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IDENTIFICATION OF NOVEL TUMOR BIOMARKERS FOR SENSITIVITY TO RADIO- AND CHEMOTHERAPY OF LUNG CANCER BASED ON GENOMIC ANALYSES

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The only source of knowledge is experience.

Albert Einstein

Dedicated to my family

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ABSTRACT

Lung cancer (LC) is one of the most common human cancers and the leading cause of cancer-related deaths worldwide. Treatment of LC is by surgery if the tumor is resectable, otherwise chemo- and/or radiotherapy (CT/RT) is given. However, development of resistance to CT/RT is very common which makes the prognosis of LC very poor with median overall survival of only about one year. This calls for discovery of novel diagnostic and/or therapeutic modalities. Biomarkers predicting response of LC to conventional CT have been identified based on some signaling alterations for a subset of NSCLC patients. The molecular aberrations for the rest of LC remain largely unclear. The main aims of this thesis were to investigate the potential of miRNAs as novel prognostic or therapeutic biomarkers of LC and to understand if some miRNAs could drive RT resistance.

In **Paper I** we found that miRNAs are widely expressed in LC cell lines and we found a link between miRNA expression and RT sensitivity. We reported that miRNA-214 and miRNA-324-5p are exclusively higher expressed in the radioresistant non-small and small cell lung cancer (NSCLC and SCLC) cell lines, respectively, as compared to the radiosensitive counterparts. Interestingly, we found that ablation of miRNA-214 in the NSCLC cells reversed RT resistance and induced senescence concomitant with up regulation of p27^{Kip1}. In line with this, we observed that overexpression of miRNA-214 in RT sensitive NSCLC cells blocked caspase-3-mediated apoptosis concomitant with activation of p38MAPK and phosphorylation of FoxO4. In conclusion we show that miRNA-214 confers resistance of NSCLC to RT.

In **Paper II** we showed that miRNA-214 is modulating invasiveness of NSCLC cells. We demonstrated that ablation of miRNA-214 enhanced while overexpression of the miRNA reduced invasiveness of NSCLC cells. Through gene expression and bioinformatics analyses, we found that 18 genes out of the predicted miRNA-214 targets were regulated in the NSCLC cells. Among these, we focused on four genes, PAPP-A, ALPK2, CDK6 and TNFAIP3, which were previously reported to be regulating metastasis in a mouse lung cancer model. Through argonaute 2 (Ago2) immunoprecipitation, we found that only ALPK2 is directly regulated by miRNA-214 whereas PAPP-A, CDK6 and TNFAIP3 are indirect targets of this miRNA. Moreover, we performed immunohistochemical analysis of these targets in tissue microarrays of about 600 NSCLC tumors to reveal their expression pattern and to examine if a correlation could be made to metastatic potential of the tumors or overall survival (OS) of the patients. We showed that NSCLC tumors express these proteins at a moderate to high level although with no correlation to OS or clinical records of metastasis. In summary, we demonstrate that miRNA-214 regulates invasiveness of NSCLC yet further studies are required to delineate the molecular components involved.

In **Paper III** the aim was to find biomarkers of cisplatin resistance in NSCLC cells. We showed that long-term NSCLC clones surviving cisplatin treatment had a heterogeneous gene expression pattern. We found DKK1, XRCC2 and LGALS9 to be linked to cisplatin resistance of NSCLC cells. Accordingly, we showed that knockdown of DKK1 sensitized NSCLC cells to cisplatin. Through Ingenuity pathway analysis we identified TCF4, EZH2, DNAJB6 and HDAC2 as altered upstream regulators of DKK1 and GSK3B as a possible downstream signaling molecule. Further work is required to demonstrate their signaling role in cisplatin resistance of NSCLC cells.

In summary, we show in this thesis that genomics techniques in combination with bioinformatics can be used to identify biomarkers of LC which could be used for prediction of prognosis or treatment response but also to reveal novel RT or CT sensitizing targets.

LIST OF PUBLICATIONS

I.   **Salim H***, Akbar NS*, Zong D, Vaculova AH, Lewensohn R, Moshfegh A, Viktorsson K and 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; 107(8):1361-73.

II. **Salim H**[#], Arvanitis A, de Petris L, Kanter L, Hååg P, Zovko A, Özata DM, Lui WO, Lundholm L, Zhivotovsky B, Lewensohn R and Viktorsson K[#]. miRNA-214 is related to invasiveness of human non-small cell lung cancer and directly regulates alpha protein kinase 2 expression. *Genes Chromosomes Cancer*. 2013; 52(10):895-911.

III. **Salim H**, Zong D, Hååg P, Mörk B, Lewensohn R, Lundholm L[#] and Viktorsson K[#]. Analysis of cisplatin-refractory non-small cell lung cancer clones reveals Dickkopf-1 as a potential novel driver of resistance. *(Submitted Manuscript)*

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LIST OF ABBREVIATIONS

AC	Adenocarcinoma
Ago2	Argonaute 2
ALK	Anaplastic lymphoma kinase
ALPK2	Alpha protein kinase 2
ATM	Ataxia telangiectasia mutated
Bcl-2	B-cell lymphoma 2
BCL2L2	BCL-2 like protein 2
BER	Base excision repair
CDK6	Cyclin-dependent kinase 6
CDKN1A	Cyklin dependent kinase inhibitor 1A
CT	Chemotherapy
DDR	DNA damage response
DKK1	Dickkopf-1
DSB	DNA double-strand break
EPHA3	Ephrin receptor A3
EGFR	Epidermal growth factor receptor
ERCC1	Excision repair cross-complementing
ESMO	European society of medical oncology
EzH2	Enhancer of zeste homolog 2
FA	Fanconi anemia
FDA	Food and drug administration
FFPE	Formalin-fixed, paraffin-embedded
FMN1	Formin 1
FoxO4	Forkhead box protein O4
GSK3B	Glycogen synthase kinase 3 beta
Gy	Gray
HIF	Hypoxia inducible factor
HR	Homologous recombination
HSP	Heat shock protein
IR	Ionizing radiation
LC	Lung cancer
LCC	Large cell carcinoma
LGALS9	Lectin galactoside-binding soluble 9
LKB1	Liver kinase B1
miR	microRNA
miRNA	microRNA
MAPK	Mitogen-activated protein kinase
MCL-1	Myeloid cell leukemia sequence 1
MMP9	Matrix metalloproteinase 9
MMR	Mismatch repair
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining

NSCLC	Non small cell lung cancer
OS	Overall survival
PAPP-A	Pregnancy-associated plasma protein A
PFS	Progression free survival
PI3K	Phosphatidylinositol 3-kinase
Pre-miRNA	Precursor miRNA
Pri-miRNA	Primary miRNA
PI	Propidium iodide
PTEN	Phosphatase and tensin homolog
RR	Radioresistant
RISC	RNA-induced silencing complex
RRM1	Ribonucleotide reductase M1
RS	Radiosensitive
RT	Radiotherapy
SBRT	Stereotactic body radiotherapy
SCC	Squamous cell carcinoma
SCLC	Small cell lung cancer
SF2	Surviving fraction after 2 gray irradiation
TKI	Tyrosine kinase inhibitor
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
XIAP	X-linked inhibitor of apoptosis
XRCC2	X-ray repair cross-complementing protein 2

1 INTRODUCTION

1.1 Lung cancer

1.1.1 Clinical background

Lung cancer (LC) is among the most frequent diagnosed cancers and the leading cause of cancer-related deaths worldwide. It is estimated that about 1.6 million new cases are diagnosed leading to about 1.38 million deaths each year which accounts for 18.2% of all cancer-related deaths worldwide [1]. LC is of two main histological subtypes which are non-small and small cell lung cancer (NSCLC and SCLC, respectively). The cornerstone of treatment for LC is surgery for localized disease; however, the majority of the cases are diagnosed at more advanced stages for which chemo- and/or radiotherapy (CT and RT, respectively) are the main curative treatment options. Despite the advances in diagnostic and surgical techniques and development of new RT and CT options, the prognosis of LC is still poor. Thus conventional CT and RT are effective in some, but most of the patients eventually develop resistance to treatment [2]. Using different approaches such as proteomics, metabolomics and genomics techniques to identify signaling networks of LC cells, certain of the molecular mechanisms underlying resistance to treatment have been revealed but still the overall picture is vague. To use a systems biology approach for analysis of signaling aberrations of tumor cells on a global scale has recently become feasible and shown great potential in identifying putative biomarkers of response and to find novel drug targets. In this thesis work, the genomic aspect of the systems biology, particularly miRNA and gene expressions in relation to RT and CT is under focus, with the aim of finding prognostic or therapeutic biomarkers for LC but also to identify novel RT/CT sensitizing targets.

Histopathology

Since many decades LC has been classified by pathologists into two main histological types, SCLC and NSCLC, with the latter accounting for about 85% of the cases and subdivided into adeno-, squamous cell- and large cell carcinoma (AC, SCC and LCC, respectively) [3]. However, after a multidisciplinary discussion including oncologists, radiologists, molecular biologists, surgical oncologists and pathologists, in which recent knowledge in molecular aberrations in LC were taken into consideration, the classification of LC has been updated [4]. It was thus agreed that pathologists should

make more distinct classification of LCs based on molecular investigations in small biopsies and cytologies which are possible to obtain from about 70% of the patients [3]. Previously, both AC and SCC were treated in a similar manner and a clear distinction was not of extra benefit, but nowadays, the practice has to be changed since certain therapies are effective against only a small subgroup of patients. For example, tyrosine kinase inhibitors like gefitinib or erlotinib and crizotinib, are recommended only for tumors with epidermal growth factor receptor (EGFR) mutations and anaplastic lymphoma kinase (ALK) rearrangements, respectively [5-7]. Further details about these mutations and targeted therapies are presented below.

Staging and prognosis

Clinically, LC is divided into four stages depending on how advanced the disease is, according to the TNM staging system which takes tumor size, lymph node involvement and metastasis to distant organs into consideration [8]. According to the latest version of TNM (i.e. 7th edition), both SCLC and NSCLC are classified into four stages with majority of NSCLC cases being of stage II and III and for SCLC, stage III and IV [9, 10].

Prognosis of LC varies according to the histological subtype and the clinical stage of the disease; however, the 5-year survival rate even for patients with early stage disease is ranging within 30-60% indicating a poor prognosis of the disease [11]. SCLC is considered as a very aggressive disease and if left untreated, the median survival will be about 2-4 months [12] and the 5-year survival rate of about 6.1% [13]. NSCLC is considered less aggressive with the 5-year survival rate being 50% for stage IA, 43% for IB, 36% for IIA, 25% for IIB, and 19% for stage IIIA [14, 15].

Management

Diagnosis of LC is often made at advanced stages of the disease since it has no characteristic clinical features and is typically presented in the form of general respiratory symptoms like dyspnoea, cough and hemoptysis or general non-specific symptoms like weight loss and fatigue [16]. The disease is suspected from the clinical features but definite diagnosis is usually made through histopathological examination of a tumor biopsy or bronchial brushing or washing. Imaging of the chest and the other parts of the body is mostly done to define the extent and the clinical stage of the disease.

