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microRNAs: Significance for Sensitivity/Resistance of Lung Cancer Cells to Treatment

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To My Family, Madre-Ilmi, and Iqbal

ABSTRACT

Intrinsic or acquired resistance of lung cancer (LC) to chemo- or radiotherapy (CT/RT) limits the treatment effectiveness, which, in turn contributes to tumor progression and ultimately increases the mortality rate of the LC patients. Although resistance to tumor treatment is a multifactorial event for which several factors were identified, still many critical features underlying the mechanisms of resistance remain elusive. One of the main mechanisms, evasion of apoptosis, was recently shown to be modulated by miRNAs, an emerging class of important regulators of various biological processes, including apoptotic cell death that essentially contributes in CT/RT response. Thus miRNAs, as oncomiRs, might regulate anti-apoptotic protein's expression or suppress a pro-apoptotic cellular response. In the current thesis we therefore, analyzed the role of miRNAs and the core proteins involved in their biogenesis in LC cells response to CT/RT.

The paramount question we addressed was to infer whether the expression of core proteins involved in miRNA biogenesis can be associated with LC resistance to RT and if so, can we sensitize resistant LC cells to RT upon silencing of these proteins. Detailed analysis of a panel of SCLC and NSCLC cell lines revealed that the major proteins of miRNA biogenesis machinery, Drosha and Dicer, were expressed at higher levels in RT resistant LC cells as compared to RT sensitive counterparts. However, knock-down of these proteins by siRNA appeared to be insufficient to sensitize for RT. Moreover, knock-down of downstream components of miRNA biogenesis pathway, Ago-2 and TSN, did not either enhance the sensitivity of NSCLC cells to ionizing radiation. These data suggest that RT resistance in LC cells cannot be reverted by modulation of a single component of the miRNA biogenesis machinery. Next, to find out whether miRNA expression can affect RT sensitivity of LC cells, a global miRNA profiling was performed using the same panel of SCLC and NSCLC cell lines with different RT sensitivity. We observed that miRNA-214 had a higher expression in radioresistant NSCLC cells as compared to their sensitive counterparts. Considering miRNA-214 as an important modifier of LC cells radioresistance capacity, expression of this miRNA was silenced in radioresistant and overexpressed in sensitive NSCLC cells, respectively. Indeed, knock-down of miRNA-214 in radioresistant NSCLC cells increased their RT sensitivity and these cells underwent senescence after irradiation. Importantly, overexpression of miRNA-214 in radiosensitive NSCLCs protected them from RT-induced apoptosis, an effect that in part was mediated by p38MAPK as downregulation of this kinase reversed the protective response of miRNA-214 overexpression.

Finally, to determine the key modifiers of LC CT resistance, we observed that downregulation of an evolutionally conserved multifunctional protein TSN increased the NSCLC cell death response either alone or in combination with CT drugs. A higher expression of TSN was detected in NSCLC cell lines than in normal lung fibroblast cells. Gene expression profiling upon silencing of TSN revealed that TSN likely contributes to NSCLC CT resistance by regulating expression of several tumor survival genes, such as S100A11, ATP6V1F, and MDC1, and simultaneously suppressing many of pro-apoptotic genes e.g., BNIP3, DRAM1, PDCD4, BCL2L13, and LAMP2 that eventually compromise tumor ability to undergo apoptosis. Altogether this suggests a potential contribution of high TSN expression towards LC malignancy and a CT resistant phenotype.

In conclusion, in this study, we demonstrate the role of some miRNAs and the regulators of their biogenesis in LC therapy response. It is anticipated that further understanding of their functional impacts on mechanism(s) of resistance of LC cells to the current treatment modalities will generate novel therapy approaches as well as biomarkers of treatment response of this tumor malignancy.

LIST OF PUBLICATIONS

I. Surova O*, **Akbar NS***, Zhivotovsky B. **Knock-down of core proteins regulating microRNA biogenesis has no effect on sensitivity of lung cancer cells to ionizing radiation.** *PloS One, 2012; 7(3): e33134.*

II. Salim H*, **Akbar NS***, Zong D, Vaculova AH, Lewensohn R, Moshfegh A, Viktorsson K, Zhivotovsky B. **miRNA-214 modulates radiotherapy response of non-small cell lung cancer cells through regulation of p38MAPK, apoptosis and senescence.** *Br J Cancer. 2012 Oct 9; 107(8):1361-73. doi: 10.1038/bjc.2012.382. Epub 2012 Aug 28.*

III. **Akbar NS***, Surova O*, Zhivotovsky B. **Down-regulation of Tudor staphylococcal nuclease in non-small cell lung carcinoma cells enhances the effect of DNA-damaging drugs.** *Manuscript.*

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CONTENTS

LIST OF ABBREVIATIONS

ADARs	Adenosine deaminases
AIF	Apoptosis inducing factor
AML	Acute myeloid leukemia
ANT	Adenine nucleotide translocase
Apaf-1	Apoptotic protease activating factor-1
ATP	Adenosine tri-phosphate
Bad	Bcl-2 associated death promoter
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2 associated X protein
Bcl	B-cell lymphoma
Bcl-2	B-cell lymphoma 2
Bcl-XL	Bcl-2 related gene, long isoform
BH	Bcl-2 homology
Bid	BH-3 interacting-domain death agonist
Bim	Bcl-2 interacting mediator of cell death
C. elegans	Caenorhabditis elegans
Calpain	Calcium-activated neutral protease
CARD	Caspase recruitment domain
Caspase	Cysteine-dependent aspartate-specific protease
CLL	Chronic lymphocytic leukemia
dATP	Deoxy-adenosine tri-phosphate
DED	Death effector domain
DGCR8	Digeorge syndrome critical region gene 8
DIABLO	Direct IAP-binding protein with low pi
DISC	Death inducing signaling complex
DNMT	DNA methyltransferase
EGFR	Epidermal growth factor receptor
EML4-ALK	Echinoderm microtubule-associated protein-like 4 - anaplastic lymphoma kinase
EndoG	Endonuclease G
ER	Endoplasmic reticulum
FADD	Fas-associated death domain
FGF	Fibroblast growth factor
FXR1	Fragile X mental retardation syndrome-related protein 1
GW182	Glycine-tryptophan protein of 182 kDa
HDAC	Histone deacetylase
HER-1/HER-2	Human epidermal growth factor receptor- 1/2
HOTAIR	Homeobox (HOX) transcript antisense RNA
HtrA2	High temperature requirement protein A2
IAP	Inhibitor of apoptosis
IGF-1R	Insulin growth factor-1 receptor
LC	Lung cancer
lncRNAs	Long non-coding ribonucleic acids (RNAs)
miRISC	miRNA induced silencing complex
ncRNA	Non-coding RNA

NSCLC	Non-small cell lung carcinoma
Omi	Omi stress-regulated endoprotease
PACT	Protein activator of the interferon induced protein kinase
PAZ	Piwi, Argonaute and Zwille
PCI	Prophylactic cranial irradiation
PDGF	Platelet-derived growth factor
PET	Positron emission tomography
piRNA	Piwi interacting RNA
PIWI	P-element-induced wimpy testis
PRC2	Polycomb chromatin remodeling complex 2
PTEN	Phosphatase and tensin homolog
q-RT-PCR	Quantitative real time polymerase chain reaction
rasiRNA	Repeat-associated small interfering RNA
RB1	Retinoblastoma 1
RIP1	Receptor-interacting protein 1
RISC	RNA-induced silencing complex
rRNA	Ribosomal RNA
SBRT	Stereotactic body radiation therapy
SCLC	Small cell lung carcinoma
siRNA	Small interfering RNA
Smac	Second mitochondria-derived activator of caspases
sncRNA	Small non-coding RNA
snoRNA	Small nucleolar RNA
snRNA	Small nuclear RNA
TNF-R1	Tumor necrosis factor receptor1
TNM	Tumor, node, metastasis
TRADD	Tumor necrosis factor receptor associated death domain
TRAIL	TNF-related apoptosis-inducing-ligand
TRBP	TAR RNA-binding protein
tRNA	Transfer RNA
Tudor-SN (TSN)	Tudor staphylococcal nuclease
VDAC	Voltage-dependent anion channel
VEGF	Vascular endothelial growth factor
XIST	X-inactive specific transcript

1 BACKGROUND

Nature has conferred living cells an enormous versatility and autonomy that is stored inside their genomic content, and is always regulated by specialized guardians. These guardians/gatekeepers ensure a strict check on cell's genomic makeup, thereby, controlling the growth and proliferation status of any given cell to only let it survive if it possesses a correct and intact genome. Nevertheless, disruption of these controlling mechanisms might turn normal cells into cells which can replicate despite a non-intact genome and promote the transformation into a cancerous cell[1].

Cancer, which has been diagnosed annually with more than 12.7 million cases and cause of 7.6 million deaths a year, is a major concern for public health. About 200 different forms of cancer have been described and among them lung cancer (LC) is listed as one of the most frequent tumor types (1.61 million cases, 12.7% of the total) along with breast (1.38 million, 10.9% of total) and colorectal cancers (1.23 million, 9.7% of total). Besides, LC causes 1.38 million (18.2% of the total) deaths worldwide, making it the most lethal malignancy[2].

1.1 Lung Cancer

LC is based on histology divided into two main categories **small cell lung carcinomas (SCLCs)** and **non-small cell lung carcinomas (NSCLCs),** respectively[3]. The former entity, which accounts for 15-20% of all LC cases is comprised of densely-packed, round to ovoid, small sized cancerous cells with scant cytoplasmic content[4]. SCLC is an aggressive type of malignancy, characterized by its neuroendocrine differentiation that initially responds well to chemo- and radiotherapy (CT/RT). However, after initial response, cells with a CT/RT refractory phenotype evolve and proliferate quickly which then can contribute to their metastatic spread. Therefore, patients with SCLC are primarily characterized by either limited stage (LS) or extensive stage (ES) tumors[5]. LS-SCLC malignancy is confined to hemithorax i.e., one side of the chest that might involve tumor spread to regional lymph nodes and can be safely contained by a single tolerable radiation port, while an ES-SCLC describes a tumor state that has spread beyond hemithorax region, and includes malignant pleural effusion and hematogenous metastases[5]. Since LC remains an insidious disease at early stage, regrettably 2 out of 3 SCLC patients are diagnosed at ES-SCLC. These tumors are treated with combined CT regimen i.e., cisplatin and etoposide but despite that, the median survival rate of these patients still hovers around 8-12 months with a 5-years

survival rate of 1-2%. In contrast, LS-SCLC patients have an overall median survival of 18-30 months and a 5-years survival rate of 10-15%[6].

The major entity of LC is designated as NSCLC which makes about 80% of all LC cases. There are three major subtypes of NSCLC namely; adeno-, squamous- and large cell carcinomas. Adenocarcinoma with glandular appearance occurs in the outer part of the lungs and is the most common subtype of NSCLC, making up about 40% of all LC cases[7]. Adenocarcinomas are mainly linked with smoking, yet it is a frequent type of NSCLCs that can be also found in people with no-smoking history[8]. The second entity, squamous cell carcinoma (SCC), is centrally located and typically detected near the bronchus. This tumor accounts for approximately 30% of all LC cases[3]. SCC has been linked with smoking history and is less frequently observed in non-smoking population in comparison to other subtypes of NSCLC. The third subtype of NSCLC is large cell carcinoma, which comprises of a heterogeneous population of either immature or undifferentiated cells. Large cell carcinomas tend to grow quite quickly with a higher tendency of metastatic spread. With least favorable diagnosis among NSCLCs, large cell carcinoma is held responsible for 10-15% of human LC cases[9]. As NSCLCs account for the major percentage of LC, the present thesis is mainly focused towards NSCLC's entity with relatively brief investigations and discussions about SCLC.

1.1.1 Lung cancer from molecular aberrations to clinical management

Indisputably, smoking remains the principle cause of LC; however, about 10% of the cases are also detected among people without any smoking history. Nearly sixty carcinogens produced during smoking are described to hold mutagenic potential[10]. The consequential mutations contribute in LC heterogeneity, essentially by the activation of oncogenes or loss of tumor suppressor gene (TSG) functions. In NSCLC, EGFR/HER1/ERBB1, HER2/ERBB2, MYC, KRAS, MET, CCND1, CDK4, EML4-ALK fusion, and BCL-2 are the most commonly observed oncogenes that get activated[11] while various tumor suppressor genes, including p53 (80-90% of cases), RB1 (60-90% of cases) and PTEN (13% of cases) lose their proper function during LC development[10]. In NSCLC, the oncogenic activation often involves the deregulation of growth factor signaling cascades as a result of increased activation of receptor tyrosine kinases (TKs), e.g., epidermal growth factor receptor (EGFR) and insulin growth factor 1 receptor (IGF-1R). Consequently, the aberrant activation of multiple signal pathways, such as RAS/RAF/MEK (mitogen-activated and extracellular signal-regulated kinase),

PI3K (phosphatidylinositol-3-kinase)/AKT/mammalian target of rapamycin (mTOR), and STAT (signal transducer and activator of transcription) leads to the uncontrolled growth of tumor and impaired cell death signaling[11, 12]. Instantaneously, decreased DNA repair capacity, cell cycle deregulations, angiogenic potential and, importantly, evasion of cell death provide tumor with limitless replicative potential and ultimately contribute in malignant progression[13, 14]. Moreover, these molecular aberrations can also impair conventional therapy response and hence they may be used as potential targets to improve the therapy response such as EGFR and vascular endothelial growth factor (VEGF) inhibiting treatments[11].

1.1.2 Conventional Therapy in lung cancer

Currently, LC therapy involves multifaceted options of surgery, radio-, chemo- or targeted therapies that are either administered alone or as combined modalities. The selection of any treatment regimens (Table I), however, is mainly restricted to tumor histology and certain clinical stage, the important parameters to define the LC prognostic and therapeutic implications. In addition, screening of molecular aberrations is essential to offer clinicians an assortment of considerable biological agents for LC targeted therapy[5].

SCLC patients show poor prognosis, and without appropriate therapy, the median survival remains about 7-14 weeks depending on tumor stage. Principally chemotherapy has remained a better option to manage SCLC with an increase ranging from 2-3-years overall survival in LS-SCLC. More often, a combined treatment modality of cisplatin and etoposide is preferred, where cisplatin can be replaced with carboplatin, if the foremost is contraindicated[5]. In LS-SCLC, in conjunction with CT, thoracic RT can also be applied. An application of thoracic RT at initial stage has yielded an improved 5% of 2-years overall survival rate as compared to the late RT[5, 6]. Though SCLC shows an initial response to chemotherapy; however, this is the type of LC that frequently shows a rapid progression to advanced stage. Up to 60% of SCLC patients are suspected to develop brain metastases, once diagnosed with LC. In such circumstances, a prophylactic cranial irradiation (PCI) has been a promising therapy in reducing 25% of brain metastases rates and a 5% increase in 3-years overall survival with primary treatment followed by PCI management[15]. It has been also encouraging in ES-SCLC patients, where PCI application has reduced the symptomatic brain metastases incidences to about 15% as compared to 40%, observed in non-PCI-treated patients and which also have doubled the 1-year survival rate to about 30%. Surgical

resection has limited application in SCLC patients that has already reached to advanced stage at the time of diagnosis and only 10% cases are found suitable for staging thoracotomy[5].

For NSCLC staging, an anatomical TNM (Tumor, Node, Metastasis) classification system is followed. This T, N, M system can well describe an extent of tumor spread at primary site, nodal region and the degree of metastases. Hence in case of NSCLC, these descriptors designate three main prognostic and treatment groups, respectively i.e., early stage (I and II), locally advanced tumor (stage III) and metastasized tumor (stage IV)[3]. Around 25% of NSCLC patients are diagnosed at stage I, II, where surgical resection is opted as first line therapy with a favorable efficacy of 60-80% and 40-50% with stage I and stage II disease. Under unresectable conditions, radiotherapy remains as the treatment of choice being practiced in clinics. A considerable improvement of RT treatment response has been observed at stage I, II when RT is used with CT and/or PET imaging techniques. For patients with medically inoperable stage I NSCLC, a more precise variant of radiotherapy approach; Stereotactic body RT (SBRT) has been applied with an excellent local control rates of 85-96% and 3-years survival rates of more than 50% [16]. Distant relapse is among the fundamental causes leading to NSCLC patient's death at large occurring within 5 years of complete surgical resection. Thus, even when the LC appears to be constrained to lung, the overlooked micrometastases pose a major concern for clinicians[5]. An improvement of 5-15% in 5-years survival rate has been observed for patients at stage II and III- NSCLC who take adjuvant platinum-based chemotherapy after complete surgical resection. A combined modality of chemotherapy and RT either administered concurrently or sequentially, is used to manage both local and distant tumors at NSCLC stage III[17]. Cisplatin, carboplatin, or etoposide are the common chemotherapy regimens that are used during chemo-radiotherapy of NSCLCs. As second line chemotherapy, the U.S. food and drug administration (FDA) has permitted docetaxel and pemetrexed to be used for advanced stage NSCLC patients. More than 40% of NSCLCs are diagnosed at advanced/metastatic stage IV, where commonly a palliative chemotherapy is applied along with a so-called targeted therapy that comprises various biological agents.

Targeted therapy in NSCLC currently refers to two main classes of anticancer drugs i.e., monoclonal antibodies-based and small TKIs-based drugs while for SCLC treatments, unfortunately, no drug has yet been approved under targeted therapy regimens by the FDA. Currently one mAb-based drug 'bevacizumab' and two TKIs

i.e., erlotinib and crizotinib have been approved and are being employed in the clinical management of NSCLCs with certain genetical aberrations[11].

