FILIPPIDIS, C., DALAMAGAS, T. & HATZIGEORGIOU, A. G. 2013. DIANA-microT web server v5.0: service integration into miRNA functional analysis workflows. *Nucleic Acids Res,* 41**,** W169-73.
PARODI, F., CAROSIO, R., RAGUSA, M., DI PIETRO, C., MAUGERI, M., BARBAGALLO, D., SALLUSTIO, F., ALLEMANNI, G., PISTILLO, M. P., CASCIANO, I., FORLANI, A., SCHENA, F. P., PURRELLO, M., ROMANI, M. & BANELLI, B. 2016. Epigenetic dysregulation in neuroblastoma: A tale of miRNAs and DNA methylation. *Biochim Biophys Acta,* 1859**,** 1502-1514.
PINTO, N. R., APPLEBAUM, M. A., VOLCHENBOUM, S. L., MATTHAY, K. K., LONDON, W. B., AMBROS, P. F., NAKAGAWARA, A., BERTHOLD, F., SCHLEIERMACHER, G., PARK, J. R., VALTEAU-COUANET, D., PEARSON, A. D. & COHN, S. L. 2015. Advances in Risk Classification and Treatment Strategies for Neuroblastoma. *J Clin Oncol,* 33**,** 3008-17.
PORTELA, A. & ESTELLER, M. 2010. Epigenetic modifications and human disease. *Nat Biotechnol,* 28**,** 1057-68.
PRATT, G. 2002. Molecular aspects of multiple myeloma. *Mol Pathol,* 55**,** 273-83.

PUGH, T. J., MOROZOVA, O., ATTIYEH, E. F., ASGHARZADEH, S., WEI, J. S., AUCLAIR, D., CARTER, S. L., CIBULSKIS, K., HANNA, M., KIEZUN, A., KIM, J., LAWRENCE, M. S., LICHENSTEIN, L., MCKENNA, A., PEDAMALLU, C. S., RAMOS, A. H., SHEFLER, E., SIVACHENKO, A., SOUGNEZ, C., STEWART, C., ALLY, A., BIROL, I., CHIU, R., CORBETT, R. D., HIRST, M., JACKMAN, S. D., KAMOH, B., KHODABAKSHI, A. H., KRZYWINSKI, M., LO, A., MOORE, R. A., MUNGALL, K. L., QIAN, J., TAM, A., THIESSEN, N., ZHAO, Y., COLE, K. A., DIAMOND, M., DISKIN, S. J., MOSSE, Y. P., WOOD, A. C., JI, L., SPOSTO, R., BADGETT, T., LONDON, W. B., MOYER, Y., GASTIER-FOSTER, J. M., SMITH, M. A., GUIDRY AUVIL, J. M., GERHARD, D. S., HOGARTY, M. D., JONES, S. J., LANDER, E. S., GABRIEL, S. B., GETZ, G., SEEGER, R. C., KHAN, J., MARRA, M. A., MEYERSON, M. & MARIS, J. M. 2013. The genetic landscape of high-risk neuroblastoma. *Nat Genet,* 45**,** 279-84.

PUSZTAI, L., WAGNER, P., IBRAHIM, N., RIVERA, E., THERIAULT, R., BOOSER, D., SYMMANS, F. W., WONG, F., BLUMENSCHEIN, G., FLEMING, D. R., ROUZIER, R., BONIFACE, G. & HORTOBAGYI, G. N. 2005. Phase II study of tariquidar, a selective P-glycoprotein inhibitor, in patients with chemotherapy-resistant, advanced breast carcinoma. *Cancer,* 104**,** 682-91.

RATNER, N., BRODEUR, G. M., DALE, R. C. & SCHOR, N. F. 2016. The "neuro" of neuroblastoma: Neuroblastoma as a neurodevelopmental disorder. *Ann Neurol,* 80**,** 13-23.

RIHANI, A., VANDESOMPELE, J., SPELEMAN, F. & VAN MAERKEN, T. 2015. Inhibition of CDK4/6 as a novel therapeutic option for neuroblastoma. *Cancer Cell Int,* 15**,** 76.

ROSS, R. A., SPENGLER, B. A., DOMENECH, C., PORUBCIN, M., RETTIG, W. J. & BIEDLER, J. L. 1995. Human neuroblastoma I-type cells are malignant neural crest stem cells. *Cell Growth Differ,* 6**,** 449-56.

ROTH, S. A., HALD, O. H., FUCHS, S., LOKKE, C., MIKKOLA, I., FLAEGSTAD, T., SCHULTE, J. & EINVIK, C. 2018. MicroRNA-193b-3p represses neuroblastoma cell growth via downregulation of Cyclin D1, MCL-1 and MYCN. *Oncotarget,* 9**,** 18160-18179.

ROTH, S. A., KNUTSEN, E., FISKAA, T., UTNES, P., BHAVSAR, S., HALD, O. H., LOKKE, C., MESTDAGH, P., JOHANSEN, S. D., FLAEGSTAD, T. & EINVIK, C. 2016. Next generation sequencing of microRNAs from isogenic neuroblastoma cell lines isolated before and after treatment. *Cancer Lett,* 372**,** 128-36.

RUBY, J. G., JAN, C. H. & BARTEL, D. P. 2007. Intronic microRNA precursors that bypass Drosha processing. *Nature,* 448**,** 83-6.

RUPAIMOOLE, R. & SLACK, F. J. 2017. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. *Nat Rev Drug Discov,* 16**,** 203-222.

RYAN, J., TIVNAN, A., FAY, J., BRYAN, K., MEEHAN, M., CREEVEY, L., LYNCH, J., BRAY, I. M., O'MEARA, A., TRACEY, L., DAVIDOFF, A. M. & STALLINGS, R. L. 2012. MicroRNA-204 increases sensitivity of neuroblastoma cells to cisplatin and is associated with a favourable clinical outcome. *Br J Cancer,* 107**,** 967-76.

SAITO, Y., LIANG, G., EGGER, G., FRIEDMAN, J. M., CHUANG, J. C., COETZEE, G. A. & JONES, P. A. 2006. Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. *Cancer Cell,* 9**,** 435-43.

SANTARIUS, T., SHIPLEY, J., BREWER, D., STRATTON, M. R. & COOPER, C. S. 2010. A census of amplified and overexpressed human cancer genes. *Nat Rev Cancer,* 10**,** 59-64.

SAUTER, E. R., NESBIT, M., LITWIN, S., KLEIN-SZANTO, A. J., CHEFFETZ, S. & HERLYN, M. 1999. Antisense cyclin D1 induces apoptosis and tumor shrinkage in human squamous carcinomas. *Cancer Res,* 59**,** 4876-81.

SAUTER, E. R., TAKEMOTO, R., LITWIN, S. & HERLYN, M. 2002. p53 alone or in combination with antisense cyclin D1 induces apoptosis and reduces tumor size in human melanoma. *Cancer Gene Ther,* 9**,** 807-12.

SCHULTE, J. H., HORN, S., OTTO, T., SAMANS, B., HEUKAMP, L. C., EILERS, U. C., KRAUSE, M., ASTRAHANTSEFF, K., KLEIN-HITPASS, L., BUETTNER, R., SCHRAMM, A., CHRISTIANSEN, H., EILERS, M., EGGERT, A. & BERWANGER, B. 2008. MYCN regulates oncogenic MicroRNAs in neuroblastoma. *Int J Cancer,* 122**,** 699-704.

SCHULTE, J. H., MARSCHALL, T., MARTIN, M., ROSENSTIEL, P., MESTDAGH, P., SCHLIERF, S., THOR, T., VANDESOMPELE, J., EGGERT, A., SCHREIBER, S., RAHMANN, S. & SCHRAMM, A. 2010. Deep sequencing reveals differential expression of microRNAs in favorable versus unfavorable neuroblastoma. *Nucleic Acids Res,* 38**,** 5919-28.

SCHWAB, M., ALITALO, K., KLEMPNAUER, K. H., VARMUS, H. E., BISHOP, J. M., GILBERT, F., BRODEUR, G., GOLDSTEIN, M. & TRENT, J. 1983. Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. *Nature,* 305**,** 245-8.

SHAHAR, T., GRANIT, A., ZRIHAN, D., CANELLO, T., CHARBIT, H., EINSTEIN, O., ROZOVSKI, U., ELGAVISH, S., RAM, Z., SIEGAL, T. & LAVON, I. 2016. Expression level of miRNAs on chromosome 14q32.31 region correlates with tumor aggressiveness and survival of glioblastoma patients. *J Neurooncol,* 130**,** 413-422.

SHEN, R., WANG, X., DRISSI, H., LIU, F., O'KEEFE, R. J. & CHEN, D. 2006. Cyclin D1-cdk4 induce runx2 ubiquitination and degradation. *J Biol Chem,* 281**,** 16347-53.

SHERVINGTON, A. & LU, C. 2008. Expression of multidrug resistance genes in normal and cancer stem cells. *Cancer Invest,* 26**,** 535-42.

SHEWEITA, S. A. 2000. Drug-metabolizing enzymes: mechanisms and functions. *Curr Drug Metab,* 1**,** 107-32.

SHIOTANI, A., MURAO, T., KIMURA, Y., MATSUMOTO, H., KAMADA, T., KUSUNOKI, H., INOUE, K., UEDO, N., IISHI, H. & HARUMA, K. 2013. Identification of serum miRNAs as novel non-invasive biomarkers for detection of high risk for early gastric cancer. *Br J Cancer,* 109**,** 2323-30.

SHUKLA, G. C., SINGH, J. & BARIK, S. 2011. MicroRNAs: Processing, Maturation, Target Recognition and Regulatory Functions. *Mol Cell Pharmacol,* 3**,** 83-92.

SI, M. L., ZHU, S., WU, H., LU, Z., WU, F. & MO, Y. Y. 2007. miR-21-mediated tumor growth. *Oncogene,* 26**,** 2799-803.

SORIANO, A., PARIS-CODERCH, L., JUBIERRE, L., MARTINEZ, A., ZHOU, X., PISKAREVA, O., BRAY, I., VIDAL, I., ALMAZAN-MOGA, A., MOLIST, C., ROMA, J., BAYASCAS, J. R., CASANOVAS, O., STALLINGS, R. L., SANCHEZ DE TOLEDO, J., GALLEGO, S. & SEGURA, M. F. 2016. MicroRNA-497 impairs the growth of chemoresistant neuroblastoma cells by targeting cell cycle, survival and vascular permeability genes. *Oncotarget,* 7**,** 9271-87.

STEPANOVA, L. A. S., B. 2015. ''Side Population'' Cells in Mouse Neuroblastoma Express Abcg2 and Are Enriched for Neuroblastoma Stem Cells. *Blood,* 108**,** 4268.

STRATHDEE, G., MACKEAN, M. J., ILLAND, M. & BROWN, R. 1999. A role for methylation of the hMLH1 promoter in loss of hMLH1 expression and drug resistance in ovarian cancer. *Oncogene,* 18**,** 2335-41.

SUGIMURA, K., MIYATA, H., TANAKA, K., HAMANO, R., TAKAHASHI, T., KUROKAWA, Y., YAMASAKI, M., NAKAJIMA, K., TAKIGUCHI, S., MORI, M. & DOKI, Y. 2012. Let-7 expression is a significant determinant of response to chemotherapy through the regulation of IL-6/STAT3 pathway in esophageal squamous cell carcinoma. *Clin Cancer Res,* 18**,** 5144-53.

SUI, X., CHEN, R., WANG, Z., HUANG, Z., KONG, N., ZHANG, M., HAN, W., LOU, F., YANG, J., ZHANG, Q., WANG, X., HE, C. & PAN, H. 2013. Autophagy and chemotherapy resistance: a promising therapeutic target for cancer treatment. *Cell Death Dis,* 4**,** e838.

SUN, F., FU, H., LIU, Q., TIE, Y., ZHU, J., XING, R., SUN, Z. & ZHENG, X. 2008. Downregulation of CCND1 and CDK6 by miR-34a induces cell cycle arrest. *FEBS Lett,* 582**,** 1564-8.

SWARBRICK, A., WOODS, S. L., SHAW, A., BALAKRISHNAN, A., PHUA, Y., NGUYEN, A., CHANTHERY, Y., LIM, L., ASHTON, L. J., JUDSON, R. L., HUSKEY, N., BLELLOCH, R., HABER, M., NORRIS, M. D., LENGYEL, P., HACKETT, C. S., PREISS, T., CHETCUTI, A., SULLIVAN, C. S., MARCUSSON, E. G., WEISS, W., L'ETOILE, N. & GOGA, A. 2010. miR-380-5p represses p53 to control cellular survival and is associated with poor outcome in MYCN-amplified neuroblastoma. *Nat Med,* 16**,** 1134-40.

TAGAWA, H. & SETO, M. 2005. A microRNA cluster as a target of genomic amplification in malignant lymphoma. *Leukemia,* 19**,** 2013-6.

TAKWI, A. A., WANG, Y. M., WU, J., MICHAELIS, M., CINATL, J. & CHEN, T. 2014. miR-137 regulates the constitutive androstane receptor and modulates doxorubicin sensitivity in parental and doxorubicin-resistant neuroblastoma cells. *Oncogene,* 33**,** 3717-29.

TARASOV, V., JUNG, P., VERDOODT, B., LODYGIN, D., EPANCHINTSEV, A., MENSSEN, A., MEISTER, G. & HERMEKING, H. 2007. Differential regulation of microRNAs by p53 revealed by massively parallel sequencing: miR-34a is a p53 target that induces apoptosis and G1-arrest. *Cell Cycle,* 6**,** 1586-93.

THOMAS, H. & COLEY, H. M. 2003. Overcoming multidrug resistance in cancer: an update on the clinical strategy of inhibiting p-glycoprotein. *Cancer Control,* 10**,** 159-65.

THORSEN, S. B., OBAD, S., JENSEN, N. F., STENVANG, J. & KAUPPINEN, S. 2012. The therapeutic potential of microRNAs in cancer. *Cancer J,* 18**,** 275-84.

TIVNAN, A., TRACEY, L., BUCKLEY, P. G., ALCOCK, L. C., DAVIDOFF, A. M. & STALLINGS, R. L. 2011. MicroRNA-34a is a potent tumor suppressor molecule in vivo in neuroblastoma. *BMC Cancer,* 11**,** 33.

TRANG, P., MEDINA, P. P., WIGGINS, J. F., RUFFINO, L., KELNAR, K., OMOTOLA, M., HOMER, R., BROWN, D., BADER, A. G., WEIDHAAS, J. B. & SLACK, F. J. 2010. Regression of murine lung tumors by the let-7 microRNA. *Oncogene,* 29**,** 1580-7.

TRANG, P., WIGGINS, J. F., DAIGE, C. L., CHO, C., OMOTOLA, M., BROWN, D., WEIDHAAS, J. B., BADER, A. G. & SLACK, F. J. 2011. Systemic delivery of tumor suppressor microRNA mimics using a neutral lipid emulsion inhibits lung tumors in mice. *Mol Ther,* 19**,** 1116-22.

TRILLER, N., KOROSEC, P., KERN, I., KOSNIK, M. & DEBELJAK, A. 2006. Multidrug resistance in small cell lung cancer: expression of P-glycoprotein, multidrug resistance protein 1 and lung resistance protein in chemo-naive patients and in relapsed disease. *Lung Cancer,* 54**,** 235-40.

TROCHET, D., BOURDEAUT, F., JANOUEIX-LEROSEY, I., DEVILLE, A., DE PONTUAL, L., SCHLEIERMACHER, G., COZE, C., PHILIP, N., FREBOURG, T., MUNNICH, A., LYONNET, S., DELATTRE, O. & AMIEL, J. 2004. Germline mutations of the paired-like homeobox 2B (PHOX2B) gene in neuroblastoma. *Am J Hum Genet,* 74**,** 761-4.

TSUBOTA, S. & KADOMATSU, K. 2018. Origin and initiation mechanisms of neuroblastoma. *Cell Tissue Res,* 372**,** 211-221.

VAN ROOIJ, E. & OLSON, E. N. 2012. MicroRNA therapeutics for cardiovascular disease: opportunities and obstacles. *Nat Rev Drug Discov,* 11**,** 860-72.

VANGIPURAM, S. D., WANG, Z. J. & LYMAN, W. D. 2010. Resistance of stem-like cells from neuroblastoma cell lines to commonly used chemotherapeutic agents. *Pediatr Blood Cancer,* 54**,** 361-8.

WANG, H. Q., HALILOVIC, E., LI, X., LIANG, J., CAO, Y., RAKIEC, D. P., RUDDY, D. A., JEAY, S., WUERTHNER, J. U., TIMPLE, N., KASIBHATLA, S., LI, N., WILLIAMS, J. A., SELLERS, W. R., HUANG, A. & LI, F. 2017a. Combined ALK and MDM2 inhibition increases antitumor activity and overcomes resistance in human ALK mutant neuroblastoma cell lines and xenograft models. *Elife,* 6.

WANG, K., JIN, J., MA, T. & ZHAI, H. 2017b. MiR-376c-3p regulates the proliferation, invasion, migration, cell cycle and apoptosis of human oral squamous cancer cells by suppressing HOXB7. *Biomed Pharmacother,* 91**,** 517-525.

WANG, Z., LEI, H. & SUN, Q. 2016. MicroRNA-141 and its associated gene FUS modulate proliferation, migration and cisplatin chemosensitivity in neuroblastoma cell lines. *Oncol Rep,* 35**,** 2943-51.

WEBER, B., STRESEMANN, C., BRUECKNER, B. & LYKO, F. 2007. Methylation of human microRNA genes in normal and neoplastic cells. *Cell Cycle,* 6**,** 1001-5.

WEBER, J. A., BAXTER, D. H., ZHANG, S., HUANG, D. Y., HUANG, K. H., LEE, M. J., GALAS, D. J. & WANG, K. 2010. The microRNA spectrum in 12 body fluids. *Clin Chem,* 56**,** 1733-41.