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The latest guidelines and recommendations for treatment of NSCLC have been proposed by the European Society of Medical Oncology (ESMO) and are used also in Sweden [17]. Treatment of early stages of NSCLC i.e. stages I and II is aimed to be curative and includes surgical removal of the tumor. However, if surgery is contraindicated for any reason or the patient refuses to get operated, then stereotactic body radiotherapy (SBRT) is an alternative with favorable survival benefit and toxicity [18, 19]. For stage II tumors, different doublets of CT should be used as adjuvant treatment, which consists mainly of cisplatin in combination with other CT agents such as gemcitabine and vinorelbine. For resectable stage III NSCLC, concurrent cisplatin-based CT and RT is usually given after surgical resection of the tumor while for non resectable stage III tumors, the treatment of choice is combined cisplatin-based CT and RT up to 60 gray (Gy) in fractions [17]. However, for advanced stages of the disease, usually palliative therapy is given in order to relieve the symptoms and consists of CT for example cisplatin, gemcitabine or pemetrexed or targeted therapies such as EGFR- or ALK tyrosine kinase inhibitors (TKI)s [10]. The role of palliative RT in advanced stage NSCLC is mainly used to relieve the metastasis-related pain or to control bleeding or airway compressions [10].

Management of SCLC is also dependent on the stage of the disease and follows the ESMO guidelines and recommendations [9]. The treatment of localized disease, with no lymph node involvement or metastasis, includes surgery followed by adjuvant CT or as an alternative concomitant chemo-radiotherapy is used. For SCLC tumors reaching the ipsilateral lymph nodes or pleura, the best treatment option is concomitant CT and RT while for more advanced stages of the disease the treatment is usually palliative in the form of CT. For those patients responding to the first line treatment, prophylactic cranial irradiation should be considered in order to prevent brain metastasis [9].

In the last decade, our knowledge of some of the molecular aberrations driving NSCLC tumors has generated favorable therapeutic options with less undesirable side effects. Examples are TKI for tumors with mutated EGFR such as gefitinib and erlotinib or for tumors with ALK rearrangement like crizotinib [14]. However, with respect to EGFR ablative therapy the problem is that it is only effective against a small subgroup of NSCLC patients of AC subtype, who have mutated EGFR [14]. Moreover, the vast majority of the cases recur because of development of resistance towards the EGFR TKIs and for this patient cohort no optimal treatment regimen is at hand. Therefore,

development of new drugs which can target a larger group of LC patients with tumors of different histological subtypes and molecular aberrations is urgently needed.

1.1.2 Molecular aberrations in lung cancer

With the complete sequencing of the whole human genome and the development of high throughput technologies, our understating of the molecular aberrations in LC that drive the processes of tumor development, progression, vascularization, invasion and the mechanisms of drug-induced cell death has improved. Focus of the scientific community in the last decade has thus been on applying these advanced techniques i.e. DNA sequencing, gene and miRNA analyses and mass spectrometry based proteomics to identify diagnostic, prognostic or therapeutic biomarkers or to discover targets that can be used for development of novel therapy. Certain of these molecular markers and their clinical applications in terms of LC will be explained below.

In a large study of lung AC, DNA sequencing has demonstrated that certain genes are more frequently mutated for example EGFR, KRAS and EPHA3 (ephrin receptor A3) [20]. Similarly, analysis of lung SCC showed frequent amplification of SOX2 and FGFR1 and mutation of DDR2 [21-23]. In the squamous subtype of NSCLC, aberrations of immunological checkpoint proteins such as programmed death-1 (PD-1) have been identified more frequently than in other subtypes and monoclonal antibodies have been developed to target these proteins with promising results [24, 25].

Certain molecular prognostic biomarkers have been identified for NSCLC and include oncogenes like KRAS, tumor suppressor genes such as p53 and DNA repair genes like excision repair cross-complementing group 1 gene (ERCC1) and ribonucleotide reductase subunit M1 (RRM1) [26-30]. KRAS mutations are frequently detected in NSCLC and found in about 25% of all such cases [26, 28]. A number of studies have shown a poor survival of NSCLC patients with KRAS mutation [27]. However in a multicenter study, mutations of p53 and KRAS were not associated with prognosis of NSCLC [31]. Thus, the predictive value of KRAS mutation in NSCLC needs to be further investigated. ERCC1 is involved in the nucleotide excision repair (NER)-mediated repair of DNA strand breaks such as those induced by CT and other DNA damaging agents. Studies have shown that NSCLC patients with higher tumor expression level of ERCC1 (mRNA or protein) have longer survival rate than patients with low ERCC1 level [29, 30]. The prognostic significance of RRM1, another DNA repair protein which is involved in DNA synthesis, has also been shown in clinical

trials [32]. Thus a higher tumor expression of RRM1 was associated with higher survival rates of NSCLC patients as compared with those having lower RRM1 expression [32, 33].

Regarding the new therapeutic drugs developed based on known underlying targets, two groups of drugs have so far been introduced into the market; these are TKIs against EGFR mutation and ALK rearrangement [5-7]. EGFR TKIs like gefitinib and erlotinib have been shown to be highly effective mainly against lung AC with mutated EGFR being associated with longer progression free survival (PFS) as compared to conventional CT [6, 34]. On the other hand, those patients having a wild type EGFR had longer PFS in the CT-treated arm as compared to the gefitinib arm [34]. Certain less common genetic aberrations have recently been explored for therapeutic purposes in NSCLC such as BRAF mutation and c-MET amplification [35, 36]. Mutations in BRAF, which is a kinase downstream of KRAS, are most common in melanoma and this tumor has shown good response to BRAF-based TKI like vemurafenib [37]. Interestingly, *in vivo* LC mouse model studies have shown promising results in tumors bearing BRAF mutation treated with vemurafenib [35]. However, random selection of NSCLC patients for treatment with sorafenib, a multikinase inhibitor targeting among others, BRAF [38], was not associated with improvement of overall survival (OS) neither as single treatment nor in combination with CT [39, 40]. Other targetable genetic aberrations in NSCLC include MET amplification, a receptor tyrosine kinase, which enhances phosphatidylinositol 3-kinase (PI3K) signaling upon activation. MET amplification has been observed in 2-20% of lung ACs [41, 42]. Besides that, development of resistance to gefitinib has partly been attributed to MET amplification [43]. A number of kinase inhibitors and monoclonal antibodies have been developed to target MET amplification in preclinical and clinical studies with promising results [36]. However, standardization of the detection methods and optimization of patient selection is needed.

Among the molecular markers being associated with response of NSCLC cells to CT are ERCC1 and RRM1 [30, 44]. It has been shown that NSCLC patients with stage I-III tumors treated with adjuvant CT (cisplatin) had prolonged survival if their tumors were ERCC1 negative as compared to the control group while in ERCC1 positive tumors, there was no significant difference between the CT and the control arm [30]. Similarly, low tumor expression of RRM1 mRNA has been shown to be associated

with higher response rate of NSCLC patients to gemcitabine as compared to high RRM1 expression [44].

1.2 MicroRNAs as potential biomarkers in lung cancer

1.2.1 Background

MicroRNAs (miRNAs, miRs) are endogenously expressed short single-stranded RNA molecules that negatively regulate gene expression. miRNAs regulate gene expression through either degrading the target mRNA or inhibiting translation of the mRNA into protein [45, 46]. The processing of miRNA is shown in **Figure 1**. First, miRNAs are transcribed from the genome by RNA polymerases in the form of long double stranded primary transcripts called pri-miRNA [47]. These transcripts are processed in the nucleus by a ribonuclease, Drosha, into shorter transcripts called precursor miRNAs (pre-miRNAs) [48]. Through the help of a transporter protein, Exportin-5, they are transferred to the cytoplasm [49] and then further processed through another ribonuclease, Dicer, which cleaves the pre-miRNA into 18-25 nucleotide long miRNA duplexes [50]. One of the strands of the duplex which becomes the mature miRNA will be directed towards the target mRNA in the RNA-induced silencing complex (RISC) complex via the help of argonaute 2 (Ago2) and the other strand will be degraded [51].
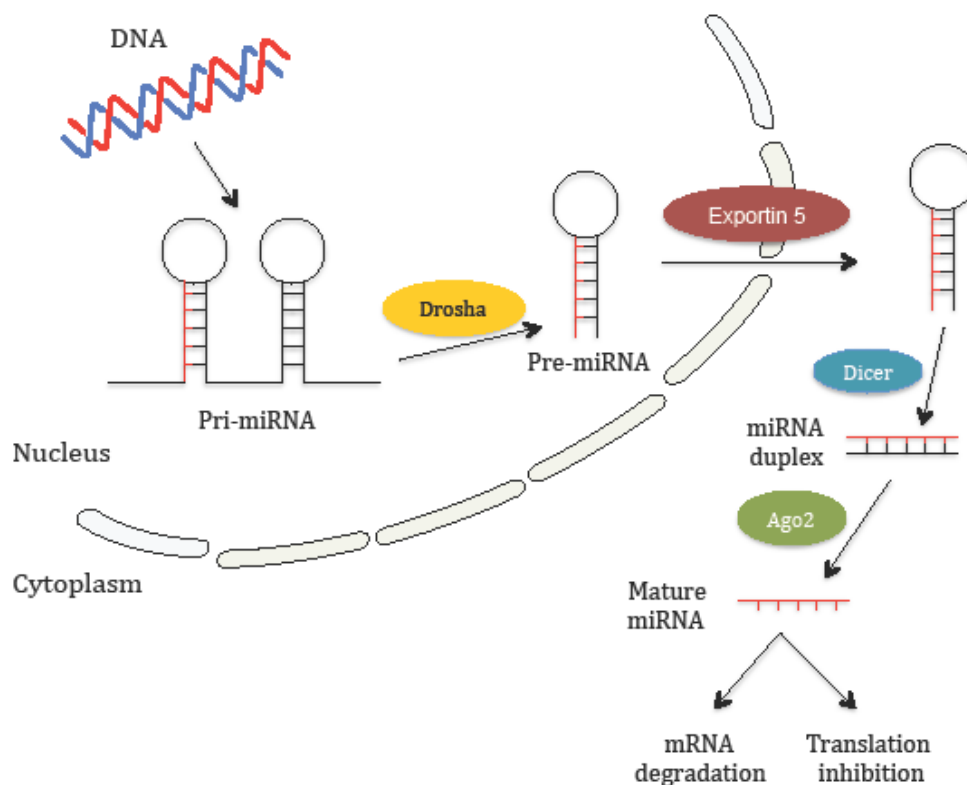

Figure 1. A simplified schematic overview of miRNA biogenesis.

1.2.2 Functions of miRNAs

miRNAs are regulating gene expression negatively and it is estimated that up to 60% of protein coding genes are regulated by miRNAs [52]. miRNAs have been shown to play important roles in regulation of different physiologic conditions such as organ development for example high miRNA-17-92 cluster expression is essential during lung development, apoptosis and differentiation [53-55]. miRNAs have also been shown to be involved in certain pathologic conditions including tumor development and in this context might function as oncogenes, often called oncomiRs, in which they are regulating tumor suppressor genes or conversely they can function as tumor suppressors upon targeting oncogenes [56].

The influence of miRNA on tumor development or progression could be due to either modifications in the miRNA expression levels or modification of interaction between miRNAs and their mRNA targets. An aberrant miRNA expression during tumor development can be due to difference in the overall miRNA expression profile [57], chromosomal rearrangements [58], defects in the miRNA biogenesis machinery [59], point mutations in miRNA coding genes [60] or defects in the transcriptional regulation of miRNAs [61]. For example, deletion in the 3'UTR of HMGA2, which is targeted by the miRNA, let-7, leads to failure of the latter to regulate the gene enhancing lung tumorigenesis [62]. Another aberration is single nucleotide polymorphism (SNP) in the target genes which alter miRNA binding as exemplified by mutation in the 3'UTR of KRAS which disables let-7 family members from regulating the gene in NSCLC [63].