Table I. Lung cancer stages and treatment modalities

Tumor types	Stages	Treatment modalities
SCLC	LD	Combined chemotherapy/ radiotherapy +/- PCI
	ES	Palliative therapy
NSCLC	I	Surgical resection, SBRT
	II	Surgery + adjuvant chemotherapy for stage IB, IIA/B
	III	Chemotherapy + radiation therapy/adjuvant chemotherapy
	IV	Palliative therapy (combined first line therapy/single second line therapy) and targeted therapy

Signaling aberrations involving the EGFR pathway have been noticed in more than 50% of NSCLC cases, leading to the stimulation of downstream cascades including RAS/RAF/MEK, PI3K/AKT/mTOR and STAT signaling[11]. EGFR TKIs have displayed encouraging response rate of 60-80% with median progression-free survivals of 9-11 months in comparison to response rate of 10% in wild type EGFR cases[11]. Upon administration of EGFR TKIs e.g. erlotinib/ gefitinib, a robust clinical response was observed in patients with EGFR gene mutations at exons 19 than 21[18]. Erlotinib (Tarceva) is a primary EGFR targeting agent that shows a competitive binding to adenosine triphosphate pocket of EGFR and inhibits EGFR phosphorylation and downstream signaling. In addition to erlotinib, gefitinib is another first generation EGFR's TKI that has also been recommended for the treatment of NSCLC patients.

Bevacizumab, a monoclonal antibody is applied in the management of abnormal VEGF signaling[19]. VEGF is the key mediator of angiogenesis along with PDGF, FGF, and several interleukins. Bevacizumab was shown to be an effective drug for treating advanced stage NSCLCs. In particular, NSCLCs with histologic profiles other than squamous cell carcinoma have shown more promising response after bevacizumab treatment. The addition of bevacizumab, to carboplatin-paclitaxel for patients with advanced non squamous NSCLC considerably enhanced the survival

rate while no positive effect was observed after its administration with cisplatin-based treatments[20].

A persistent mitogenic signaling and malignant transformation as a result of the EML4-ALK fusion protein is found in 4-5% of NSCLC cases[21]. Crizotinib, a potent inhibitor of the ALK kinase is an approved targeted therapy to treat ALK mutation-positive NSCLCs patients[12]. With an objective response rate of about 60% and median progression-free survival of 10 months, the results achieved with crizotinib has been impressive in patients with relapsed NSCLC and in which the ALK gene rearrangement is driving the tumor.

These and many other novel small molecule inhibitors currently under clinical trials for NSCLCs have brought hope that in the near future, an increasing number of agents will be available to improve treatment outcome in this tumor malignancy.

1.2 APOPTOSIS

When normal cells enter a neoplastic state, along many tumorigenic characteristics that enable their tumor growth and metastatic dissemination[22], they also acquire a deceptive ability to evade 'programmed cell death', a barrier to restrict tumor cells uncontrolled growth[13]. Several different forms of cell death have been devised by the Nomenclature Committee on Cell Death, which more distinctly define the morphological and/or biochemical events, characteristic for each of the cell death modalities[23]. In a broader context these forms have been classified as 'atypical' and 'typical' modes of cell death in which e.g., mitotic catastrophe, anoikis, paraptosis, pyroptosis, pyronecrosis, represent atypical, whereas apoptosis, autophagy and necrosis are considered the modes of typical cell death[24, 25].

Out of these, apoptosis is the best characterized form of programmed cell death. An enigmatic phenomenon that was observed in mid of 1800 by Carl Vogt[26] but came to lime light in the middle of the 20th century. Alfred Glüksmann was the first researcher who assembled[27] and John Saunders who revealed certain instances of cell death[28]. However, R.A. Lockshin and C.M. Williams quoted the term 'programmed cell death' in 1964-66, labeling a specific sequence of events leading to cellular demise[29]. Later John Kerr, an Australian pathologist, after joining Alastair Currie's group in Edinburg, together with Andrew Wyllie coined the term 'apoptosis' in 1972, as a basic biological phenomenon with wide-ranging implications in tissue kinetics[30]. This mode of cell death is characterized by membrane blebbing, cellular shrinkage, chromatin marginalization (pyknosis), nuclear fragmentation (karyorrhexis)[23] and finally cellular

disintegration in apoptotic bodies. The word 'Apoptosis' borrowed from the Greek language, refers to 'falling or dropping of leaves'. It is a distinctive energy-dependent process that maintains normal cellular homeostasis, largely by the proficient clearance of obsolete/dysfunctional cells. Within the intact organism, an apoptotic cell is readily recognized by macrophages with integral cellular organelles and cleared out of the body, avoiding any harm to the organism. A defined pattern of morphological features readily distinguish apoptosis from pathological necrosis, a passive form of the cellular demise, preceded by accidental and unregulated cellular events. Among a multicellular entity, these events result in an intense inflammatory response within the tissue as a consequence of plasma membrane rupturing and leakage of cytoplasmic content. Apoptosis and pathological necrosis represent a specific and non-specific mode of cellular demise, respectively; however, accumulating evidences have now delineated another mode that lies tightly connected with both forms of cell death, termed as necroptosis or programmed necrosis[31]. This mechanism is orchestrated by regular and tightly controlled events, mainly indicated by the serine/threonine kinase activity of receptor-interacting protein 1 (RIP1) and RIP3, designate a physiological form of necrosis that seemingly acts as a backup of apoptosis, to eliminate the defective cells under impaired apoptotic conditions[32].

The process of apoptosis is intrinsic to each cell of the body and involves a cascade of events, regulated by appropriate apoptosis-mediating machinery. This apoptotic signaling machinery comprised of many constituents, briefly illustrated in Figure 1, guarantees the normal cellular development and homeostatic functions, regulating this endogenous suicide program[33]**.**

1.2.1 Important mediators of apoptotic signaling

A family of cysteinyl aspartate proteases – caspases[34, 35], Bcl-2-[36] and p53-[37] protein family members predominantly constitute an intricate network that substantially contributes to proper implementation of apoptosis (Figure 1). The foremost constituents, caspases, hold a dominant role in programmed cell death. Fourteen members (11 of them are found in human) of this family that include both initiator and effector enzymes have been recognized so far. All caspases are initially present as zymogens, an inactive pro-enzyme that harbors an N-terminal pro-domain (initiators hold a long N-terminal domain, while effectors have short N-terminal domains), which upon apoptotic stimuli are proteolytically cleaved. Caspase-1, -2, -4, -5, -8, -9, -10 and -12 belong to the proximal group of enzymes while caspase-3, -6, -7, -11, and -13

represent the effector or executioner group of enzymes[38, 39]. The majority of these enzymes are located in cytosol, however, certain caspases can also be found in different intracellular compartments, such as endoplasmic reticulum e.g., caspase-12[40], Golgi apparatus[41] or inside the nucleus i.e., caspase-2[42]. Caspase enzymatic function is usually determined by specificity for aspartate-residue in their substrate, while a signatory cysteine-residue located inside a conserved sequence of pentapeptide, QACRG, is assumed to be important for their catalytic activity. Although overexpression of all caspases lead to apoptosis, they also fulfill other biological activities like control of T cell proliferation, cell-cycle regulation and neural functions[43, 44].

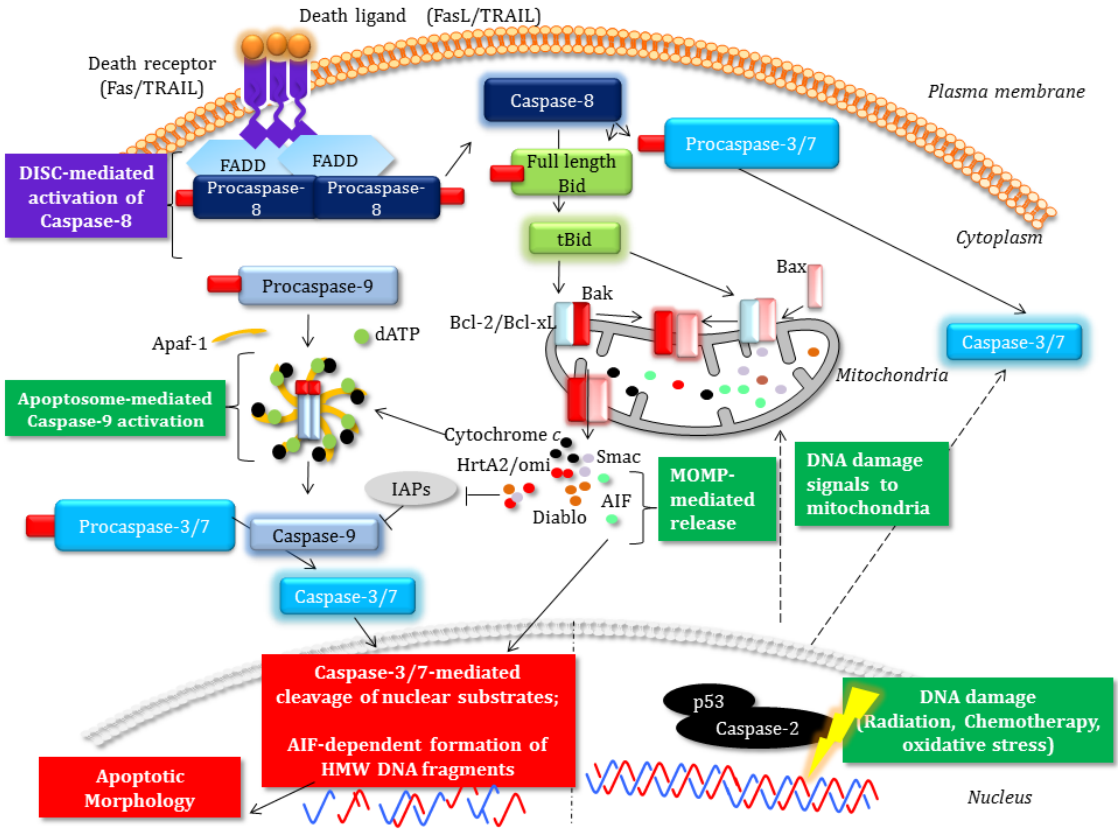

Figure 1. A general overview of apoptosis pathways

Inappropriate activation of caspases is detrimental for cellular fate. Therefore, well-controlled caspase activation is a prerequisite for an efficient apoptotic process. Proximal caspases harbor specific motifs in their long prodomains such as death effector domain (DED), found in procaspases-8 and -10 and caspase recruitment domain (CARD) in procaspases-9 and -2. These specific domains facilitate the

recruitment of procaspases to death inducing signaling complex (DISC), apoptosome or PIDDosome, followed by self-activation of these enzymes[45]. Proximal caspases usually get activated by a proteolytic cleavage and formation of heterodimers consisting of 2 long and 2 short fragments. These active enzymes later stimulate terminal or effector caspases by cleaving their short N-terminal domains. This can either be initiated as a response of signals originating from cell surface death receptors upon interaction with their respective ligands, leading to an extrinsic apoptotic reaction or as a consequence of stimuli emanating from inside of cells, triggering an intrinsic or mitochondria mediated apoptotic pathway (Figure 1). Both pathways can function independently, but a cross-talk has been observed among these cascades that ultimately amplifies the apoptotic response [46].

The extrinsic (receptor-mediated) apoptotic signaling

Extrinsic apoptotic signaling is initiated upon ligation of cell surface death receptors (DRs) with their specific ligands that subsequently induce the formation of DISC and activation of caspase-8/-10. Fas (Apo-1/CD-95), TNF receptor-1 (TNF-R1/DR1), TNF-related apoptosis-inducing-ligand **(**TRAIL) receptor R1 (DR4) and -R2 (DR5/Killer) are the major cell surface DRs that belong to the tumor necrosis factor (TNF) superfamily[45]. All members of this receptor family possess a cysteine rich extracellular domain that helps them to bind their specific ligands, following trimerization of receptors in the cytoplasmic region and subsequent recruitment of adaptor proteins like Fas-associated death domain (FADD) and tumor necrosis factor receptor associated death domain **(**TRADD). Recruitment of adaptor proteins to activated death receptors death domain (DD) constitutes DISC (Figure 1). Adaptor proteins in combination with DISC sequester procaspase-8/-10 through their DEDs. When bound to DISC, a number of procaspase-8/-10 molecules come in close proximity to each other which help in self-processing of procaspase-8 and eventually lead to the activation of caspase-8/-10. These activated initiator caspases trigger the downstream targets in a cell-specific manner; they convert procaspase-3, and -7 to active forms in Type I cells that is followed by an apoptotic cascade. In Type II cells, active caspase-8 cleaves the Bcl-2 family protein Bid and its truncated form, tBid, subsequently initiates mitochondria-mediated intrinsic apoptosis[47], an event where a cross talk takes place between receptor-mediated and mitochondrial-mediated cell death pathway.

The intrinsic (mitochondria-mediated) apoptotic signaling

Apoptotic signal emanating from inside the cell as a response to a wide range of stimuli such as oxidative stress, DNA damage, or ischemic injury can induce the non-receptor mediated intrinsic apoptotic pathway[48]. Mitochondria, besides serving as energy store-house, also contain many apoptosis-associated factors/elements[49]. Induction of mitochondrial permeability transition (MPT) and disruption of its inner transmembrane potential ($\Delta\psi$) lead to the release of several apoptogenic factors (apoptosis-promoting factors) from the intermembrane space[50] (Figure 1). Of these the most important one is cytochrome *c* which is released to the cytosol and takes part in the activation of the apoptosome complex in the presence of dATP, apoptotic protease activating factor-1 (Apaf-1) and procaspase-9[50]. Procaspase-9 dimerizes at the Apaf-1 scaffold in an energy-dependent manner and results in the activation of caspase-9, which then mediates the caspase cascade by cleaving the effector caspases-3 and -7, thereby amplifying the downstream apoptotic signaling. Cytochrome *c* is also released upon pore formation in the mitochondrial membrane by Bcl-2 family protein members Bak/Bax, either by tBid-mediated activation of Bak/Bax or oligomerization of Bak/Bax[51] (Figure 1). Many other apoptotic factors (Smac/ DIABLO (second mitochondria-derived activator of caspases/direct IAP-binding protein with low pI)), a serine protease HtrA2/Omi (high temperature requirement protein A2/stress-regulated endoprotease), apoptosis inducing factor (AIF), and endonuclease G (endoG) are released from the mitochondrial intermembrane space and effectively contribute to apoptosis signaling[50]. Family of serine proteases represented by Granzyme B also mediate apoptosis both in caspase-dependent and -independent manner[52]. Another example is calpain, a calcium activated cysteine protease that mediates apoptosis in response to elevated intracellular calcium[53], illustrating that apoptosis is executed at various organelle levels with a wide range of regulators. Among these regulators are the heat shock proteins, protein kinases, that involve in both pro- and anti-survival mechanisms and Bcl-2 family proteins[54], the essential gatekeepers of the apoptotic pathways.

Bcl-2 family proteins

A set of pro- and anti- apoptotic modulators have been identified among cells that tightly regulate cell death[54]. Bcl-2, described as an oncogene in follicular lymphoma, was the first oncogene shown to inhibit cell death rather than promoting proliferation[55]. Today, approximately 30 members of this family and associated

proteins have been identified in mammals which either can act as pro- or anti-apoptotic proteins depending on the presence of specific sequence motifs; Bcl-2 homology domains 1- 4 (i.e., BH-1-BH-4). For pro-apoptotic proteins a BH-3 domain holds a signatory value, while BH-4 domain is of key importance for anti-apoptotic family members. Pro-apoptotic proteins comprise of Bax, Bak, Bad, Bim, Noxa, Puma, etc., while Bcl-xL, Bcl-w, Mcl-1, and Bcl-2, etc., constitutes the anti-apoptotic, pro-survival Bcl-2 family proteins[36]. Furthermore, the pro-apoptotic group of Bcl-2 family proteins can be sub-categorized as Bax-subfamily comprising Bax, Bak, and Bok, containing all three domains of BH-1, BH-2, and BH-3, while BH-3-only proteins have only BH-3 domains e.g., Bid, Bim, Bad, Bilk, Bmf, Noxa, and Puma. These proteins are mainly destined either in the cytosol within some complexes, or in the mitochondria, where they positively or negatively regulate mitochondria-dependent apoptotic processes. Certain members of this family have been localized in the ER, lysosome or nuclear membrane[51].

The function of these proteins in controlling a cytochrome *c*-mediated intrinsic route to apoptosis can be defined by several non-exclusive models. Thus, BH-3 only proteins interact with cardiolipin and induce cytochrome *c* release from mitochondria[56]. BH-3 only proteins may also take part in the activation of Bak/Bax or induce mitochondrial permeabilization upon interaction with voltage-dependent anion channel (VDAC) and in this way mediate cytochrome *c* release[57, 58]. BH-3 only proteins can also interact with pro-survival proteins and silence their activity regulating pro-apoptotic response[59].

After initial interaction, Bax translocates to mitochondria and moves towards the outer mitochondrial membrane, where Bak is already residing. Eventually, Bax and Bak upon conformational changes and oligomerization are ready to induce pore formation allowing cytochrome *c* and other apoptogenic factors to be released and to propagate the pro-apoptotic effect[60]. The underlying mechanistic details of such activation, however, are yet to be fully defined.

p53 family proteins

In addition to its role as a transcription- and cell cycle regulator, p53 is involved in apoptosis induction where it participates in regulation of both the extrinsic and intrinsic pathways. p53 interaction with Bak/Bax confers conformational changes in both proteins whereas p53-dependent activation of pro-apoptotic genes Puma and Noxa are of key importance in p53-induced apoptosis[61]. p53 also participates in receptor-

mediated apoptosis by regulating the expression of Fas/Apo-1/CD-95 or DR5 receptors[62]. As a transcriptional factor, p53 has been shown to repress the transcription of survivin and anti-apoptotic protein Bcl-2[63]. A substantial role of other p53 family members (p63 and p73) has also been observed in apoptosis. p73 actively participates in the transcriptional regulation of many known p53-regulated promoters like Noxa, Puma, and Bax[64]. Elevated level of Puma was observed and Bax activation was seen among cells overexpressing p73γ[65]. p53 and p73 are not restricted to mitochondria-mediated apoptosis, but both have been shown to be involved in ER-mediated apoptosis while p63 in addition to p53-dependent apoptosis has also been reported to mediate a p53-independent apoptosis under ER stress by the activation of Puma[66]. This altogether signifies the functional diversity of these proteins in regulation of apoptosis.