WEI, J. S., SONG, Y. K., DURINCK, S., CHEN, Q. R., CHEUK, A. T., TSANG, P., ZHANG, Q., THIELE, C. J., SLACK, A., SHOHET, J. & KHAN, J. 2008. The MYCN oncogene is a direct target of miR-34a. *Oncogene,* 27**,** 5204-13.

WELCH, C., CHEN, Y. & STALLINGS, R. L. 2007. MicroRNA-34a functions as a potential tumor suppressor by inducing apoptosis in neuroblastoma cells. *Oncogene,* 26**,** 5017-22.

WENZEL, A., CZIEPLUCH, C., HAMANN, U., SCHURMANN, J. & SCHWAB, M. 1991. The N-Myc oncoprotein is associated in vivo with the phosphoprotein Max(p20/22) in human neuroblastoma cells. *EMBO J,* 10**,** 3703-12.

WENZEL, A. & SCHWAB, M. 1995. The mycN/max protein complex in neuroblastoma. Short review. *Eur J Cancer,* 31A**,** 516-9.

WIESTNER, A., TEHRANI, M., CHIORAZZI, M., WRIGHT, G., GIBELLINI, F., NAKAYAMA, K., LIU, H., ROSENWALD, A., MULLER-HERMELINK, H. K., OTT, G., CHAN, W. C., GREINER, T. C., WEISENBURGER, D. D., VOSE, J., ARMITAGE, J. O., GASCOYNE, R. D., CONNORS, J. M., CAMPO, E., MONTSERRAT, E., BOSCH, F., SMELAND, E. B., KVALOY, S., HOLTE, H., DELABIE, J., FISHER, R. I., GROGAN, T. M., MILLER, T. P., WILSON, W. H., JAFFE, E. S. & STAUDT, L. M. 2007. Point mutations and genomic deletions in CCND1 create stable truncated cyclin D1 mRNAs that are associated with increased proliferation rate and shorter survival. *Blood,* 109**,** 4599-606.

WILLIAMS, M., CHENG, Y. Y., BLENKIRON, C. & REID, G. 2017. Exploring Mechanisms of MicroRNA Downregulation in Cancer. *Microrna,* 6**,** 2-16.

WINTER, J., JUNG, S., KELLER, S., GREGORY, R. I. & DIEDERICHS, S. 2009. Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat Cell Biol,* 11**,** 228-34.

WONG, N. & WANG, X. 2015. miRDB: an online resource for microRNA target prediction and functional annotations. *Nucleic Acids Res,* 43**,** D146-52.

XIN, C., BUHE, B., HONGTING, L., CHUANMIN, Y., XIWEI, H., HONG, Z., LULU, H., QIAN, D. & RENJIE, W. 2013. MicroRNA-15a promotes neuroblastoma migration by targeting reversion-inducing cysteine-rich protein with Kazal motifs (RECK) and regulating matrix metalloproteinase-9 expression. *FEBS J,* 280**,** 855-66.

XIONG, Y., CONNOLLY, T., FUTCHER, B. & BEACH, D. 1991. Human D-type cyclin. *Cell,* 65**,** 691-9.

XUE, H., HUA, L. M., GUO, M. & LUO, J. M. 2014. SHIP1 is targeted by miR-155 in acute myeloid leukemia. *Oncol Rep,* 32**,** 2253-9.

YANG, J. H., SHAO, P., ZHOU, H., CHEN, Y. Q. & QU, L. H. 2010. deepBase: a database for deeply annotating and mining deep sequencing data. *Nucleic Acids Res,* 38**,** D123-30.

YANG, X., LIANG, L., ZHANG, X. F., JIA, H. L., QIN, Y., ZHU, X. C., GAO, X. M., QIAO, P., ZHENG, Y., SHENG, Y. Y., WEI, J. W., ZHOU, H. J., REN, N., YE, Q. H., DONG, Q. Z. & QIN, L. X. 2013. MicroRNA-26a suppresses tumor growth and metastasis of human hepatocellular carcinoma by targeting interleukin-6-Stat3 pathway. *Hepatology,* 58**,** 158-70.

YANG, Z. & KLIONSKY, D. J. 2010. Eaten alive: a history of macroautophagy. *Nat Cell Biol,* 12**,** 814-22.

YE, G., FU, G., CUI, S., ZHAO, S., BERNAUDO, S., BAI, Y., DING, Y., ZHANG, Y., YANG, B. B. & PENG, C. 2011. MicroRNA 376c enhances ovarian cancer cell survival by targeting activin receptor-like kinase 7: implications for chemoresistance. *J Cell Sci,* 124**,** 359-68.

YI, R., QIN, Y., MACARA, I. G. & CULLEN, B. R. 2003. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev,* 17**,** 3011-6.

YU, H., LEE, H., HERRMANN, A., BUETTNER, R. & JOVE, R. 2014. Revisiting STAT3 signalling in cancer: new and unexpected biological functions. *Nat Rev Cancer,* 14**,** 736-46.

YU, H., PARDOLL, D. & JOVE, R. 2009. STATs in cancer inflammation and immunity: a leading role for STAT3. *Nat Rev Cancer,* 9**,** 798-809.

YU, Z., PESTELL, T. G., LISANTI, M. P. & PESTELL, R. G. 2012. Cancer stem cells. *Int J Biochem Cell Biol,* 44**,** 2144-51.

ZALCBERG, J., HU, X. F., SLATER, A., PARISOT, J., EL-OSTA, S., KANTHARIDIS, P., CHOU, S. T. & PARKIN, J. D. 2000. MRP1 not MDR1 gene expression is the predominant mechanism of acquired

multidrug resistance in two prostate carcinoma cell lines. *Prostate Cancer Prostatic Dis,* 3**,** 66-75.

ZEHAVI, L., AVRAHAM, R., BARZILAI, A., BAR-ILAN, D., NAVON, R., SIDI, Y., AVNI, D. & LEIBOWITZ-AMIT, R. 2012. Silencing of a large microRNA cluster on human chromosome 14q32 in melanoma: biological effects of mir-376a and mir-376c on insulin growth factor 1 receptor. *Mol Cancer,* 11**,** 44.

ZHANG, H. F., CHEN, Y., WU, C., WU, Z. Y., TWEARDY, D. J., ALSHAREEF, A., LIAO, L. D., XUE, Y. J., WU, J. Y., CHEN, B., XU, X. E., GOPAL, K., GUPTA, N., LI, E. M., XU, L. Y. & LAI, R. 2016. The Opposing Function of STAT3 as an Oncoprotein and Tumor Suppressor Is Dictated by the Expression Status of STAT3beta in Esophageal Squamous Cell Carcinoma. *Clin Cancer Res,* 22**,** 691-703.

ZHAO, G., WANG, G., BAI, H., LI, T., GONG, F., YANG, H., WEN, J. & WANG, W. 2017. Targeted inhibition of HDAC8 increases the doxorubicin sensitivity of neuroblastoma cells via up regulation of miR-137. *Eur J Pharmacol,* 802**,** 20-26.

ZHONG, Z., WEN, Z. & DARNELL, J. E., JR. 1994. Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science,* 264**,** 95-8.

ZHUANG, G., WU, X., JIANG, Z., KASMAN, I., YAO, J., GUAN, Y., OEH, J., MODRUSAN, Z., BAIS, C., SAMPATH, D. & FERRARA, N. 2012. Tumour-secreted miR-9 promotes endothelial cell migration and angiogenesis by activating the JAK-STAT pathway. *EMBO J,* 31**,** 3513-23.

Appendix (Paper I, II & III)

PAPER-I

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Original Articles

Next generation sequencing of microRNAs from isogenic neuroblastoma cell lines isolated before and after treatment

Sarah Andrea Roth [a], Erik Knutsen [b], Tonje Fiskaa [b], Peter Utnes [c], Swapnil Bhavsar [a], Øyvind H. Hald [c], Cecilie Løkke [a], Pieter Mestdagh [d], Steinar D. Johansen [b,e], Trond Flægstad [a,c], Christer Einvik [a,c,*]

[a] Pediatric Research Group, Department of Clinical Medicine, Faculty of Health Science, The Arctic University of Norway – UiT, NO-9037 Tromsø, Norway
[b] RNA and Molecular Pathology (RAMP), Department of Medical Biology, Faculty of Health Sciences, The Arctic University of Norway – UiT, NO-9037 Tromsø, Norway
[c] Department of Pediatrics, Division of Child and Adolescent Health, UNN – University Hospital of North-Norway, NO-9038 Tromsø, Norway
[d] Center for Medical Genetics Ghent, Ghent University, Ghent, Belgium
[e] Marine Genomics Group, Faculty of Biosciences and Aquaculture, University of Nordland, Bodø, Norway

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ABSTRACT

Neuroblastoma is a pediatric cancer of the developing sympathetic nervous system. High risk neuroblastoma patients typically undergo an initial remission in response to treatment, followed by recurrence of aggressive tumors that have become refractory to further treatment. Recent works have underlined the involvement of microRNAs (miRNAs) in neuroblastoma development and evolution of drug resistance.

In this study we have used deep sequencing technology to identify miRNAs differentially expressed in neuroblastoma cell lines isolated from 6 patients at diagnosis and at relapse after intensive treatments. This approach revealed a panel of 42 differentially expressed miRNAs, 8 of which were upregulated and 34 were downregulated. Most strikingly, the 14q32 miRNA clusters encode 22 of the downregulated miRNAs. Reduced expression of 14q32 miRNAs in tumors associated with poor prognosis factors was confirmed in a cohort consisting of 226 primary neuroblastomas. In order to gain insight into the nature of the genes that may be affected by the differentially expressed miRNAs we utilized Ingenuity Pathway Analysis (IPA). This analysis revealed several biological functions and canonical pathways associated with cancer progression and drug resistance.

The results of this study contribute to the identification of miRNAs involved in the complex processes of surviving therapeutic treatment and developing drug resistance in neuroblastoma.

Introduction

Neuroblastoma is a neoplasm of the sympathetic nervous system representing the most frequently diagnosed solid tumor in infants [1]. As neuroblastomas originate from neuroblasts of the sympathoadrenal lineage of the neural crest, the tumor may be located anywhere along the sympathetic ganglia and the adrenal medulla [2]. One hallmark of neuroblastoma is its extremely heterogeneous behavior ranging from spontaneous regression or differentiation into benign histological variants to aggressive metastatic behavior with poor prognosis in children older than 18 months at the time of diagnosis [3,4]. Despite continued improvements in cancer treatment, the overall survival of patients with high risk neuroblastoma is still only 40–50% [5].

Low risk neuroblastomas, characterized by potential spontaneous regression, often require no treatment or may be treated by surgical resection. Nevertheless, even in low risk neuroblastoma, chemotherapy cannot be avoided in all cases [6]. Irrespective of risk factors, neuroblastomas generally respond well to initial therapy. However, the majority of high risk patients relapse with tumors refractory to standard chemotherapeutic agents [7]. Treatment failures have been associated with acquired multidrug resistance in response to initial therapy but also with selection and expansion of intrinsic resistant tumor clones [7].

Beside genetic (mutation, amplification) and epigenetic changes (DNA hypermethylation, histone-modification), recent evidence has revealed a substantial role of microRNAs (miRNAs) in multidrug resistance in various cancers [8]. MiRNAs are small (~22 nucleotides) non-coding RNA molecules that regulate the expression of genes at the post-transcriptional level by either direct cleavage of target mRNAs or repression of translation [8,9]. It has been estimated that 20–30% of all human encoding genes are regulated by miRNAs. Thus, miRNAs have been implicated to be involved in the regulation of a

* Corresponding author. Tel.: +47 77 66 97 26; fax: +47 77 64 53 50.
E-mail address: christer.einvik@uit.no (C. Einvik).

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wide variety of cellular processes such as proliferation, differentiation, apoptosis, and survival [9].

Whole-genome miRNA profiling revealed aberrant expression of miRNAs in most human cancers indicating that miRNAs have critical functions in carcinogenesis and tumor progression through acting as either oncogenes or tumor suppressors [10,11]. Furthermore, there are increasing number of publications providing new candidate miRNAs whose deviant expressions have been shown to influence cellular responses to anticancer agents through modulation of survival pathways and apoptotic responses. Moreover, aberrant expression of miRNAs targeting drug transporters and drug metabolizing enzymes may also modulate drug efficiency [8].

In recent years, miRNA-based anticancer therapy has emerged as a promising approach. The aim of miRNA-based therapy is to restore the activity of tumor-suppressive miRNAs or to inhibit oncogenic miRNAs [12].

Several studies indicate that deviant expression of certain miRNAs correlates with poor clinical outcome in neuroblastoma [13]. However, the role of miRNAs in neuroblastoma cell resistance to chemotherapeutic drugs is limited and poorly understood.

In this study, we used next generation sequencing technology (SOLiD ligation sequencing) to determine miRNA expression profiles in neuroblastoma cell lines established from patients at diagnosis and at relapse after treatment.

Materials and methods

Neuroblastoma cell lines

We used a unique panel of twelve neuroblastoma cell lines (Table 1) isolated from six neuroblastoma patients before (SK-N-BE(1), SMS-KAN, SMS-KCN, CHLA-15, CHLA-122, NBL-W) and after (SK-N-BE(2), SMS-KANR, SMS-KCNR, CHLA-20, CHLA-136, NBL-WR) treatment with combination chemotherapy regimens.

CHLA cells were grown in Iscove's modified Dulbecco's medium supplemented with 20% fetal bovine serum, 4 mM L-Glutamine and 1× ITS (5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenous acid). The other cell lines were grown in RPMI-1640 supplemented with 10% fetal bovine serum and 2 mM L-Glutamine (final concentrations).

All cell lines were cultured at 37 °C in a humidified incubator containing a 95% air/5% CO_2 atmosphere without antibiotics. The identity of all cell lines was authenticated by short tandem repeat analysis at the Center of Forensic Genetics, The Arctic University of Norway – UiT, Norway. Cells were tested and confirmed negative for mycoplasma contamination.

Total RNA isolation and small RNA enrichment

Total RNA was isolated from 8×10^6 cells using TRIzol (Invitrogen, Life Technologies, Carlsbad, USA), with prolonged precipitation and centrifugation steps in order to preserve the small RNA fraction. Total RNA quantification and integrity assessment were performed using Quant-iT assay (Life Technologies) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. All RNA used in this study had RNA integrity number (RIN value) above 9.5. The small RNA fraction was enriched from total RNA samples by PureLink™ miRNA Isolation Kit (Life Technologies) according to the manufacturer instructions.

Table 1
Overview of neuroblastoma cell lines used in this study.

Cell line isolated at diagnosis	Cell line isolated at relapse following therapy	Therapy given to the patients
SMS-KAN	SMS-KANR	DOX, CTX, RAD
CHLA-15	CHLA-20	DOX, CTX, CDDP, VM-26
SK-N-BE (1)	SK-N-BE (2)	DOX, CTX, VCR, RAD
CHLA-122	CHLA-136	DOX, ETOP, autoSCT with TBI
NBL-W	NBL-WR	DOX, CTX, DAU, VCR, CDDP, DTIC, ETOP
SMS-KCN	SMS-KCNR	DOX, CTX

Abbreviations: DOX: Doxorubicin; CTX: Cyclophosphamide; VCR: Vincristine; CDDP: Cisplatin; ETOP: Etoposide; DAU: Daunorubicin; DTIC: Dacarbazine; VM-26: Teniposide; RAD: local radiation; TBI: total body irradiation; autoSCT: autologous stem cell transplantation.

Reverse transcription of miRNAs

Purity and concentration of TRIzol-isolated RNA used for real-time quantitative PCR was estimated photometrically by measuring the absorbance at 260 nm (A260) using a NanoDrop™ 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). cDNA was synthesized from isolated total RNA using the miScript II RT Kit (Qiagen, Crawley, UK). In short, 1 μg of isolated total RNA was brought to a total volume of 12 μl with deionized water. 2 μl of miScript Reverse Transcriptase Mix and 2 μl of miScript Nucleics Mix diluted in 4 μl of 5× miScript HiSpec buffer were added to each sample. The reverse transcription was performed at 37 °C for 60 minutes followed by 95 °C for 5 minutes to terminate the reaction. The resulting cDNA was diluted with deionized water to achieve a concentration of 1 ng/μl. The cDNA was stored at –20 °C.

Real-time quantitative PCR

miScript SYBR® Green PCR Kit (Qiagen) was used for quantitative real-time PCR. The PCR was performed in a 10 μl reaction volume according to the following protocol: Each 10 μl reaction was composed of 1 μl of cDNA equivalent to 1 ng of cDNA, 2 μl of water, 5 μl of QuantiTect SYBR Green, 1 μl of 10× Universal primers and 1 μl of 10× specific miScript primers. The following miScript Primer Assays (Qiagen) were used: Hs_miR-337-3p_1 (MS00007651), Hs_miR-381_1 (MS00004116), Hs_miR-375_2 (MS00031829), Hs_miR-21_2 (MS00009079).

Hs_RNU6-2_11 (MS00033740), Hs_SNORD38B_11 (MS00007490), Hs_miR-149_1 (MS00003570) and Hs_miR-4286_1 (MS00021371) were used as internal controls for normalization.

Amplifications were carried out according to manufacturer's recommendations using the ABI 7300 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). To confirm amplification specificity, a melt curve was generated after the completion of the amplification reaction. Expression levels of mature miRNAs were evaluated using the ΔΔCT comparative cycle threshold method. To determine differential expression of miRNAs between matched cell line pairs, miRNA expression levels of cell lines isolated after therapy were calculated relative to miRNA expression levels of cell lines isolated before therapy whose mean miRNA concentration was set to 1.

SOLiD4 next generation sequencing

Small RNA sequencing was performed according to Ref. 14 using the SOLiD4 platform. In short, 100 ng of small RNA enriched samples was subjected to adaptor ligation and subsequently cDNA synthesis. Size selected cDNAs were then barcoded, purified, and analyzed for size and concentration, before pooled into a single library that was used in emulsion PCR. Approximately 700 million enriched beads were deposited on a full glass slide for SOLiD4 sequencing.