1.2.3 miRNAs and hallmarks of cancer

miRNAs can regulate almost all hallmarks of tumor development which include sustaining proliferative capacity, irresponsiveness to apoptosis, evading suppressors of cell growth, enabling replicative immortality and inducing angiogenesis **(Figure 2)** [64]. For instance, miRNA-221/222 is targeting the cell cycle regulator, p27^{Kip1} and thus influencing cell growth [65]. Several miRNAs have been shown to regulate different components of both the intrinsic and the extrinsic pathways of apoptosis and thus either promote or inhibit tumor development **(Figure 3)**. In the intrinsic pathway, Bcl-2 and Bim mRNAs have been shown to be targeted by miRNA-15a/16 and miRNA-17/92 cluster, respectively [66, 67]. The pro-apoptotic protein, Puma, has been shown to be targeted by miRNA-221/222 [68] which is also targeting p27^{Kip1} [65]. In

the extrinsic pathway, miRNA-21 has been shown to regulate the expression of FAS-L [69].
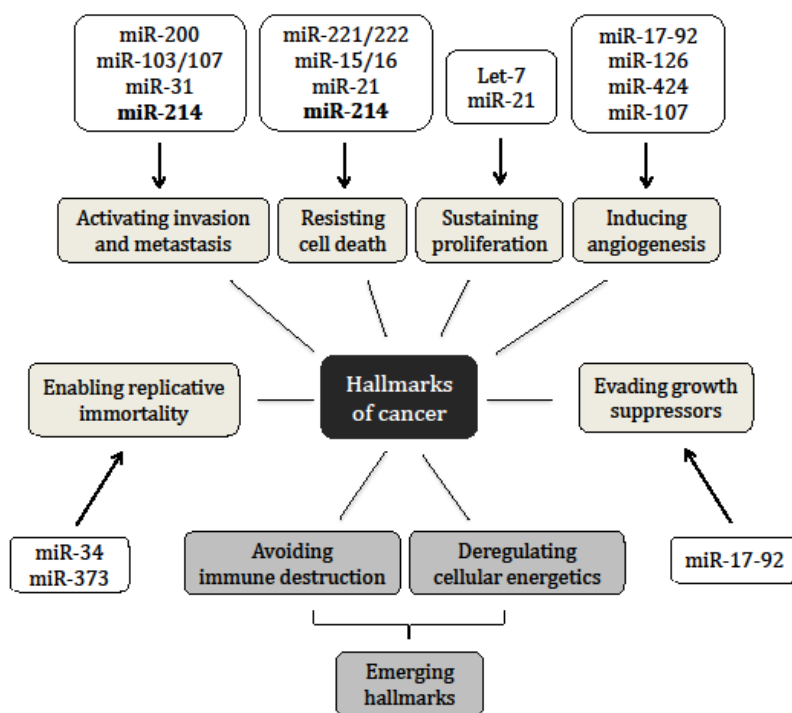

Figure 2. Examples of miRNAs involved in regulation of hallmarks of cancer.

With respect to sustained angiogenesis, miRNA-424 has been shown to enhance angiogenesis via up regulation of HIF-α1 and HIF-α2 [70]. miRNA-200 family has been shown to enhance invasion and metastasis in LC mouse models by regulating epithelial mesenchymal transition (EMT) [71]. Different components of the angiogenesis pathway have also been targeted by miRNAs. Thus, down regulation of miRNA-126, which is an endothelial cell-specific miRNA, has been associated with angiogenesis reduction through negative regulation of a component of VEGF pathway, SPRED1 [72]. Other miRNAs also have anti-angiogenic property such as miRNA-15a/16 which target VEGF mRNA [73].

Similarly, miRNAs have been shown to regulate different components involved in invasion and metastasis of tumor cells such as high miRNA-103/107 being associated with high invasiveness and metastasis of breast cancer via regulation of Dicer and miRNA-200 [74].
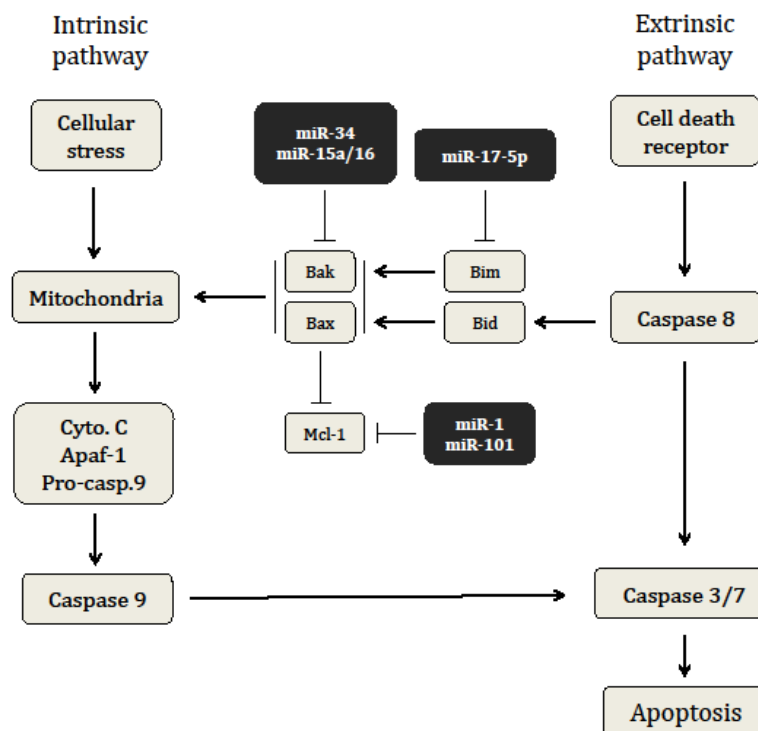
Figure 3. A simplified schematic outline of intrinsic and extrinsic apoptotic pathways. Some examples of miRNAs involved in regulation of these pathways are shown.

1.2.4 miRNAs as diagnostic and prognostic biomarkers

miRNAs can serve as new diagnostic tools and even for classification of tumor subtypes as illustrated by a list of 56 differentially expressed miRNAs in lung tumors as compared to normal lung tissue [75]. It is even possible to use non-invasive methods to detect the level of miRNAs in different body fluids including blood, plasma, serum, urine, saliva and amniotic fluid [76]. In the circulation, miRNAs are protected inside micro vesicles called exosomes which are secreted to the circulation from different cell types [77]. A number of studies have shown that miRNAs are differentially secreted into the serum or blood of LC patients [78, 79]. Using miRNA sequencing, it was thus shown that 76 miRNAs have a significantly higher level in serum of NSCLC patients as compared to healthy controls followed by q-RT-PCR validation of the highest expressed miRNAs i.e. miRNA-223 and miRNA-25 [78]. In another study, miRNA expression profiling in relation to OS of NSCLC patients, stage I-III AC and SCC, was tested using solexa sequencing [79]. It was found that miRNA-499, miRNA-1, miRNA-486 and miRNA-30d had a higher expression in patients with longer OS

(mean survival time 49.5 months) than those with shorter OS (mean survival time 9.5 months) showing that circulating miRNAs can serve as prognostic biomarkers.

Several studies have shown that miRNAs can be suitable prognostic biomarkers of tumors in general and of LC in particular. High expression of miRNA-146b and miRNA-155 in lung SCC and AC, respectively, was associated with poor OS of the patients after surgical resection of the tumor [80, 81]. miRNA expression profiling of formalin-fixed, paraffin-embedded (FFPE) NSCLC tumors of stage I-III has shown that miRNA-221, 137, 372, 182* and let-7a have prognostic value [82]. However, until till now their utility as clinical biomarkers has been hampered by the inconsistence of miRNAs revealed in the different studies. This can probably be explained by the fact that the miRNA-mRNA interacting network is more complex and that different factors can influence the downstream signaling pathways. Moreover usage of current techniques for analyzing miRNAs may also influence the results and generate inconsistent profiles.

1.2.5   miRNAs as therapeutic targets

Since miRNAs can function as oncogenes or tumor suppressors and are involved in the regulation of different cellular processes important for tumor cell survival and death, they are indeed suitable as therapeutic targets. In this respect, miRNAs have a number of advantages. First, miRNAs can reduce the concentration and not only the activity of the target oncogenic proteins as they can degrade the targets. Second, miRNAs can target multiple oncogenes simultaneously which might reduce the need for combination therapies.

Drugs based on targeting miRNAs can be used in the form of replacement therapy such as mimics of miRNAs which can minimize the effect of certain oncogenes. A good example of miRNA replacement therapy is the intra-tumoral introduction of the miRNA, let-7, into mouse models of NSCLC which reduced the tumor size [83]. Antagonists of miRNAs can also be used to reduce the tumor expression level of oncogenic miRNAs and in turn increasing the level of specific tumor suppressor genes. One such example was the use of miRNA-21 antagonists in glioma cell lines which activated caspase-induced apoptotic pathway [84]. Another approach to introduce miRNAs into the therapeutic field is called indirect miRNA-based therapy. As some tumors have a low level of certain miRNAs as compared to normal cells, thus introducing vectors which carry miRNA-regulated suicide genes into the tumor cells may lead to high abundance of the suicide gene in the cancer cells not in the normal

cells. Albeit these therapies are promising in preclinical and animal settings, further refinement of vehicles or administration routes are required to enable entrance to the clinical practice.

1.2.6   miRNAs as regulators of radiotherapy response

Radiotherapy (RT) is one of the main curative therapeutic options of both SCLC and NSCLC patients with locally advanced disease [9, 17]. The principal signaling of ionizing radiation involves triggering of different types of DNA damages of which DNA double strand break (DSB)s are the most critical. These are formed either through indirect ionizations within the DNA but more important through the formation of free radicals. In response to these DNA damages certain major cellular response pathways are started which include the DNA damage detection response (DDR), DNA damage repair systems, cell cycle regulating networks and different cell death signaling cascades including apoptosis **(Figure 4)**. Accordingly, putative biomarkers of RT responsiveness as well as RT sensitizing strategies may be found among all these pathways but so far no biomarker has clinically been established for such purpose. Given that miRNAs may interfere with tumor signaling at multiple levels, it is not surprising that they also impact on RT response of tumors. It has thus been shown that miRNAs can regulate several genes involved in various steps of RT signaling. For example, the influence of lin-28/let-7 expression on RT sensitivity was tested in NSCLC cell lines with mutated KRAS and wild type KRAS. It was shown that overexpression of let-7a or down regulation of lin-28, a repressor of let-7, led to down regulation of KRAS and sensitized the cells to RT in KRAS mutated but not KRAS wild type cells [85]. In another study, high level of let-7g and miRNA-9 was associated with enhanced sensitivity of NSCLC cell lines to gamma irradiation through negative regulation of NFκB1 [86]. It was also shown that NSCLC cell lines with different response to RT had different miRNA expression pattern and that miRNA-449a was down regulated in the radioresistant subset of cell lines. Further, overexpression of the miRNA-449a sensitized the RT refractory NSCLC cells to IR through enhancing DNA damage, cell cycle arrest and apoptosis [87]. The role of miRNA-210 in RT response has been shown in NSCLC cell lines, where it was found that high levels of the miRNA in hypoxic conditions enhances radioresistance through induction of hypoxia-induced factor 1 (HIF-1) [88]. miRNA-101 has also been shown to enhance radiosensitivity of most of the NSCLC cell lines after ectopic expression through targeting the DDR protein, ataxia telangectasia mutated (ATM), and the non homologous end joining