1.3 NON-CODING RNAs

Delineated by the central dogma of life theory, DNA is transcribed to RNA and the message encoded in RNA is translated into the catalytic players of the cell, the proteins. About 20,000-25,000 protein-coding genes from human genome, encapsulated in 23 chromosomes, have been estimated. This number of genes only account for 1.5% of the total genome and it can be extended to 2% if untranslated regions (UTRs) are included[67]. During 1990's, the long believed simplicity of the Watson and Crick's life theory was confronted by the innovative findings about RNAs by Lee, Feinbaum, Ambrose, Ruvkun, and Reinhart[68-71]. The successive discovery of RNA interference (RNAi) then further questioned the fact that RNA is merely bridging the gap between the stable conformation of the genes 'DNA' and the catalytic players, 'the proteins'. To date, several evidences indicate that non-protein-coding regions of the genome, mainly non-coding RNAs (ncRNAs), appear to be evolved and developed alongside proteins and DNA and comprise a huge class of RNAs. These ncRNAs can, based on their size, mainly be divided into **long** and **small ncRNAs** (lncRNA/snRNA), whose importance and active role in metazoan life, from normal developmental/physiological processes to pathophysiological conditions has clearly been manifested in recent years. Some of the major types of ncRNAs have been summarized in Table II.

Long non-coding RNAs (lncRNAs) that arbitrarily comprise of > 200bp - 100Kbp in length can usually be transcribed from the intergenic stretches or even from intronic regions of protein coding sequences by RNA polymerase II (Pol II) and in some cases by RNA polymerase III (Pol III)[72]. To date, ~118 human **lncRNAs** have been annotated in database **lncRNAdb** with high probability of being expanded in the near

future[73]. Among the major subtypes of lncRNAs analyzed so far e.g., XIST[74], HOTAIR[75], Evf-2[76], T-UCRs[77] and lncRNA-p21[78] interpret an extensive functional repertoire ranging from chromatin regulation, telomere biology to gene expression management at genomic- and epi-genetics levels[79-81]. Concurrently aberrant expressions of lncRNAs have been strongly linked to various diseases ranging from neurodegeneration to cardiovascular and tumorigenesis, respectively[72, 82, 83].

The second major class of ncRNAs termed as **sncRNAs** consist of molecules with a size of ≤ 200 base pairs. To date, a high throughput sequencing technology has allowed the uncovering of the hidden layer of many such sncRNAs that have been shown to span almost all domains of life. Of all sncRNAs identified so far snRNAs, snoRNAs, piRNAs, siRNAs and miRNAs exemplify the major subtypes of sncRNAs[67, 72, 84]. In mammals thousands of piRNA sequences have been found, and these sncRNAs are characteristically produced independently of Dicer, a dsRNA-specific RNase III family endoribonuclease[67]. siRNAs or so-called small information carrying RNAs, constitute a dominant class of ncRNAs that mediate post transcriptional silencing of target genes by a naturally conserved mechanism, essentially known as RNA interference (RNAi)[85]. These small silencing RNAs are principally derived from longer dsRNAs of either exogenous or endogenous origin through a Dicer mediated processing and in close association with Argonaute-2 (Ago-2), mediate their target regulation[86]. Two distinct types of siRNA exist: exo and endo, a biological division realized after observing the diversity in their mode of biogenesis, regulations and size specificity[85, 87]. In general, the most important functions of the so far identified sncRNAs include RNA splicing, telomere maintenance, gene expression regulation and ensuring genomic integrity and stability by acting as forefront defense against transposons and viruses[81, 88, 89].

miRNAs designate the type of sncRNAs that have been studied at large in contrast to their counterparts. The size of 'miRNome' narrating miRNAs expressed in human genome is growing and nearly 2000 miRNAs have been annotated at public database miRBase by December, 2012[90]. An intensive research to elaborate their role in genomic regulations, cellular metabolism and developmental processes has already been initiated and yet far is to go to explore the functional richness and regulatory vastness provided by these tiny regulators of the genome[67, 70, 91, 92]. A detailed description of all aspects of miRNAs functions is beyond the scope of this work and hence focus will be kept on their role in carcinogenesis in particular in LC and their impact on therapeutic response of the same tumor malignancy.

Table II. Types of non-coding RNAs

ncRNAs	Characteristics/ Biological activities	References
Long ncRNAs [72, 82, 93]		
HOTAIR	A 2.2 kb long trans-acting lncRNA, localized to the nucleus and associates with chromatin to regulate gene expression mostly at epigenetics level; in cooperation with histone modifying enzymes like polycomb chromatin remodeling complex 2 (PRC2), and lysine-specific demethylase1 (lSD1) CoReST-ReST complex. Has been linked with metastasis, breast and hepatocellular carcinomas.	[67, 75, 81, 94, 95]
Xist	A 17 kb long lncRNA that mainly acts as determinant of organism's developmental fate by X-chromosome inactivation.	[81, 96]
Evf-2	Acts as cofactor and is involved in ventral forebrain and craniofacial development.	[81]
T-UCRs	Transcribed from ultra-conserved regions (UCRs), and is assumed to modulate miRNA regulation. Putatively linked with apoptosis in colon cancer cells whereas abnormal expression has been reported in CLL.	[97, 98]
lncRNA-p21	lncRNA-p21 is induced upon DNA damage and regulates the expression of various downstream targets of p53 pathways.	[78]
Small ncRNA[72]		
snRNAs	A mid-sized ncRNA containing 100-300 nucleotides, and typically found in nucleoplasm. Predominantly involves RNA splicing, telomere maintenance and regulation of many transcriptional factors. Has been sub-classified as Sm-class RNAs and LSm-class RNAs.	[88]
snoRNA	ncRNAs of 60-300 nucleotides in length that are named after their nucleolar localization. Mainly involved in ribosomal RNA (rRNA) processing and can regulate gene expression by giving rise to other regulatory RNA species, such as miRNAs. Two distinctive classes; C/D box and H/ACA RNAs are reported. Their differential expression has been linked with tumorigenesis.	[67, 88, 99]
piRNAs	Small ncRNAs with 24-30 nucleotides, distinctively generated by independently of Dicer. Designated as Piwi (P-element-induced wimpy testis) interacting RNAs (piRNAs) that are predominantly found in germline and immediately coupled somatic cells. Mainly acts as forefront defense against transposons while their deregulation has been putatively linked with carcinogenesis.	[72, 86]
siRNAs	~21-22 nucleotides small interfering RNAs, processed by Dicer and found as exo- or endo- siRNAs. In cooperation with Ago proteins involved in gene regulation, transposon control and viral defense.	[86]
miRNAs	Small ncRNAs of 19-22 nucleotides in length. Can regulate their targets in association with Ago proteins, leading either to their repression, degradation or occasionally activation. Have widely been investigated for their diverse role from physiological processes to pathophysiological conditions.	[100]

1.4 microRNAs

microRNAs (miRNAs or miRs) comprise of approximately 19-25 nucleotides in length[100, 101]. These small ncRNAs mediate translational regulation either by repression, degradation or even activation of their target genes, depending on interaction with corresponding mRNAs. It is anticipated that miRNAs take part in the regulation of approximately 60% of all protein coding genes within the human genome, thereby regulating almost all cellular processes including developmental timings, stem cell functions, cellular differentiation, proliferation, and cell death[70, 102-104]. Aberrant expression or irregular activities of miRNAs can contribute to several diseases including cardiovascular- and metabolic disorders but in addition also to tumor initiation and cancer progression[105-107].

1.4.1 Discovery of miRNA

miRNA discovery came through a screen of genes, involved in larval developmental timings. lin-4, a small ~22-nt RNA, responsible for larval development of *Caenorhabditis elegans* (*C. elegans*) was identified as a founding member of this family[69, 71]. It was observed that *lin-4* rather than coding for a protein generates a small RNA that held an antisense complementary sequence to multiple, roughly 7 conserved sites at *lin-14* gene's 3´ UTR region. *lin-14* encodes a protein named LIN-14 whose downregulation is critical for the transition from larval stage L1 to L2. To mediate LIN-14 silencing, presence of active lin-4 RNA and intact 3`UTR region of *lin-14* was found indispensable to abrogate LIN-14 expression without noticeable reduction in lin-14 mRNA levels. The discovery of lin-4-based translational repression of *lin-14* gene revealed a new phenomenon of gene regulation at developmental stages that was later established with the discovery of let-7 by G. Ruvkun and colleagues in the year 2000[70]. Let-7 translationally repressed *lin-41* and *hbl-1*(*lin-57*) by binding to their 3´UTR regions during larval development, therefore driving the progression from L4 to adult stage. Initially lin-4 and let-7 were termed as small temporal RNAs (stRNAs); however, subsequent identification of let-7 homologues in mollusks, sea urchins, fly and human genome made it obvious that these small ncRNAs are evolutionary conserved throughout metazoans. In broader perspective, they were then termed as microRNAs or miRNAs to describe precisely, this new class of small ncRNAs[108]. Although the initial set of miRNAs was identified through a forward genetics approach,

subsequent identifications by directional cloning and bioinformatics approaches cited in Table III have contributed immensely in finding new members of miRNAs[109].

miRNAs are scattered in the human genome where majority of the miRNAs originate from the independent transcription units, yet others are derived from the intronic regions of pre-mRNA. Almost half of all miRNAs are confined in clusters which are transcribed as multi-cistronic primary transcripts[110]. The current view is that miRNAs which are clustered together can target the same gene or a set of genes involved in a particular pathway thereby providing an enormous regulatory potential of the miRNome.

1.4.2 miRNA Biogenesis

miRNAs residing in intronic regions constitute 40% of all miRNAs[111] and the majority of these miRNAs share the regulatory elements with their host genes, while rest are transcribed under their own promoters. miRNA genes are commonly transcribed by RNA Pol II and in certain cases by RNA Pol III into primary transcripts (pri-miRNA) of a size of 1-kb or even more[100] (Figure 2). The pri-miRNAs are usually polyadenylated and 5´-capped, which is a characteristic of Pol II transcription. Nonetheless both RNA Pol II and Pol III recognize specific promoters and terminators that provide a breadth of regulatory options during miRNA biogenesis.

In accordance with conventional biogenesis, pri-miRNAs are endonucleolytically processed by a microprocessor complex, whose subunits are constituted by RNase III endonuclease **Drosha** (RNASEN) and its partner **DGCR8/Pasha**, a double-stranded RNA-binding protein (dsRBP)[112] (Figure 2, nuclear processing). In addition to these, certain auxiliary factors including DEAD box RNA helicases p68 (DDX5) and p72 (DDX17), heterogeneous nuclear ribonucleoproteins (hnRNPs) are assumed to provide the specificity and activity of Drosha-mediated cleavage of pri-miRNA[113]. **DGCR8** ensures the precise cleavage site at primary transcript where Drosha cleaves both strands ~11-nt away from the base of stem loop of pri-miRNA[114], leaving its typical staggered cut and producing a precursor-miRNA (pre-miRNA) transcript. This ~60-70-nt strand of pre-miRNA has 5´phosphate and 2-nt overhang at 3`end after primary processing. Then the pre-miRNA along its 3`overhang is recognized by a Ran-GTP-dependent export receptor, the **Exportin-5 (XPO-5)** which promotes the export of correctly processed pre-miRNA form the nucleus to the cytoplasm[115] (Figure 2).

Once in cytoplasm, pre-miRNAs are diced into a ~22-nt miRNA duplex by **Dicer**, a second RNase III enzyme of the miRNA biogenesis pathway[116]. Two proteins, a HIV-

1 TAR RNA-binding protein (**TRBP)** in collaboration with protein activator of the interferon-induced protein kinase **(PACT)** facilitate Dicer-dependent cleavage of pre-miRNAs[117-119] (Figure 2, cytoplasmic processing). Dicer cleaves almost two helical turns away from the base of the stem loop of pre-miRNA yielding a ~22-nt miRNA/miRNA*duplex, leaving again 5´phosphate and 3`overhang of the molecule[120]. One strand in this duplex is termed "guide strand" and the other is known as "passenger strand". Either strand is then incorporated into a large protein complex known as RNA-induced silencing complex (**RISC**), the core component of which is the Ago proteins alongside Fragile X mental retardation syndrome-related protein 1 **(FXR-1)** and Tudor staphylococcal nuclease (**Tudor SN**)[121-123]. The strand selection of any given miRNA during maturation is based on the thermodynamic stability of each strand, where the strand having less stable 5′- base pairing is selected while the other is degraded[68, 124]. Loading of the mature miRNA strand to the RISC complex also referred to as miRNA-induced silencing complex (miRISC) leads to the translational regulation of target mRNA, which is either cleaved, translationally silenced or even sometimes results in its activation, depending on the degree of sequence complementarity of the miRNA to its cognate mRNA[125].

The canonical miRNA biogenesis and processing pathway involves two RNase III enzymes, Drosha and Dicer that yield pre-miRNA. Interestingly, small RNA sequencing has revealed that about 10% of all miRNA species produced in mammals are generated via a non-canonical biogenesis route[120, 126]. One subset of such miRNA is termed 'mirtron' which after spliceosomal-excision of the introns becomes a direct target of Dicer[127]. In addition, some miRNAs are also produced independent of the spliceosome, e.g., miRNA-1225 and miRNA-1228, termed as 'simtrons' and which are characterized by bypassing the canonical miRNA biogenesis components e.g., DGCR8, Dicer, Exportin-5 or Ago-2, but require Drosha for their apical processing[126]. Also a Dicer-independent, non-canonical biogenesis has been observed, for pre-miRNA-451 maturation, which was shown to be dependent on Ago-2 endonucleolytic slicer activity alone[120, 128]. No matter how the miRNA are produced, for their target regulations, mature miRNAs from both mirtrons and simtrons have to be incorporated in the RISC to mediate their regulatory effects on potential targets.

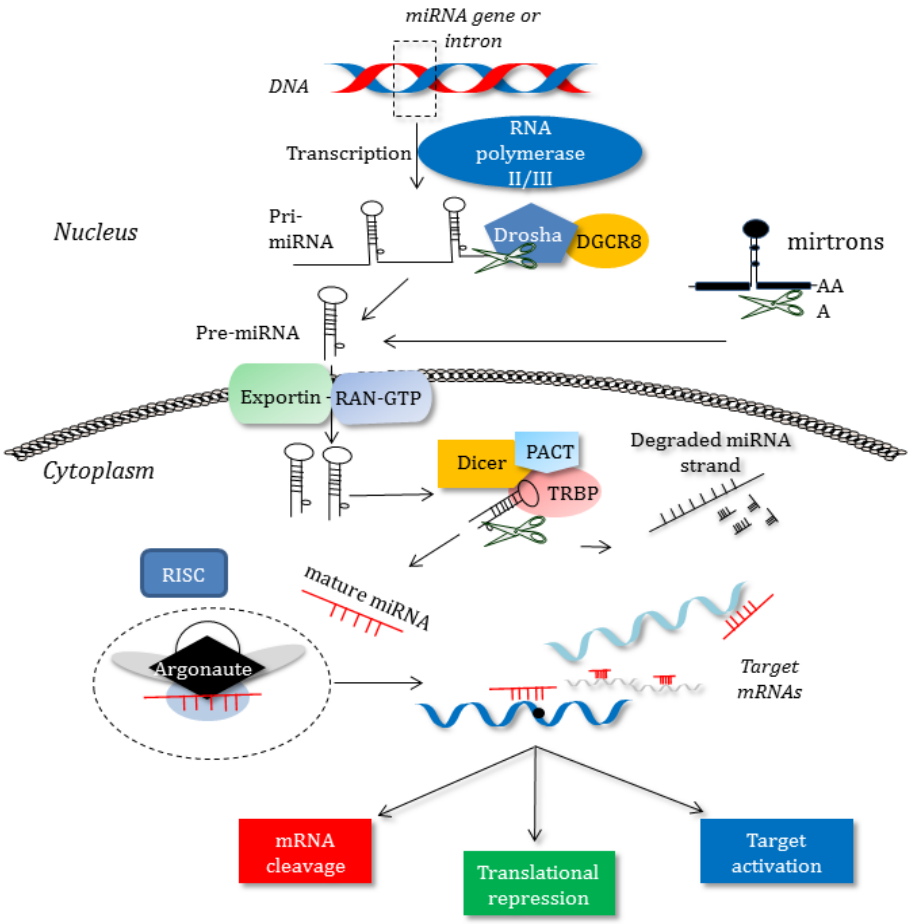

Figure 2. microRNA biogenesis and target regulation. For details see text.