Data analyses

The obtained raw color-space data from SOLiD4 sequencing were analyzed in CLC Genomic Workbench (CLCbio, Aarhus, Denmark). Adaptors were trimmed, sequences were grouped, counted and selected according to length. Unique small RNA sequences were mapped to the human genome (Ensembl GRCh37.74) and annotated using miRBase V21. Mapping and annotation were done in color space and only a single mismatch was allowed. Total linear count scaling was used for normalization of the unique small RNA sequences, where the read numbers are given as reads per million (RPM).

miRNA expression data from 226 primary neuroblastoma tumors were obtained through the Neuroblastoma Research Consortium (NRC). Differential miRNA expression was analyzed using a Mann–Whitney test; *p*-values were corrected for multiple testing using the Benjamini–Hochberg procedure.

Ingenuity Pathway Analysis

MiRNAs with a range of at least 100 in four of the five cell line pairs and a fold change of at least two or minus two in three of the five cell line pairs (adjusted *p*-value < 0.01) were analyzed with Ingenuity Pathway Analyses (IPA) (Ingenuity Systems, Inc., Redwood City, CA, USA). Using IPA's microRNA target filter, a list of experimentally validated mRNA targets for the most significant upregulated and downregulated miRNAs was generated. This mRNA target list was then uploaded into the ingenuity knowledge base to determine affected molecular and cellular functions and the enrichment of canonical pathways.

The *p*-value associated with a function or a pathway is a measure of the likelihood that the association between a set of focus genes (IPA Global Molecular Network eligible molecules) in an experiment and a given process or pathway is due to random chance. The *p*-values were calculated using the right-tailed Fisher's exact test.

Results

Deep sequencing of miRNAs from neuroblastoma cell lines

To investigate the role of miRNAs in drug resistance in neuroblastoma, we used twelve neuroblastoma cell lines, consisting of six isogenic matched pairs isolated from neuroblastoma patients at the time of initial diagnosis and at the time of relapse following treatment (Table 1).

Small RNA libraries were generated from all twelve cell lines and subsequently sequenced by SOLiD (Sequencing by Oligonucleotide Ligation and Detection) on the SOLiD4 platform.

An average of 27.3 million reads were generated from the twelve libraries, ranging from 11.5 to 45.0 million reads (Supplementary Table S1). Raw reads were trimmed for adaptor sequence and unique sequences reads (tags) with less than 10 read counts were discarded as noise. In addition, reads with read length above 29 nucleotides were removed in order to prevent inclusion of untrimmed reads. This gave an average of 9.7 million reads per sample, ranging from 5.0 to 14.0 million reads (Supplementary Table S1). Processed sequencing reads were mapped to the human genome as a quality control for the library preparation. Out of 116 million reads, 81 million were mapped to either genomic or intergenomic regions of the human genome (69.4%), and 51% of the uniquely mapped reads corresponded to miRNAs (Fig. 1).

To determine the relationship between all small RNA profiles across all cell lines, we performed a multivariate correlation (Pearson's) analysis on the processed sequence reads and represented the data with a correlation matrix heat map (Supplementary Table S2). Mapping data in this fashion clearly revealed that the small RNA expression profile from SMS-KCN correlated poorly to all other cell lines (average r = 0.238/SD = 0.052, average between all other cell lines r = 0.774/SD = 0.123), including the isogenic paired SMS-KCNR cell line (r = 0.176, average between other isogenic pairs: r = 0.843/SD = 0.152). The reason for this difference is most likely due to selection of a substrate-adherent variant of SMS-KCN during cultivation in our laboratory. This variant, similar to previously reported SMS-KCNs cells [15], showed complete loss of N-myc protein expression (Supplementary Fig. S1, lane 2). The loss of N-myc expression, in this otherwise "*MYCN*-addicted" cell line, could explain the extensive difference in small RNA expression. Therefore, the SMS-KCN/SMS-KCNR pair was excluded from further analysis.

Differentially expressed miRNAs between neuroblastoma cell lines deriving from patients before and after therapy

In order to identify differentially expressed miRNAs, unique small RNA sequencing tags were compared between all cell lines. 157.899 unique tags were identified by at least one cell line. To avoid the problem of high fold change values generated from low expressed tags, only tags with a difference in expression of at least 100 RPM (reads per million) between the highest and lowest expressed cell lines were included. This reduced the number of unique small RNA sequence tags to 2.449. Out of these, 908 were annotated as miRNA sequences. However only about 1/3 of sequence tags (37.1%) were annotated as miRNAs; this corresponded to close to 3/4 of sequence reads (74.2%).

Both upregulation and downregulation of miRNAs were evident when the expression level in cell lines isolated at diagnosis was compared to the corresponding matched cell line isolated after treatment. To restrict our analysis to the most robustly expressed miRNAs, we considered only miRNAs with a range of at least 100 in four of the five matched cell line pairs, and a fold change of at least ± two in three of the five cell line pairs (adjusted p-value < 0.01). Using these strict criteria, we identified 34 downregulated and 8 upregulated miRNAs (Table 2). Interestingly, 22 of the 34 downregulated miRNAs are located on chromosome 14q32. However, only four miRNAs were found to be downregulated (*miR-136-3p*, *miR-337-3p*, *miR-34a-5p* and *miR-381-3p*) in all five neuroblastoma cell lines isolated after therapy, three of which are located on chromosome 14q32 (*miR-136-3p*, *miR-337-3p* and *miR-381-3p*).

RT-qPCR validation analysis

To independently validate the miRNA deep sequencing data, we used RT-qPCR to examine the expression of selected miRNAs (*miR-337-3p*, *miR-381-3p*, *miR-375* and *miR-21-5p*) that were both

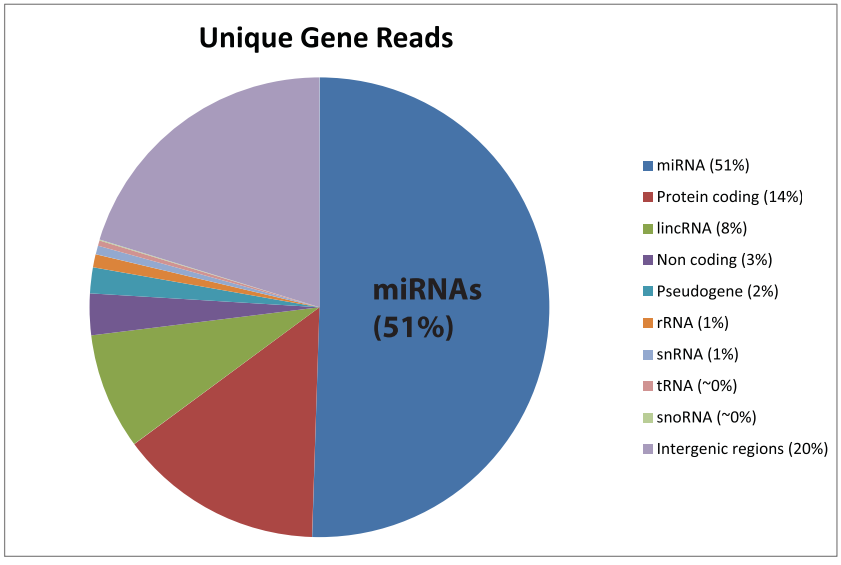

Fig. 1. Pie chart with average percentage distribution of the uniquely mapped reads. The percentage was calculated by dividing uniquely mapped read from each subcategory by the total uniquely mapped reads from all sequence cell lines.

Table 2
MiRNAs differentially expressed in neuroblastoma cell lines isolated before and after treatment.

miRNA	Change	Location	Fold change (range)				
			SMS-KAN vs. SMS-KANR	CHLA-15 vs. CHLA-20	SK-N-BE1 vs. SK-N-BE2	CHLA-122 vs. CHLA-136	NBL-W vs. NBL-WR
mir-181a-1-5p	↑	1q32.1	3.0 (2,410.2)	3.1 (2,848.6)	–1.4 (754.1)	4.4 (1,173.6)	1.4 (1,287.3)
mir-181a-2-5p		9q33.3					
mir-181b-1-5p	↑	1q32.1	1.7 (177.8)	4.5 (1,321.0)	7.1 (602.6)	13.9 (430.5)	–3.3 (815.5)
mir-181b-2-5p		9q33.3					
mir-193b-3p	↑	16p13.12	3.8 (577.9)	1.1 (19.0)	4.7 (103.1)	2.9 (247.3)	2.7 (347.6)
mir-21-5p	↑	17q23.1	11.7 (3,129.3)	2.0 (1,676.9)	–2.8 (3,098.1)	15.8 (2,646.3)	4.2 (208.5)
mir-30a-5p	↑	6q13	14.0 (1,347.0)	3.9 (217.6)	–2.3 (50.9)	–8.3 (332.4)	4.6 (128.1)
miR-375	↑	2q35	3.0 (181.7)	9.3 (252.8)	1.3 (5.4)	–1.4 (107.2)	95.7 (204.8)
mir-424-5p	↑	Xq26.3	–4.1 (2,135.4)	2.1 (3,544.5)	33.7 (3,763.3)	2.7 (2,751.8)	–1.8 (2,266.1)
mir-99b-5p	↑	19q13.41	2.7 (31,388.9)	1.3 (4,789.8)	14.6 (11,368.3)	48.8 (28,895.5)	1.1 (2,915.4)
mir-100-5p	↓	11q24.1	–21.3 (1,759.8)	–3.1 (3,653.3)	4.4 (167.8)	3.3 (49.5)	–4.9 (391.6)
mir-10b-5p	↓	2q31.1	–11.8 (6,303.5)	–1.3 (1,696.8)	–4.4 (3,597.3)	–10.0 (1,952.7)	1.3 (1,801.3)
mir-136-5p	↓	14q32.2	–5.7 (154.8)	–7.3 (284.8)	–70.9 (3,319.2)	2.0 (731.6)	–220.4 (538.7)
mir-136-3p	↓	14q32.2	–5.0 (131.3)	–7.5 (111.6)	–3.9 (469.9)	–2.4 (521.6)	–4.8 (67.0)
mir-138-1-5p mir-138-2-5p	↓	3p21.32 16q13	–20.8 (2,671.1)	1.0 (52.5)	–5.5 (249.3)	–13.1 (2127)	1.7 (484.7)
mir-154-3p	↓	14q32.31	–10.5 (293.3)	–6.4 (236.7)	3.8 (324.0)	–4.9 (370.6)	–6.0 (28.1)
mir-16-2-3p	↓	3q25.33	6.2 (1,290.3)	3.8 (289.1)	–19.8 (547.3)	–3.5 (781.9)	–65.6 (411.7)
mir-181a-1-3p	↓	1q32.1	–38.3 (724.2)	1.1 (91.4)	1.6 (583.6)	–71.5 (417.2)	–5.0 (2,049.6)
mir-199a-1-3p mir-199a-2-3p	↓	19p13.2	–68.5 (6,139.3)	–25.4 (7,230.1)	8.0 (3,106.5)	3.3 (194.4)	–2.3 (5,723.0)
mir-199b-3p		1q24.3 9q34.11					
mir-29b-1-3p mir-29b-2-3p	↓	7q32.3 1q32.2	–2.0 (806.7)	1.7 (1,154.1)	1.2 (324.9)	–2.5 (756.9)	–3.1 (1,903.6)
mir-30e-5p	↓	1p34.2	1.4 (480.3)	1.2 (230.0)	–4.0 (769.2)	–2.2 (1,519.2)	–2.8 (694.4)
mir-323a-3p	↓	14q32.31	–34.3 (627.4)	–5.9 (440.1)	–1.3 (89.2)	1.3 (339.1)	–3.0 (372.9)
mir-323b-3p	↓	14q32.31	–49.5 (713.1)	–7.2 (443.6)	–1.2 (54.5)	–2.0 (753.1)	–3.9 (88.4)
mir-329-3p	↓	14q32.31	–12.7 (644.5)	–5.6 (412.0)	1.3 (157.5)	–1.5 (571.9)	–6.2 (317.4)
mir-337-3p	↓	14q32.2	–10.6 (795.1)	–5.9 (783.6)	–1.4 (436.0)	–2.5 (1505.1)	–15.1 (423.6)
mir-33a-5p	↓	22q13.2	2.1 (199.6)	1.0 (2.2)	–3.7 (216.4)	–3.1 (388.7)	–6.0 (117.3)
mir-34a-5p	↓	1p36.22	–44.0 (1245.8)	–5.3 (491.5)	–5.3 (40.1)	–4.2 (728.5)	–4.5 (1684.0)
mir-369-3p	↓	14q32.31	–7.8 (101.0)	–7.7 (192.3)	–1.7 (70.5)	–6.2 (182.5)	–4.0 (14.3)
mir-376a-3p	↓	14q32.31	–19.9 (12,039.2)	–6.9 (8,317.8)	1.3 (1,776.5)	53.6 (4,975.9)	–4.0 (1,617.0)
mir-376b-3p	↓	14q32.31	–10.3 (866.4)	–5.4 (416.2)	–1.2 (176.3)	211.8 (783.8)	–11.7 (221.3)
mir-376c-3p	↓	14q32.31	–13.9 (19,313.9)	–4.9 (9,416.1)	2.8 (7,216.9)	57.3 (11,853.6)	–2.4 (1,195.9)
mir-379-5p	↓	14q32.31	–16.0 (385.6)	–9.6 (389.7)	–3.3 (975.8)	–1.4 (179.5)	9.0 (33.3)
mir-380-3p	↓	14q32.31	–8.1 (202.0)	–6.0 (165.0)	3.0 (145.8)	–2.3 (177.4)	–5.5 (43.6)
mir-381-3p	↓	14q32.31	–2.8 (146.0)	–8.0 (149.1)	–5.4 (781.9)	–6.3 (729.5)	–3.2 (16.4)
mir-409-3p	↓	14q32.31	–29.3 (3,316.0)	–9.6 (2,853.3)	16.8 (2,853.6)	–1.6 (323.8)	–32.8 (195.5)
mir-410-3p	↓	14q32.31	–9.1 (173.0)	–6.4 (109.7)	6.2 (142.1)	–8.5 (232.3)	–2.9 (3.4)
mir-4454-5p	↓	4q32.2	1.0 (39.2)	–2.6 (576.8)	–2.4 (2,019.0)	–2.1 (1,938.4)	–3.8 (2,440.5)
mir-487a-3p	↓	14q32.31	–19.5 (424.9)	–5.7 (335.9)	6.9 (489.3)	–3.3 (283.8)	–3.7 (25.2)
mir-487b-3p	↓	14q32.31	–13.7 (2,097.6)	–7.1 (1,957.0)	2.1 (1,472.5)	–1.7 (1,377.1)	–3.4 (478.7)
mir-494-3p	↓	14q32.31	–9.8 (903.2)	–5.9 (715.8)	4.4 (655.8)	–2.3 (310.8)	–5.4 (134.0)
mir-495-3p	↓	14q32.31	–12.4 (5,963.3)	–3.6 (3,744.1)	16.5 (5,721.3)	–3.5 (1,566.7)	–6.5 (284.2)
mir-543-3p	↓	14q32.31	–13.3 (819.5)	–4.3 (578.7)	11.1 (589.8)	–2.7 (173.1)	–5.3 (29.1)
mir-654-3p	↓	14q32.31	–6.0 (1,695.6)	–469.9 (2,033.0)	6.6 (1,727.3)	–1.6 (567.1)	–39.0 (607.0)
mir-7-2-3p	↓	15q26.1	–1.6 (119.7)	–35.8 (393.6)	–7.5 (129.1)	–2.9 (219.5)	7.9 (48.8)

Considered are miRNAs with a range of at least 100 in four of the five cell line pairs and a fold change of at least minus two or two in three of the five cell line pairs (adjusted p-value < 0.01).

up- and downregulated according to the SOLiD sequencing data. A total of four small RNAs (*RNU6* and *SNORD38B*), including two miRNAs (*miR-149* and *miR-4286*) that showed constant expression between cell lines, were used as internal controls for normalization.

As illustrated in Table 3, the normalized expression values obtained from RT-qPCR data are comparable to the sequencing data, verifying the reliability of the SOLiD sequencing based expression analysis.

Table 3
Validation of the SOLiD sequencing data by qRT-PCR.

miRNA	SMS-KAN vs. SMS-KANR		CHLA-15 vs. CHLA-20		SK-N-BE1 vs. SK-N-BE2		NBL-W vs. NBL-WR		CHLA-122 vs. CHLA-136	
	SOLiD	RT-PCR	SOLiD	RT-PCR	SOLiD	RT-PCR	SOLiD	RT-PCR	SOLiD	RT-PCR
hsa-miR-381-3p	0.36	0.5547	0.12	0.3008	0.19	0.4285	0.31	0.3241	0.16	1.3104
hsa-miR-375	3.0	3.8966	9.3	2.6631	1.3	1.4702	95.7	60.1543	0.7	0.8936
hsa-miR-21-5p	11.7	3.2547	2.0	3.2340	0.3618	0.4620	4.2	1.7716	15.8	2.0768
hsa-miR-337-3p	0.0940	0.6791	0.1683	0.5139	0.6947	1.1014	0.0663	0.1987	0.4038	1.2062

Expression levels of mature miRNAs were evaluated using the ΔΔCT comparative cycle threshold method. MiRNA expression levels of cell lines isolated after treatment were calculated relative to miRNA expression levels of cell lines isolated before therapy whose mean miRNA concentration was set to 1. *RNU6*, *SNORD38B*, *miR-149* and *miR-4286* were used as housekeeping genes. Results are given as mean from three independent reverse transcription reactions and triplicate qPCR reactions.

Reduced expression of 14q32 miRNAs correlates with poor prognosis factors in neuroblastoma tumors

To investigate whether the expression of 14q32 miRNAs is associated with prognostic markers of poor outcome in neuroblastoma patients, the expression levels of 14q32 miRNAs were evaluated in a cohort of 226 primary neuroblastoma tumors with known *MYCN* status and International Neuroblastoma Staging System (INSS). As can be seen from Fig. 2, the mean expression levels of 14 of the 22 mature 14q32 miRNAs from Table 2 were lower in *MYCN*-amplified (MNA) tumors compared to *MYCN* single-copy (MNSC) tumors. Similarly, these miRNAs were also lower expressed in INSS stage 4 tumors compared to the tumors belonging to stage 1 and 2 (Supplementary Fig. S2).