(NHEJ) repair protein, DNA-PKcs; however, it failed to do so in some other cell lines with high endogenous level of the miRNA-101 and even in xenograft models [89]. In another *in vitro* study, the importance of high levels of miRNA-155 and 20a in IR-induced senescence was shown in lung fibroblasts and for miRNA-155, the downstream signaling pathway was shown to be p38 mitogen-activated protein kinase (p38MAPK) [90].
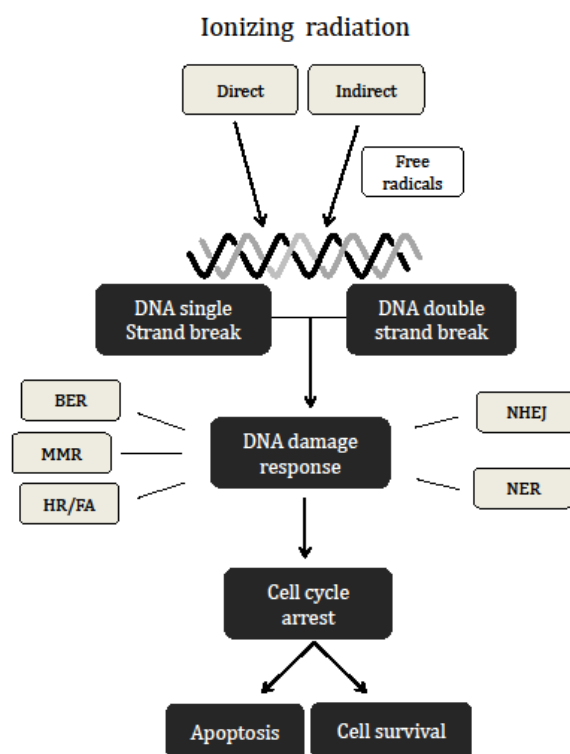

Figure 4. A diagrammatic overview of IR-induced DNA damage induction and cellular response.

Apart from regulation of the different steps induced by IR, several miRNAs have been linked to the sensitivity or resistance of LC, both *in vitro* and *in vivo* [85-94]. High expression level of certain miRNAs through targeting different pathways were shown to be associated with enhanced sensitivity of LC cells to IR such as let-7a [85], let-7g and miRNA-9 [86], miRNA-449a [87], miRNA-34b [93] and miRNA-101 [89]. On the other hand, low level of certain miRNAs was shown to be associated with better response to IR in normal lung fibroblasts for example miRNA-210 [88] or in LC cell lines such as miRNA-155 [90]. However, the direct downstream targets of these miRNAs were not identified.

Among the *in vivo* studies, Lussier and colleagues have shown that NSCLC patients with oligometastasis (i.e. ≤ 5 metastatic sites) have a different miRNA expression pattern compared to those who develop polymetastasis (i.e. > 5 metastatic sites) and thus have a poorer chance of response to SBRT. These oligometastatic NSCLC tumors were found to express higher level of miRNA-200 family members [91]. miRNA expression patterns have also been used to classify NSCLC patients into radio-sensitive (RS) and radio-resistant (RR) groups and a 12-miRNA signature was revealed [92]. Five of the miRNAs (miRNA-126, let-7a, 495, 451 and 128b) were up regulated and the remaining seven miRNAs (miRNA-130a, 106b, 19b, 22, 15b, 17-5p and 21) were down regulated in the RS group [92]. The role of miRNA-126, the most up regulated miRNA, in modulating RT sensitivity was further shown *in vitro* to be modulating PI3K-Akt pathway illustrating that this miRNA has the potential to be used as a therapeutic target [92].

1.3 **Chemotherapy**

Different principal groups of CT agents with different mechanism of action are used in LC therapy and these include alkylating agents, microtubule targeting agents, DNA synthesis inhibitors and topoisomerase inhibitors. However, the DNA crosslinking platinum compounds are the most widely used ones usually in combination with another drug. Out of these platinum-containing compounds, cisplatin is the most well-known and the most frequently used one for treatment of many solid tumors, including LC, being first approved by the FDA for the treatment of testicular- and bladder cancer in 1978 [95]. However, development of serious side effects such as bone marrow suppression and neuro- and nephrotoxicity, and development of resistance to the treatment forced the development of two other derivatives of platinum compounds, carboplatin and oxaliplatin. Carboplatin was first approved by FDA in 1989 for the treatment of ovarian cancer [96] and oxaliplatin in 2002 for treatment of colorectal cancer [97]. These two compounds have less serious side effects but still the major obstacle in using these drugs is the development of therapy resistance as they have similar chemical structure as cisplatin. However, in a number of clinical trials, it was shown that doublets composed of cisplatin with different other CT agents had better therapeutic potential in terms of OS when compared to carboplatin or oxaliplatin [17]. Therefore, cisplatin combined with etoposide or vinorelbine is still the recommended doublet for LC treatment.

1.3.1 Mechanism of action of platinum compounds

Cisplatin as well as the structurally related compounds, carboplatin and oxaliplatin, mediate antitumor effects via binding to the DNA helix, forming intra- and inter-strand crosslinks thereby inducing DNA single and double strand breaks (SSB and DSB, respectively) [98]. The consequence of these DNA adducts is dependent on the extent of DNA damage which dictates either a DNA damage repair response of the tumor cell and thus survival of the tumor cells or leads to activation of cell death signaling networks including apoptosis [99]. Thus, if the amount of DNA damage is less extensive, the cells will be arrested at S and G2/M phase of cell cycle and the DNA repair machinery will be activated, allowing cell survival [99]. However, if the DNA damage is extensive, apoptotic cell death will occur as a result of cytochrome c release from the mitochondria followed by activation of procaspase-9 and then caspase-3 and subsequently cell demise **(Figure 3)** [99]. Accordingly, a number of proteins are activated in response to DNA damage and mediate the downstream signaling pathways involved in cell cycle arrest, DNA repair and apoptosis. Hence platinum-induced formation of inter- and intra-strand adducts in DNA will recruit sensors of DNA damage which in turn activates components of DNA repair machinery. The most commonly recognized DNA repair pathways for platinum compounds are nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination (HR) and non-homologous end joining (NHEJ) repair **(Figure 5)** [100].
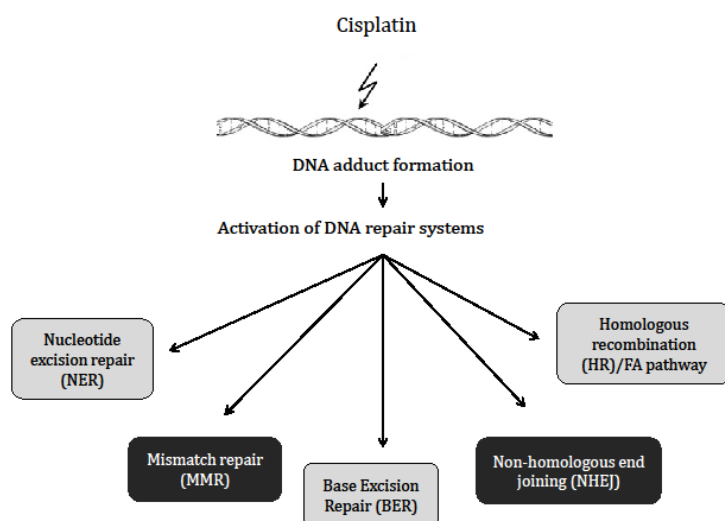

Figure 5. A schematic overview of principal DNA repair pathways induced upon treatment with cisplatin.

1.3.2   Role of miRNAs in chemotherapy response

Several studies have shown that miRNAs can regulate the response of LC cells to cisplatin, gemcitabine, paclitaxel and docetaxe, the most clinically used CT agents for LC [101-115]. Among the miRNAs regulating response of LC cells to cisplatin is miRNA-98 which inhibits p53 and enhances up regulation of Blc-2 causing drug resistance [101]. On the other hand, overexpression of miRNA-497 through down regulating Bcl-2 [102] and miRNA-451 possibly via targeting Akt signaling pathway has been shown to sensitize NSCLC cells to cisplatin [103]. In another study, it was demonstrated that up regulation of miRNA-181a and down regulation of miRNA-630 enhanced cisplatin-induced cell death in NSCLC cell lines [104]. Moreover, overexpression of miRNA-200bc/429 was also demonstrated to sensitize NSCLC cells to cisplatin by targeting Bcl-2 and XIAP [106].

Among the miRNAs regulating response of LC cells to paclitaxel, which is used as a first line treatment of advanced NSCLC, is miRNA-135a which enhances resistance to the drug through down regulation of adenomatous polyposis coli (APC), *in vitro* and *in vivo* [109]. miRNA-101 has also been reported to sensitize NSCLC cells to paclitaxel via targeting enhancer of zester homolog 2 (EzH2) and subsequently enhancing apoptosis as visualized by high Bim-2 expression and PARP cleavage [110].

Certain miRNAs have been linked to response of LC to anthracyclins such as doxorubicin. In an *in vitro* study, it was shown that high expression levels of miRNA-134, miRNA-379 and miRNA-495 increase the sensitivity of SCLC cells to doxorubicin [108]. It was also shown that miRNA-128-2 confers resistance of NSCLC cells to doxorubicin through negative regulation of the transcription factor, E2F5 [111], while miRNA-1 overexpression sensitized the cells to doxorubicin by increased caspase-3 and -7 mediated apoptotic signaling [112]. In relation to gemcitabine, low level of miRNA-133b, which regulates pro-survival proteins, myeloid cell leukemia sequence 1 (MCL-1) and BCL-2-like protein 2 (BCL2L2), has been shown to be associated with drug resistance [113]. The response of SCLC to etoposide has also been shown to be modulated by miRNA-134, miRNA-379 and miRNA-495 [108].

In summary, the above mentioned studies indicate that miRNAs play an important role in the response of LC to CT through targeting different signaling pathways and in a cell dependent manner.

2 AIMS

The overall aim of this thesis was to find novel biomarkers which can be used to predict the response of NSCLC to conventional chemo- or radiotherapy or be used as sensitizers of these conventional therapies. The specific aims of each project were:

➢ To identify miRNAs which can be linked to RT response of NSCLC and SCLC (**Paper I**).

➢ To analyze miRNA-214 targets and their significance for invasion and metastatic spread of NSCLC (**Paper II**).

➢ To find novel cisplatin resistance mechanisms of NSCLC and determine pathways to be targeted for improvement of cisplatin response (**Paper III**).

3   MATERIALS AND METHODS

3.1 Cell lines

A panel of SCLC and NSCLC cell lines was used in this thesis. Their intrinsic radiotherapy sensitivity which has been tested through colony formation assay and measured as surviving fraction after two grays (SF2) are given in **Table 1**.

Table.1 Histology and SF2 values of the cell lines used.

Histology	Cell line	SF2 value	References
NSCLC	A549 (AC)	0.62-0.82	[116, 117]
	H23 (AC)	0.17-0.20	[116, 117]
	H157 (LCC)	0.38-0.8	[116, 117]
	H661 (LCC)	0.93	[116]
	H1299 (AC)	0.3	[118]
	U-1810 (LCC)	0.80-0.88	[119, 120]
SCLC	H69	0.23-0.25	[116, 121]
	H82	0.58-0.73	[116, 121]
	U-1285	0.25-0.30	[119, 120, 122]
	U-1690	0.57	[123, 124]
	U-1906	0.45-0.59	[119, 120]

Abbreviations: SF2: surviving fraction after 2-Gy irradiation, NSCLC: non-small cell lung cancer, SCLC: small cell lung cancer, AC: adenocarcinoma, SCC: squamous cell carcinoma, LCC: large cell carcinoma.