1.4.3 Distinction between siRNA and miRNA

siRNAs, that facilitate RNAi phenomenon in animals and also be known as regulators of post-transcriptional gene silencing (PTGS) in plants, are twin sibling of miRNA. Apparently, it is hard to distinguish between these two non-coding RNAs on the basis of their function or chemical composition. Both are functionally compatible, being involved either in translational repression or degradation of their cognate mRNA targets. Nevertheless, a subtle distinction can be made on the basis of their biogenesis/origin or on the mechanisms through which they regulate various target genes in animals[129].

miRNAs are transcribed from distinctive loci in the genome, whereas siRNAs can originate from heterochromatic DNA, transposons and mRNAs. As described above, small hairpin transcripts from where miRNA are derived are recognized by nuclear processing machinery that usually specify the mature miRNA end product. While each miRNA primary hairpin results in a single miRNA/miRNA* duplex that yields a specific mature miRNA involved in the regulation of multiple target genes[100], siRNAs

are generally derived from long dsRNAs either of exogenous or endogenous origin[125, 130]. A large number of small siRNA duplexes are derived from both strands of each precursor siRNA molecule. In most cases, small siRNAs, with certain exceptions, are involved in the silencing of genes from the same locus of their origin, while miRNAs act broadly by silencing varied and distant genes. Remarkably, accumulated data show that miRNAs also participate in the activation of certain transcripts other than silencing or translational repression of their target mRNAs[131]. Besides, a mutation in the siRNA sequence can affect the recognition sequence of its target, whereas miRNAs exhibit a higher grade of conservation and are being rarely affected by this kind of mutation[100].

1.4.4 Enhanced miRNA functional diversity by RNA editing

RNA editing provides the functional diversity to RNA molecules by manipulating the structural properties of the transcripts, a process that characterizes miRNA processing as well. In mammals, A-to-I editing is the predominant type of RNA editing where adenosine deaminases (ADARs) reform RNA structures post-transcriptionally by modifying mainly adenosine (A) to inosine (I) through deamination. miRNAs are also targeted by ADARs which process dsRNAs and stem loop structure of pre-miRNA. miRNA-22, one of the first miRNA entity shown to undergo editing has thereafter been followed by many other molecules, like miRNA-151, miRNA-376a, and miRNA-99a, which all are the prime candidates for RNA editing[132-134]. miRNAs processing by ADARs not only determine their fate at biogenesis level but also confers target specificity, e.g., a conversion of A-to-I in pre-miRNA form of miRNA-376 directs it to different targets that eventually results in altered protein expression. Moreover, this editing also influences processing by the miRNA biogenesis proteins. Thus, enhanced processing of miRNA by Drosha mainly associates with the edited form even though the opposite is also reported as in the case of pri-miRNA-142, where a conversion skips Drosha processing and makes it available to another ribonuclease TSN, that ultimately degrades the miRNA[132, 133]. Another pri-miRNA-151 avoids Dicer-mediated cleavage as editing affects its interaction with the Dicer-TRBP complex[134]. In conclusion, miRNA editing not only increases the diversity but also adds up another processing control in the accurate regulation of miRNAs[133].

1.4.5 Target recognition and regulation by miRNAs

The most important task in understanding the function of miRNAs is to comprehend how these ncRNAs recognize and then mediate their target regulation. To resolve this

issue, several newly developed extensive genome-based approaches like ultraviolet crosslinking/immunoprecipitation (CLIP)[135, 136] has provided a clue to recognize miRNA binding sites while methods like ribosomes profiling[137, 138] has helped in defining gene regulatory patterns adopted by miRNAs. Primarily miRNAs regulate the transcriptome by exercising the effect on cognate mRNAs[139]. This regulation, except for a handful evidences of target activation[131, 140], generally leads to either target cleavage/degradation, or translational repression. Thus, miRNAs are assumed to be stable transcripts which in mammals usually regulate their target mRNA by interacting with the 3`UTR region of the mRNA. In miRNAs, a noteworthy feature is the presence of a 7-nt 'seed sequence' between 2-8 nucleotides at 5`end of the miRNA[141] that in principal regulates target mRNA at 3`UTR. However, in certain cases, a characteristic base pairing at 3` becomes insignificant when coupling with seed sequence turns to be insufficient to suppress target mRNAs[142]. Hence, further regulation is then endowed with a supplementary mode of target regulation by bulges and certain defined mismatches in the miRNA/miRNA duplex along the 'centered sites'[143].

Repression of protein translation at various stages also highlights the concealed potential held by miRNAs to regulate their targets. Thus, miRNAs either terminate the translation initiation step or even obstruct ribosomal assembly formation, the latter, which is required for proper translation into a functional protein, indicating that miRNAs can add up a further regulatory step in their target processing as well[129].

miRNAs regulate their targets in close cooperation with RISC and the proteins associated to this complex which indeed are quite important for the regulatory functions of miRNAs. The two most important proteins in this respect, Ago and GW182 (glycine (G)-tryptophan (W) repeats containing protein with 182 kDa MW), in combination with several other auxiliary proteins hence assist miRNA-induced regulation of target sequences[129, 144].

Ago proteins are characterized by two homologous domains, PAZ (Piwi, Argonaute and Zwille) and PIWI[145]. The PIWI domain has been shown to specifically bind to the 5´end of small RNA, whereas the PAZ domain mainly targets the 3`end of ssRNAs. In mammals, four Ago proteins (Ago-1, Ago-2, Ago-3 and Ago-4) have been characterized, which have redundant functions and in a cell type dependent manner are of importance in miRNA repression of their targets[145]. Among them, Ago-2 is largely considered as a major contributor in miRNA-mediated silencing or degradation. Heat shock protein (HSP) 90, as a major regulator of Ago proteins, ensures their proper stabilization, localization and functionality in various cells and, thereby, indirectly

influences the regulation of miRNA target repression[146]. GW182 is the second major protein that contributes to the miRISCs repressive function. It has been demonstrated that GW182 proteins offer a docking platform by which deadenylase complexes get access to the poly(A) tail of miRNA targets, thereby stimulating deadenylation[147]. After deadenylation, miRNAs mainly undermine their target mRNAs by a proper compartmentalization and sequestration in processing bodies (called as p-bodies)[148], a site for mRNA degradation. Several other miRISCs-interacting proteins, e.g., a family of DexD/H RNA helicases[149], RBPs[150], and nuclease TSN, besides Ago and GW182, function as accessory proteins in miRNA-mediated mRNA regulation.

miRNA-dependent regulation is not always ending up in translational repression, as studies have indicated that miRNAs may also cause activation of their targets, a property exemplified by miRNA-10 which activates ribosomal protein translation after binding its mRNA's 5′ UTR[131]. Similarly, miRNA-122 in hepatitis C virus (HCV) is shown to be involved in activation of its specific targets[151, 152]. Beside these miRNAs, the complex 'Ago-2-miRISCs' itself has been found to shift from a repressor to an activator of transcription under serum deprivation[140].

1.4.6 Tools and resources applied to identify miRNA targets

A blend of complementary approaches from genetic[70] to computational[153], and biochemical[154, 155] methods have been applied to define and recognize specific miRNA target sequences.

Initially, loss-of-function phenotype during genetic screening of particular miRNAs made it possible to detect certain miRNA target genes. As stated above, Lin-41, for instance, was recognized among a particular set of let-7 targets after mutating *let-7* in *C. elegans*. Concomitant appearance of specific phenotype unlocked a promising window to physiologically identify relevant target genes at that time; however, defining a direct or indirect target of a particular miRNA by this approach was more difficult. Later algorithmic-based *in silico* approaches (cited in Table III), such as miRanda and TargetScan, were adopted to identify candidate mRNAs. With certain exceptions, requirement of 5´Watson and Crick base pairing between miRNA and mRNA, consideration of seed sequence and accessibility to AU-rich content at 3´ UTR has largely augmented miRNA target recognition sites[156, 157].

A number of online sources have accumulated the validated targets of miRNA (miRecords, Tarbase)[158, 159]. To track down putative target elements, various target prediction tools like TargetScan/TargetScanS[141, 160], MiRDB[161], TargetMiner[162],

Microcosm, microrna.org[163] and PicTar[164] are available and which in most cases can be accessed online.

Concisely, **TargetScan** is the most widely used target prediction tool. To classify a particular target of certain miRNAs this tool uses an algorithm that looks for the presence of conserved 8-mer and 7-mer sites that match the seed region of each miRNA[141]. Moreover, a conserved 3' pairing is taken into account, in case if mismatches are located in the seed region. **PicTar**, also considers evolutionary conservation of target sequence along perfect or imperfect complementarity with 7-nt seed sequence[164]. However, strict complementarity at miRNA seed sequence is demanded by the **Microcosm** to consider particular mRNA as a putative target of miRNA. Microcosm applies miRanda algorithm[163] to identify potential targets and then finally a mRNA is considered to be a target of a particular miRNA, if at least one conservation criterion among two species is fulfilled.

Table III. Major miRNA databases/target prediction tools.
A comprehensive list is available at; http://www.ncrna.org/KnowledgeBase

Databases/ Target prediction tools	Organisms included	Web site	References
mirBase	human, mouse, worm, fly, plant	http://microrna.sanger.ac.uk/	[165]
miRNA Map	human, rat, mouse, other metazoan	http://mirnamap.mbc.nctu.edu.tw	[166]
Tarbase	human, rat, mouse, worm, fly, plant	http://www.diana.pcbi.upenn.edu/tarbase.html	[167]
TargetScan, TargetScanS	human, mouse, worm, zebrafish, fly	http://genes.mit.edu/targetscan/	[141, 160]
miRTar	human	http://mirtar.mbc.nctu.edu.tw/html/predictV3.html	[168]
DIANA-microT	human, rat, mouse, worm, fly, plant	http://diana.pcbi.upenn.edu/DIANA-microT/	[169]
miRanda	human, zebrafish, fly	http://www.microrna.org/	[163]
PicTar	human, mouse, worm, fly	http://pictar.bio.nyu.edu/	[164]
RNAhybrid	mammals	http://bibiserv.techfak.unibielefeld.de/rnahybrid/	[170]
miRDB (MirTarget2)	human, dog, chicken, rat, mouse	http://mirdb.org/miRDB	[161]
PITA	human, mouse, worm, fly	http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html	[171]
RNA22	human, mouse, worm, fly	http://cbcsrv.watson.ibm.com/rna22.html	[172]

Diana-microT also combines conserved and non-conserved miRNA recognition elements and considers many other parameters, defined individually for each miRNA[169]. **PITA** mainly focuses on structural accessibility of the binding site without much considering the conservation among these sites in order to identify genome-wide miRNA targets[171].

The access to high-throughput techniques including those referred above might allow a wide-range of miRNA targets to be identified but at the same time the use of these different tools has met with number of discrepancies due to the different set of rules adopted by each algorithm. Likewise, high false-positive/negative ratio strongly stresses the need of an adaptive algorithm that can encompass miRNA interaction with targets genes under a broader set of rules.

Although, the identification of predicted miRNA targets has been robust after applying *in silico* approaches including those cited in Table III, the major challenge faced is the target validation, which requires the biochemical techniques. A reporter gene assay fused to target 3´UTR is considered as the major follow-up approach to certify the mounting quantity of miRNA projected targets after *in silico* analysis. However, natural regulatory settings are somehow compromised by this approach as miRNA regulation of target genes in genetic and natural cellular environment plausibly differ from the *in vitro* settings. Recently, more comprehensive approaches, using both biochemical and bioinformatics applications, has made it possible to identify not only mRNA target transcripts but also target sequences along mRNA at large scale. A co-immunoprecipitation technique CLIP along with high-throughput sequencing (CLIP-seq) is applied for this purpose to identify endogenous target sites bound within the miRISCs[135]. Thereafter, next-generation sequencing of target sites further provides a nucleotide level resolution of target sequences. The CLIP technique is carried out using UV light exposure, which enables crosslinking and stabilizes the protein-RNA complex. After immunoprecipitation this complex is readily separated from the unbound sequences and subjected to deep-sequencing and bioinformatics analysis. A modified version of this approach is also practiced by the addition of a photo-activatable ribonucleoside analogue (like 4-thiouridine) in the culturing conditions (PAR-CLIP)[135]. This induces a single-nucleotide mutation at the site of the crosslink, which can be used as a location marker of successful crosslinking[173, 174]. A transcriptome profiling by either microarray or RNA sequencing approach like stable isotope labeling with amino acids in culture (SILAC) has indeed provided a

great potential to validate and certify the CLIP data in the context of mRNA and protein regulations[154, 175].

1.4.7 miRNA Nomenclature

To establish a nomenclature system for miRNAs, a precise annotation criterion is charted to recognize a definite miRNA from a particular species [108]. Thus, a three letter prefix 'miR' that is followed by a unique identifying number with dash in between them, e.g. miR-1, miR-2, . . . miR-214, etc. is used. The uncapitalized 'mir' refers to pre-miRNA, while 'miR' denotes a mature entity. miRNAs with one or two nucleotides difference are denoted with additional lower case letters, such as miR-181a and miR-181b[135] while miRNAs that end up as identical but originate from different loci hold an additional dashed number suffix, like miR-194-1 and miR-194-2. Two mature miRNAs originating from opposite arms of the same pre-miRNA are denoted with a -3p or -5p suffix. Species prefix is added at the very beginning to delineate miRNAs so that **'hsa'** suffix will be added for miRNAs of human origin while '**mmu**' will be a prefix for miRNAs of mouse origin. The genes encoding these miRNAs are also named with identical three-letter prefix, capitalization, hyphenation, and italics according to the specific conventions for particular organism. The numeric categorization is done sequentially, with identical miRNAs having the same number, irrespective of the organism[90, 108].

1.4.8 Methods to decipher miRNA-mediated target regulation

miRNA-mediated target regulation can be detected either at mRNA or protein level, and for this purpose various methods have been chosen to assess the ultimate effect.

As miRNAs normally carry translational regulation by targeting mRNAs, a compromised mRNA level can be a possible consequence of miRNA regulations. The most systematic way to assess the mRNA expression level is q-RT-PCR (quantitative real-time polymerase chain reaction)[176, 177]. It is an invaluable and highly sensitive tool to assess the miRNA effect at the mRNA level; however, a customary method; northern blot analysis can as well be applied to quantify mRNA expression levels. For genome-wide studies of changes in mRNA abundance upon miRNA up- or- downregulation, microarrays and RNA sequencing may also be used to perform the detailed analysis at a global scale. miRNAs may carry deadenylation of their target mRNAs without much affecting the mRNA levels. In addition of probing mRNA levels, poly (A) tail-length analysis by using RNase H cleavage of the mRNA 3′ ends can be performed[178]. Then,

a follow up analysis by northern blot or PCR-based methods is applied to compare adenylation status which can ultimately provide quantitative estimate of miRNA regulation. Overall, these approaches can confirm whether or not the anticipated target of a miRNA is undergoing regulation at mRNA level.

As miRNA may regulate the entire genome at various levels, absence of any detectable changes at mRNA level does not guarantee that mRNA can avoid miRNA-mediated regulation since considerable effect might be observed at protein level rather than at mRNA level.

Generally, the impact of miRNA target regulation turns out in altered protein expression. The most straight forward approach to assess this is by using western blot, a routine laboratory method applied to determine the changes in protein levels. Western blot can however not provide detailed information regarding the mechanism of translational regulation. In that case, a large number of proteins can be subjected to mass spectrometry to get an overview of sporadic protein levels after miRNA regulations.

Furthermore, for comprehensive information about proteins that appear at lower abundance a more sensitive method is required. Polysome profiling is such a method that is applied to study the efficiency of mRNA translation *in vivo*[179]. Likewise with high sequence resolution, ribosome profiling[137, 180] delivers an ancillary method for assaying protein production. This method helps in determining a ratio between specific mRNAs and respective proteins, providing an approximate measure of gene expression at the translational level. Moreover, ribosome profiling can reveal the physical location site of certain mRNAs inside the ribosomes, so that an accumulation of ribosomes at the 5′ end of any mRNA can indicate stalled translational initiation. Thus, ribosome profiling is perceived as a prevailing approach in determining the individual mRNAs regulatory mechanism by miRNA.

1.4.9 The role of miRNAs in health and disease

miRNAs dynamically contribute to cellular fates in both physiological homeostasis as well as in pathological conditions[103]. Since their discovery miRNAs have now been recognized as important players in development, stem cell and organ differentiation[104, 181, 182]. Instantaneously, their disrupted role in various systems e.g., neurodegenerative[183], metabolic[184], and cardiovascular[185] functioning has been discerned (Table IV). Besides, their contribution to tumorigenesis has been elucidated in depth in a large number of studies[91].

Table IV. Involvement of miRNAs in different processes or diseases apart from tumor malignancies.
A comprehensive information can be accessed at; http://www.mir2disease.org/

Process/Disease	Functions	Implicated miRNAs	References
Cell/ Tissue Development and differentiation	Embryonic development and differentiation, skin differentiation, hind limb development, adipogenesis, lung development, and pulmonary hemostasis	Let-7, miRNA-203, miRNA-196, miRNA-2, miRNA-143, miRNA-155, miRNA-21	[186-189]
Metabolism	Di-, tri-acylglycerol regulation, cholesterol metabolism, insulin secretion, glucose homeostasis, and pancreatic β-cell development/differentiation	miRNA-124, miRNA-33a/b, miRNA-103, miRNA-107, miRNA-122, miRNA-34a, miRNA-14, miRNA-375, miRNA-204, miRNA-141	[106, 190]
Cardiovascular	Cardiac- homeostasis, -hypertrophy, -myopathy, -oxidative stress, -ischemia/ reperfusion (I/R), and vascular remodeling	miRNA-1, miRNA-2, miRNA-33, miRNA-320, miRNA-155, miRNA-24, miRNA-208, miRNA-29, miRNA-21, miRNA-221/222	[191-193]
Immune and neuronal systems	Immune response, B cell and myloed differentiation, neuronal development and dendritic spine formation, Huntington's disease, Schizophrenia, Parkinson's and Alzheimer's diseases	miRNA-1, miRNA-155, miRNA-150, miRNA-223, miRNA-17-92, miRNA-9, miRNA-24, miRNA-200, miRNA-10, miRNA-33, miRNA-146, miRNA-29, miRNA-107, miRNA-132	[183, 189, 192, 194, 195]

1.4.10 The role of miRNAs in tumorigenesis and tumor cell signaling

miRNAs have certainly been associated with tumorigenesis[91, 196, 197]. Anomalous miRNA expression and/or mutations both at somatic-and germ line levels have been

related to several processes that are hallmarks of cancer including from increased proliferation to the loss of cell death capacity as illustrated by Figure 3[102]. Interestingly, nearly 50% of all annotated miRNAs have been found in or close proximity of fragile sites in the human genome and common breakpoints which are linked to tumor development[198]. Accordingly, dysregulation of miRNAs expression has been observed in many types of tumors, including Burkitt's lymphoma, glioblastoma, skin-, colorectal-, prostate-, breast- and also lung tumors[91, 199]. Hence, studies have highlighted their role both as oncomiRs or as tumor suppressors[107]. Some of the oncomiRs or tumor suppressors associated specifically with LC have been shown in Figure 4.