Functional enrichment analysis on predicted miRNA targets

To analyze miRNA expression data in the context of known biological functions, a functional enrichment analysis using Ingenuity Pathway Analysis (IPA) was performed.

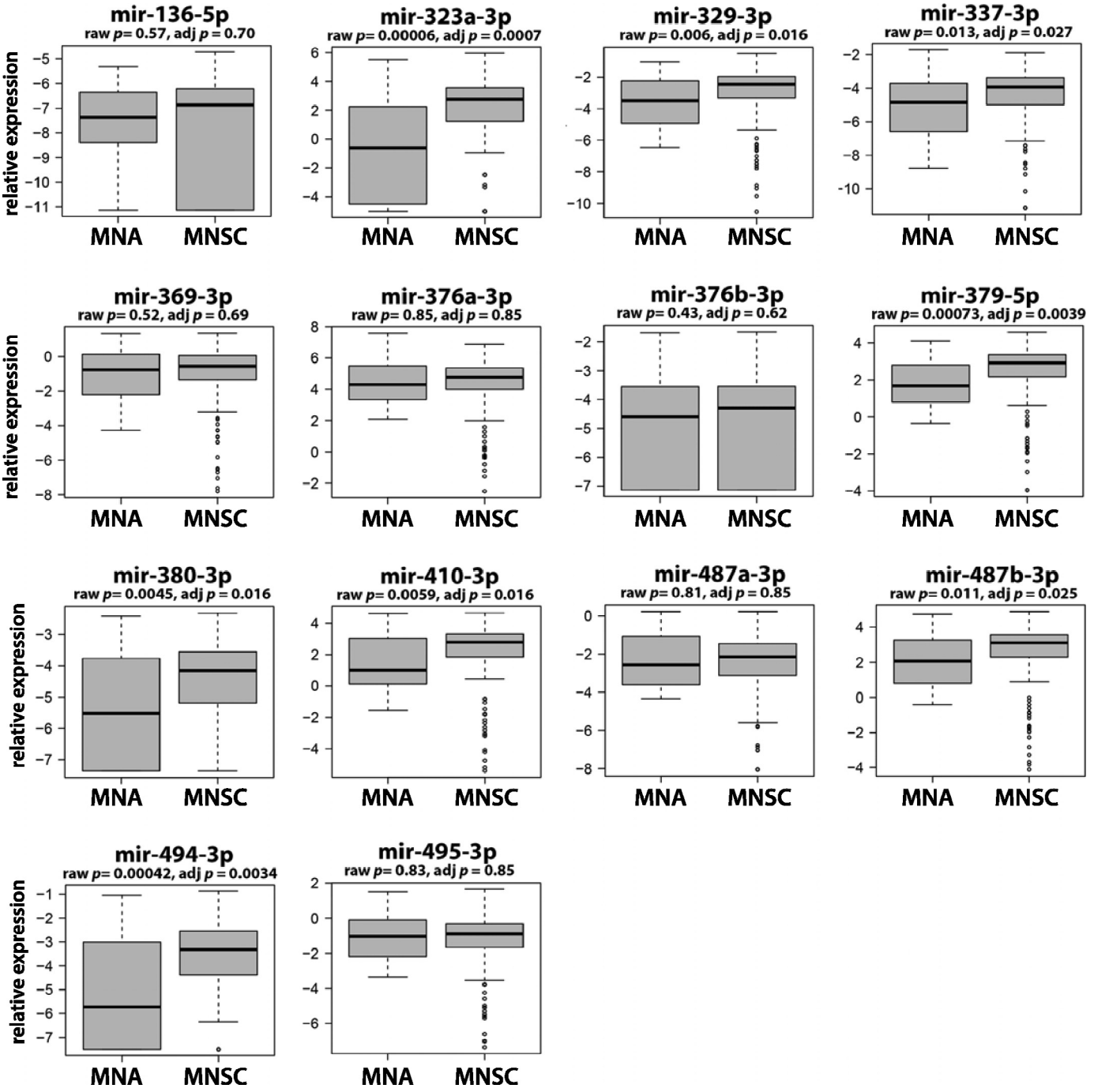

Fig. 2. Reduced expression of 14q32-miRNAs in MNA neuroblastoma tumors. Box plot presentations showing relative expression levels of mature 14q32-miRNAs in *MYCN*-amplified (MNA, n = 40) and *MYCN* single-copy (MNSC, n = 160) primary neuroblastoma tumors. The boxes represent the 25%–75% quartiles. The horizontal line in the boxes represents the median level. Whiskers represent the non-outlier range. Open circles represent the outliers. Adjusted *p*-values (adj *p*) were calculated using the Benjamini–Hochberg procedure.

Table 4
Functional categories containing enriched molecular and cellular functions of predicted miRNA target genes differentially expressed between neuroblastoma cell lines isolated before and after treatment.

Category	*p*-value range	Number of targets
Cell Death and Survival	2.70×10^{-32}–3.07×10^{-06}	167
Cellular Growth and Proliferation	9.79×10^{-30}–3.77×10^{-06}	177
Cellular Development	3.88×10^{-29}–3.77×10^{-06}	159
Cellular Movement	3.00×10^{-27}–2.54×10^{-06}	121
Cell Cycle	7.51×10^{-22}–3.77×10^{-06}	97
Gene Expression	1.25×10^{-20}–2.01×10^{-08}	122
Cell Morphology	2.54×10^{-18}–2.97×10^{-06}	71
Cell-To-Cell Signaling and Interaction	1.31×10^{-10}–2.45×10^{-06}	59
DNA Replication, Recombination, and Repair	4.52×10^{-10}–3.77×10^{-06}	63
Post-Translational Modification	7.68×10^{-10}–1.41×10^{-07}	41

The *p*-value range (Fisher's exact test) indicates the *p*-values of the various pathways and processes belonging to that category. The number of targets indicates the total number of predicted miRNA targets associated with the functional category.

The 34 downregulated and 8 upregulated miRNAs were predicted to target 425 unique experimentally observed mRNAs (Supplementary Table S3). This resulting list of mRNAs was used to determine affected molecular and cellular functions (threshold p-value < 0.05) that are predicted to be affected by the differentially expressed miRNAs. As expected, IPA identified several functional categories with molecular and cellular functions, like the top scoring functions 'Cell Death and Survival' and 'Cellular Growth and Proliferation', associated with chemo- and radiotherapy resistant phenotypes (Table 4). The full list of predicted miRNA target genes associated with the ten most affected IPA functional categories is shown in Supplementary Table S4.

Furthermore, the predicted targets were enriched for several canonical pathways known to be involved in drug resistance, including PI3K/AKT signaling, STAT3 signaling, G1/S checkpoint regulation, p53 signaling (Fig. 3). The complete list of putative targets related to the ten most affected canonical pathways is shown in Supplementary Table S5.

Discussion

Most drug resistance cell model systems for neuroblastoma, as well as many other cancers, are based on *in vitro* selection of resistant cell lines by continuous or intermittent exposure of cells to a single chemotherapeutic agent [16–21]. To investigate the role of miRNAs during drug resistance development in neuroblastoma, we determined miRNA expression profiles by SOLiD deep-sequencing in clinically relevant neuroblastoma models, consisting of cell lines established from the same patients at diagnosis (pre-treatment) and at relapse after therapy (post-treatment). In these model systems, drug resistance was established in the patients during treatment, and the resistance patterns have been shown to generally correlate with the treatment intensities and the drugs that the patients were exposed to [22,23].

We identified a panel of miRNAs that are either downregulated (34 miRNAs) or upregulated (8 miRNAs) in the cell lines isolated after treatment. In concordance with previously published studies on miRNA expression in cancer cell lines, we observed downregulation of miRNAs to be more common than upregulation [24]. The differential expression of a subset of these miRNAs was validated using RT-qPCR.

Most strikingly, 22 of the 34 (65%) downregulated miRNAs are encoded by miRNA clusters located at chromosome 14q32. This locus harbors two miRNA clusters (14q32.31 and 14q32.2 clusters), of which the 14q32.31 cluster is the largest miRNA cluster identified in the human genome [25]. The two miRNA clusters encode a total of 47 different miRNAs. Our sequencing data identify 32 (68%) different miRNAs from these clusters, 6 of 8 miRNAs from the 14q32.2 cluster and 26 of 39 miRNAs from the 14q32.32 cluster (Fig. 4).

miRNA expression data from a cohort consisting of 226 primary neuroblastoma tumors confirmed the lower expression of most of

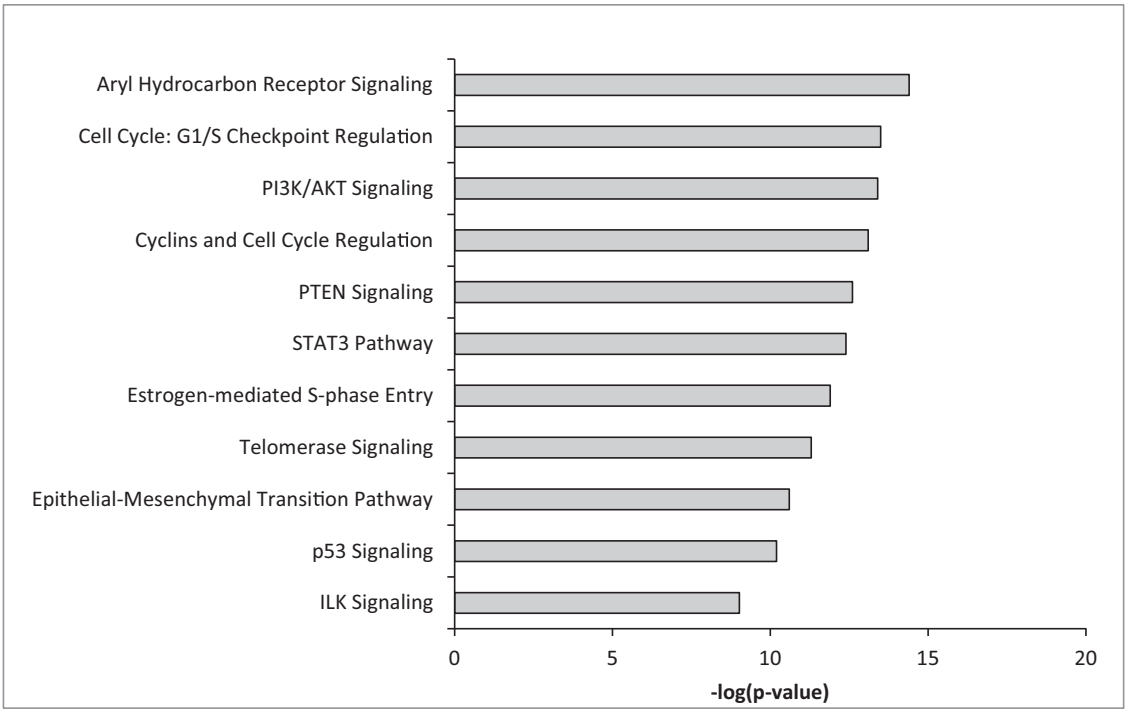

Fig. 3. Canonical pathways predicted to be affected by differential miRNA gene expression. The diagram shows the ten most significantly enriched canonical pathways identified by IPA. The x-axis displays the –log of *P* value, which is calculated by Fisher's exact test.

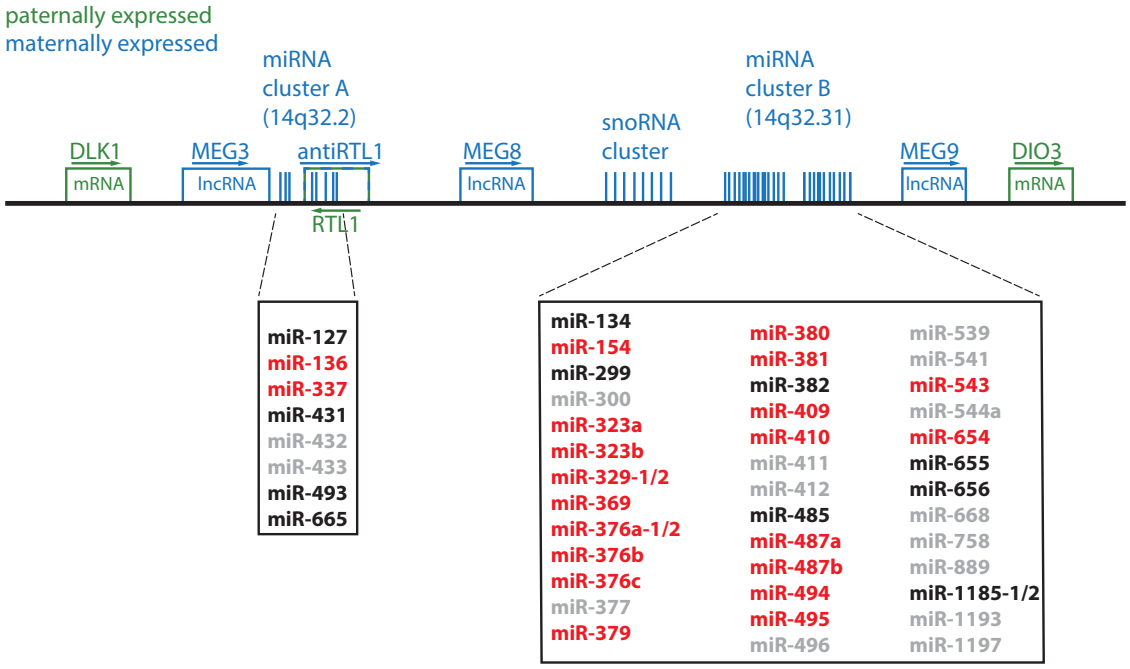

Fig. 4. Schematic overview of the DLK1-DIO3 imprinted domain located on chromosome 14q32. Green and blue represent paternally and maternally expressed genes, respectively. Boxed miRNA names are listed according to the miRNA IDs and not chromosomal location. MiRNAs shown in red and black are differentially expressed, but only miRNAs labeled in red meet our strict criteria to represent the panel of differentially expressed miRNAs. MiRNAs shown in gray were not detected. DLK1: delta-like 1 homolog (Drosophila); MEG3: maternally expressed 3; RTL1: retrotransposon-like 1; MEG8: maternally expressed 8; MEG9: maternally expressed 9; DIO3: Type III iodothyronine deiodinase.

our identified 14q32 miRNAs in tumors exhibiting poor prognosis factors like MNA and INSS stage 4. We also identified several additional 14q32 miRNAs (all observed to be repressed in cell lines isolated after treatment but not following our strict criteria) that were lower expressed in the tumors associated with poor prognosis (Supplementary Fig. S3).

The 14q32.31 miRNA cluster has previously been shown to be downregulated in multiple human cancers, including glioblastoma multiforme, ovarian cancer, breast invasive carcinoma, kidney renal clear cell carcinoma, stomach adenocarcinoma, prostate adenocarcinoma and bladder urothelial cancer [26]. Using genome-wide miRNA expression profiling, Xu et al. found strong downregulation of all miRNAs coded by the 14q32.31 cluster in doxorubicin-resistant K562 leukemia cells when compared to their drug-sensitive counterparts [27]. Furthermore, *miR-381* and *miR-495*, which also in our study were significantly downregulated in the post-treatment cell lines, were previously shown to specifically repress expression of *MDR1/p-gp* through direct targeting of the 3'UTR. Similar to our results, most aberrantly expressed miRNAs were downregulated in drug resistant cells, while *miR-375* was upregulated.

miR-337-3p, encoded by the 14q32.2 miRNA cluster, is one of the miRNAs that was downregulated in all cell lines isolated from patients after treatment. *miR-337-3p* has previously been reported to sensitize lung cancer cells to taxanes by direct targeting and repression of *signal transducer and activator of transcription 3* (*STAT3*) and *Ras-related Protein 1A* (*RAP1A*) expression [28]. *STAT3* is well known for its positive regulation of several survival proteins including *cyclin D1*, *survivin*, *c-myc*, *Bcl-XL* and *Bcl-2* [29]. Unlike many other cancers, *STAT3* is not constitutively activated in neuroblastoma; however, *in vitro* studies demonstrate that bone marrow-derived IL-6 increases the proliferation and decreases etoposide-induced apoptosis through activation of *STAT3* in neuroblastoma cells [30].

Very recently, Xiang et al. showed that *miR-337-3p* is downregulated in neuroblastoma tumor samples and that *miR-337-3p* expression is inversely correlated with advanced neuroblastoma stages, tumor progression and *MYCN* amplification [31]. Exogenous overexpression of *miR-337-3p* inhibited growth, invasion, metastasis and angiogenesis in neuroblastoma cell lines. *miR-337-3p* was shown to directly bind the matrix metalloproteinase 14 (*MMP-14*) promoter to repress its transcription. These studies indicate that *miR-337-3p* could be a critical regulator of several processes involved in survival and progression of aggressive neuroblastomas.

Allelic loss of the long arm of chromosome 14 (14q) has been reported to be quite common (20–30%) in neuroblastoma patients despite no correlation between 14q loss of heterozygosity (LOH) and clinicopathologic features [32,33]. However, thirteen miRNAs belonging to the 14q32.31 cluster were significantly downregulated in high-risk neuroblastoma tumors and two of these (*miR-410* and *miR-487*) were proposed as prognostic markers since they were able to discriminate high-risk from low-risk neuroblastoma [34].

Several non-14q32 differentially expressed miRNAs from this study have previously been associated with poor clinical outcome in neuroblastoma and chemoresistance in various other cancers. For instance, we found that the tumor suppressor miRNA *miR-34a* is downregulated in cell lines isolated from patients after treatment. Loss or downregulation of *miR-34a* has been reported in several cancers including neuroblastoma, breast and bladder cancer [35–37]. Furthermore, *miR-34a* overexpression sensitizes various cancer cell lines to a number of chemotherapeutic drugs and to radiotherapy [37–47].