3.2 Patient tumor material

A cohort of 598 tumors obtained from NSCLC patients was used in **Paper II** for analysis of biomarker expression using immunohistochemistry (IHC) on tissue microarrays (TMA)s. The patients were operated for curative resection of the tumor at Karolinska University Hospital, Sweden during 1997-2004. The tumors were formalin-

fixed paraffin embedded (FFPE) and archived. Ethical permit for research usage of the material was taken from Karolinska Institutet ethical committee (2005/588-31/4). For details of the patient characteristics and the exclusion criteria of the samples in the study see Materials and methods section of **Paper II**.

3.3 Treatments

In this thesis two commonly used treatment options in NSCLC were applied which were IR (Co^{60} source) and cisplatin. In **Paper I** the aim was to study effect of radiation-induced cell death and how that could be manipulated. A dose of 8-Gy IR was chosen as the NSCLC cell line used, U-1810, was previously shown to be refractory to this dose during a 72h period [125, 126]. For H23 cells also, 8 Gy was used in order to analyze the protective role of miRNA against induction of RT-induced cell death. For clonogenic survival in **Paper III** a range of cisplatin doses was applied (2.5-20 µM) whereas for gene expression profiling and the rest of experiments, 10 µM cisplatin was chosen. The doses applied in clonogenic survival assays are clinically relevant as these doses have been measured in plasma of cancer patients after cisplatin administration [127].

In **Paper I and II**, we used mimic and antagomir of miRNA-214 to increase or decrease the endogenous level of this miRNA in NSCLC cells, respectively, and thereafter study the effect on RT response and invasion capacity. Antagomir of miRNA is composed of RNA oligonucleotides with novel secondary structure which improves the efficacy and longevity of the molecule [128]. Antagomirs are used for loss-of-function studies. Mimic of miRNA is a double-stranded oligonucleotide chemically enhanced to preferentially program RISC with active miRNA strand [129]. Mimics of miRNAs are used for gain-of-function studies.

3.4 Cell death and survival assays

Assessment of apoptotic nuclear morphology
DAPI (4',6-diamidino-2-phenylindole), a fluorescent dye which can pass through intact cellular membrane and bind to the DNA, was used to stain the cellular nuclei after treatment with IR in order to detect apoptotic features of cell nuclei such as chromatin condensation and fragmentation **(Paper I)**.

Analysis of cell cycle distribution and caspase-3 activity

Flow cytometry is a broadly used technique to measure cellular DNA content in each phase of cell cycle and the fraction of cells with apoptosis [130]. This technique was used to measure the cell cycle progression and percentage of cells with sub-G1 (**Paper I**). In order to permeabilize the cell membrane, digitonin which facilitates entry of the stain into the cells, was applied. Propidium iodide (PI) which is a fluorescent dye was used to assess DNA content. The cells were fixed in ethanol and permeabilized by digitonin and then treated with RNase-A followed by staining with PI. To measure apoptotic signaling, a FITC-conjugated anti-caspase 3 antibody which recognizes active caspase-3 was applied in flow cytometry. The amount of fluorescence emitted from the stained cells was then captured by a flow cytometer and the percentage of cells for each phase of the cell cycle or stained with FITC-conjugated antibody was then revealed [130].

Senescence assay

Senescence is a cellular state recognized by inability of cells to divide which is associated with morphological changes and gene expression alteration. We used a commercially available kit to measure the activity of β-galactosidase enzyme, a marker of this phenotype (**Paper I)** [131].

Colony formation assay

Colony formation assay is a commonly used method to measure cytotoxicity of treatments such as CT or RT. It is based on the assessment of the capability of single cells to undergo cell division and form colonies over a period of 1-3 weeks [132]. The colonies are thereafter stained and the relative survival proportion to untreated cells is used to describe the cytotoxicity of treatment. We used this assay to measure the response of cells to cisplatin treatment and also isolated these clones for gene expression profiling **(Paper III)**.

3.5 Invasion assay

Invasiveness of tumor cells is commonly measured via transwell invasion chambers *in vitro*. We used this technique to assess the invasiveness of NSCLC (**Paper II**). Because of the high invasive potential of the cells used, difficulties in accurate counting of the number of invading cells was experienced therefore another more recently established technique, real-time cell invasion and migration (RT-CIM) system [133], was applied.

A special plate is used for this purpose which has electrodes attached to the under surface measuring the electrical impedance imparted by the invasive cells upon time. The advantage of this method over the transwell assay is that the invasiveness can be measured in real time as the electrodes are continuously attached to the plate and automatically registers the impedance as it is set up [134].

3.6 MicroRNA and gene expression assays

Microarray

MicroRNA microarray is one of the broadly used techniques for assessment of miRNA expression but there are also other alternatives such as bead-based technology and RT-PCR-based techniques [135]. In **Paper I** we applied microRNA microarray technology (Affymetrix Inc., Santa Clara, CA, USA) to profile miRNA expression in the NSCLC and SCLC cell lines panels on a global scale. We used this technique for miRNA profiling as it has the capacity of high-throughput profiling covering a large number of miRNAs and it is a golden standard technique in the field [136]. The total RNA from the cells was labeled with biotin and then hybridized onto the microarray chip. After washing and staining steps, the array chips were scanned using LASER scanner. The signal intensities for each probe were background extracted and normalized to the spike-in controls. For gene expression profiling in **Paper II** and **Paper III** the same technique was used but instead of miRNA probes, multiple transcript probes covering different lengths of about 22 000 genes were applied [137].

Quantitative real-time polymerase chain reaction (q-RT-PCR)

q-RT-PCR is a technique measuring the copy number of a gene of interest in the cells via its amplification and detection in real time [138]. The principle of the technique is based on ordinary polymerase chain reaction except for the fact that the end product of amplification is detected and quantified at the end of each reaction. The product used for this purpose is total RNA which is reverse transcribed to cDNA via a transcriptase enzyme and through the help of specific primers the gene of interest is amplified and detected. For q-RT-PCR analysis of miRNA expression, TaqMan miRNA primers and probes designed by Applied Biosystems were used [139]. This looped structure of the primer allows for more specific and efficient detection of the mature miRNAs. This technique was used in both **Paper I and II** for validation of the microarray findings and the efficiency of transfection with antagomirs or mimics of miRNA. For

amplification and quantification of other genes than miRNA, ordinary q-RT-PCR was applied (**Paper I-III**).

3.7 **Bioinformatics analyses**

We used a number of bioinformatics tools to analyze the miRNA and gene expression data. For prediction of miRNA targets, three commercially available softwares, Targetscan, PicTar and MicroCosm (previously called miRBase) were applied in order to find the targets of miRNA-214 in an unbiased way (**Paper I**). Each of these tools uses a different background algorithm to predict the targets of miRNAs [140-142]. PicTar predicts the targets of miRNAs based on sequence match between the seed region of 5´end of miRNAs and the 3´ UTR of the mRNAs [141]. TargetScan also uses the sequence match between the seed region of miRNA and the 3´ UTR of the mRNAs but also takes the conservation of miRNA targets between different species into consideration [142]. MicroCosm takes the sequence similarity and conservation across species into account for predicting the targets of miRNAs and finding multiple sequence matching across the 3´ UTR of the mRNAs and besides that it gives a P-value for each predicted target [140]. To integrate each gene of interest into signaling pathways and find relevant testable hypothesis, we used Ingenuity Pathway Analysis (IPA) tool (**Paper I, II and III**). IPA is one of the three most frequently used network analysis tools in systems biology field and is based on manual annotations about genes and proteins and thereby building networks of interactions [143]. In **Paper II**, another function of IPA called ingenuity miRNA target filter was applied to find the targets of miRNA-214. This tool is based on findings of Targetscan and Tarbase tool, the latter describes the experimentally proven targets of miRNAs [144], and Ingenuity intelligence center to identify the targets of miRNAs.

4 RESULTS AND DISCUSSION

4.1 Paper I

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

In this study we aimed to understand if miRNA expression could be linked to RT responsiveness of LC and identify signaling events that could be targeted for RT sensitization purposes. For that purpose, we used a panel of SCLC and NSCLC cell lines with different intrinsic RT sensitivity (measured via colony formation assay and indicated by SF2 (**Table 1**). The outline of the study is shown in **Figure 6**.
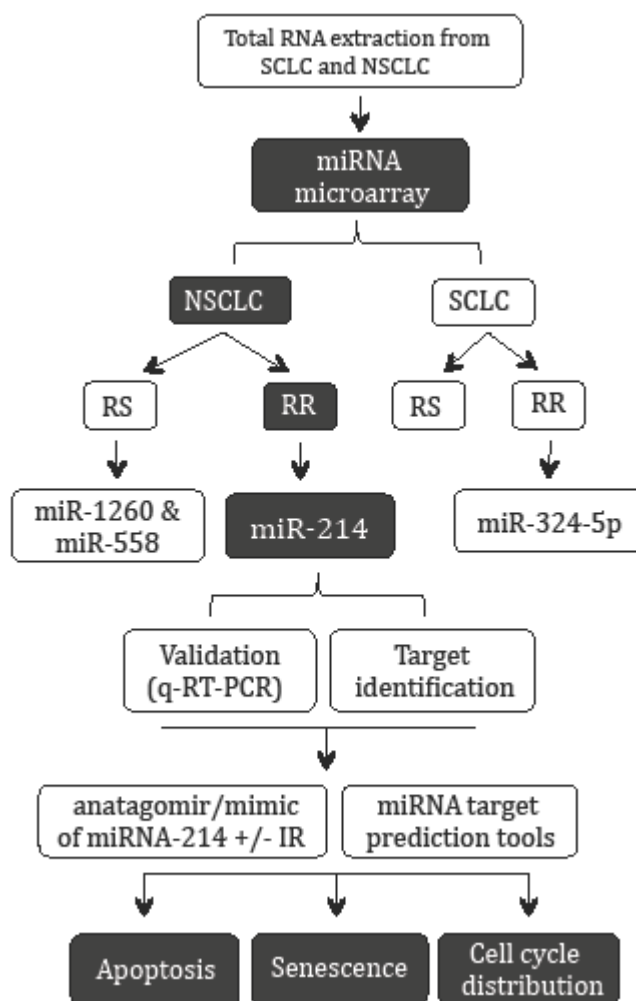
Figure 6. Schematic outline of experimental setup (**Project I**). RR and RS represent radioresistant and radiosensitive, respectively.

Through miRNA profiling, we showed that each LC cell line expressed about 100-200 miRNAs at a level which was significantly over background. All the NSCLC cell lines had 69 miRNAs in common while the SCLC cell lines shared 56 miRNAs. The NSCLC cells differentially expressed 25 miRNAs compared to the SCLC cell lines which in turn differentially expressed 10 miRNAs.