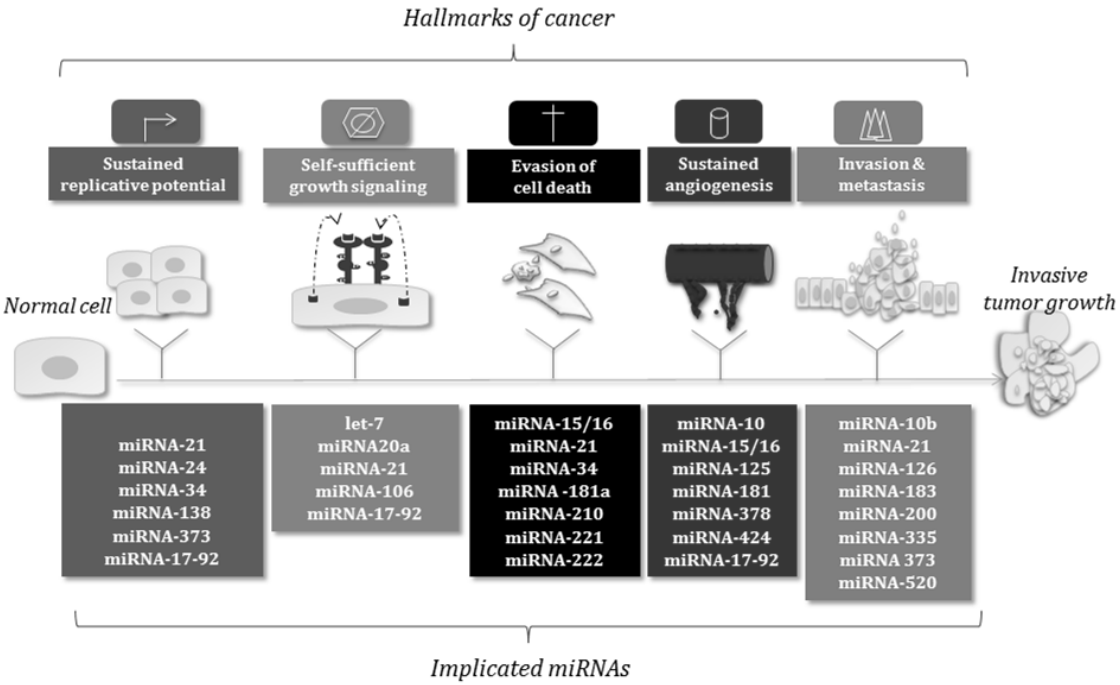

Figure 3. A link between miRNAs and different hallmarks of cancer[200-202]

miRNAs as OncomiRs

A number of miRNAs, known as oncomiRs, have been reported to possess intrinsic oncogenic properties or to directly regulate one or several oncogenes. The most well-known example is the miRNA-17-92 cluster, which was termed as oncomiR-1 and comprises six miRNAs: miRNA-17, miRNA-18a, miRNA-19a, miRNA-20a, miRNA-19b-1, and miRNA-92-1[203]. This cluster is believed to directly influence c-*myc*, an oncogene whose function is deregulated in multiple tumors. Accordingly, multiple members of the miRNA-17-92 cluster are highly expressed in a variety of solid tumors

and hematological malignancies, including cancers of the breast, colon, lung, pancreas, prostate, and stomach as well as in lymphomas[204]. Indeed miRNA-17-92 participates in the control of tumor proliferation, apoptosis inhibition, tumor angiogenesis, and cooperates with *c-myc* to cause lymphomas in mice[203]. miRNA-155 is another oncomiR which is found in the B cell integration cluster (*BIC*)[205], and which in cooperation with *c-myc* is involved in oncogenesis. Higher expression of miRNA-155 has been reported in many cancers e.g. lung and breast cancer, CLL (Chronic lymphocytic leukemia) and AML (acute myeloid leukemia)[206, 207].

miRNA-31 holds oncogenic properties and downregulation of this miRNA repressed LC cell's clonal growth and *in vivo* tumorigenicity in mice. A number of tumor suppressor genes, such as LATS2 and PPP2R2A are reported as putative targets of this miRNA[208].

In parallel, miRNA-21's role as oncogene has been reported and upregulation of this miRNA has been detected in many hematological malignancies, as well as solid tumors including NSCLCs[206, 207]. In fact, two well-known tumor suppressors that control tumor cell growth and proliferation have been validated to be targets of miRNA-21, namely PTEN in NSCLC[209] and PDCD4 (programmed cell death) in breast and colon carcinomas, respectively[207]. Moreover, miRNA-21 has also been observed to be an anti-apoptotic miRNA in glioblastoma[207]. Thus, overexpression of miRNA-21 led to apoptosis inhibition, while upon silencing of this miRNA an increased cell death and caspase activation were observed[207]. In addition augmented expression of miRNA-21 was associated with undifferentiated, prometastatic potential in mouse and human tumors characterized by p53 mutations and in which induction of epithelial-mesenchymal transition (EMT) was instrumental[210].

Yet several other miRNAs including miRNA-10b[211], miRNA-141, miRNA-200s and miRNA-429 have been linked with tumor metastasis[212]. Augmented expression of miRNA-221 and miRNA-222 has been detected in different types of human malignancies in which they have been implicated in the regulation of cell growth and cell cycle progression by targeting p27[213] and p57, respectively[214]. A recent report has shown that these two miRNAs also suppressed apoptosis by targeting the pro-apoptotic gene PUMA in human glioma cells, positively contributing to survival and progression of the tumor[212]. Besides their overexpression in NSCLC cell lines, miRNA-221/222 have been demonstrated to play a role in resistance to TRAIL and to enhance migration through activation of the AKT signaling pathway[212]. Though

further *in vivo* validation is required but it's becoming increasingly clear that miRNA-221/222 are frequently upregulated in several solid tumors, including LC.

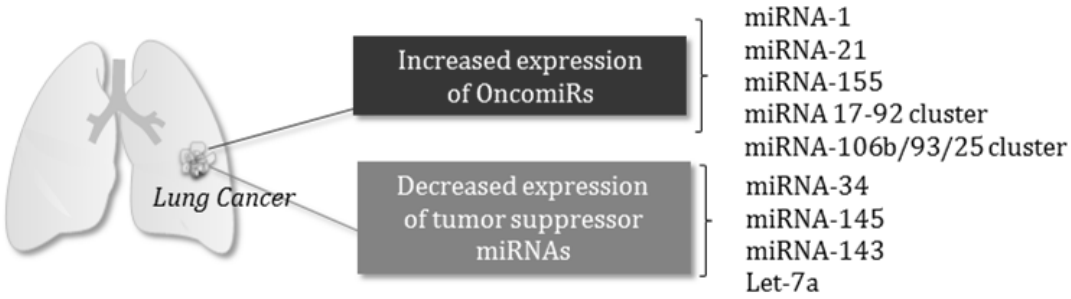

Figure 4. Several oncomiRs and tumor suppressors are associated with lung cancer

miRNAs with tumor suppressing capacity

A number of miRNAs have been found to hold tumor suppressor properties. Hence, absence of their functions as a consequence of either genomic deletions/mutations, epigenetic silencing, and/or miRNA machinery alterations, ultimately promote tumor development.

Let-7 levels have been reported to be very low in several cancers, including LC. It has been documented that many of the let-7 family members are located in fragile genomic regions which are lost in lung, breast, and cervical tumor development[206, 215]. A significantly shortened survival of LC after surgical-resection was associated with reduced expression level of let-7, while its induced overexpression in lung adenocarcinoma A549 cell line decreased tumor cell growth[216]. Similarly, in other tumors including colon and Burkitt's lymphoma cells, let-7 overexpression was found to induce apoptosis and cell cycle arrest[217]. Several well-characterized oncogenes, such as the *Ras* family , *HMGA2* [207], and *c-myc*[218] have been proposed as candidate targets of the miRNAs of let-7 family.

A tumor suppressor function of the miRNA-34 family (miRNA-34a, miRNA-34b and miRNA-34c) became apparent when restoring miRNA-34 levels in neuroblastoma cell lines led to reduced cells proliferation and induction of caspase-dependent apoptotic cell death[219]. A study has shown that the abundance of the miRNA-34 family is directly regulated by p53 and restoration of this miRNA in NSCLC resulted in LC

cell's growth inhibition. Several targets of this miRNA have been discovered including BCL-2, BIR3, CCNE2, CDK4, CDK6, DCR3 and E2F3[207]. miRNA-15a and miRNA-16-1 cluster targets *Bcl-2* oncogene and interrupt tumorigenesis. This cluster is frequently deleted or downregulated in CLL when compared to normal CD5+ lymphocytes from healthy tissues. Moreover, miRNA-15/16 in close synergy with miRNA-34 was shown to prompt cell cycle arrest in a RB-dependent fashion in NSCLC cells[212].

The miRNA-29 family, comprised of three isoforms is arranged in two clusters: miRNA-29b-1/miRNA-29a on chromosome 7q32 and miRNA-29b-2/miRNA-29c on chromosome 1q23. Interestingly, chromosome 7q32 is a frequent region of deletion in myelodysplasia and AML[220]. In fact, miRNA-29 family members have been shown to be downregulated in CLL, LC, invasive breast cancer, and AML[206, 221, 222]. In LCs, expression of miRNA-29 is inversely correlated with the level of two DNA methyltransferases named DNMT3A and DNMT3B[127]. Accordingly, forced overexpression of this miRNA has been shown to induce apoptosis and reduced tumorigenicity in a LC xenograft model[223, 224].

Two other miRNA, miRNA-143 and miRNA-145 are downregulated in colon cancer and upon re-establishing miRNA-143 expression, a higher growth inhibitory potential was observed with inhibition of ERK5[225]. miRNA-145 has also been shown to regulate cell cycle and cause G1 arrest by targeting CDK4 in LCs, while partial inhibition of MUC1 by this miRNA lead to decrease in LC metastasis[212].

A number of studies have reported that miRNAs can play a crucial role in promoting metastatic progression of different malignancies. A fundamental route for the metastases development is EMT which enables tumor cells to lose their cell-to cell contact, invade the surrounding tissue and enter the circulation. A number of miRNAs which suppress EMT and metastatic process have recently been identified as exemplified by the miRNAs belonging to the miRNA-200 family, e.g. miRNA-200a, miRNA-200b, miRNA-429, miRNA-200c, and miRNA-141[212]. This family together with miR-205 inhibits EMT through targeting ZEB1 and ZEB2[97]. In LCs, miRNA-200c overexpression reduced ZEB1 expression and repressed the transcriptional target of ZEB1, E-cadherin, in A549 cells. Studies in mouse models of lung adenocarcinoma have further confirmed the involvement of miRNA-200 family members in the regulation of EMT and in the modulation of pulmonary metastatic potential of LC cells. Apart from the miRNA-200 family[226], several other miRNAs

have been identified with metastasis-regulating potential, such as miRNA-126, miRNA-429, miRNA-335, miRNA-373 and miRNA-520c [227].

miRNA and epigenetic regulation of tumors

In the genome, the intronic region from where miRNAs are transcribed, also contain CpG islands. Since hypermethylation of CpG island in promoter regions has been shown to trigger carcinogenesis by the transcriptional silencing of tumor suppressor genes, it was anticipated that miRNAs might also be epigenetically controlled. In fact, miRNA-124a in various human cancer cell types was shown to be affected by transcriptional inactivation through CpG island hypermethylation[228]. In another study, an epigenetic control of miRNA expression was observed in breast cancer cell lines, as silencing of histone deacetylase (HDAC) expression resulted in altered expression levels of about 30 different miRNAs including let-7[229]. While a combinatorial treatment with DNA-demethylating agent and HDAC inhibitors raised the expression level of 17 out of 313 miRNAs by >3-fold in human bladder cancer cells[230]. A cross-talk between miRNAs and epigenetic control is also evident as miRNAs in turn regulate the expression of proteins that epigenetically repress certain genes like *DNMT, HDAC, HMGA2* and *PcG* [231-233].

Yet another way to control miRNA expression, which is used by tumors, is hypermethylation of their promoters. Thus, hypermethylation of miRNA-9 loci is observed in various malignant tumors, including gastric-[234], breast-[235] and several other cancers and is associated with metastasis[236].

1.4.11 miRNAs as therapeutic targets and their clinical implications

miRNAs have an altered expression in tumors in comparison to normal tissues and their expression can be linked to clinico-biological features of the neoplastic tissues. miRNAs have, therefore, attracted interest as potential therapeutic targets. Their higher stability in tissues and fluids samples, their detection range in a given amount of clinical materials even in highly degradable samples poses an advantage over mRNAs and makes them more suitable to be used as predictors of diagnostic/ prognostic values of malignant tumors.

Analysis of sputum cytology has been used for the diagnosis of LC. It was shown that miRNA-21 was highly expressed in sputum specimens of cancer patients as compared to healthy counterparts. Moreover, elevated levels of miRNA-21 provided 70% sensitivity and 100% specificity in the diagnosis of LC through sputum

cytology[237]. Further, miRNA-21 along with miRNA-205 was found to accurately differentiate adenocarcinomas from different SCC subtypes. miRNAs expression profile have also been successfully employed to distinguish primary lung tumors from metastasized tumors. Thus, miRNA-182 overexpression was found to be specific for primary lung tumors while elevated miRNA-126 expression was frequently associated with metastatic tumors[212], illustrating that miRNAs expression might indeed be helpful for diagnostic purposes. miRNAs have also been considered promising prognostic factors. The higher expression levels of miRNA-155, let-7, and miRNA-221 have been associated with tumor protective tendency while high levels of miRNA-137, miRNA-372, and miRNA-182 have been shown to be associated with a worse clinical outcome. Moreover, reduced let-7 expression levels in human LCs have been associated with decreased postoperative survival, whereas similar alterations of miRNA-34a in combination with p53 mutations were associated with a high probability of tumor relapse[237]. Furthermore, low expression of miRNA-143 was correlated with smoking status and higher expression of miRNA-21 and lower expression of miRNA-181a were associated with poor survival, independently from TNM staging and lymph node status[237].

Given that certain miRNAs influence tumor treatment responses one may likely also use miRNA expression profiles for patient treatment stratification. For instance, miRNA-21 expression was significantly higher in LC with EGFR mutations from smokers compared with non-smokers, and increased expression of let-7a, miRNA-126 and miRNA-145 enhanced the cytotoxicity induced by gefitinib in LC cells, suggesting that miRNAs hold an increased potential in elevating the targeted therapy response that can be administered along the approved biological agents in treating LC[237].

Importantly, miRNAs can regulate multiple genes which can in turn simultaneously be regulated by many different miRNAs. Therefore altering miRNA expression for therapeutic purposes might concomitantly impair several tumor cell aberrations at the same time[238, 239]. Thus, targeting or restoring a specific miRNA might even impair the function of a complete oncogenic pathway within tumors[240]. Some pioneering groups in specialized pharmaceutical companies have already begun their quest for the identification of valid therapeutic candidates. Consequently, a number of mimetics and antagomirs has already been formulated and efficient antagonizing potential has been attained by using complex technologies such as locked–nucleic acid modified oligonucleotide (LNA-antimiR)[241, 242]. miRNA-122, as a lead discovery in miRNA

based therapeutics has set the stage for being targeted in chronic HCV-infection. It has already entered in clinical phase II studies and is being perceived to have a significant impact on the discovery of miRNA based future therapeutics[243]. In miRNA diagnostics, a test, 'miRInform Pancreas LDT' (laboratory based developed test), has been devised to detect differential level of miRNA-196 and miRNA-217, which can help in distinguishing chronic pancreatitis from pancreatic ductal adenocarcinomas in formalin-fixed-paraffin-embedded specimens[244]. While for the LC, two assays namely 'miRview squamous' and 'miRview lung' were described recently. The first assay, based on differential expression levels of miRNA-205, has allowed to distinguish between squamous- and non-squamous LCs with higher degree of sensitivity whereas the latter has been instrumental in classifying the four type of LCs i.e., squamous-, non-squamous NSCLC, carcinoid, and small cell carcinoma with overall accuracy of 94% among 90% of samples[9, 245].

Thus, it is strongly anticipated that miRNAs will soon find a way to clinics either as biomarkers or tumor targets by virtue of novel therapeutic approaches. However, an imminent research to devise proper formulation and precise delivery to cancer cells is highly demanded to avoid unwanted miRNA effects on genes in healthy tissues[246-253].