Although only few miRNAs were found to be upregulated in our study, several of these have previously been reported to be associated with clinical outcome in neuroblastoma.

miR-21 is an established oncomir that promotes growth, metastasis, chemo-/radioresistance and angiogenesis in several cancers

by direct targeting known tumor suppressor genes [48]. We found *miR-21* to be upregulated in all cell line pairs except SK-N-BE(1)/(2). SK-N-BE(2) has acquired a *p53* mutation that we speculate is responsible for the major difference in several differentially expressed miRNA in this cell line compared to the other post-treatment isolated cell lines. No previous reports have shown *miR-21* to be transcriptionally modulated by p53. Increased *miR-21* expression has been linked to *in vitro* selected cisplatin-resistant neuroblastoma cell lines. Treatment with antagomirs *of miR-21* sensitized resistant cells to cisplatin treatment [18]. Furthermore, a study using deep sequencing of neuroblastoma tumors identified miR-181 family members (*miR-181a-5p* and *miR-181b-5p*) to be overexpressed in unfavorable neuroblastomas [49]. In concordance with this, we observed upregulation of both *miR-181a-5p* and *miR-181b-5p* in cell lines isolated from patients after treatment. A recent report including *in vitro* and *in vivo* experiments revealed that *miR-181a-5p* confers chemoresistance in cervical squamous cell carcinoma by directly targeting and decreasing *PRKCD* expression [50]. PRKCD is a protein kinase C isoform critical for DNA damage-induced apoptosis and chemotherapeutic drug resistance in several cancers, including neuroblastoma [51–53]. A search through the public available R2 database (http://r2.amc.nl) using the two largest neuroblastoma datasets (Kocak; n = 649 and SEQC; n = 498) showed a highly significant correlation between low expression levels of PRKCD and poor survival of neuroblastoma patients (Supplementary Fig. S4).

Increased expression of *miR-375* has also been associated with neuroblastoma patients with unfavorable outcome and metastatic dissemination [54] and *miR-375* was one of ten miRNAs whose increased expression was associated with advanced stage disease [55]. Recently, high *miR-375* expression was also associated with tumorigenic undifferentiated neuroblastoma cell lines and *miR-375* was shown to target HuD, a gene involved in neuronal differentiation [56].

In order to gain insight into biological functions and pathways that may be affected by the miRNAs differentially expressed between the pre- and post-treatment isolated cell line pairs, we utilized Ingenuity Pathway Analysis (IPA). Predicted targets belong to several molecular and cellular functions well known to be implicated in drug resistant cancer cells. The top scoring functions were 'cell death and survival' and 'cellular growth and proliferation', both of which were recently identified as the top scoring functions affected in several cisplatin-resistant neuroblastoma cell lines [21]. For the canonical pathway analysis of the predicted miRNA targets, all pathways are known to play important functions during cancer progression and drug resistance. The roles of cancer-associated PI3K/Akt-, PTEN-, STAT3- and p53 pathways have recently been extensively reviewed [57–60]. Furthermore, the aryl hydrocarbon receptor (AHR) was recently suggested to be an important upstream regulator of *MYCN* in neuroblastoma implying the involvement of AHR signaling in many aspects of neuroblastoma tumorigenesis [61]. Epithelial–mesenchymal transition (EMT) and telomerase signaling have been implicated as important processes during development of chemoresistance in neuroblastoma [21,62,63]. Functional enrichment analysis using IPA-predicted up- and downregulated miRNA target genes separately (separate-DE), or the strong-evidence target genes (all-DE and separate-DE) from miRTarBase, revealed similar results (Supplementary Table S6).

Our functional enrichment analysis of differentially expressed miRNAs has several limitations and must be interpreted with some caution. First, IPA is a threshold-dependent tool where a user-preselected list of interesting genes (here the criteria-selected differentially expressed miRNAs) is used as input data. Second, the miRNA target gene prediction is based on predetermined database knowledge and was not functionally validated by experimental testing. Third, the functional enrichment analysis considers that each individual gene is of equal importance, which is often not the situation in biological systems. Finally, the enrichment analysis is based on the current state of knowledge. Since new relationships between gene products and biological functions/pathways are expected to be unveiled in the future, the presented results capture only a subset of the biological processes affected in the analyzed model systems.

Our work may serve as a model on which to base future investigations on the role of miRNAs in the complex processes of surviving therapeutic treatment and developing drug resistance in neuroblastoma cell lines and also in clinical patient samples. However, the applicability of the study to a clinical setting remains to be determined.

Conflict of interest

None.

Acknowledgments

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2015.11.026.

References

[1] J.R. Park, A. Eggert, H. Caron, Neuroblastoma: biology, prognosis, and treatment, Hematol. Oncol. Clin. North Am. 24 (1) (2010) 65–86.
[2] T.A. Ishola, D.H. Chung, Neuroblastoma, Surg. Oncol. 16 (3) (2007) 149–156.
[3] S. Modak, N.K. Cheung, Neuroblastoma: therapeutic strategies for a clinical enigma, Cancer Treat. Rev. 36 (4) (2010) 307–317.
[4] C. Owens, M. Irwin, Neuroblastoma: the impact of biology and cooperation leading to personalized treatments, Crit. Rev. Clin. Lab. Sci. 49 (3) (2012) 85–115.
[5] J.M. Maris, Recent advances in neuroblastoma, N. Engl. J. Med. 362 (23) (2010) 2202–2211.
[6] G.P. Tonini, A. Nakagawara, F. Berthold, Towards a turning point of neuroblastoma therapy, Cancer Lett. 326 (2) (2012) 128–134.
[7] J.I. Fletcher, et al., Targeting multidrug resistance in neuroblastoma, in: M.A. Hayat (Ed.), Pediatric cancer, vol. 1: Neuroblastoma, Springer, New York, USA, 2012, pp. 115–123.
[8] E. Giovannetti, et al., Molecular mechanisms underlying the role of microRNAs (miRNAs) in anticancer drug resistance and implications for clinical practice, Crit. Rev. Oncol. Hematol. 81 (2) (2012) 103–122.
[9] F. Chen, S.J. Hu, Effect of microRNA-34a in cell cycle, differentiation, and apoptosis: a review, J. Biochem. Mol. Toxicol. 26 (2) (2012) 79–86.
[10] W.C. Cho, MicroRNAs as therapeutic targets and their potential applications in cancer therapy, Expert Opin. Ther. Targets 16 (8) (2012) 747–759.
[11] Y.W. Kong, et al., microRNAs in cancer management, Lancet Oncol. 13 (6) (2012) e249–e258.
[12] S.B. Thorsen, et al., The therapeutic potential of microRNAs in cancer, Cancer J. 18 (3) (2012) 275–284.
[13] J. Buechner, C. Einvik, N-myc and noncoding RNAs in neuroblastoma, Mol. Cancer Res. 10 (10) (2012) 1243–1253.
[14] E. Knutsen, et al., Performance comparison of digital microRNA profiling technologies applied on human breast cancer cell lines, PLoS ONE 8 (10) (2013) e75813.
[15] B.A. Spengler, et al., Cell lineage and differentiation state are primary determinants of MYCN gene expression and malignant potential in human neuroblastoma cells, Oncol. Res. 9 (9) (1997) 467–476.

[16] R. Kotchetkov, et al., Development of resistance to vincristine and doxorubicin in neuroblastoma alters malignant properties and induces additional karyotype changes: a preclinical model, Int. J. Cancer 104 (1) (2003) 36–43.
[17] J. Bedrnicek, et al., Characterization of drug-resistant neuroblastoma cell lines by comparative genomic hybridization, Neoplasma 52 (5) (2005) 415–419.
[18] Y. Chen, et al., Micro-RNA-21 regulates the sensitivity to cisplatin in human neuroblastoma cells, J. Pediatr. Surg. 47 (10) (2012) 1797–1805.
[19] S.V. Govindan, et al., Establishment and characterization of triple drug resistant head and neck squamous cell carcinoma cell lines, Mol. Med. Rep. 12 (2) (2015) 3025–3032.
[20] D. Ayers, et al., Identification of miRNAs contributing to neuroblastoma chemoresistance, Comput. Struct. Biotechnol. J. 13 (2015) 307–319.
[21] O. Piskareva, et al., The development of cisplatin resistance in neuroblastoma is accompanied by epithelial to mesenchymal transition in vitro, Cancer Lett. 364 (2) (2015) 142–155.
[22] N. Keshelava, R.C. Seeger, C.P. Reynolds, Drug resistance in human neuroblastoma cell lines correlates with clinical therapy, Eur. J. Cancer 33 (12) (1997) 2002–2006.
[23] N. Keshelava, et al., Loss of p53 function confers high-level multidrug resistance in neuroblastoma cell lines, Cancer Res. 61 (16) (2001) 6185–6193.
[24] N. Dahiya, et al., MicroRNA expression and identification of putative miRNA targets in ovarian cancer, PLoS ONE 3 (6) (2008) e2436.
[25] E.A. Glazov, et al., Origin, evolution, and biological role of miRNA cluster in DLK-DIO3 genomic region in placental mammals, Mol. Biol. Evol. 25 (5) (2008) 939–948.
[26] S.V. Laddha, et al., Genome-wide analysis reveals downregulation of miR-379/miR-656 cluster in human cancers, Biol. Direct 8 (2013) 10.
[27] Y. Xu, et al., Changes in the expression of miR-381 and miR-495 are inversely associated with the expression of the MDR1 gene and development of multi-drug resistance, PLoS ONE 8 (11) (2013) e82062.
[28] L. Du, et al., miR-337-3p and its targets STAT3 and RAP1A modulate taxane sensitivity in non-small cell lung cancers, PLoS ONE 7 (6) (2012) e39167.
[29] A. Xiong, et al., Transcription factor STAT3 as a novel molecular target for cancer prevention, Cancers (Basel) 6 (2) (2014) 926–957.
[30] T. Ara, et al., Interleukin-6 in the bone marrow microenvironment promotes the growth and survival of neuroblastoma cells, Cancer Res. 69 (1) (2009) 329–337.
[31] X. Xiang, et al., miRNA-337-3p suppresses neuroblastoma progression by repressing the transcription of matrix metalloproteinase 14, Oncotarget 6 (26) (2015) 22452–22466.
[32] M. Hoshi, et al., Detailed deletion mapping of chromosome band 14q32 in human neuroblastoma defines a 1.1-Mb region of common allelic loss, Br. J. Cancer 82 (11) (2000) 1801–1807.
[33] P.M. Thompson, et al., Loss of heterozygosity for chromosome 14q in neuroblastoma, Med. Pediatr. Oncol. 36 (1) (2001) 28–31.
[34] C.H. Gattolliat, et al., Expression of miR-487b and miR-410 encoded by 14q32.31 locus is a prognostic marker in neuroblastoma, Br. J. Cancer 105 (9) (2011) 1352–1361.
[35] G. Feinberg-Gorenshtein, et al., Reduced levels of miR-34a in neuroblastoma are not caused by mutations in the TP53 binding site, Genes Chromosomes Cancer 48 (7) (2009) 539–543.
[36] A. Javeri, et al., Downregulation of miR-34a in breast tumors is not associated with either p53 mutations or promoter hypermethylation while it correlates with metastasis, Med. Oncol. 30 (1) (2013).
[37] X. Wang, et al., microRNA-34a sensitizes lung cancer cell lines to DDP treatment independent of p53 status, Cancer Biother. Radiopharm. 28 (1) (2013) 45–50.
[38] Y. Akao, et al., Dysregulation of microRNA-34a expression causes drug-resistance to 5-FU in human colon cancer DLD-1 cells, Cancer Lett. 300 (2) (2011) 197–204.
[39] W. Cao, et al., miR-34a regulates cisplatin-induce gastric cancer cell death by modulating PI3K/AKT/survivin pathway, Tumour Biol. (2013). doi:10.1007/s13277-013-1171-7.
[40] W. Duan, et al., Ectopic expression of miR-34a enhances radiosensitivity of non-small cell lung cancer cells, partly by suppressing the LyGDI signaling pathway, J Radiat Res (2013). doi: 10.1093/jrr/rrs136.
[41] K. Kojima, et al., MiR-34a attenuates paclitaxel-resistance of hormone-refractory prostate cancer PC3 cells through direct and indirect mechanisms, Prostate 70 (14) (2010) 1501–1512.
[42] X.J. Li, et al., MicroRNA-34a modulates chemosensitivity of breast cancer cells to adriamycin by targeting notch1, Arch. Med. Res. 43 (7) (2012) 514–521.
[43] F. Nakatani, et al., miR-34a predicts survival of Ewing's sarcoma patients and directly influences cell chemo-sensitivity and malignancy, J. Pathol. 226 (5) (2012) 796–805.
[44] P. Neri, et al., Mir-34a sensitizes multiple myeloma (MM) cells to the proteasome inhibitor bortezomib, Blood 118 (21) (2011) 66–67.
[45] R.L. Vinall, et al., MiR-34a chemosensitizes bladder cancer cells to cisplatin treatment regardless of p53-Rb pathway status, Int. J. Cancer 130 (11) (2012) 2526–2538.
[46] S.D. Weeraratne, et al., miR-34a confers chemosensitivity through modulation of MAGE-A and p53 in medulloblastoma, Neuro Oncol. 13 (2) (2011) 165–175.
[47] F. Yang, et al., MicroRNA-34a targets bcl-2 and sensitizes human hepatocellular carcinoma cells to sorafenib treatment, Technol. Cancer Res. Treat. (2013). doi:10.7785/tcrt.2012.500364.
[48] S.R. Pfeffer, C.H. Yang, L.M. Pfeffer, The role of miR-21 in cancer, Drug Dev. Res. (2015). doi:10.1002/ddr.21257.
[49] J.H. Schulte, et al., Deep sequencing reveals differential expression of microRNAs in favorable versus unfavorable neuroblastoma, Nucleic Acids Res. 38 (17) (2010) 5919–5928.
[50] Y. Chen, et al., MicroRNA-181a enhances the chemoresistance of human cervical squamous cell carcinoma to cisplatin by targeting PRKCD, Exp. Cell Res. 320 (1) (2014) 12–20.
[51] A. Basu, Involvement of protein kinase C-delta in DNA damage-induced apoptosis, J. Cell. Mol. Med. 7 (4) (2003) 341–350.
[52] T.W. Day, C.H. Wu, A.R. Safa, Etoposide induces protein kinase Cdelta- and caspase-3-dependent apoptosis in neuroblastoma cancer cells, Mol. Pharmacol. 76 (3) (2009) 632–640.
[53] B. Marengo, et al., PKCdelta sensitizes neuroblastoma cells to L-buthionine-sulfoximine and etoposide inducing reactive oxygen species overproduction and DNA damage, PLoS ONE 6 (2) (2011) e14661.
[54] J. Guo, et al., Identification of miRNAs that are associated with tumor metastasis in neuroblastoma, Cancer Biol. Ther. 9 (6) (2010) 446–452.
[55] V.R. Chu, I. Lee, MicroRNA target signatures in advanced stage neuroblastoma, in: Neuroblastoma – present and future, Intech, 2012, pp. 271–286.
[56] L. Samaraweera, et al., MicroRNAs define distinct human neuroblastoma cell phenotypes and regulate their differentiation and tumorigenicity, BMC Cancer 14 (2014) 309.
[57] M.C. Hollander, G.M. Blumenthal, P.A. Dennis, PTEN loss in the continuum of common cancers, rare syndromes and mouse models, Nat. Rev. Cancer 11 (4) (2011) 289–301.
[58] T. Soussi, K.G. Wiman, TP53: an oncogene in disguise, Cell Death Differ. 22 (8) (2015) 1239–1249.
[59] H. Yu, et al., Revisiting STAT3 signalling in cancer: new and unexpected biological functions, Nat. Rev. Cancer 14 (11) (2014) 736–746.
[60] D.A. Fruman, C. Rommel, PI3K and cancer: lessons, challenges and opportunities, Nat. Rev. Drug Discov. 13 (2) (2014) 140–156.
[61] P.Y. Wu, et al., Aryl hydrocarbon receptor downregulates MYCN expression and promotes cell differentiation of neuroblastoma, PLoS ONE 9 (2) (2014) e88795.
[62] J.A. Naiditch, et al., Mesenchymal change and drug resistance in neuroblastoma, J. Surg. Res. 193 (1) (2015) 279–288.
[63] S. Wesbuer, et al., Association of telomerase activity with radio- and chemosensitivity of neuroblastomas, Radiat. Oncol. 5 (2010) 66.

PAPER-II

Hsa-miR-376c-3p targets Cyclin D1 and induces G1-cell cycle arrest in neuroblastoma cells

SWAPNIL PARASHRAM BHAVSAR[1], CECILIE LØKKE[1], TROND FLÆGSTAD[1,2] and CHRISTER EINVIK[1,2]

[1]Pediatric Research Group, Department of Clinical Medicine, Faculty of Health Science, The Arctic University of Norway-UiT, NO-9037 Tromsø; [2]Department of Pediatrics, Division of Child and Adolescent Health, University Hospital of North-Norway, NO-9038 Tromsø, Norway

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Abstract. High-risk neuroblastoma is the most aggressive form of cancer in children. The estimated survival of children with high-risk neuroblastoma is 40-50% compared with low and intermediate risk neuroblastoma, which is >98 and 90-95%, respectively. In addition, patients with high-risk neuroblastoma often experience relapse following intensive treatments with standard chemotherapeutic drugs. Therefore alternative strategies are required to address this problem. MicroRNAs (miRNAs/miRs) are small, endogenously expressed non-coding RNAs, which when deregulated have been demonstrated to serve significant roles in the tumorigenesis of a number of different types of cancer. Results from a previous deep sequencing study identified 22 downregulated miRNAs from the 14q32 miRNA cluster differentially expressed in neuroblastoma cell lines isolated from 6 patients at diagnosis and at relapse following intensive treatments. miR-376c-3p is one of the 22 miRNAs that was downregulated in the majority of the cell lines isolated from patients post treatment. The present study employed reverse transcription-quantitative polymerase chain reaction (RT-qPCR) to quantify the basic expression of miR-376c-3p in 6 neuroblastoma cell lines. The functional role of miR-376c-3p in the neuroblastoma cell lines was evaluated by alamar blue-cell viability and propidium iodide-flow cytometric assays. In addition, luciferase reporter assays, RT-qPCR and western blotting were performed to identify and quantify the targets of miR-376c-3p in neuroblastoma cell lines. Ectopic expression of miR-376c-3p led to significant inhibition of cell viability and G1-cell cycle arrest in multiple neuroblastoma cell lines by reducing the expression of cyclin D1, an oncogene critical for neuroblastoma pathogenesis. The results of the present study provide novel insights into the functional role of miR-376c-3p and suggest new approaches for the treatment of neuroblastoma.