The cell lines were divided into two groups with a cutoff SF2 value of 0.3 to differentiate radioresistant (RR) cells (SF2 value $\geq$ 0.3) from the radiosensitive (RS) one (SF2 value < 0.3). With this approach, a number of miRNAs were found to be expressed in one or more of the RR NSCLC or SCLC cell lines and not in the RS ones as illustrated in **(Figure 7)**.
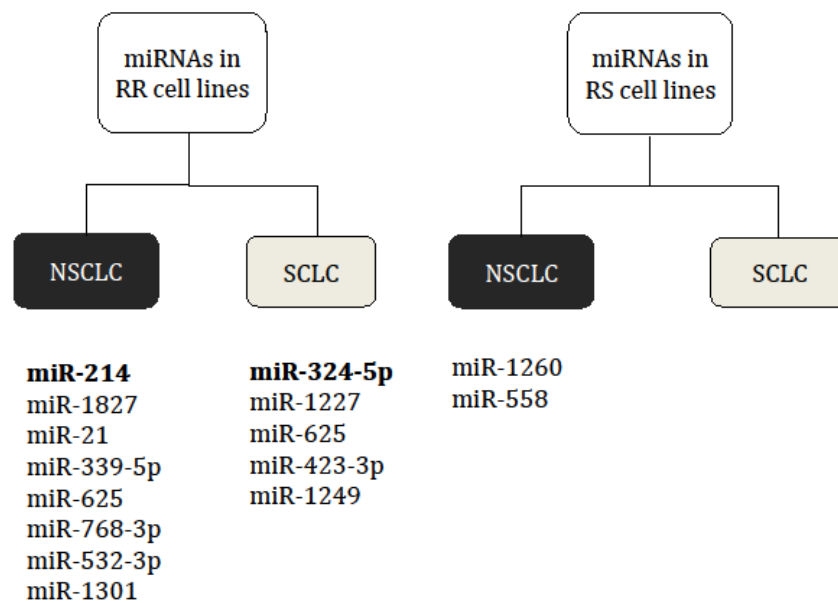
Figure 7. miRNAs differentially expressed in radioresistant (RR) and radiosensitive (RS) NSCLC and SCLC cell lines.

In the clinical setting, in general, SCLCs are known to be responsive to both CT and RT in the first line but majority of the patients develop resistance to treatment resulting in tumor progression and poor outcome [9]. Previous reports from our group have suggested that a RR phenotype of SCLC is in part attributed to defective apoptotic propensity possibly due to lack of components of apoptotic cascade e.g. pro-apoptotic caspase-8 and high Bcl-2/Bax ratio [145] but also as a consequence of increased DDR and DNA repair signaling [146]. Yet, the role of miRNAs in this context remains

elusive. In our study, we found miRNA-324-5p to have a differentially higher expression in the RR subtype of SCLC **(Figure 7)**. Expression of this miRNA in other tumor types has been shown to be associated with tumor development and metastasis [147, 148]. Thus, overexpression of miRNA-324-5p was found to prevent medulloblastoma development via down regulation of the transcription factor, Gli1 [147] and up regulation of miRNA-324-5p is reported to be associated with distant metastasis of oropharyngeal carcinoma [148]. However, the functional role of miRNA-324-5p in SCLC and in their RT response has not been addressed. Interestingly, when analyzing the predicted targets of miRNA-324-5p, a number of proteins involved in apoptosis such as apoptotic peptidase activating factor 1 (Apaf-1), Diablo and calpain as well as DNA repair components like Rad52 and Runx1 were identified. Thus, alteration in miRNA-324-5p may explain the observed alteration in apoptosis and DNA repair in SCLC but further studies are required to prove if this is the case.

In NSCLC, we found miRNA-21 among the miRNAs which were linked to radioresistance **(Figure 7)**. Interestingly, miRNA-21 has previously been shown to have a lower expression in NSCLC tumors responding to postoperative RT as compared to poor responders indicating a potential role of this miRNA in radioresistance [92]. *In vitro* studies have also linked miRNA-21 to RT and both cell cycle check point proteins like CDC25A [149] and DNA repair proteins like mismatch repair (MMR), such as human mutS homolog 2 and 6 (hMSH2 and hMSH6, respectively) are influenced by this miRNA [150].

One of the important findings of this study was that miRNA-214 was linked to a RT refractory phenotype of NSCLC. In order to substantiate a role for miRNA-214 in RT response, we used antagomirs towards this miRNA and could demonstrate that ablation of miRNA-214 expression in RR NSCLC sensitized for RT-induced senescence but not apoptosis. Moreover, through overexpression of miRNA-214 in a RT sensitive NSCLC, we observed decreased RT-induced apoptotic response **(Figure 8)**. Thus, we proved that miRNA-214 is a regulator of RT response of NSCLC cells. The significance of miRNAs in regulating apoptotic pathways has been described previously such as negative effect of miRNA-17-5p on apoptosis via targeting the pro-apoptotic protein, Bim [151], or miRNA-1 and miRNA-101 through targeting the anti-apoptotic protein, Mcl-1 **(Figure 3)** [152]. However, modulation of different modes of cell death i.e. apoptosis and senescence in response to manipulation of miRNA-214 may have different explanations. First, the cell type-specific effects of miRNAs cannot

be ignored as it has been shown in many studies that the same miRNA could target different pathways in different tumor types. For example miRNA-214 is influencing metastatic potential of malignant melanoma through regulating transcription factor AP2 gamma (TFAP2C) while in cervical cancer via targeting plexin-B1 [153, 154]. Second, the higher magnitude of miRNA-214 overexpression after introducing mimic-214 in the RS cells which was even higher than the baseline level of the miRNA-214 in the RR cells could possibly influence other targets than just senescence. We observed increased senescence after miRNA-214 ablation in RT resistant NSCLC cells and in order to further analyze possible molecular determinants involved, we checked the expression level of two well-known cell cycle and senescence regulators, p21$^{WAF1/Cip1}$ and p27^{Kip1} [155-157] using western blot. We observed no change in the p21$^{WAF1/Cip1}$ expression in the RR NSCLC cells after miRNA-214 knockdown in combination with IR. One possible explanation could be that the primary transcriptional regulator of p21$^{WAF1/Cip1}$, p53 [158], is mutated resulting in lack of its expression in these cells [159] which may cause this signaling circuit to be impaired. Although senescence is mainly occurring during the G0 phase of the cell cycle, induction of senescence at G2/M arrest has also been reported in human tumor cells through a mechanism involving the anti-apoptotic protein Bcl-XL inhibition of cyclin-dependent kinase 1 (CDK1) which is controlling G2/M phase of the cell cycle [160]. Such a mechanism is in line with our data as we found that miRNA-214 knockdown sensitizes the NSCLC cells to IR through G2 cell cycle arrest and induction of senescence with a concomitant increase in p27^{Kip1} expression. A role of p27^{Kip1} in G2/M arrest has been reported in different studies [161, 162], further supporting this explanation. Hence elucidation of the downstream target of p27^{Kip1} involved in the G2/M arrest and induction of senescence in these NSCLC cells would therefore be interesting.

To identify the potential targets of miRNA-214 which could govern a RR phenotype of the NSCLC cells in an unbiased way, we used three common miRNA target prediction tools, Target scan, PicTar and MicroCosm [141, 142]. The target genes in common were further investigated using IPA with respect to connection to apoptosis and/or senescence. One of the reported targets of miRNA-214 was phosphatase and tensin homolog (PTEN), a tumor suppressor which has previously been reported to confer RT sensitivity via inhibition of the pro-survival PI3K/Akt signaling pathway [163, 164]. PTEN has been demonstrated in ovarian cancer cells and monocytes to be a direct target of miRNA-214 and in these cells, ablation of miRNA-214 expression was found

to increase PTEN expression leading to inhibition of Akt phosphorylation and induction of apoptosis [165, 166]. In contrast to this study, we did not observe a significant alteration in the PTEN/Akt signaling cascade in response to miRNA-214 manipulation pointing towards a minor role of this pathway in the RT response of at least these NSCLC cells. Albeit it is not surprising that the same miRNA is regulating treatment response of different tumors by targeting different genes [167], there are a number of possible explanations to this discrepancy. First of all, we cannot rule out that miRNA-214 has mutation in part of the sequence binding to PTEN mRNA such mechanism has previously been shown for other miRNAs e.g. mutation in the sequence of miRNA-499 leads to failure of the miRNA to regulate mRNA targets in cardiac cells [168]. Another possible explanation is that PTEN mRNA is not expressed in sufficient amount to be targeted by miRNA-214 in our NSCLC cells. In support of this explanation is a report which demonstrated that at least a sub fraction of NSCLC tumors expresses low levels of PTEN [169]. Yet, another possibility is that PTEN is mutated rendering it insensitive to miRNA-214 regulation and in support for this is a study which demonstrated PTEN mutations in about 6% of NSCLC tumors [170]. DNA sequencing of both miRNA-214 and PTEN would reveal if any of these explanations are correct in this NSCLC system.

Another predicted target of miRNA-214 was forkhead box protein O4 (FoxO4), a transcription factor which has been demonstrated to regulate different cellular processes including apoptosis and oxidative stress via the c-Jun N-terminal kinase (JNK) pathway [171]. We examined FoxO4 upon irradiation of the RS cells with forced overexpression of miRNA-214. Interestingly, we observed a reduction of apoptosis concomitant with increased phosphorylation of FoxO4 rather than a decrease which one would expect if miRNA-214 was directly targeting the FoxO4 mRNA. Our current hypothesis is that the increased FoxO4 phosphorylation is probably due to inhibitory effect of miRNA-214 on upstream regulators of FoxO4. However, an inhibitory effect of FoxO4 phosphorylation on apoptosis has been shown in endothelial progenitor cells via negative regulation of Bim [92]. Moreover, it has been demonstrated that FoxO4 confers resistance to oxidative stress-induced apoptosis via p38MAPK pathway [172]. Given that we observed increased phosphorylation of p38MAPK upon miRNA-214 overexpression and irradiation and that inhibiting p38MAPK activation re-sensitized the cells to RT, an effect of FoxO4 on p38MAPK is a plausible explanation that would be interesting to further examine. Furthermore, previous reports from our group also

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support a role for p38MAPK in RT response as it was demonstrated that activation of this pathway via insulin-like growth factor 1 receptor (IGF-1R) enhances RT resistance in NSCLC [173]. However, how miRNA-214 regulates p38MAPK needs to be further explored as so far it has not been reported. Moreover, we observed alteration mainly in the phosphorylated form of p38MAPK whereas the total expression level was rather modest. This suggests that the regulation of p38MAPK by miRNA-214 most likely is indirect. Further studies are required to elucidate these possibilities.

In conclusion, we show that knockdown of miRNA-214 sensitizes NSCLC cells to RT through induction of senescence with concomitant $p27^{Kip1}$ up regulation. Moreover, we demonstrate that overexpression of miRNA-214 activates a FoxO4 and p38MAPK signaling pathway in response to RT in RS NSCLC cells thereby prevents RT-induced apoptosis. Modulation of different cell death/survival pathways by miRNA-214 in these NSCLC cells supports the concept that miRNA-214 is regulating multiple cellular processes and in a cell type-dependent manner. These findings are summarized in **Figure 8**.
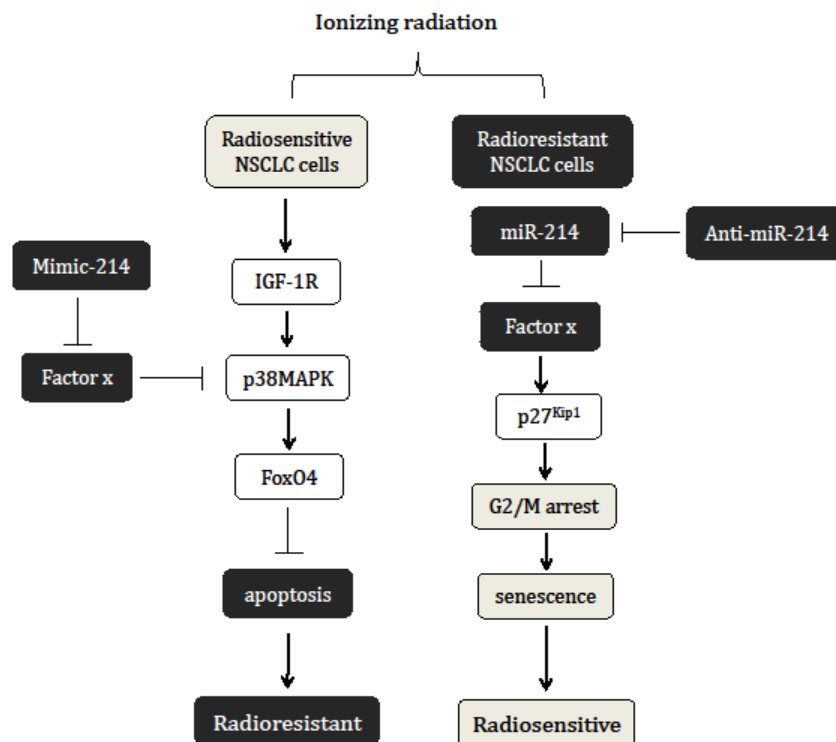

Figure 8. Mechanism of miRNA-214 regulation of RT response in NSCLC cells.