1.5 INTRODCUTION TO CURRENT STUDY

LC RT/CT resistance on molecular level is a major obstacle that hampers the successful treatment response and is counted as a prominent factor in tumor recurrence and cancer related mortalities. Resistance can be either intrinsic or acquired and is often multifactorial. In NSCLCs resistance is mainly intrinsic and due to the presence of some *de novo* signaling aberrations the treatment outcome is impaired while SCLCs though initially show treatment responsiveness, gradually acquire a resistant phenotype which is considered to be the main contributor in SCLC treatment futility. Careful selection and conjunction of available chemo- and radiotherapies along targeted modalities can improve considerably tumor response rates; however, the majority of patients are susceptible to develop progressive disease even after showing initial response to treatment. Therefore, understanding the core mechanisms involved in tumor resistance is critical in order to achieve a comprehensive control against lung tumor.

Disparate mechanisms are known to be involved in CT/RT resistance including genetical aberrations that alter CT target expression, detoxification systems, increased DNA damage repair capacity as well as evasion of cell death.

The principal therapeutic effect of CT/RT is the formation of different DNA damages which in turn trigger the endogenous suicidal program in tumor cells. In solid tumors, including LC, an increased potential of avoiding programmed cell death (apoptosis) remains the fundamental obstacle to treat cancer effectively. Significant progress has been made to identify apoptotic pathways which are altered in tumor cells, unfortunately, malfunction in apoptotic pathways appear to be more complex than ever thought before. Thus, more than 200 genes are known or suspected to be involved in the regulation of apoptosis, and their deregulated expression is a major hurdle to properly execute this physiological mode of cell death in tumor cells upon CT/RT. Indeed, in LC a number of defects in apoptotic signaling pathways have been reported. Thus, in SCLC loss of expression of procaspase-8, an essential regulator of receptor-mediated signaling, was reported as a result of promoter methylation[254]. Importantly, re-establishment of procaspase-8 expression by methylation impairment re-sensitized cells to CT-induced apoptosis. Similarly, failure in intrinsic apoptotic singling mainly by Bcl-2 family proteins abnormal functions either by increased activity of prosurvival proteins or compromised function of pro-apoptotic proteins likely influence LC propensity to CT/RT-induced apoptosis. A correct conformation of Bak and Bax is critical for the proper execution of apoptosis upon death-inducing

stimuli. In fact, a failure of Bak and Bax to undergo conformation changes was observed in RT resistant LC cells and contributed to their failure of executing RT-induced apoptosis[255, 256]. Similarly, increased signaling from growth factor regulated kinases e.g., AKT and ERK was demonstrated to impair RT-induced apoptosis in NSCLC and inhibition of these kinases indeed was demonstrated to restore a proper apoptotic response[255, 257].

1.5.1 miRNAs as apoptomirs and their role in lung cancer therapeutic efficacy

miRNAs have recently gained immense scientific interests as regulators of a variety of oncogenic signaling cascades whereby tumor cells maintain growth, proliferation, invasion but also avoids apoptosis induction. The involvement of miRNAs in regulation of apoptosis was first reported in 2003[258, 259] when miRNA-14 and *bantam* were shown to regulate cell death in Drosophila. *Bantam*, originally identified as a gene that causes overgrowth of wing and eye tissue, was demonstrated to be a miRNA and was shown to promote proliferation and inhibit apoptosis by targeting the pro-apoptotic gene *hid1*[259]. Similarly, miRNA-14, which was identified in a screen for genes that alter cell death in the Drosophila eye, was demonstrated to inhibit cell death by regulating the effector caspase, Drice, essential for execution of apoptosis in Drosophila[258] . To date, miRNAs have been shown to affect one or simultaneously multiple mRNAs controlling apoptotic signaling for example, miRNA-1 and miRNA-133 produce opposing effects on apoptosis induced by oxidative stress in H9c2 rat ventricular cells; with miRNA-1 being pro-apoptotic and miRNA-133 acting as anti-apoptotic[260]. Post-transcriptional repression of HSP60 and HSP70 by miRNA-1 as well as miRNA-133-dependent inactivation of caspase-9 was shown to be responsible for their opposing actions. It has been observed that miRNA-1 can also achieve its pro-apoptotic function by the regulation of Bcl-2 expression at both mRNA and protein levels as well[261, 262]. Moreover, computer-based sequence analysis revealed that 3′UTR of caspase-3 contains the putative recognition sequence for let-7a as evidently an ectopic let-7a expression resulted in the deceased caspase-3 activity, without effecting caspase-8 or -9[262]. Consistently, it has become obvious that decreased expression of pro-apoptotic miRNAs and/or increased expression of anti-apoptotic miRNAs in human tumors could be associated with a high apoptosis threshold. miRNA-29 has been reported to target two anti-apoptotic molecules Mcl-1 and Bcl-2. This miRNA is frequently downregulated in LC[206]. Moreover, overexpression of miRNA-29b was

demonstrated to make tumor cells more sensitive to TRAIL, suggesting that miRNA-29b/Mcl-1 interaction is extremely important and could serve therapy sensitizing purposes[223]. On the other hand, overexpression of miRNA-221 and miRNA-222 in TRAIL-sensitive cells increased resistance to TRAIL-induced cell death by 40% and also weakened caspase-3 and -8 activation, demonstrating that multiple miRNAs likely determine the apoptotic propensity of tumor cells to TRAIL[263] and their affiliation with the apoptotic machinery.

The role for miRNAs in CT resistance has been studied, whereas their function in regulating LC tumor resistance is less well characterized[264]. Yet in an *in vitro* study Galluzzi et al.,[265] demonstrated that miRNA-630 inhibits p53-regulated pro-apoptotic signaling response after cisplatin and carboplatin treatment. A chemoresistant phenotype along with tumor invasiveness in NSCLC cells was observed with loss of miRNA-200c expression and restoring its expression re-sensitized NSCLC cells to both cisplatin and EGFR ablative therapy i.e., cetuximab[266]. Similarly, miRNA-1 was linked to anti-apoptotic signaling in LC cells in which ablation of its expression was accompanied with increased doxorubicin-induced apoptosis involving proper activation of caspase-3, -7, PARP-1 cleavage and reduced expression of the anti-apoptotic protein Mcl-1[267]. Similarly, miRNA-17-92 cluster, miRNA-147 and miRNA-574-5p have been documented to contribute to chemotherapeutic response of LC[268].

A few miRNAs have so far been demonstrated to regulate RT resistance of LC. Thus, ectopic miRNA-101 was reported to regulate two components of the cellular DNA damage and repair response i.e., ATM and the DNA-PK catalytic subunit (DNA-PKcs) and in this way modulates tumor response to radiation both *in vitro* and *in vivo*[269]. Another study conducted on LC cells demonstrated that miRNA-20a expression was up-regulated after radiation and dramatically inhibited clonogenic survival, suggesting this miRNA as a potential candidate for regulating RT-induced cell death[270].

In summary, this all illustrates that miRNAs have now become vital players in carcinogenesis and therapy response. For the aforementioned reasons and since by the time of our current project to highlight miRNA's putative role in LC radio- and chemoresistance commenced, very few information was available about miRNAs role in tumor's therapeutic response in general and, particularly, in LC. Therefore, to achieve an ultimate therapeutic efficacy of RT/CT in LC, the current investigations were performed. Our study **in paper II,** along existing information has shown the feasibility of using these small ncRNAs to alter resistance to anticancer therapy. A

higher therapeutic efficacy is promised with miRNA therapy since each miRNA affects multiple targets and potentially allows us to correct the entire pathway.

1.5.2 miRNA biogenesis-related core proteins and their role in apoptotic signaling propensity

Albeit miRNAs have been linked with altered apoptotic propensity of tumor cells, inferring a link between the core proteins involved in their biogenesis and apoptosis thresholds within tumor cells have yet only partly been elucidated. Nevertheless, their aberrant expression has been demonstrated in various tumor types[271-273].

Thus, high expression levels of Drosha, a principal enzyme of the miRNA biogenesis pathway, were observed in basal cell skin carcinomas (BCC) and SCC of skin, cervical, and esophagus[274-277]. In esophagus SCC, abnormal Drosha expression caused enhanced cells motility, invasion and metastasis with adverse survival. Moreover, the major RNAIII enzyme, Dicer, involved in miRNA maturation, was found deregulated along with Drosha in triple-negative breast cancers, where Drosha mRNA levels were found significantly higher than those in the surrounding normal breast tissues[278]. An increased expression of Dicer was detected in head and neck squamous cell carcinoma cell lines. Subsequent analysis revealed that Dicer overexpression was correlated with let-7b and an increased cell proliferation rate[279]. Different expression of Dicer, Drosha and major RISC component Ago both at mRNA and protein level was observed at distinct metastatic sites in ovarian carcinoma, in which an increased level of Ago-2 mRNA in pre-chemotherapy tumor lesions was closely related to shorter progression-free survival[280].

Similarly, a link between anomalous Dicer expression and poor prognosis, survival and reduced progression-free survival has been shown in prostate and colorectal carcinomas[281]. Also LC gene expression array data demonstrate an upregulation of Dicer along with another accessory component of miRNA biogenesis, PACT, in bronchioloalveolar carcinoma (BAC) and adenocarcinoma while the rest of other genes encoding miRNA machinery proteins were downregulated[271]. However, another study has shown that Dicer along with SND1, PACT, and FXR1 had a higher expression in lung SCC cells as compared to adenocarcinoma cells[271].

A role of Dicer in therapy resistance has also become evident. Enriched Dicer ERa-positive MCF-7 human breast cancer cells showed a concomitant increase in the expression of breast cancer resistance protein (BCRP) while elevated levels of Dicer were seen to contribute in resistance to tamoxifen treatments in tumor xenograft

cells[282]. As a response to the DNA damaging agent, cisplatin, silencing of Dicer resulted in remarkable G1 arrest and increased sensitivity in MCF-7 cells[283]. In various carcinomas TSN has also been implicated in tumor resistance and progression. It has been found as a strong candidate protein that interacts with metadherin (MTDH) and promotes LC metastasis[284]. Silencing of Ago-2 was found to induce apoptosis in myeloid leukemia cells (HL-60)[285], while downregulation of Dicer, Drosha, or XPO-5 reduced cell proliferation and induced apoptosis in bladder urothelial carcinomas [286]. Yet, the role of these proteins in LC therapy response is not known but was addressed in the current study (**papers I and III).**

The Present Study

Albeit analyses of the LC signalome have generated targeted therapy approaches which in some LC patients improved outcome; CT/RT still is of major importance for clinical management of LC patients. Unfortunately, intrinsic or acquired resistance to CT/RT remains a clinical obstacle. Even after decades of deciphering the underlying mechanisms which have demonstrated a role for increased growth factor receptor signaling, deregulated DNA repair capacity and impaired cell death pathways, a complete picture still remains elusive. This project aims to understand signaling aberrations that contribute to LC CT/RT resistance, focusing on miRNAs and the proteins involved in their biogenesis and their relation to RT/CT-induced cell death signaling.

Specific aims of this study are:

1. To evaluate the expression of core proteins responsible for miRNA biogenesis in a panel of small- and non-small cell lung carcinomas and their impact on tumor sensitivity to chemo- or radiotherapy.

2. To analyze and validate the expression of miRNAs in a panel of small- and non-small cell lung carcinomas in relation to radiotherapy sensitivity and delineate their mechanism of action with respect to RT-induced cell death signaling.

2 MATERIALS AND METHODS

Comprehensive description of all the materials and methods employed throughout this study has been listed in **papers I - III** constituting the thesis. A brief description of materials and methods and the rationale for applying them is given below.

2.1 Mammalian cell cultures

In the present thesis, a panel of SCLC and NSCLC cell lines of different histologies as well as normal human lung fibroblasts was used[287-292]. The NSCLC and SCLC cell lines (achieved from ATCC or a gift from Uppsala University, Uppsala, Sweden) has previously been demonstrated to have different radiotherapy sensitivity as illustrated by their different surviving fraction at 2 Gy (SF2) in clonogenic survival assay. In all the papers the NSCLC cell lines U-1810 (**papers I-III**), A549, and H661 (**papers I and III**) were taken as models of RT/CT-resistant LC cells, while the H23 cell line was considered a representative of RT/CT-sensitive cell lines. All cell lines, except human lung fibroblasts, were grown in RPMI-1640 medium and supplemented with 10% (v/v) of heat-inactivated fetal bovine serum (FBS), L-glutamine (2mM), penicillin (100 U/ml) and streptomycin (100µg/ml). For human lung fibroblasts DMEM was supplemented with pyruvate and MEM-non essential amino acids, in addition to the reagents as stated above. During experiments cells were maintained at 37^{o}C with 95% humidity and 5% CO_2 supply.

2.2 Irradiation and chemotherapy treatments

In **paper I and II,** cells were irradiated by 8 Gy using a Co^{60} source either at room temperature or on ice. In **paper III,** chemotherapeutic drugs which induces different types of DNA damages were applied including the DNA crosslinking, platinum-compound cisplatin, and an inhibitor of topoisomerase-I, camptothecin.

2.3 siRNA transfections

siRNA methodology has been recognized as an imperative experimental strategy to efficiently knock-down mRNA expression of a particular gene and hence abolish protein expression, thereby enabling elucidation of its function. In **papers I** and **III,** cells were transfected with siRNA against Drosha, Dicer-1, Ago-2, and TSN, whereas scramble siRNA was used as negative control. 25-50 nM siRNA was used for 24 or 48 hours along with Dharmafect-1 transfection reagent from Dharmacon. **In paper II,**

miRNA-214 expression was downregulated in NSCLC U-1810 cells by miRNA-214 antagomir, whereas in NSCLC H23 cells miRNA-214 mimics were consumed to re-establish miRNA-214 expression. In **paper II,** p38MAPK-alpha was silenced using custom designed siRNA obtained from Dharmacon. For all experiments, transfection efficiency was confirmed by analyzing the targets sequence expression using q-RT-PCR or western blot.

2.4 Microarray analysis of miRNA and mRNA expression

In **paper II,** a miRNA expression profiling of five SCLC and six NSCLC cell lines with different SF2 values was carried out using Affymetrix miRNA microarray platform. The miRNA Array genechip used consisted of 46,228 different probes comprising 7,815 probe sets. Total RNA was extracted from cells using Trizol Reagent and labeled with Biotin through FlashTag^{tm}Biotin RNA Labeling kit. The target RNA was subjected to microarray hybridizations and the resulting hybridization signal of biotin binding to a streptavidin-PE conjugate from the bound miRNA was detected with an Affymetrix scanner. Subsequently for array analysis, miRNA QC Tool (Affymetrix Inc. USA) software was applied and a p-value was taken as major parameter to consider miRNA expression. miRNAs with p-value of < 0.06 were considered as expressed, whereas the rest were designated as under-expressed miRNAs.

In **paper III,** a gene array was performed on the Agilent platform. TSN was silenced in the NSCLC cell line A549 and the influence of this on gene expression, consequentially, an altered gene expression profile was assessed through Agilent SurePrint G3 Human Gene Expression 8x60K Microarray Kit (Design ID 028004). This platform is equipped with 27,958 target gene RNAs and 7,419 lincRNAs, respectively. In these experiments four biological replicates of A549 cells transfected with mock siRNA and siRNA against TSN were executed. The arrays were scanned on Agilent Microarray Scanner (G2565BA, Agilent Technologies) and analysis was performed as defined by Agilent Feature Extraction software 9.5.3.1 (Agilent Technologies) and Gene Spring GX12.0.2 (Agilent Technologies) software. Transcripts with an average cut-off value of 2.0-fold deviation were considered as TSN-regulated genes.

2.5 Validation of microarray and miRNA results

In order to confirm the miRNA and mRNA results, q-RT-PCR was used to monitor the amount of amplicon generated in real time as the reaction proceeds. In this method the initial amount of template DNA is considered as inversely proportional to a parameter measured for each reaction, the threshold cycle (Ct) value [293].

TaqMan® MicroRNA Assay, based on Taq-polymerase 5' nuclease activity, and fluorophore-based detection assay were applied. These assays were selected since they can detect only specific miRNA targets with high sensitivity (within the range of 1-10 ng of total RNA) and with high specificity and only detects mature miRNAs. For these assays RNA was isolated either though TRIzol reagent or by a Pure Link™ RNA Mini Kit. cDNA was synthesized using miRNA specific primers and TaqMan MicroRNA Reverse Transcription Kit. U6 small RNA expression was used as loading control for the different samples. Using miRNA specific assays and control U6 snRNA assay, q-RT-PCR was performed on the ABI 7900 HT Thermal cycler in standard mode for 40 cycles. All assays were performed in triplicate. Relative miRNA expression values (Target miRNA vs. U6) were calculated with the $2^{-(\Delta\Delta Ct)}$ method [293].

In order to access the mRNA levels and validate the gene expression results, q-RT-PCR based on SYBR Green detection method was applied in all papers constituting the thesis. This method is a flexible and sensitive way to detect mRNA expression levels. Reverse-transcribed cDNAs from the samples were used as templates and target gene's mRNA expression was compared with the 18S mRNA/β-actin levels to correct for loading differences among the samples. The expression results were calculated with the $2^{-(\Delta\Delta Ct)}$ formula[293].

2.6 Target prediction and ingenuity pathways analysis

To find possible targets of shortlisted candidate miRNAs/genes, various system biology tools were implemented in the different papers of the thesis. In **paper II,** several miRNA target prediction databases/registries were used to sort out putative miRNA targets i.e., miRBase predicted mRNA targets of miRNAs (http://www.mirbase.org/), Targetscan (http://www.targetscan.org/), microrna.org (http://www.microrna.org/microrna/home.do), microcosm (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) and PicTar (http://pictar.mdc-berlin.de/).