Correspondence to: Professor Christer Einvik, Pediatric Research Group, Department of Clinical Medicine, Faculty of Health Science, The Arctic University of Norway-UiT, Hansine Hansens veg 18, NO-9037 Tromsø, Norway
E-mail: christer.einvik@uit.no

Abbreviations: miRNA, microRNA; NC, negative control; miR-376c-3p, microRNA-376c-3p; CCND1, Cyclin D1

Key words: high-risk neuroblastoma, microRNA, deep sequencing, 14q32 microRNA cluster, microRNA-376c-3p, Cyclin D1

Introduction

Neuroblastoma is the most common childhood malignancy derived from the primitive cells of the sympathetic nervous system (1). It accounts for ~15% of all pediatric oncology deaths (2). The treatment of patients with advanced neuroblastoma or high-risk neuroblastoma is still a challenge due to several factors including, dose-limiting toxicity to standard chemotherapeutic agents, disease heterogeneity and tumor regression (3). Moreover, these patients have a very poor prognosis with the overall ten-year survival rates of less than 10% (4). Therefore, the development of innovate alternate treatment strategies is necessary to increase the treatment effectiveness and lower toxicity.

MicroRNAs (miRNAs/miRs) are a large family of small (~22-25 nucleotides), endogenous, non-coding RNAs, which binds the partial or perfect complementary sequences in the 3'untranslated region (3'UTR) of target messenger RNAs (mRNAs) leading to translational repression or mRNA degradation (5). They regulate the expression of genes involved in proliferation, apoptosis, development and stress response. Thus, miRNAs have shown to play an important role in the initiation and progression of cancer (5). Depending on their respective target, miRNAs can act as oncogenes and or tumor suppressors (6). miRNAs are differentially expressed across cancer types and microRNA-profiling studies have revealed a general downregulation in tumors as compared with normal tissues (7). Interestingly, growing evidence have now shown the potential of aberrant expression of miRNAs to use as the prognostic and diagnostic biomarkers of human malignancies (6).

miR-376 family of miRNAs comprises of *miR-376a2*, *miR-376b*, *miR-376c* (earlier known as *miR-368*), *miR-376b1* and *miR-376b2* and their sequence identity is highly similar to mouse *miR-376a-c* miRNAs (8). miR-376 family members are located on chromosome 14 in humans and at the distal end of mouse chromosome 12 (9). They are expressed in

placenta, developing embryos, and adult tissues (8). The expression of *miR-376c* is downregulated in many human malignancies including cervical cancer (10), prostate cancer (11), oral squamous cell carcinoma (12), intrahepatic cholangiocarcinoma (13), melanoma (14), osteosarcoma (15) and gliomas (16). However, it has been shown that *miR-376c* is upregulated and it act as oncogenic in acute myeloid leukemia (17) and gastric cancer (18). In addition, forced expression of *miR-376c* enhance ovarian cancer cell survival by targeting activing-receptor like kinase 7 (19). Whereas, forced overexpression of *miR-376c* suppressed growth and invasion of non-small cell lung cancer (20). According to our knowledge, there is no report regarding the role of *miR-376c* in neuroblastoma. Thus uncovering the mechanisms of *miR-376c* function is critical to both the fundamental understanding of neuroblastoma pathogenesis and novel therapeutic treatments.

The Cyclin D1 (*CCND1*) is one of the extensively documented oncogene in human cancers. Functionally, *CCND1* binds with cyclin-dependent kinases (CDK 4/6) which phosphorylate pRB family proteins, which in turn transactivates genes necessary for cell cycle progression (21). Dysregulated expression of *CCND1* is a common feature in various human cancers (22,23). Inhibitors targeting *CCND1* are thoroughly studied but no results have yet been proven effective (22,23). The *CCND1* gene has one of the longest 3'UTR (~3.1 kb), suggesting a strong functional relevance (24). To date, many experimentally validated miRNAs targeting *CCND1* in different cancers are identified. For example, *let-7e*, *miR-9-5p*, *miR-15a-5p*, *miR-16*, *miR-17, miR-20a*, *miR-106b*, *miR-34a* and *miR-206* (25-31). However, no miRNA directly targeting *CCND1* 3'UTR is yet identified in neuroblastoma.

In this study, we examined the relationship between *miR-376c-3p* expression and neuroblastoma tumorigenesis. In our previous deep sequencing study, we have analyzed *miR-376c-3p* expression in neuroblastoma cell lines with different genetic characteristics (32). *miR-376c-3p* was downregulated in most of the cell lines tested and therefore we overexpressed *miR-376c-3p*, which have had significant effects on cell growth and survival of neuroblastoma cells.

In addition, we demonstrated that *CCND1* is a direct target of *miR-376c-3p* in neuroblastoma and overexpression of *miR-376c-3p* might have a significant influence on inhibition of neuroblastoma tumorigenesis.

Materials and methods

Cell culture. Human neuroblastoma cell lines, CHLA-15 and CHLA-20 were cultivated in Iscove's Modified Dulbecco's Medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 20% fetal bovine serum, 4 mM L-Glutamine and 1X ITS (5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml selenous acid). CHLA-15 and CHLA-20 were obtained from the same neuroblastoma patient prior to and following treatment with combination chemotherapy regimens, respectively. SKNAS, BE(2)-C, Kelly and SHSY-5Y cells were grown in RPMI-1640 medium (Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal bovine serum and 2 mM L-Glutamine (final concentrations). All cells were split before confluence and maintained at 37˚C in a humidified incubator with 4.5 to 5% CO_2 atmosphere. The cell lines were authenticated by short tandem repeat profiling at the Center of Forensic Genetics, The Arctic University of Norway-UiT, Norway and tested negative for mycoplasma contamination. CHLA-15 and CHLA-20 cell lines were kindly provided by Children's Oncology Group, Cell Culture and Xenograft Repository, Texas Tech University Health Science Centre (Lubbock, TX, USA). BE(2)-C, SKNAS, Kelly and SHSY-5Y were provided by Dr. John Inge Johnsen (Childhood Cancer Research Unit, Department of Women's and Children's Health, Karolinska Institutet, Stockholm, Sweden).

Transfections. MicroRNA *miR-376c-3p* mimics or negative control (NC) mimics were purchased from GenePharma Co., Ltd. (Shanghai, China) and Ambion (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Transfections of miRNA and NC mimics (25-40 nM) were carried out using Lipofectamine® 2000 reagent (Thermo Fisher Scientific, Inc.) according to manufacturer's instructions.

Cell viability assay. CHLA-15, CHLA-20, BE(2)-C, Kelly and SHSY-5Y cells were seeded in 24-well plates and reverse transfected with 25 nM *miR-376c-3p* or NC mimics using Lipofectamine® 2000 reagent (Thermo Fisher Scientific, Inc.). Every 24 h post-transfection, alamarBlue® (Thermo Fisher Scientific, Inc.) cell viability assay was performed according to manufacturer's instructions. Ten percent of the alamarBlue reagent was added to the cultured cells, mixed gently and incubated at 37˚C for three hours. 100 μl of medium was then transferred to black-walled 96-well plate and fluorescence was monitored at 540 nm excitation wavelength and 590 nm emission wavelength in a microplate reader (CLARIOstar; BMG Labtech GmbH, Offenburg, Germany). Cell viability was calculated as the percentage of NC transfected cells set to 100 percent.

Flow cytometric analysis of cell cycle distribution. The BE(2)-C, Kelly and SHSY-5Y cells were seeded in 25 cm^2 culture flasks and reverse transfected with 25 nM mimics as described previously. 24 h post-transfection cells were trypsinized and washed with 1X phosphate-buffered saline (PBS). The cells were then fixed in ice-cold 70% ethanol and incubated overnight at -20˚C. Next day, the ethanol fixed cells were centrifuged for 10 min at 0.8 x g and washed twice with 1X PBS and resuspended in the propidium iodide (PI) (Thermo Fisher Scientific, Inc.) staining solution (PBS with 100 μg/ml RNase, 50 μg/ml PI). The cells were then incubated for 30 min protected from light and stored on ice until analyzed. Fluorescence emitted from the PI-DNA complex was analyzed by flow cytometry using BD LSRFortessa™ cell analyzer (BD Biosciences, Franklin Lakes, NJ, USA). FlowJo 7.6.5 software was used to analyze the cell cycle data using the Dean-Jett-Fox model for cell cycle evaluation.

Bioinformatics target prediction. TargetScan (version 6.2; www.targetscan.org/) target prediction software was used to predict miR-376c-3p targets related to cell cycle.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The Kelly, SHSY-5Y and BE(2)-C cells were seeded in 6-well plates and transfected with 40 nM of *miR-376c-3p* or

NC mimics. Cells were harvested 48 h post-transfection and total RNA was isolated with QIAzol®Lysis reagent (Qiagen Sciences, Inc., Gaithersburg, MD, USA) according to manufacturer's instructions and quantified by NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Inc.).

For miRNA expression analysis, complementary DNA (cDNA) was synthesized from isolated total RNAs using the miScript II RT kit (Qiagen Sciences, Inc.) according to manufacturer's instructions. The reaction mixture (1X) (20 μl reaction volume) for reverse transcription was as follows: Total RNA, 1 μg (diluted in RNase Free Water up to 12 μl); 5X miScript HiSpec Buffer, 4 μl; 10X miScript Nucleics Mix, 2 μl; miScript Reverse Transcriptase Mix, 2 μl; The cycling conditions were 37˚C for 60 min followed by 95˚C for 5 min. The cDNA obtained was diluted with 80 μl RNase Free Water to achieve 10 ng/μl concentration and stored at -20˚C until use. To quantitate *miR-376c-3p* levels, quantitative polymerase chain reaction was performed with miScript primer assay for miR-376c (cat. no MS00004046) using miScript SYBR®Green PCR kit (Qiagen Sciences, Inc.,). The miScript primer assay for miR-4286 (cat. no MS00021371) was used as an internal control for normalization. The reaction mixture (1X) (20 μl reaction volume) for real time PCR was as follows: cDNA (1 ng/5 μl), 5 μl; QuantiTect SYBR-Green PCR Master Mix, 10 μl; Specific miScript primer assay, 2 μl; 10X miScript Universal Primer, 2 μl; RNase Free Water, 1 μl.

For basic miRNA expression levels in neuroblastoma cell lines, the following method was used for calculations. Raw fluorescence values (non-baseline corrected) generated from RT-qPCR reactions were used to calculate mean PCR efficiencies in the LinRegPCR software (Version 11.0; http://LinRegPCR.HFRC.nl.). N0 values (starting concentrations calculated by LinRegPCR software, N0=threshold/(mean amplicon efficiency^{Cq})) were used to calculate the expression of *miR-376c-3p* relative to the stably expressed *miR-4286* (32). qPCR reactions were performed in triplicates on 2 independent biological replicates. Standard deviations were calculated taking into account the principle of error propagation (including technical and biological replicates).

For mRNA expression analysis, complementary DNA (cDNA) was synthesized from isolated total RNAs using the maxima reverse transcriptase (Thermo Fisher Scientific, Inc.) according to manufacturer's instructions. The reaction mixture (1X) (20 μl reaction volume) for reverse transcription of mRNAs was as follows: Oligo DT primer (20 μM), 1 μl; dNTP (10 mM each), 1 μl; Total RNA, 1 μg (diluted in RNase Free Water up to 13.75 μl); Incubate 65˚C for 5 min followed by addition of 5x RT Buffer, 4 μl; Maxima Reverse Transcriptase, 0.25 μl; The cycling conditions were 60˚C for 30 min followed by 85˚C for 5 min. The cDNA obtained was diluted with 80 μl RNase Free Water to achieve 10 ng/μl concentration and stored at -20˚C until use. To quantitate *CCND1* levels, quantitative polymerase chain reaction was performed with *Power* SYBR-Green PCR Master Mix (Thermo Fisher Scientific, Inc.). SDHA housekeeping gene was used as an internal control for normalization. The reaction mixture (1X) (20 μl reaction volume) for real time PCR was as follows: cDNA (1 ng/1 μl), 10 μl; Power SYBR-Green PCR Master Mix, 5 μl; Forward Primer (10 μM), 0.4 μl; Reverse Primer (10 μM), 0.4 μl; RNase Free Water 4.2 μl.

Amplifications were carried out using Light Cycler 96 SW 1.1 (Roche Diagnostics GmbH, Mannheim, Germany) and expression levels of miRNAs and mRNAs were evaluated using the comparative ΔΔCq comparative cycle threshold method (33). The primers used were *CCND1* (forward: 5'-CCGTCCATG CGGAAGATC-3'; reverse: 5'-ATGGCCAGCGGGAAGAC-3') and *SDHA* (forward: 5'-CTGATGAGACAAGATGTGGTG-3'; reverse: 5'-CAATCTCCCTTCAATGTACTCC-3').

Western blot analysis. Cells were trypsinized and lysed in 40 μl RIPA buffer (50 mM Tris-HCL pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 1X Protein Inhibitor Cocktail (Roche Diagnostics GmbH) and 1 mM dithiothreitol (DTT). Lysate was cleared with centrifugation (21.1 x g) and the total protein concentrations were determined using DC™Protein Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to manufacturer's instructions. 40 μg protein was then loaded in each well and separated on a NuPAGE® Novex 4-12% Bis-Tris precast polyacrylamide gel (Thermo Fisher Scientific, Inc.). The separated proteins were transferred on Immobilon-FL PVDF membrane (EMD Millipore, Billerica, MA, USA) and blocked for 1 h at room temperature in 5 ml Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE, USA). The PVDF membrane was then incubated overnight at 4˚C with primary antibodies anti-Cyclin D1-(H-295)-Human Cyclin D1 Rabbit, polyclonal; (1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti-actin-(ab3280)-Human Actin Mouse, monoclonal (1:1,000; Abcam, Cambridge, UK). The secondary antibodies used were goat anti-rabbit-IRDye80^{0C}W, (1:5,000) (Rockland Immunochemicals, Inc., Gilbertsville, PA, USA) and goat anti-mouse-Alexa Fluor 680, (1:5,000; Thermo Fisher Scientific, Inc.). Antibody binding was detected using the Odyssey CLx Infrared Imaging System (LI-COR Biosciences). ImageJ software was used to quantify western blot results.

Luciferase reporter assay. The pMIR-Report-Cyclin D1-UTR-WT construct was a generous gift from Dr. Laura Barkley (30) and pMIR-Report-Cyclin D1-UTR-MUT construct with a mutation in the putative *miR-376c-3p* seed sequence (mutant) was generated using QuikChange® Multi Site-Directed Mutagenesis kit (Agilent Technologies, Inc., Santa Clara, CA, USA). The primers used for mutagenesis were CCND1_3'UTR_miR-376c-3p (forward: 5'-CACATC TTGGCATACTAATTCTTG-3'; reverse: 5'-CAAGAATTA GTATGCCAAGATGTG-3'). To validate for mutation in seed sequence the mutant plasmid was sequenced using sequencing primer 5'-CATCTGATTGGACAGGCATG-3'. The cells seeded at a density of 5x10^{4}cells/well in a 12-well plate were co-transfected with 40 nM NC or *miR-376c-3p* mimics, 20 ng pRL-SV40 construct (Promega Corporation, Madison, WI, USA) and 100 ng wild type or mutant luciferase constructs using Lipofectamine® 2000 reagent (Thermo Fisher Scientific, Inc.). 24 h post-transfection, firefly and renilla luciferase activities analyzed using the Dual-Luciferase Reporter Assay (Promega Corporation), according to manufacturer's instructions. Firefly luciferase activity was normalized against renilla luciferase

activity and luciferase activities of *miR-376c-3p* transfected cells were calculated relative to NC transfected cells set to 100 %.

MicroRNA expression data from neuroblastoma tumors. miRNA expression data from 226 primary neuroblastoma tumors were obtained through the Neuroblastoma Research Consortium (NRC). Differential miRNA expression was analyzed using a Kruskal-Wallis test.

Statistical analysis. The data was expressed as mean ± standard deviation (SD). Unless otherwise stated, all experiments were performed at least three times independently. Statistical analysis was performed using the software GraphPad Prism version 5.00 for Windows (GraphPad Software, Inc., La Jolla, CA, USA; available at www.graphpad.com). Statistical differences between means were determined using Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Multiple miRNAs located at 14q32 chromosomal region are downregulated and their lower levels are associated with poor prognosis factors in neuroblastoma. Results from our previous study, where we used next generation sequencing technology (SOLiD ligation sequencing) to determine miRNA expression profiles in neuroblastoma cell lines established from patients at diagnosis and at relapse after treatment, identified 22 downregulated microRNAs from 14q32 miRNA cluster. The expression of these downregulated miRNAs was confirmed in a cohort consisting of 226 primary neuroblastomas (32). *miR-376c-3p*, was one of the 22 miRNAs that was downregulated in most of the cell lines isolated from patients after the treatment (Fig. 1A). When *miR-376c* expression was compared in neuroblastoma primary tumors of different stages from the 226-cohort, we observed a trend towards lower expression in advanced stage disease compared to tumors in stage 1 (Fig. 1B). Thus, we sought out to focus on the functional role of *miR-376c-3p* in neuroblastoma.