4.2 Paper II

miRNA-214 is related to invasiveness of human non-small cell lung cancer and directly regulates alpha protein kinase 2 expression

Metastasis is considered the main etiological factor of poor prognosis of NSCLC as illustrated by shorter overall survival of the advanced stage of the disease as compared to early stages [174]. Therefore, finding biomarkers in the primary tumor which can predict metastatic spread or predict treatment response is urgently needed to sort out the NSCLC patients that should be given CT upfront as an adjuvant treatment. Interestingly miRNAs have previously been linked to metastasis of NSCLC for example overexpression of miRNA-143 has been shown to inhibit invasion and metastasis of NSCLC possibly via CD44v3 [175] and miRNA-335 has been shown to reduce metastasis of NSCLC cell lines via regulation of BCL2L2 and specificity protein 1 (SP1) [176]. In **paper I** we identified miRNA-214 as a regulator of NSCLC RT response. Interestingly, a role for miRNA-214 in regulating metastasis of other human tumors has been reported i.e. in esophageal, cervical, hepatocellular, breast and gastric cancer where miRNA-214 inhibits metastasis by altering different signaling cascades [154, 177-182]. However, a role for miRNA-214 in driving NSCLC metastasis remained elusive. Therefore, we aimed to explore the role of miRNA-214 in the invasiveness of NSCLC *in vitro*, to identify putative targets involved in this process and to analyze their function in NSCLC clinical specimen as illustrated in **Figure 9**.

We found that knockdown of miRNA-214 expression enhances invasiveness of NSCLC cells and vice versa, overexpression of the miRNA reduces invasive potential of the cells. Thus our results are in line with those reported in cervical, hepatocellular, breast and gastric cancer where miRNA-214 is described to inhibit metastasis [154, 177-182]. However, our results are in contrast to what has been observed in metastatic melanoma where miRNA-214 was found to enhance metastasis [153]. These results illustrate that miRNA-214-induced regulation of metastasis is cell type-dependent and that in NSCLC cells miRNA-214 is a negative regulator of invasion capacity.

Besides increasing the invasiveness of the cells, we found that miRNA-214 knockdown also reduced the proliferation rate of the NSCLC cells yet overexpression of the miRNA did not increase proliferation capacity which indicates that the effects of miRNA-214 are likely regulated by different pathways and in a cell type-dependent manner.

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In order to identify putative genes involved in the miRNA-214 regulation of NSCLC invasion and metastasis we carried out gene expression profiling of NSCLC cells with ablated miRNA-214 expression. Results showed that blockade of miRNA-214 expression in these NSCLC cells caused a direct or indirect up regulation of 297 and down regulation of 33 genes by at least 1.5-fold. As miRNAs are known to negatively regulate the expression of genes, the observed down regulation of 33 genes in the NSCLC cells with blocked miRNA-214 expression is likely due to indirect interaction of the miRNA-214 with upstream regulators of these genes. In order to search for reported miRNA-214 target genes among the observed gene expression alterations, we made use of Ingenuity miRNA target filter which identified 1360 genes to be potential targets of miRNA-214. After overlapping these potential targets with the 297 up regulated genes, a list of 18 common genes was found in our model system **(Table 2)**.

Table 2. List of genes up regulated after miRNA-214 ablation by at least 1.5-fold.

Number	Gene symbol	Fold change
1	EFCAB4B	2.0
2	HDAC9	1.8
3	ZBTB20	1.8
4	TRAF1	1.7
5	ALPK2*	1.6
6	CDK6*	1.6
7	TNFAIP3*	1.6
8	CPEB4	1.6
9	CYLD	1.6
10	PDE5A	1.6
11	MECOM	1.6
12	MTMR7	1.6
13	PAPPA*	1.5
14	MYO1D	1.5
15	NLGN1	1.5
16	QTRTD1	1.5
17	SPIRE1	1.5
18	FAM122A	1.5

Metastasis-related targets of miRNA-214 in NSCLC.

To filter out metastasis-related targets of miRNA-214 in our data set, we made use of a previously published metastasis-associated gene signature identified in a metastatic mouse LC model with deleted liver kinase B1 (LKB1) and mutated KRAS [183]. We overlapped this signature with the list of 18 target genes of miRNA-214 in our model **(Figure 9)**. Four genes were found to be in common with the metastasis signature. These were pregnancy-associated plasma protein A (PAPP-A), alpha protein kinase 2 (ALPK2), cyclin-dependent kinase 6 (CDK6) and tumor necrosis factor, alpha-induced protein 3 (TNFAIP3).
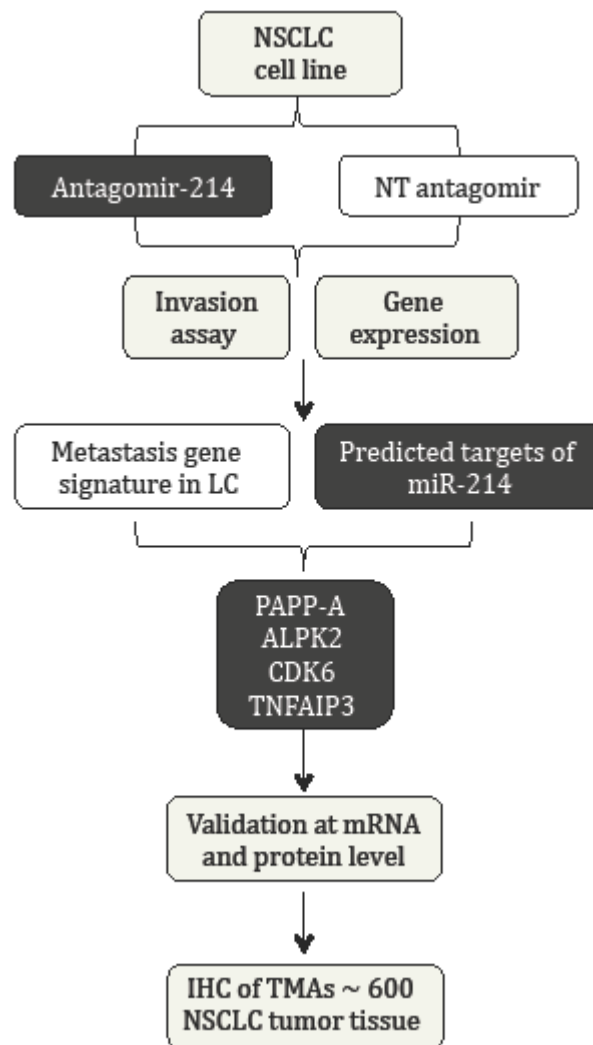
Figure 9. Schematic outline of the experimental setup (**Project II**).

The four identified candidates were all validated to be altered at mRNA level using q-RT-PCR. At protein level; however, PAPP-A, ALPK2 and CDK6 but not TNFAIP3 were found to be regulated by miRNA-214 using a combination of different techniques (western blot, ELISA and immunofluorescence). With overexpression of miRNA-214, the level of these targets was reduced significantly, further supporting the results

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achieved by the microarray technique. To test the direct or indirect regulation of these genes by miRNA-214, we used Ago2 immunoprecipitation as it has been shown that human miRNAs are directed towards their targets through Ago2 protein and that miRNA and their genes both can be recovered within this complex by such an approach [184]. Our results show that only ALPK2 is directly regulated by the miRNA-214 and the rest are likely indirect targets as they were not enriched in the Ago2 immunoprecipitates. Hence, our results suggest that miRNA-214 regulates PAPP-A gene expression by influencing the expression of some of its upstream regulators e.g. transcription factors. In support for such hypothesis, we observed that besides PAPP-A, other members of metalloproteases were also up regulated after miRNA-214 knockdown, such as matrix metalloproteinase 9 (MMP9) and leucyl/cystinyl aminopeptidase (LNPEP). Moreover, we identified that other regulatory factors of metalloproteases such as phospholipase D (PLD), interleukin 1 (IL1) and G-protein-coupled receptor (GPCR) [185-187] were concomitantly up regulated after miRNA-214 knockdown. This indicates that miRNA-214 is targeting upstream regulators of metalloproteases including PAPP-A. In conclusion, our data show that miRNA-214 is directly regulating the expression of ALPK2 while PAPP-A, CDK6 and TNFAIP3 are indirect targets of this miRNA. The possible role of miRNA-214 in regulating invasiveness of NSCLC is summarized in **Figure 10**.

In order to understand the role of these targets in inducing metastasis and thus their correlation to survival of NSCLC patients, the protein expression level of PAPP-A, ALPK2, CDK6 and TNFAIP3 was examined through immunohistochemistry (IHC) of tissue microarrays (TMAs). For this purpose, a cohort of about 600 NSCLC specimens was used. We found that all these proteins were expressed in NSCLC clinical tumor specimen at moderate to high level as compared to normal tissue. However, there was no correlation between the expression level of these targets and the clinical records of metastasis or the OS of the NSCLC patients. There are at least two putative explanations to the lack of these associations. The first is presence of micro-metastasis in the clinically recorded cases as non-metastatic. The second explanation is technical and relates to the TMA construction and the heterogeneity of expression in different parts of the tumor specimen. As these proteins are involved in invasion, one can hypothesize that the expression level is higher in the invasive front of the tumor specimen. Since TMAs cover only a small piece of clinical tumor specimen and is taken from the core of the tumor, it is plausible that it does not cover the invasive front.

Hence our data call for extended analyses where the whole tissue or at least part of the tumor tissue including the invasive front is confined. For PAPP-A, the secreted form to the tumor environment rather that its intra tumor cell expression may play a role in induction of metastasis [188]. Therefore, it would be interesting to examine if the level of PAPP-A in the serum of these NSCLC patients is predictive of metastasis. Interestingly, high level of PAPP-A has been reported in serum of LC patients as compared to healthy controls albeit not in the context of metastasis [189].