Among the putative targets for miRNA-214 in **paper II,** only those candidates were considered which overlapped by at least three of databases, mentioned above and

thereafter candidates being involved in cell death related pathways were chosen for further analysis. Similarly, in **paper III,** the transcripts under TSN regulation, being implemented in apoptosis, autophagy, and Ca^{2+} signaling were picked for subsequent studies. To find the closely related networks of targeted genes/proteins and possible protein-protein interactions, Ingenuity Pathway Analysis (IPA) (©2011 Ingenuity Systems Inc., CA, USA) along KEGG pathways were applied in **papers II** and **III,** respectively. Various pathways and networks were generated with the help of the IPA database. The resulting networks enabled the identification of candidate genes and corresponding proteins for biological validation.

2.7 Evaluation of apoptosis

In all papers different methods were applied to assess apoptotic cell death which was induced as a consequence of various DNA damaging treatments. Cells undergoing apoptosis exhibit typical features like cellular shrinkage, chromatin condensation and DNA fragmentation. These features can be visualized under fluorescence microscopy after nuclei staining with DNA-specific fluorochromes. In order to differentiate viable cells from those having condensed nuclei (**paper II**), 4, 6′-diamidino-2-phenylindole dihydrochloride (DAPI) staining was used. Cells nuclei, which showed chromatin condensation and DNA fragmentation, were considered as apoptotic and the percentage of cells with apoptotic nuclei among 200 cells examined was quantified.

Caspase activity is an important measurement of apoptosis induced by different stimuli. In **papers I and III,** a fluorometric assay was performed to measure caspase-3-like activity in which DEVD-AMC, a synthetic substrate conjugated with fluorochrome 7-amino-4-methylcoumarin (AMC), was applied in a 96-well plate. The cleavage rate of the fluorogenic peptide substrate was monitored by AMC liberation in a Fluoroscan II plate reader using 355-nm excitation and 460-nm emission wavelengths and obtained results were recalculated and corrected to the amount of total protein in each sample. Expression of caspase-3, - 9 in active and pro-forms as well as its target proteins were examined using western blot analysis in **papers I** and **III**.

Antibody-based caspase-3 activity was measured from a fraction of ethanol-fixed cells after irradiation treatment by flow cytometry **(Paper II)**. Following washing steps, digitonin-permeabilized cells were incubated at room temperature in dark with mild agitation with FITC-conjugated antibody recognizing active caspase-3 (BD Pharmingen, Franklin Lakes, NJ, USA) for 1 hour. The amount of active caspase-3 was quantified in the FL1 channel on a FACS-Calibur flow cytometer (BD Biosciences,

San Jose, CA, USA). Ten thousand cells were analyzed for each sample with the Cell Quest Program and the percentage of cells with active caspase-3 was taken as readout and, subsequently, compared with scrambled (non-targeting) and transfected samples with or without irradiation.

2.8 Evaluation of senescence

In **Paper II,** the fraction of U-1810 cells exhibiting a senescent morphology was ascertained by measuring the β-galactosidase activity using a histochemical method. Cells were post miRNA transfection and irradiation washed, fixed and stained overnight at 37^{o}C. Cells showing β-galactosidase activity were recognized by the appearance of blue color and counted under a light microscope. A total of 100 cells from various fields of the plates were taken into account to get the final percentage of senescent cells from both non-targeting (NT) and miRNA-214 antagomir-treated samples before and after irradiation.

2.9 Analysis of cell cycle distribution and subG1 population

Fractions of cell population with different DNA content as a consequence of applied treatments can be evaluated by flow cytometric analysis using Propidium Iodide (PI). This dye, which intercalates into DNA base pairs, is membrane impermeant and normally is excluded by viable cells. Upon binding to double stranded DNA it produces highly fluorescent adducts that can be excited at 488 nm with a broad emission around 600nm wavelength. PI-staining was used to assess the cell population in subG1 and also in G1, S and G2M phases of the cell cycle by FACS following different treatments after siRNA manipulation of varied cellular components in **papers I and III**. Degraded DNA in apoptotic cells was denoted by subG1 population on DNA histogram. Cells for these analyses were fixed in 70% ethanol and after an overnight incubation at -20^{o}C, subjected to RNA degradation using RNase A. Finally, cells were stained with PI for 30 minutes and subjected to analysis on a FACScan cytometer and the data were processed with the Cell Quest software. In **paper II,** for cell cycle analysis the distribution of cells in different phases of the cell cycle (G1, S and G2/M) was measured on a FACS Calibur cytometer (BD Biosciences, San Jose, CA, USA) and calculated with ModFitLT V2.0 software (Verity Software House Inc., Topsham, ME, USA).

2.10 Western blot analyses

A conventional analytical technique, western blot, was used for the immunodetection of proteins, in the different papers of this thesis. Following harvesting, cells were lysed with either urea buffer (6M urea, 200mM ammonium bicarbonate, 2% SDS) or complete lysis-M reagent (Roche Diagnostic GmbH, Mannheim, Germany) containing protease inhibitor cocktail. Amount of protein in the resulting lysates were quantified with BCA protein assay and then samples were mixed with gel loading Laemmli buffer. Proteins were separated on SDS-PAGE gels and were subsequently transferred to nitrocellulose membranes by electro-blotting. Thereafter membranes were rinsed and blocked with 5% BSA/dry milk and probed with primary antibodies for overnight incubation. In **papers I** and **III**, horseradish peroxidase-labeled anti-mouse or anti-rabbit secondary antibodies were applied to raise a signal which was obtained on X-ray films following chemiluminescence while additionally in **paper II**, an Odyssey system was also applied to obtain a signal for desired proteins. Here Alexafluor 680- or Alexafluor 800-labeled secondary antibodies were used to probe primary antibody labeled membranes. Then membranes were scanned on Odyssey scanner and an evolved signal was analyzed by an Odyssey Sa-infrared imaging system application software (version 1.0.12). Image J software (http://rsbweb.nih.gov/ij/) was used to compare equal protein loadings.

3 RESULTS AND DISCUSSION

3.1 PAPER I:

Knock-down of core proteins regulating microRNA biogenesis has no effect on sensitivity of lung cancer cells to ionizing radiation

The apoptotic machinery normally controls cellular proliferation and homeostasis. It is, therefore, not surprising that deregulation of apoptotic signaling circuits is one of the hallmarks of cancers. Such deregulation of apoptosis not only offers a growth advantage of tumor cells but also greatly influences the outcome of CT/RT response since apoptosis at least in part is responsible for the execution of cell death. Hence, identification of candidate genes or molecular pathways in tumor cells that are critical for the induction of proper apoptosis signaling during CT/RT may reveal ways of sensitizing for CT/RT as well as identify biomarkers of response to these therapies. In **paper I**, therefore, we aimed to investigate the role of core proteins responsible for miRNA biogenesis in the efficacy of RT-induced apoptosis induction in LC cell lines. Although a role of Drosha, XPO-5, Dicer1, PACT, TSN, Ago-2 and FXR1 have been demonstrated in the development and progression of several other tumors e.g., lung, hepatocellular, skin, colon, esophagus, prostate and ovarian carcinomas[271-275, 280, 284, 286], their influence on RT response of LC remained elusive.

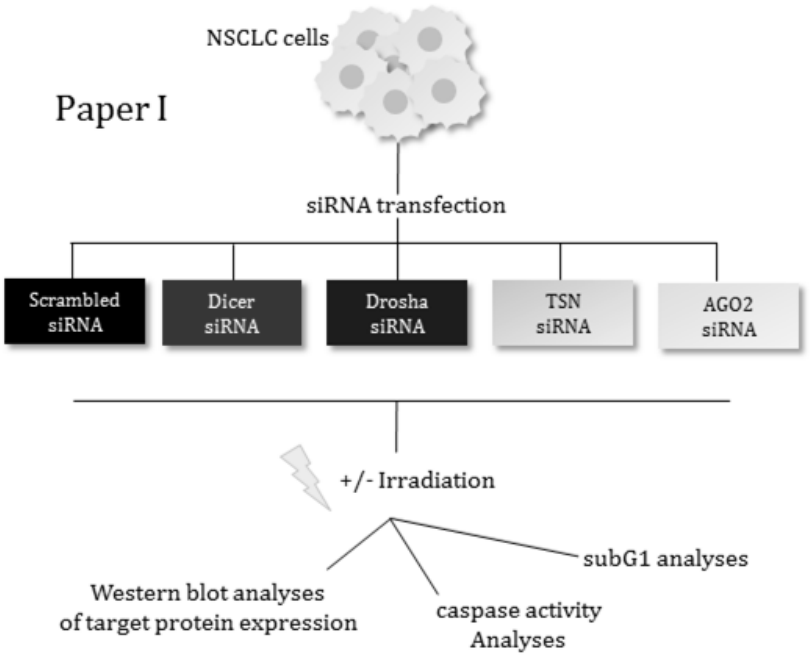

Figure 5. An experimental strategy used in paper I

A panel of human SCLC cell lines (H69, H82, U-1285, U-1690, U-1906, U-2020) and NSCLC cell lines (H23, H157, H661, A549, H1299, U-1810) was selected to analyze the expression of above-mentioned proteins and their impact on tumor resistance/sensitivity towards radiotherapy (Figure 5). The cell lines were divided into RT resistant (RR) and sensitive (RS) groups according to their surviving fraction 2 Gy (SF2) values in a clonogenic assays and were regarded as RR if SF2 ≥ 0.3 and RS with SF2 < 0.3.

Both the RNase III enzymes Drosha and Dicer were expressed at higher levels within the NSCLC cell lines as compared to SCLC cell lines. A member of the karyopherins protein family, XPO-5, which is involved in the nuclear export of miRNAs, was expressed at a higher level in H661, while lower expression levels of this protein were detected in H69 and U-1690 cells in comparison to the rest of cell lines. The expression level of TSN, PACT, FXR1 and Ago-2 proteins did not show profound differences in expression levels among all the cell lines analyzed, with the exception of H69 that exhibited lower expression of all miRNA core biogenesis proteins analyzed.

The cell line panel used consisted of both RS and RR cells, but for the subsequent analysis H23 cell line was taken as a representative of the RS cells, whereas U-1810 and H661 cells were considered as model RR cell lines. The fact that the core proteins Drosha and Dicer were expressed at higher levels in RR cells in contrast to their RS counterparts strongly suggested that an elimination of either of these two proteins might reduce miRNA production which in turn could affect RT responsiveness. In order to address this, we efficiently downregulated Dicer by siRNA approach and validated that silencing of this protein was accompanied with the reduced levels of several miRNAs such as miRNA-1301, miRNA-1249, miRNA-1227, miRNA-532-3p, miRNA-625, miRNA-1827, miRNA-324-5p. Surprisingly, the cellular response to RT-induced apoptosis, as assessed by the cleavage of PARP, caspase-3 and -9 into apoptotic active forms, and accumulation of cells in subG1, another indicator of cell death, was not profoundly different in U-1810 and H661 cells with or without expression of Dicer. Similarly, downregulation of Drosha did not sensitize resistant NSCLC cells to irradiation. It is clear that miRNAs carry out their target regulation in close cooperation with the RISC complex as they become more stable once they enter into this effector complex[115, 294-296]. Next, we therefore explored the possibility of modulating the RT sensitivity and resistance of NSCLC cells by depleting the cells of two of the main RISC-associated proteins, Ago-2 and TSN respectively. Indeed siRNA effectively

knocked-down the expression of either Ago-2 or TSN, however, abrogation of their expression did not either enhance the sensitivity of NSCLC cells to radiation. All in all these results led us to conclude that the magnitude of decrease in miRNA production and activity caused by the elimination of a single protein from a miRNA pathway is not sufficient to affect the mechanisms responsible for the RT resistance of NSCLC cells. It should be noted that analysis of the protein expression of all core components of the miRNA machinery in U-1810 cells performed after downregulation of Dicer, Drosha, TSN or Ago-2 revealed that neither of their knock-down had a significant effect on the expression of other proteins in the pathway but redundancy in their activity might have still enabled them to maintain a RT resistant phenotype.

The heterogeneity of Dicer and/or Drosha expression in tumors other than LC is evident and has been associated with different outcomes[274, 286]. For example, upregulation of Drosha expression has not only been observed in various cancers (skin, breast and cervical SCC)[274, 277, 278] but stringently, irrespective of tumor stage, been linked with metastasis and adverse survival of esophagus SCC. Dicer is also proposed to hold both tumor suppressor and oncogenic properties. Augmented expression level of Dicer has been associated with poor survival, enhanced cell proliferation and the development of peripheral lung adenocarcinomas[271]. It is thus perceived that within each histological entity of RR U-1810 and H661 and RS H23 NSCLC cell lines, a heterogeneous expression of Drosha, Dicer might be a consequence of tumor cells different genetic make-up. Moreover, inspite the fact that Dicer knock-down in various tumors has increased sensitivity to drugs[282, 283], it is likely that the sensitivity of tumor cells to impairment of Dicer expression can be a tumor- and/or cell type specific function. A most recent study has in fact supported this idea[297] as it was demonstrated that the genetic ablation of Dicer1 in murine sarcoma cells impaired but did not prevent tumor formation. Thus, indeed Dicer1$^{-/-}$ cells were without recovery of miRNA processing able to survive, proliferate, and form tumors in mice as well. This strongly suggests, that although siRNA-mediated silencing of Drosha and Dicer significantly reduced miRNA expression, it may not have led to the complete loss of expression of miRNAs critical for the RT protective effects in NSCLC cells. Moreover, the presence of a non-canonical pathway for miRNA biogenesis can also be suspected to contribute in miRNAs turnover that can compensate any miRNA regulatory disruption upon silencing of the canonical pathway protein[128]. Indeed, a Dicer-independent pathway has been shown where pre-miRNA-451 requires Ago-2 for its maturation but not Dicer[298]. Moreover, Drosha-independent processing of certain miRNAs, namely,

mirtrons has been observed in mammals, *C. elegans* and *D. melanogaster*[127], which increases the diversity of this system. Besides, it was also recently reported that miRNA biogenesis can be globally induced upon DNA damage in an ataxia-telangiectasia mutated (ATM) kinase-dependent manner in MEF cells upon treatment with neocarcionostatin[299]. A KH type splicing regulatory protein (KSRP) was found to be a key player in this mechanism as it translates the DNA damage signaling to the miRNA biogenesis machinery. However, whether KSRP is activated and contributes to miRNA processing in NSCLC cells upon downregulation of core components of the miRNA machinery and treatment with RT remains to be clarified. Consistently, miRNAs likely regulate multiple mRNAs and each mRNA can be a target of several miRNAs simultaneously, suggesting to analyze the particular sets of miRNAs to obtain an altered response LC cells to radiation therapy.

Likewise, Ago-2, an important member of RISC, also FXR1, TSN, PACT, and accessory proteins TRBP, DGCR8 have an altered expression in SCLC[271] and other tumors of breast, colon and epithelial skin[300-302]. In summary, in **paper I,** we for the first time analyzed the expression of a set of core proteins involved in miRNA biogenesis in a panel of human LC cell lines. Even though their expression correlated with the resistance to RT, their knock-down was not sufficient to sensitize LC cells to ionizing radiation. Thus, suggesting that RT resistance of LC cells cannot be overcome by modulation of a single component of the miRNA biogenesis machinery. Hence, other strategies such as targeting of multiple components in both canonical and non-canonical miRNA biogenesis pathways at the same time should be explored further as a way to sensitize LC to RT.

3.2 PAPER II:

miRNA-214 modulates radiotherapy response of non-small cell lung cancer cells through regulation of p38MAPK, apoptosis and senescence

Altered miRNA expression signatures have been connected with oncogenic phenotypes of different tumors[198, 303] and various forms of LC[206]. Thus, in different reports miRNA-155 and miRNA-21 exemplify those miRNAs that are up-regulated, while miRNA-198, miRNA-33, miRNA-124, and miRNA-101 illuminate downregulated miRNAs in LCs[206]. Moreover, in LC, aberrations in growth factor signaling and subsequent deregulation of downstream signaling cascades, including RAS/RAF/MEK, pI3K/AKT and mTOR[11] have indeed been shown to contribute to tumor cell survival,

and protection against RT/CT response, thereby limiting the therapeutic outcome. Importantly, identification of these growth factor receptor aberrations have indeed generated novel therapeutic strategies that either alone or in combination with CT/RT have improved the outcome for a sub-group of LC patients as exemplified by EGFR-ablative therapies[11]. Nevertheless, for NSCLC patients which do not harbor growth factor signaling aberrations and for SCLC other mechanism likely contributes to their RT refractory phenotype and should be characterized and targeted to improve RT outcome. Given that miRNA expression is presently assumed to be a key modifier of CT- and RT sensitivity of tumors and given that their role in regulating RT response of tumors remains poorly understood in general and in LC, in particular, we in **paper II,** focused on identifying miRNAs which may be linked to RT responsiveness, and, hence constitute targets which can be used for RT sensitizing purposes. Therefore, global scale miRNA profiling in a panel of SCLC and NSCLC cell lines with different RT sensitivity was performed with the aim to find a correlation between expression of certain miRNAs and RT sensitivity, and further to delineate their mechanism of action with respect to cellular RT response. For this purpose five human SCLC and six NSCLC cell lines, sorted as RT resistant and sensitive (RR and RS) based on their SF2 values (RR SF2 $\geq$ 3 < RS SF2), were investigated with respect to miRNA expression using miRNA Genechips Array (Affymetrix) (Figure 6). These analyses revealed a number of miRNAs which showed differential expression within the RS and RR NSCLC and SCLC cell lines panel, for example, miRNA-625/ miRNA-523-3p/ miRNA-214/ miRNA-1827 etc., in RR NSCLC, miRNA324-5p in RR SCLC, and miRNA-1260/ miRNA-558 in RS NSCLC cells were expressed differentially. A q-RT-PCR based validation by using TaqMan® MicroRNA Assays (Applied Biosystems) demonstrated that miRNA-214 was highly expressed in RT-resistant NSCLC cells in relative to RS counterparts and therefore the subsequent analyses were then focused on delineating miRNA-214 mechanism of action with respect to RT signaling in NSCLC cells.