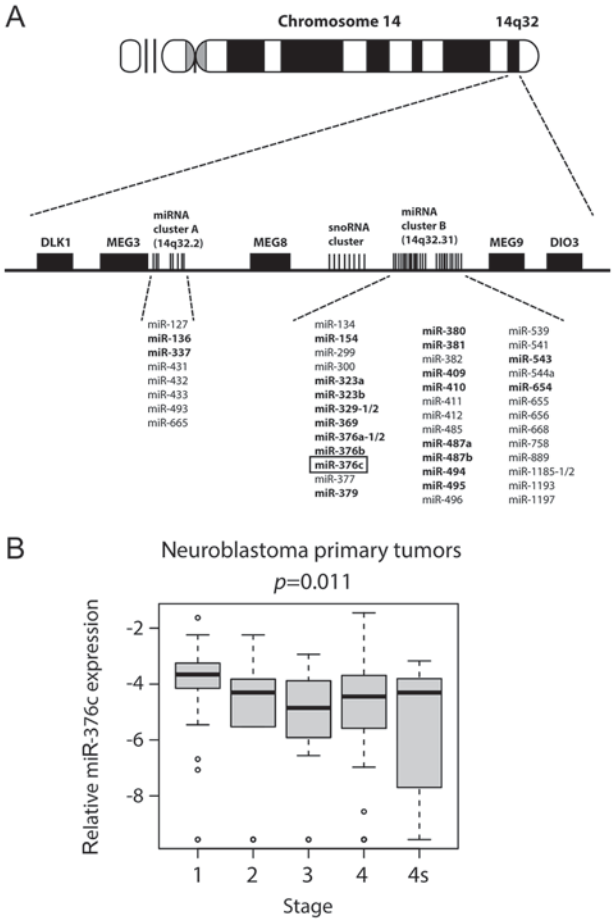

Figure 1. (A) Schematic representation of the miRNA-cluster at 14q32 chromosomal region. Multiple miRNAs located at 14q32 chromosomal region are downregulated (bold type) and associated with poor prognosis factors in neuroblastoma. The miRNA of interest, miR-376c-3p is shown in bold and a rectangular box. (B) Box-plots of miR-376c expression in tumors from neuroblastoma patients at different stages. The boxes represent the 25-75% quartiles. The horizontal line in the boxes represents the median level. Whiskers represent the non-outlier range. Open circles represent the outliers. P=0.011, comparing the median expression level of miR376c-3p across all pathological stages. DLK1, Delta Like Non-canonical Notch Ligand 1; MEG, maternally expressed; snoRNA, small nucleolar RNAs; DIO3, iodothyronine deiodinase 3; miR/miRNA, microRNA.

miR-376c-3p reduces cell viability in neuroblastoma cell lines. Even though *miR-376c-3p* has been shown to play either the oncogenic or the tumor suppressive role in different cancers (10-20) the functional role of *miR-376c-3p* is not yet determined in neuroblastoma. Therefore, we first used RT-qPCR to measure *miR-376c-3p* basic expression levels relative to *miR-4286* in 6 neuroblastoma cell lines. *miR-4286* was previously showed to be stably expressed in neuroblastoma cell lines (32). Among the cell lines, SKNAS and BE(2)-C cells have the highest expression level of *miR-376c-3p*, whereas SHSY-5Y, Kelly, CHLA-15 and CHLA-20 cells showed barely detectable levels of expression (Fig. 2A). In order to find out the potential role of *miR-376c-3p* in neuroblastoma, cell viability assay was performed on several neuroblastoma cell lines by overexpressing NC or *miR-376c-3p* mimics. The expression of *miR-376c-3p* was significantly increased in *miR-376c-3p* transfected cell lines compared with NC transfected cells, as validated and confirmed by RT-qPCR (Fig. 2B). Cell viability alamarBlue assay was performed at 24, 48, 72 and 96 h post-transfection, which showed that neuroblastoma cell lines transfected with *miR-376c-3p* mimics, had significantly reduced cell viability as compared to NC transfected cells. Thus, ectopic expression of *miR-376c-3p* reduced the growth of SKNAS, CHLA-15, CHLA-20, SHSY-5Y, Kelly and BE(2)-C cells as compared to NC transfected cells (Fig. 2C). These results indicate that cell growth of neuroblastoma cell lines is significantly affected by overexpression of *miR-376c-3p*.

miR-376c-3p induces a G1-cell cycle arrest in neuroblastoma cells. We did not detect significant apoptosis in *miR-376c-3p* transfected neuroblastoma cells using Annexin V and PARP cleavage assay (data not shown). Thus, we investigated the effects of *miR-376c-3p* overexpression on cell cycle distribution of representative neuroblastoma cell lines BE(2)-C, Kelly and SHSY-5Y by flow cytometric assay. Ectopic expression of *miR-376c-3p* in BE(2)-C, Kelly and SHSY-5Y resulted in increased percentage of cells in G1-phase of cell cycle as compared to NC transfected cells by 13% ($^{**}P=0.0023$),

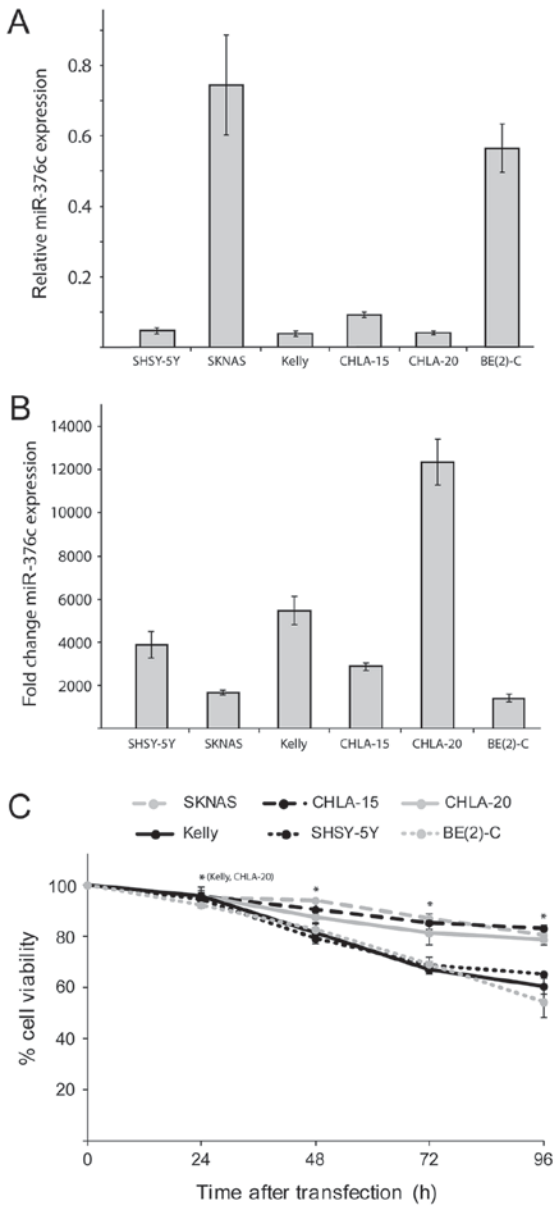

Figure 2. miR-376c-3p reduces cell viability in neuroblastoma cell lines. (A) RT-qPCR analysis was performed to quantitate the basic expression of *miR-376c-3p* in 6-neuroblastoma cell lines. *miR-4286* served as an endogenous control for miRNAs. Error bars indicate mean ± SD of two independent experiments, each repeated in triplicate. (B) RT-qPCR analysis for confirmation of *miR-376c-3p* overexpression in 6-neuroblastoma cell lines transfected with NC or *miR-376c-3p* mimics. Expression of *miR-376c-3p* in SHSY-5Y cell line was set to 1 and *miR-4286* served as an endogenous control for miRNAs. Error bars indicate mean ± SD. of two independent experiments, each repeated in triplicates. (C) Cell growth analysis of neuroblastoma cells transfected with NC or *miR-376c-3p* mimics. BE(2)-C, Kelly and SHSY-5Y were more sensitive compared with SKNAS, CHLA-15 and CHLA-20. Error bars indicate mean ± SD of three independent experiments repeated in triplicate. The results were statistically significant at 48, 72 and 96 h for all cell lines. *P<0.05. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NC, negative control; SD, standard deviation; miR, microRNA.

16% (***P=0.0001) and 9% (*P=0.0106), respectively with a corresponding reduction in the percentage of cells in the S and G2/M-phase (Fig. 3). Thus, this observation led us to the conclusion that decrease in cell viability might be due to induction of G1-cell cycle arrest in neuroblastoma cells and not apoptosis.

CCND1 is a direct target of miR-376c-3p in neuroblastoma. In order to investigate the underlying molecular mechanisms of *miR-376c-3p* induced suppression of the cell viability and G1-cell cycle arrest, a bioinformatics analysis was performed using miRNA target prediction algorithm TargetScan (Release 6.2; www.targetscan.org/) to predict the target genes of *miR-376c-3p* mainly associated with the cell cycle progression. TargetScan revealed 254 potential downstream targets with the conserved sites for *miR-376c-3p* (data not shown). It was theoretically demonstrated that *miR-376c-3p* had single binding site in the 3'UTR of *CCND1* oncogene. Thus, to determine whether *miR-376c-3p* could directly target the predicted 3'UTR of *CCND1*, a dual-luciferase reporter assay was performed in BE(2)-C and SHSY-5Y cells. Here, we used a luciferase reporter containing full-length (~3.1 kb), wild type *CCND1* 3'UTR (wt) or mutant *CCND1* 3'UTR (MUT) construct having a mutation of the putative *miR-376c-3p* target site shown in bold and italics (Fig. 4A).

Transient co-transfection of BE(2)-C and SHSY-5Y cells with *miR-376c-3p* mimics and the wild type *CCND1* 3'UTR (wt) reporter construct suppressed the luciferase activity as compared to NC transfected cells by 35% (**P=0.0091) and 38% (*P=0.0135), respectively. However, the activity of the reporter construct mutated at the specific *miR-376c-3p* target site is unaffected (Fig. 4B). These data indicated that *miR-376c-3p* represses *CCND1* expression by directly binding to the 3'UTR sequence of *CCND1* mRNA.

miR-376c-3p reduces mRNA and protein levels of CCND1 in neuroblastoma cells. To further confirm whether *miR-376c-3p* directly targets *CCND1* gene, neuroblastoma cells were transfected with *miR-376c-3p* or NC mimics and the expression levels of *CCND1* was analyzed by quantitative RT-qPCR analysis. The levels of *CCND1* mRNA was markedly decreased by *miR-376c-3p* overexpression in BE2-(C), Kelly and SHSY-5Y cells by 57% (*P=0.0110), 57% (**P=0.0017), and 53% (*P=0.0134), respectively as compared to NC transfected cells (Fig. 5A). Moreover, we also performed western blot analysis and observed significant decrease in levels of cyclin D1 proteins upon *miR-376c-3p* overexpression in BE2-(C), Kelly and SHSY-5Y cells by 41% (**P=0.0089), 67% (**P=0.0032), and 69% (**P=0.0069), respectively as compared to NC transfected cells (Fig. 5B and C). Taken together, these results demonstrates that endogenous expression of *CCND1* gene is directly regulated by *miR-376c-3p* and suggest that overexpression of *CCND1* gene could be reduced by enforced expression of *miR-376c-3p* in neuroblastoma cells.

In addition, to test whether *CCND1* could also counteract the effect of *miR-376c-3p* induced G1 cell cycle arrest, we overexpressed *CCND1* in SHSY-5Y cells. However, we only saw a modest and statistically insignificant effect of *CCND1* overexpression on reducing the effect of miR-376c-3p (Fig. 6).

Discussion

Current treatment strategies for high-risk neuroblastoma patients have limitations due to the refractory nature of the disease (2-4). Hence, alternative strategies are necessary for diagnosis and treatment of this disease. Mounting evidence have shown the potential of miRNAs as key regulators of

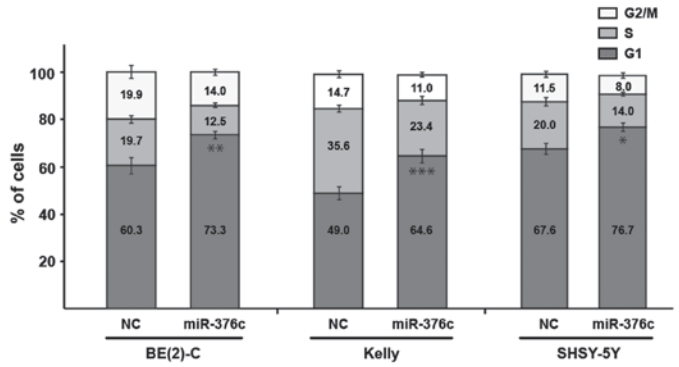

Figure 3. *miR-376c-3p* induces G1-cell cycle arrest in neuroblastoma cells. BE(2)-C, Kelly and SHSY-5Y cells were transfected with NC or *miR-376c-3p* mimics and the cell cycle analysis was evaluated by a propidium iodide-flow cytometric assay. The percentage of G1-arrested cells increased after *miR-376c-3p* overexpression. Error bars indicate mean ± standard deviation of three independent experiments repeated in triplicates. NC, negative control; miR, microRNA *P<0.05, **P<0.01 and ***P<0.001 vs. NC.

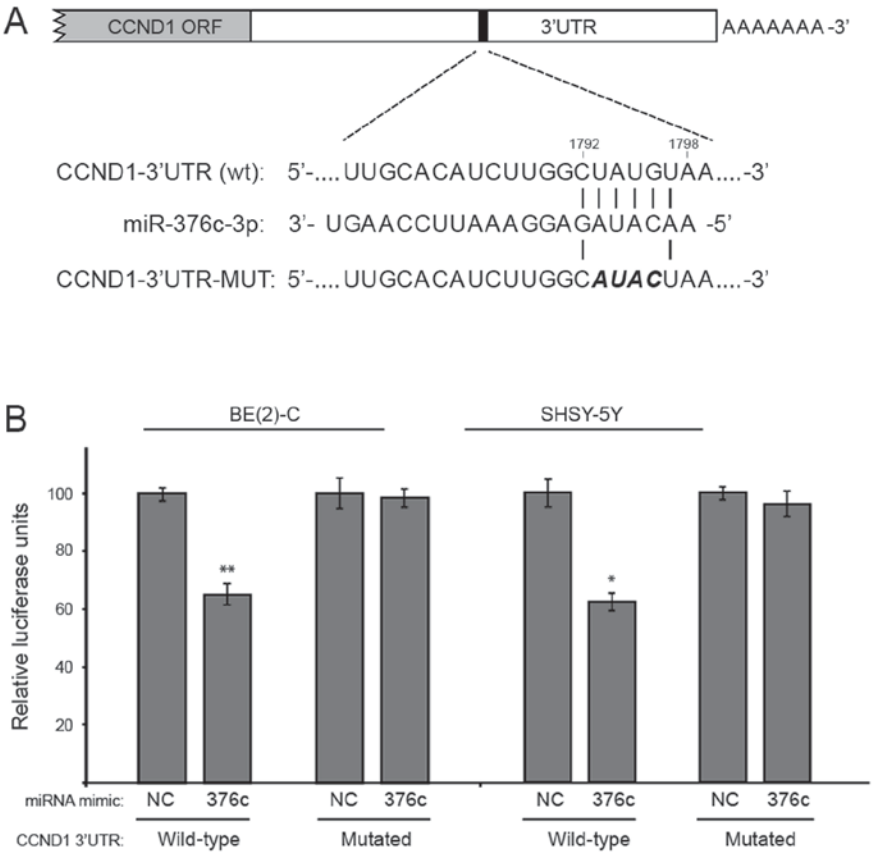

Figure 4. *CCND1* is a direct target of *miR-376c-3p* in neuroblastoma. (A) The *CCND1* 3'UTR contain one predicted *miR-376c-3p* binding site (nucleotides 1,792 to 1,798). In the figure, the alignment of the seed region of *miR-376c-3p* with *CCND1* and the site of target mutagenesis are shown in bold and italics. (B) pMIR-Report-CCND1 luciferase constructs containing a full-length wt or mutated *CCND1* 3'UTR and *miR-376c-3p* or NC mimics, were co-transfected into BE(2)-C and SHSY-5Y cells. Luciferase activity of wt construct was significantly reduced compared with mutated constructs. Relative repression of firefly luciferase activity is normalized with *renilla* luciferase activity. Error bars indicate mean ± standard devaition of three independent experiments repeated in triplicates. *P<0.05, **P<0.01 vs. the adjacent NC. miR, microRNA; NC, negative control; CCND1, cyclin D1; ORF, open reading frame; UTR, untranslated region; wt, wild type; MUT, mutated.

cancer pathogenesis by acting as oncogenes or tumor suppressors (6). Researchers have thus exploited these properties for the development of the novel anticancer therapies. miRNAs act by inhibiting the expression of its one or more target genes. Hence, identification of specific miRNAs and their targets is very important for the diagnosis and therapy of cancer (34).

Results from our previous deep sequencing analysis study identified 22 downregulated microRNAs from 14q32 miRNA cluster differentially expressed in neuroblastoma cell lines isolated from six patients at diagnosis and at relapse after intensive treatments. *miR-376c-3p*, is one of the 22 miRNAs that was downregulated in most of the cell lines isolated from patients after treatment (32). Moreover, the expression of *miR-376c-3p* was reduced in International Neuroblastoma Staging System (INSS) stage 4 compared to stage 1-2 in a cohort of 226 primary neuroblastoma tumors (32). However,

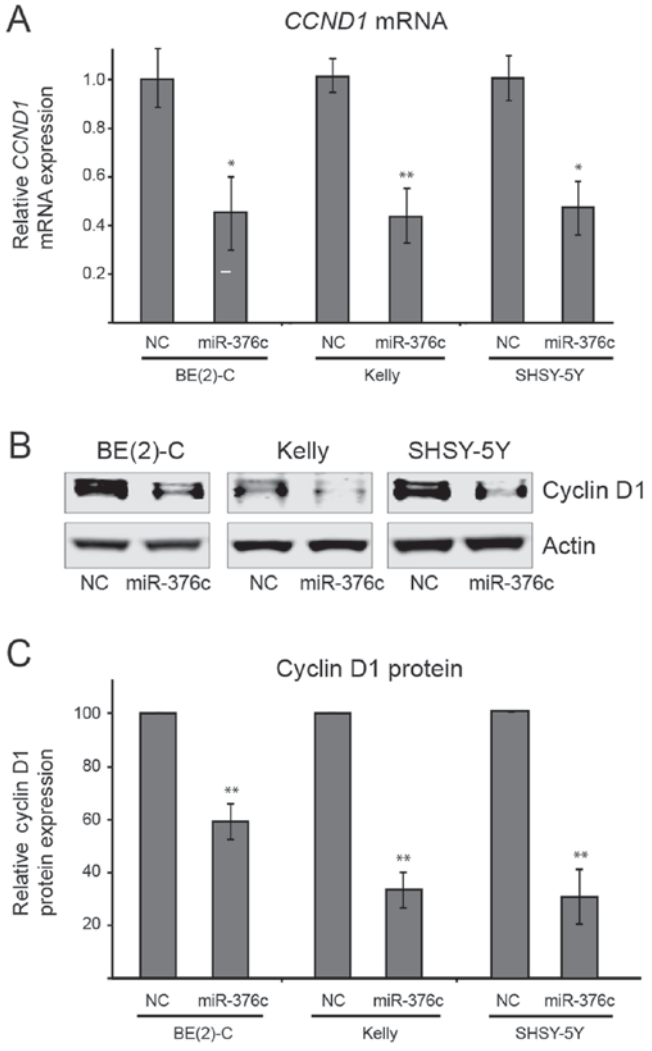

Figure 5. *miR-376c-3p* reduces mRNA and protein levels of *CCND1* in neuroblastoma cells. (A) *miR-376c-3p* overexpression significantly decreases *CCND1* mRNA levels in BE(2)-C, Kelly and SHSY-5Y. These cells were transfected with NC or *miR-376c-3p* mimics for 48 h and *CCND1* levels were assessed by reverse transcription-quantitative polymerase chain reaction. (B) *miR-376c-3p* overexpression significantly decreases endogenous levels of *Cyclin D1* proteins in BE(2)-C, Kelly and SHSY-5Y. These cells were transfected with NC or *miR-376c-3p* mimics for 96 h and *Cyclin D1* expression assessed by western blot analysis. The β-actin antibody used as a loading control. (C) Quantitative analysis of *Cyclin D1* protein expression on the western blots (n=3). Error bars indicate mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01 vs. the adjacent NC. miR, microRNA; NC, negative control; CCND1, cyclin D1.

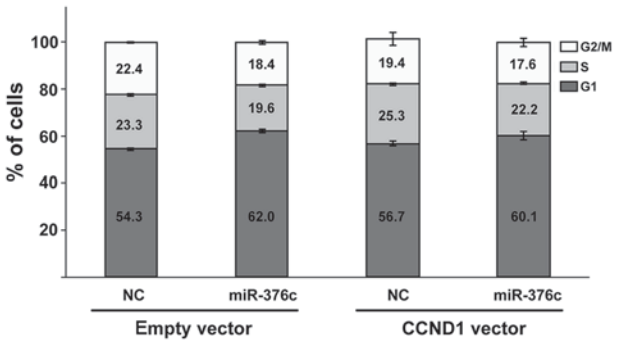

Figure 6. CCND1 overexpression partially rescues neuroblastoma cells from *miR-376c-3p* induced G1 cell cycle arrest. SHSY-5Y cells were co-transfected with NC or *miR-376c-3p* mimics and an empty or cyclin D1 overexpression plasmid. 96 h after the transfection, cells were ethanol fixed and cell cycle analysis was evaluated by the PI-flow cytometric assay. The results of the cell cycle analysis are depicted as percentage of cells in particular phases of cell cycle. Error bars indicate mean ± standard deviation of at least three repeats. miR, microRNA; NC, negative control; CCND1, cyclin D1.

the functional role of *miR-376c-3p* in neuroblastoma is not yet established.

Earlier reports from various cancers have demonstrated the dual nature of *miR-376c-3p* to act as either the oncogenic or the tumor suppressive miRNA depending on the cellular contexts (10-20). *miR-376c-3p* is downregulated in multiple human cancers, including prostate cancer (11), cervical cancer (10), oral squamous cell carcinoma (12), intrahepatic cholangiocarcinoma (13), melanoma (14), osteosarcoma (15) and gliomas (16). In contrast, *miR-376c-3p* was upregulated in acute myeloid leukemia (17) and gastric cancer (18). In these cancers, *miR-376-3p* has been shown to target a set of genes including B-cell-specific moloney murine leukemia virus insertion site 1 (BMI1), homeobox B7 (HOXB7), growth factor receptor-bound protein 2 *(GRB2),* transforming growth factor-alpha (TGFA), liver receptor homolog-1 (LRH-1), insulin growth factor 1 receptor (IGF1R) and activin receptor-like kinase 7 (ALK7). Other than affecting cell growth and proliferation, these genes act as important mediators of cell invasion (10,12,15, 20), migration (11-14), cell cycle arrest (10), apoptosis (12) and chemoresistance (19).

In order to find out how *miR-376c-3p* affects the growth and viability of neuroblastoma cells we first performed alamarBlue cell viability assay and demonstrated that transient overexpression of *miR-376c-3p* resulted in inhibition of cell viability in neuroblastoma cell lines. Consistent with this finding, we also observed that *miR-376c-3p* induced G1-cell cycle arrest suggesting that cell cycle genes may be affected and could serve as *miR-376c-3p* targets. To test this hypothesis, we used miRNA bioinformatics algorithms to predict *miR-376c-3p* targets related to the cell cycle regulation. TargetScan algorithm predicted the *CCND1* gene as a probable target of *miR-376c-3p*. *CCND1* is an important cell cycle regulator whose mRNA contains a conserved *miR-376c-3p* binding site on the 3'UTR. By dual-luciferase reporter assay, we further demonstrated that *miR-376c-3p* could significantly reduce luciferase activity of wild type constructs but not mutated *CCND1* 3'UTR construct confirming the direct regulation on *CCND1* by *miR-376c-3p*. Our experimental data further showed that the expression of *CCND1* mRNA and protein levels were significantly reduced after transfection with *miR-376c-3p* mimics as compared to NC mimics transfected cells. Additionally, we also performed flow cytometric rescue experiment by overexpression of *CCND1* in neuroblastoma cells to see if *CCND1* counteracts the effect of *miR-376c-3p* induced G1 cell cycle arrest. However, we only saw a modest and statistically insignificant effect of *CCND1* overexpression on reducing the effect of *miR-376c-3p* overexpression.

Cyclin D1, Cyclin D2 and Cyclin D3 belongs to the class of cyclins, which activates the cyclin-dependent kinases (CDKs). These cyclins through the phosphorylation of the substrates at specific cell stages co-ordinates the sequential completion of DNA replication and cell division (23). Unlike other cyclins, *CCND1* is induced by extracellular signals, including

growth factor receptor activation and integrin-derived adhesion signaling (23,35). Functionally, *CCND1* activates CDK4/6, *which* phosphorylates pRB family proteins causing the release of E2F transcription factors, which are essential for transcription of genes necessary for G1-S transition of cell cycle. Hence, the expression of cyclins are tightly regulated by variety of signaling pathways at the transcriptional as well as post-transcriptional levels (21).

The *CCND1* gene has been proposed as an important oncogene in various cancers. Numerous studies have documented the relationship between deregulation of *CCND1* and onset of tumorigenesis in wide variety of cancers. For instance, Molenaar *et al* (36,37) found that *CCND1* levels were increased in neuroblastoma tumors and high expression of *CCND1* led to tumorigenesis in neuroblastoma. In addition, Rihani *et al* (22) found that knockdown of *CCND1* reduced cell proliferation, induced G1-cell cycle arrest and inhibited the cyclin D1-Rb pathway in neuroblastoma cells. Similarly, Sun and colleagues also found that inhibition of *CCND1* and *CDK6* by *miR-34a* resulted in G1-cell cycle arrest in non-small cell lung cancer (38). In line with these reports, our data indicate that ectopic expression of *miR-376c-3p* leads to the suppression of endogenous *CCND1* gene resulting in the reduced cell growth and G1-cell cycle arrest in the neuroblastoma cells.

Taken together, our study for the first time demonstrates that miR-*376c-3p* directly target *CCND1* gene leading to reduced cell growth and G1-cell cycle arrest in neuroblastoma. Therefore, we suggest that '*miR-376c-CCND1*' axis could be a potential molecular target for preventing neuroblastoma tumorigenesis.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Author's contributions

SPB, TF and CE designed the research. SPB performed the experiments. CL assisted with the experiments. CE, CL and TF supervised the experimental work. SPB wrote the manuscript. CE, CL and TF critically amended the manuscript. The final manuscript was read and approved by all authors.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Brodeur GM: Neuroblastoma: Biological insights into a clinical enigma. Nat Rev Cancer 3: 203-216, 2003.
2. Maris JM, Hogarty MD, Bagatell R and Cohn SL: Neuroblastoma. Lancet 369: 2106-2120, 2007.
3. Garaventa A: High risk neuroblastoma: Small steps towards cure. Pediatr Blood Cancer 61: 964-965, 2014.
4. Garaventa A, Parodi S, De Bernardi B, Dau D, Manzitti C, Conte M, Casale F, Viscardi E, Bianchi M, D'Angelo P, *et al*: Outcome of children with neuroblastoma after progression or relapse. A retrospective study of the Italian neuroblastoma registry. Eur J Cancer 45: 2835-2842, 2009.
5. Croce CM and Calin GA: miRNAs, cancer, and stem cell division. Cell 122: 6-7, 2005.
6. Berindan-Neagoe I, Monroig Pdel C, Pasculli B and Calin GA: MicroRNAome genome: A treasure for cancer diagnosis and therapy. CA Cancer J Clin 64: 311-336, 2014.
7. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, *et al*: MicroRNA expression profiles classify human cancers. Nature 435: 834-838, 2005.
8. Kawahara Y, Zinshteyn B, Sethupathy P, Iizasa H, Hatzigeorgiou AG and Nishikura K: Redirection of silencing targets by adenosine-to-inosine editing of miRNAs. Science 315: 1137-1140, 2007.
9. Seitz H, Royo H, Bortolin ML, Lin SP, Ferguson-Smith AC and Cavaillé J: A large imprinted microRNA gene cluster at the mouse Dlk1-Gtl2 domain. Genome Res 14: 1741-1748, 2004.
10. Deng Y, Xiong Y and Liu Y: miR-376c inhibits cervical cancer cell proliferation and invasion by targeting BMI1. Int J Exp Pathol 97: 257-265, 2016.
11. Formosa A, Markert EK, Lena AM, Italiano D, Finazzi-Agro' E, Levine AJ, Bernardini S, Garabadgiu AV, Melino G and Candi E: MicroRNAs, miR-154, miR-299-5p, miR-376a, miR-376c, miR-377, miR-381, miR-487b, miR-485-3p, miR-495 and miR-654-3p, mapped to the 14q32.31 locus, regulate proliferation, apoptosis, migration and invasion in metastatic prostate cancer cells. Oncogene 33: 5173-5182, 2014.
12. Wang K, Jin J, Ma T and Zhai H: miR-376c-3p regulates the proliferation, invasion, migration, cell cycle and apoptosis of human oral squamous cancer cells by suppressing HOXB7. Biomed Pharmacother 91: 517-525, 2017.
13. Iwaki J, Kikuchi K, Mizuguchi Y, Kawahigashi Y, Yoshida H, Uchida E and Takizawa T: miR-376c down-regulation accelerates EGF-dependent migration by targeting GRB2 in the HuCCT1 human intrahepatic cholangiocarcinoma cell line. PLoS One 8: e69496, 2013.
14. Zehavi L, Avraham R, Barzilai A, Bar-Ilan D, Navon R, Sidi Y, Avni D and Leibowitz-Amit R: Silencing of a large microRNA cluster on human chromosome 14q32 in melanoma: Biological effects of mir-376a and mir-376c on insulin growth factor 1 receptor. Mol Cancer 11: 44, 2012.
15. Jin Y, Peng D, Shen Y, Xu M, Liang Y, Xiao B and Lu J: MicroRNA-376c inhibits cell proliferation and invasion in osteosarcoma by targeting to transforming growth factor-alpha. DNA Cell Biol 32: 302-309, 2013.

16. Huang Q, Wang C, Hou Z, Wang G, Lv J, Wang H, Yang J, Zhang Z and Zhang H: Serum microRNA-376 family as diagnostic and prognostic markers in human gliomas. Cancer Biomark 19: 137-144, 2017.
17. Dixon-McIver A, East P, Mein CA, Cazier JB, Molloy G, Chaplin T, Andrew Lister T, Young BD and Debernardi S: Distinctive patterns of microRNA expression associated with karyotype in acute myeloid leukaemia. PLoS One 3: e2141, 2008.
18. Shiotani A, Murao T, Kimura Y, Matsumoto H, Kamada T, Kusunoki H, Inoue K, Uedo N, Iishi H and Haruma K: Identification of serum miRNAs as novel non-invasive biomarkers for detection of high risk for early gastric cancer. Br J Cancer 109: 2323-2330, 2013.
19. Ye G, Fu G, Cui S, Zhao S, Bernaudo S, Bai Y, Ding Y, Zhang Y, Yang BB and Peng C: MicroRNA 376c enhances ovarian cancer cell survival by targeting activin receptor-like kinase 7: Implications for chemoresistance. J Cell Sci 124: 359-368, 2011.
20. Jiang W, Tian Y, Jiang S, Liu S, Zhao X and Tian D: MicroRNA-376c suppresses non-small-cell lung cancer cell growth and invasion by targeting LRH-1-mediated Wnt signaling pathway. Biochem Biophys Res Commun 473: 980-986, 2016.
21. Arand J and Sage J: G1 cyclins protect pluripotency. Nat Cell Biol 19: 149-150, 2017.
22. Rihani A, Vandesompele J, Speleman F and Van Maerken T: Inhibition of CDK4/6 as a novel therapeutic option for neuroblastoma. Cancer Cell Int 15: 76, 2015.
23. Musgrove EA, Caldon CE, Barraclough J, Stone A and Sutherland RL: Cyclin D as a therapeutic target in cancer. Nat Rev Cancer 11: 558-572, 2011.
24. Deshpande A, Pastore A, Deshpande AJ, Zimmermann Y, Hutter G, Weinkauf M, Buske C, Hiddemann W and Dreyling M: 3'UTR mediated regulation of the cyclin D1 proto-oncogene. Cell Cycle 8: 3592-3600, 2009.
25. Mitra D, Das PM, Huynh FC and Jones FE: Jumonji/ARID1 B (JARID1B) protein promotes breast tumor cell cycle progression through epigenetic repression of microRNA let-7e. J Biol Chem 286: 40531-40535, 2011.
26. Zheng L, Qi T, Yang D, Qi M, Li D, Xiang X, Huang K and Tong Q: microRNA-9 suppresses the proliferation, invasion and metastasis of gastric cancer cells through targeting cyclin D1 and Ets1. PLoS One 8: e55719, 2013.
27. Bonci D, Coppola V, Musumeci M, Addario A, Giuffrida R, Memeo L, D'Urso L, Pagliuca A, Biffoni M, Labbaye C, *et al*: The miR-15a-miR-16-1 cluster controls prostate cancer by targeting multiple oncogenic activities. Nat Med 14: 1271-1277, 2008.
28. Trompeter HI, Abbad H, Iwaniuk KM, Hafner M, Renwick N, Tuschl T, Schira J, Müller HW and Wernet P: MicroRNAs miR-17, miR-20a, and miR-106b act in concert to modulate E2F activity on cell cycle arrest during neuronal lineage differentiation of USSC. PLoS One 6: e16138, 2011.
29. Hermeking H: The miR-34 family in cancer and apoptosis. Cell Death Differ 17: 193-199, 2010.
30. Elliman SJ, Howley BV, Mehta DS, Fearnhead HO, Kemp DM and Barkley LR: Selective repression of the oncogene cyclin D1 by the tumor suppressor miR-206 in cancers. Oncogenesis 3: e113, 2014.
31. Buechner J, Tømte E, Haug BH, Henriksen JR, Løkke C, Flægstad T and Einvik C: Tumour-suppressor microRNAs let-7 and mir-101 target the proto-oncogene MYCN and inhibit cell proliferation in MYCN-amplified neuroblastoma. Br J Cancer 105: 296-303, 2011.
32. Roth SA, Knutsen E, Fiskaa T, Utnes P, Bhavsar S, Hald ØH, Løkke C, Mestdagh P, Johansen SD, Flægstad T and Einvik C: Next generation sequencing of microRNAs from isogenic neuroblastoma cell lines isolated before and after treatment. Cancer Lett 372: 128-136, 2016.
33. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
34. Thorsen SB, Obad S, Jensen NF, Stenvang J and Kauppinen S: The therapeutic potential of microRNAs in cancer. Cancer J 18: 275-284, 2012.
35. Witzel II, Koh LF and Perkins ND: Regulation of cyclin D1 gene expression. Biochem Soc Trans 38: 217-222, 2010.
36. Molenaar JJ, Ebus ME, Koster J, van Sluis P, van Noesel CJ, Versteeg R and Caron HN: Cyclin D1 and CDK4 activity contribute to the undifferentiated phenotype in neuroblastoma. Cancer Res 68: 2599-2609, 2008.
37. Molenaar JJ, van Sluis P, Boon K, Versteeg R and Caron HN: Rearrangements and increased expression of cyclin D1 (CCND1) in neuroblastoma. Genes Chromosomes Cancer 36: 242-249, 2003.
38. Sun F, Fu H, Liu Q, Tie Y, Zhu J, Xing R, Sun Z and Zheng X: Downregulation of CCND1 and CDK6 by miR-34a induces cell cycle arrest. FEBS Lett 582: 1564-1568, 2008.

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