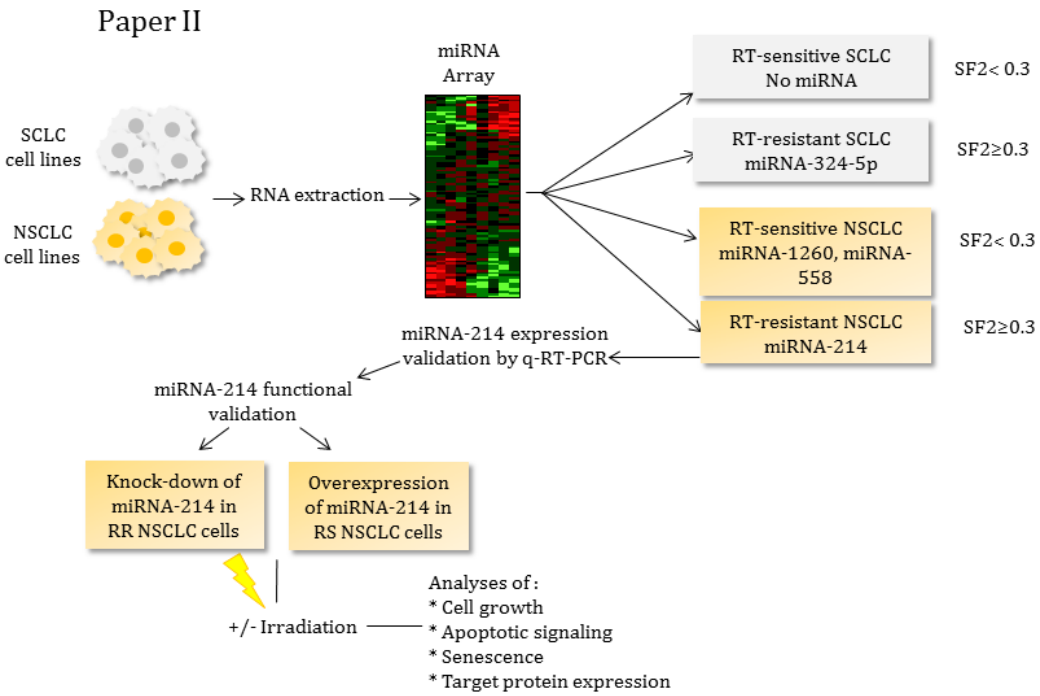

Figure 6. The experimental approach for paper II

We observed an inhibition of proliferation upon miRNA-214 silencing as compared with NT-antagomir counterparts and cell counting experiments revealed a 30% growth inhibition. Importantly, a more pronounced growth inhibition (of about 60%) was found when miRNA-214 was knocked-down in combination with irradiation, suggesting that knock-down of miRNA-214 sensitizes at least these NSCLC cells to radiation. Detailed analyses then revealed that cells instead of dying via apoptosis underwent senescence which was confirmed by high level of β-galactosidase activity. In line with observation an increase in $p27^{Kip1}$ level, a component previously associated with senescence[304], by 2.2-fold was detected as compared to NT and irradiated treated NSCLC cells. This led us to conclude that miRNA-214 downregulation in resistant NSCLC cells drive them to senescence upon irradiation. However, whether the effect of miRNA-214 on $p27^{Kip1}$ expression is direct or indirect, still remains to be investigated.

In parallel, overexpression of miRNA-214 in RS NSCLC H23 cells was found to protect them from RT-induced apoptosis as evident by a reduced proportion of cells with nuclear apoptotic morphology. To a large extent this inhibition of RT-induced apoptosis was a consequence of a block in RT-induced caspase-3 activation as a three-fold reduction in the activity of this caspase was observed when miRNA-214 mimic was overexpressed in these RS NSCLC H23 cells. After confirming the impact of

miRNA-214 in modulation of NSCLC cells RT response, next, we investigated the possible molecular factors involved. We observed that miRNA-214 overexpression in H23 cells increased total p38MAPK level as well as its phosphorylation at the Thr180/Tyr182 site. Additionally, further increase in p38MAPK phosphorylation was detected upon combined miRNA-214 overexpression and irradiation as compared to scrambled mimic transfected cells.

Analyses of predicted targets of miRNA-214 revealed PTEN, a major negative regulator of the cell survival PI3K-Akt pathway to be a candidate. Next, we examined PTEN protein level after miRNA-214 modulation in these NSCLC cells. We observed that ablation of miRNA-214 expression caused an up-regulation of PTEN at the protein level which, in turn, reduced the phosphorylation of Akt independently of irradiation in RR NSCLC U-1810 cells. However, overexpression of miRNA-214 in RS H23 cells did neither affected PTEN at protein nor at mRNA level, forcing us to conclude that miRNA-214 may not be involved in PTEN regulation in NSCLC cells which however was previously observed in ovarian carcinoma cells[305], largely suggesting that miRNA-214 mediated PTEN modulation can be cell type or tissue specific function. In NSCLC H23 cells we could clearly observe a significant increase in total and phosphorylation level of p38MAPK after miRNA-214 upregulation and irradiation. Accordingly, we found an upregulation of the transcriptional factor FoxO4 along p38MAPK. FoxO transcription factors regulation by IGF-1/IGF-1R, Akt- mediated FoxO4 regulation and involvement of p38MAPK signaling in increased FoxO4 expression has previously been shown to cause resistance to oxidative stress in liver cells[306-309]. This made us to hypothesize that p38MAPK may have a function in the RT protective effect by miRNA-214 in NSCLC cells. Indeed this increase in p38MAPK phosphorylation appeared to be responsible for the observed RT resistance conferred by miRNA-214 overexpression, since knock-down of p38MAPK by siRNA reversed the miRNA-214 overexpression-induced protection against RT-induced apoptosis. Thus, the observed critical role of p38MAPK in protecting against RT-induced cell death RT responsiveness also is in accordance with our previous results, in which p38MAPK activity conferred RT resistance through IGF-1R signaling in NSCLC cells[310]. To summarize **paper II,** miRNA profiling of NSCLC and SCLC cell lines revealed that miRNAs may inflict RT responsiveness. We demonstrated a functional role for miRNA-214 in this respect and showed that by manipulating miRNA-214 expression the RT response of NSCLC cells can be significantly altered.

In NSCLC signaling of EGFR-TKs has been among the major molecular aberrations identified and EGFR ablative therapy has shown promising results in at least a sub-fraction of NSCLC patients[311]. Interestingly, a role of miRNA-214 in the regulation of EGFR-TKs signaling was recently highlighted where a significantly increased expression of this miRNA was detected in the gefitinib-resistant NSCLC cell line-HCC827/GR[312]. It was revealed that the miRNA-214 expression level was inversely correlated with PTEN expression and cell survival upon treatment with gefitinib. Moreover, the suppression of miRNA-214 expression was found to be able to reverse the acquired resistance to EGFR-TKIs therapy in NSCLC cells. Thus, these results corroborate our findings in **paper II,** demonstrating that miRNA-214 likely is of importance of NSCLC cells with various oncogenic signaling aberrations including those conferring RT resistance. Nevertheless, further analysis of miRNA-214 and its targets are still of considerable interest to reveal novel radiotherapy sensitizing strategies. All in all, in **paper II** we clearly demonstrate that profiling of miRNAs may reveal putative targets with RT sensitizing capacity in LC cells. We showed this to be true for miRNA-214, yet the other identified miRNAs i.e. miRNA-9/ let-7g[313] , miRNA-155[314], and miRNA-101[269, 315] have been linked to RT responsiveness in NSCLC and SCLC cells respectively awaits to be analyzed for their RT sensitizing capacity but still holds promise to identify further novel RT sensitizing targets of NSCLC and SCLC, respectively.

3.3 PAPER III:

Down-regulation of Tudor staphylococcal nuclease in non-small cell lung carcinoma cells enhances the effect of DNA-damaging drugs

Acquired or intrinsic resistance of SCLC and NSCLC tumor cells to CT leads to disseminated disease and metastasis, a state where treatment options are limited and in which no remedy yet have been effective. The underlying causes of CT resistance are multifactorial and include altered DNA repair capacity, increased detoxification capacity as well as deregulated cell survival and apoptotic signaling circuits. Therefore, a better understanding of these processes and associated factors contributing to resistance may warrant significant therapeutic benefits. In **paper III,** we aimed to find molecular targets that contribute to LC chemotherapy resistance and apoptosis abrogation. For this purpose we investigated NSCLC cell lines, H23, A549, H661, U-1810, and normal human lung fibroblast cell line AG06814 (WI-38) to find putative

targets supporting tumor resistance and apoptosis evasion. We could observe that a multifunctional protein TSN was highly expressed in three out of four examined NSCLC cell lines as compared to noncancerous human lung fibroblasts cell line.

To clarify whether high expression of TSN in NSCLC cells might contribute to malfunctioning of the apoptotic machinery as well as to their CT response, we silenced the TSN expression in cells that later were treated with cisplatin and camptothecin (CPT) (Figure 7). The NSCLC cell lines A549, H661 and U-1810, all exhibited weak apoptotic response upon treatment with 5 ug/mL of cisplatin and 2.5 uM CPT alone for 24 and 48 hours as assessed by absence or weak cleavage of PARP. In addition, no processing of caspase-3 and -9 was detected in response to both drugs in all examined NSCLC cell lines. However, when TSN expression was knocked-down by siRNA, a strong induction of apoptotic cell death was observed upon treatment with either cisplatin or CPT, associated with activation of caspase-3 and -9 and increased PARP cleavage. A concomitant effect was further confirmed by the quantitative analysis of apoptotic cell death in A549 cells. Moreover, in A549 cells a significant 2-fold increase in subG1 population in response to treatment with cisplatin after TSN downregulation as compared to A549 cells that maintained endogenous level of TSN was observed. In line, silencing of TSN led to the appearance of even higher percentage of subG1 cells (from 8.4 to 25.8%) when A549 cells were treated with CPT. Similarly, a pronounced sensitizing effect of TSN knock-down was observed in H661 cells. Thus, the number of apoptotic cells was increased up to 3-times upon treatment of TSN-knocked-down cells with cisplatin as compared to TSN-expressing cells. A fluorometric analysis of caspase-3-like activity also verified the sensitization effect of TSN knock-down in A549, H661 and U-1810 cells following cisplatin treatment. Overall, these data indicate that high expression of TSN contributes NSCLC cells resistance to CT and knock-down of its expression promotes CT sensitization.

In contrast, the knock-down of another component of the miRNA biogenesis pathway, Dicer, did not affect the sensitivity of A549, H661 and U-1810 cells to treatment with cisplatin, suggesting that the effect, observed upon silencing of TSN, most likely is not related to its function as a component of miRNA system, but is rather mediated through TSN transcriptional activity. Indeed, apart from a role in miRNA biogenesis TSN is reported to act as a transcriptional co-activator[284]. Therefore to identify the molecular targets of TSN in NSCLC cells, a global gene expression analysis was performed on A549 cells after silencing of TSN and thereafter using the Agilent SurePrint G3 Human Gene Expression 8x60K Microarray Kit. Results from this analysis revealed

widespread transcriptional changes in TSN-knocked-down cells. About 391 unique genes were detected that showed a greater than 2-fold average change in overall expression after TSN silencing. Among them 234 transcripts were under- and 157 genes were overexpressed as compared to scrambled transfected A549 cells. As TSN silencing affected the expression of a large number of genes, we next investigated which functional classes of genes were altered in response to TSN-knocked-down. The Ingenuity Pathways Analysis (IPA) program (Ingenuity Systems, Mountain View, CA, USA; http://www.ingenuity.com) and Gene ontology category enrichment analyses indicated several major networks containing genes that were closely associated with autophagy and apoptotic cell death, as well as survival, DNA damage response and Ca^{2+} signaling.

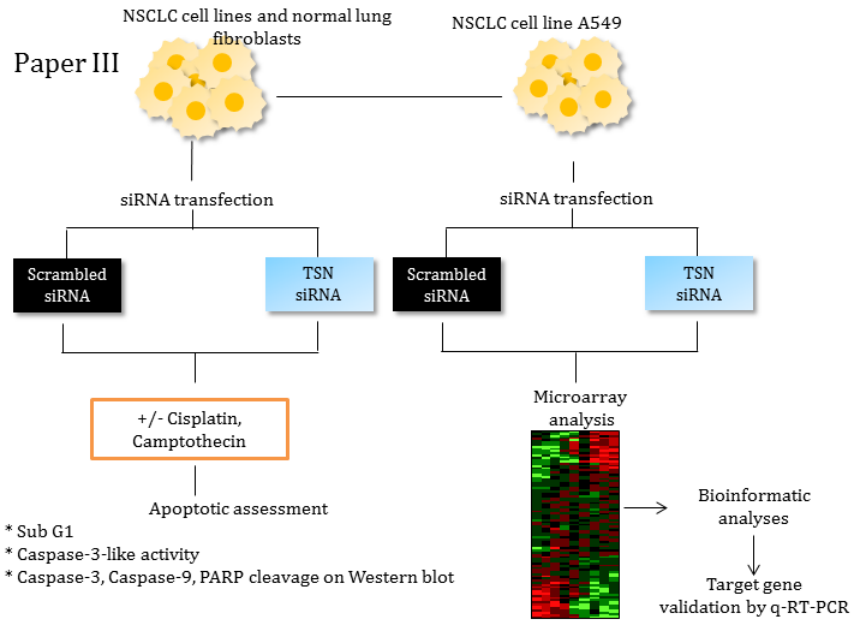

Figure 7. The experimental scheme used in paper III

The expression of shortlisted genes was further analyzed using q-RT-PCR to validate the changes established by microarray. Indeed, the expression of S100A11, ATP6V1F and MDC1 was strongly suppressed by TSN silencing, whereas the expression of BNIP, IGFBP2, ATG10, DRAM1, PDCD4, LAMP2 and BCL2L13 was significantly augmented compared to control samples. Remarkably, none of these identified genes has previously been shown as possible TSN-interacting candidates but many have been implicated in tumorigenesis, metastasis and cell death related mechanisms. For instance, S100A11 has been linked to DNA repair processes[316], MDC1 is also

involved in DNA damage response and has been noted as a potent target for caspase-3[317]. BNIP3 and BCL2L13 are known pro-apoptotic proteins[318, 319] and PDCD4 is linked with cell death and survival mechanisms[320].

This study highlights the fact that TSN, an evolutionally conserved multifunctional protein, is an important mediator of CT-induced cell death signaling in NSCLC cells. A higher expression of TSN has been implemented in the tumorigenesis of colon and of breast cancer[284, 321-324]. This prompted us to assume that a higher expression of TSN can potentially contribute towards NSCLC CT resistance. Although cisplatin as well as CPT are routinely used in therapy of LC, their therapeutic efficacy is largely compromised by LC's resistant nature and still is a major clinical problem. Therefore, we investigated the role of TSN in response to cisplatin and CPT treatment, and indeed our obtained results suggest that TSN overexpression could contribute towards the regulation of expression of some tumor survival genes and simultaneously play a role in suppression of pro-apoptotic genes that eventually compromise tumor ability to undergo apoptosis. In short, in **paper III**, we demonstrate a role of TSN in cisplatin and CPT-induced cell death. Moreover, novel TSN targets were identified and their potential role in modulation of cell death response in NSCLC was suggested. Nonetheless, a functional link between these identified TSN-regulated genes and TNS-knock-down-induced cell death upon treatment with DNA-damaging drugs remains to be established.

4 CONCLUSION AND FUTURE PROSPECTS

Evasion of cell death is accounted as the major hallmarks of cancer. Resistance to CT/RT is the main obstacle in LC clinical managing and impaired induction of cell death including apoptosis in part accounts for this treatment failure. A newly revealed class of non-coding RNAs, microRNAs, is thought to regulate a variety of biological mechanisms by modulating the effect of mRNA and subsequent protein activity. However, our understating of their comprehensive potential is still in its infancy. Nevertheless, the potential exhibited by miRNAs so far and their imperative role in the pathogenesis of various tumor types has convinced that these tiny regulators may help us in elucidating the alterations of apoptotic machinery during carcinogenesis and find novel targets to overcome cancer resistance to treatment. Thus, one can anticipate that further understanding of functional impacts of miRNAs in the mechanism(s) of CT/RT resistance of LC will provide novel targets which can improve therapeutic outcome. In this thesis work we have attempted to expand our knowledge to understand CT/RT resistance mechanisms and apoptosis perturbations in LCs.

According to our finding we can conclude that:

- miRNA biogenesis-related core proteins **Dicer, Drosha, Tudor-SN** or **Ago-2** silencing is inadequate to restore proper apoptotic response and sensitize NSCLC to radiotherapy.
- miRNA profiling in a panel of NSCLC and SCLC cells with different RT sensitivity identifies **miRNA-214** as a candidate miRNA linked to RT resistance.
- Downregulation of **miRNA-214** drives RT resistant NSCLC cells to senescence, while upregulation of miRNA-214 in RT sensitive NSCLC cells results in decreased apoptotic signaling mainly through p38MAPK signaling.
- Knock-down of **Tudor-SN** sensitizes NSCLC cells to DNA-damaging CTs by potentiating apoptosis.
- Extensive gene expression profiling upon **Tudor-SN** silencing revealed that it likely contributes to NSCLC CT resistance by regulating the expression of several tumor survival genes, such as S100A11, ATP6V1F, and MDC1, and simultaneously suppressing many pro-apoptotic genes e.g., BNIP3, DRAM1, PDCD4, BCL2L13, and LAMP2, that eventually compromise tumor ability to undergo apoptosis.

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