In conclusion, we show in this study, that miRNA-214 is playing a significant role in regulation of invasiveness of NSCLC *in vitro* and it is directly regulating ALPK2 but indirectly regulating PAPP-A, CDK6 and TNFAIP3. Furthermore, we show that NSCLC tumors express these proteins at moderate to high level but their significance in predicting metastasis needs to be further explored.
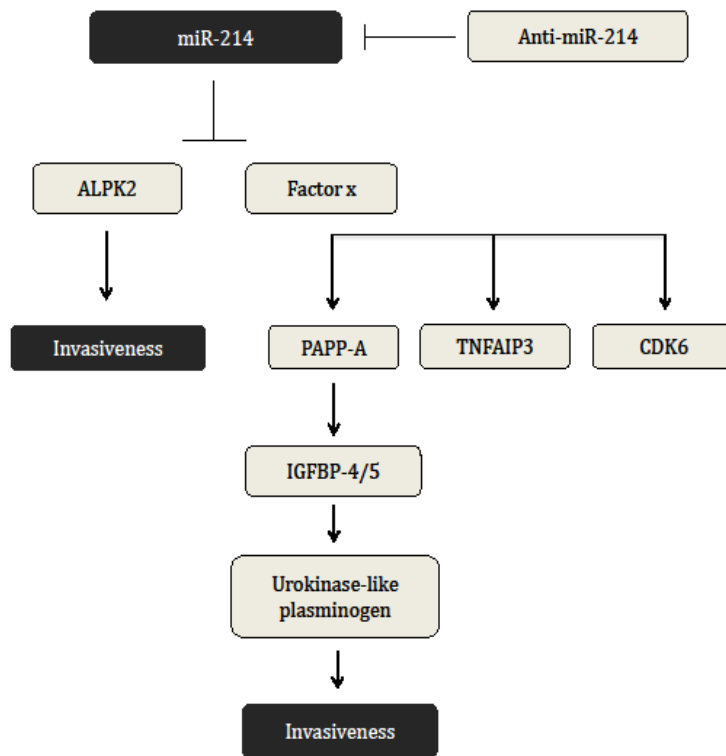
Figure 10. Possible mechanism of miRNA-214-mediated regulation of invasiveness of NSCLC cells *in vitro*. The influence of PAPP-A on regulation of IGFBP-4/5 has been shown before and the latter is inducing invasiveness through activation of urokinase-like plasminogen.

4.3 **Paper III**

Gene expression profiling of cisplatin-refractory NSCLC residual clones reveals DKK1 as a potential novel driver of resistance

Cisplatin is the mainstay CT used in the treatment of NSCLC; however, a large proportion of the patients show intrinsic resistance and only about 20% of patients with advanced disease respond to the drug in the first line [190]. Therefore, identifying biomarkers predicting cisplatin responsiveness has clear clinical relevance so that unnecessary exposure of patients to the detrimental side effects of cisplatin can be minimized. Indeed biomarkers predicting response or prognosis of NSCLC patients treated with cisplatin have been investigated during the last decade. A number of potential predictive biomarkers have been described in NSCLC such as ERCC1, RRM1, BRCA1, RAP80 and KRAS [191-194]. Thus, low expression of ERCC1 has been shown to be associated with poor response to CT in patients with advanced NSCLC [193]. However, the results from other studies have shown that ERCC1 as well as the other biomarkers can only predict cisplatin- or combined CT response in a sub group of NSCLC patients i.e. those with certain tumor histology or mutations [191, 192]. Sometimes the expression of more than one gene is necessary to predict the response to CT, for example the protein expression of BRCA1 *per se* failed to be associated with clinical outcome in NSCLC patients treated with cisplatin [195]. However, low mRNA expression of BRCA1 together with low RAP80 level was linked to a better outcome of advanced NSCLC patients treated with combined CT [192]. This suggests that multiple markers are likely required to be considered predictive to CT response. Therefore, identification of pathway aberrations rather than single genes will likely have greater potential to sort out CT responders from non-responders. For that purpose, we performed gene expression profiling of NSCLC cell clones surviving cisplatin treatment using a dose achievable in plasma [127]. We used only a single dose in order to identify the deregulated genes related to intrinsic resistance rather than acquired resistance property and performed the gene expression profiling of the surviving clones after 9 days. The results showed that these NSCLC had a heterogeneous response both in terms of survival and gene expression patterns. One may speculate that the heterogeneity of the clones in terms of gene expression is a result of survival of selective subgroups of cells with certain gene expression alterations rendering them resistant to treatment.

With a cutoff value of 1.5-fold up- or down regulation, we found that formin 1 (FMN1), which has been shown to regulate the cell-cell junction [196], was the only gene which was down regulated in all of the three biological replicates. Cisplatin has been shown to disrupt cell-cell interaction followed by induction of apoptosis [197]. As FMN1 is regulating the cell-cell interaction and it is down regulated in the treatment resistant clones, cisplatin will be unable to disrupt the cellular junction and thus fail in inducing cell death. Further studies are required to prove this concept.

Among the top de-regulated genes after cisplatin treatment, we found components of different DNA repair pathways which have been shown to enhance cisplatin resistance such as XRCC2 [198]. Apart from the DNA repair genes, we found a member of the Wnt signaling pathway, dickkopf-1 (DKK1), and a member of galectins, Lectin galactoside-binding soluble 9 (LGALS9), among the top up regulated genes in the cisplatin treatment surviving clones. LGALS9 is a member of galectins which have been shown to be linked to CT resistance [199, 200]. Thus overexpression of galectin-1 has been shown to induce resistance of lung AC cells to cisplatin via interaction of Cox-2 and RAS and thereby activating p38MAPK, ERK and NFκB pathway [199]. In another study, the role of galectin-3 was found to be instrumental in pancreatic cancer cell resistance to gemcitabine-induced apoptosis [200]. These reports show the significance of galectins in CT resistance.

In this study we identified DKK1, a negative regulator of Wnt signaling pathway, as a potential novel biomarker of cisplatin response in NSCLC. DKK1 has been shown to have a diverse oncogenic or tumor suppressive effect in different tumor types [201, 202]. Thus down regulation of DKK1 has been shown to be associated with cisplatin resistance in head and neck cancer [201] and overexpression of DKK1 has been shown to sensitize glioma cells to the same agent [202]. Interestingly, in patients with NSCLC and esophageal cancer as compared to healthy controls, high serum level and high tumor expression of DKK1 have been observed, with the latter being associated with poor prognosis [203-205]. In order to prove a role of DKK1 as a driver of cisplatin resistance in NSCLC cells, we examined if siRNA-mediated suppression of DKK1 in these cells could sensitize for cisplatin treatment. Indeed, we could show that cells which had a reduced DKK1 expression become more sensitive to cisplatin as compared to the parental cells and had a reduced clonogenic capacity. Through Ingenuity Pathway Analysis (IPA) tool, we identified 16 upstream transcriptional regulators and 3 downstream targets of DKK1. Among the upstream regulators of DKK1 we observed

up regulation of TCF4, EZH2, DNAJB6 and HDAC2 in the cisplatin treatment surviving clones. Among the downstream targets of DKK1, we found CDKN1A, EPHB2 and GSK3B to be altered and we also showed that knockdown of DKK1 was associated with reduction in phosphorylation of GSK3B at serine 9 residue. Interestingly, an increased phosphorylation of GSK3B at serine 9 residue has been shown to confer resistance of ovarian cancer cells to cisplatin [73]. These data show that, DKK1 in part drives cisplatin resistance in NSCLC cells likely by regulating GSK3B signaling.

In summary, we show that long term survival from cisplatin is associated with a heterogeneous survival response and gene expression pattern. Moreover, we found DKK1 as a potential novel marker for cisplatin resistance and a possible cisplatin sensitizing target.

5   GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

The overall aims of this thesis were to find novel biomarkers for predicting response to conventional RT/CT and/or prognosis of LC patients as well as to identify novel sensitizing targets for such therapies. A systems biology approach was taken and gene and miRNA expression analyses in *in vitro* models of NSCLC were performed for this purpose. The future implications of our findings in the clinical setting and the challenges of these approaches are summarized below.

Implications of miRNAs as targets for sensitization or as biomarkers of response to RT in NSCLC

In **paper I** miRNA microarray on a panel of NSCLC and SCLC cell lines with different radiotherapy sensitivity identified only miRNA-214 to be associated with RT responsiveness across the NSCLC panel. However, a number of other miRNAs was found to have an expression level above noise and thereby might be of importance for RT response for each of these particular NSCLC cell lines. Connection of only one miRNA to RT phenotype may not be surprising as the NSCLC cell lines used were of various histological subtypes which have recently been shown to possess different miRNA expression patterns [206]. The differential expression of miRNAs in each NSCLC subtypes is possibly a result of activation of different signaling pathways in these tumors which require the compensatory amplification or deregulation of the miRNAs. Such co-evolution between expression of miRNAs and alterations on the DNA level could be achieved by mutations in the genes regulating the miRNA [207]. My conclusion after studying miRNAs in LC is therefore that it is more interesting to focus on the whole signaling network in which the miRNA is acting rather than just a single miRNA and thereby be able to understand the underlying driving machinery of each tumor subtype.

Although findings in my thesis and work from others [208] illustrate that using miRNAs in the clinical practice is challenging, they may still have a role as biomarkers since miRNAs are stable in circulation and techniques are available to detect them [78]. However, even with this approach there are still certain difficulties. First, lack of a consistent internal control for normalization of miRNA expression level between different patient samples, although other studies have used equal serum volume as a normalization factor [78, 79]. Second, is difficulty in finding the upstream regulators of

miRNAs and the target genes regulated by miRNAs in a defined pathway which could be targeted therapeutically.

p38MAPK as a RT sensitizing target for NSCLC

From the RT point of view, an important finding of the thesis work is that miRNA-214 alters p38MAPK signaling which influences RT response of NSCLC cells. A role of p38MAPK in RT response of NSCLC is also evident in previous studies from our group and others [126, 173, 209]. It has been shown that IR induces activation of p38MAPK pathway in NSCLC cells followed by induction of apoptotic signaling via the pro-apoptotic protein, Bak [126]. Furthermore, activation of p38MAPK pathway by IGF-1R was shown by our group to be associated with RR [173]. Another study in our group has shown that high linear energy transfer IR induces apoptosis in NSCLC cells by down regulating p38MAPK and in this way can circumvent conventional RT resistance [209]. Results from other groups have in contrast shown that IR induces apoptosis of NSCLC cells via p38MAPK activation which in turn leads to induction of mitochondrial apoptotic pathway and furthermore, inhibition of p38MAPK blocked the IR-induced apoptosis [210]. Interestingly, it has been shown that NSCLC tumors express activated p38MAPK as compared to normal lung tissue, albeit with no correlation to overall survival of the patients [211, 212]. It has further been shown in the same cohort that expression of ERCC1 is significantly associated with activation of p38MAPK [213]. Given our findings and these clinical reports in NSCLC, it might be worth testing p38MAPK inhibitors as RT sensitizers of NSCLC.

Cisplatin resistance in NSCLC- from profiling of model systems to clinically relevant biomarkers

Expression of ERCC1 and RRM1 has been shown to predict response of advanced NSCLC to cisplatin [30, 44] but still they are not used to guide treatment. Moreover, results show that these markers most likely will not be universal to all patients. In this context, the observation I made in **paper III** that cells surviving cisplatin treatment are heterogeneous in terms of gene expression highlights the necessity for individualized biomarkers of NSCLC rather than an approach where one marker fits all. It would therefore be interesting to identify biomarkers which predict response to treatment in selective patients based on gene expression analysis. We identified DKK1 as a potential novel biomarker of cisplatin resistance in NSCLC cell lines *in vitro*. It is thus

interesting to look for the protein expression of DKK1 in NSCLC tumor tissue before and after treatment to further clarify if DKK1 expression is indeed related to survival benefit of NSCLC patients to cisplatin.

Concluding remarks

miRNAs are negative regulators of a wide range of genes involved in dictating different cellular processes and thereby playing a master role in tumor development, progression and metastasis as well as in regulation of tumor cell response to treatment as illustrated in this thesis work. The use of miRNAs in the clinical setting is still not in practice as the translation from bench to bedside has been associated with certain challenges among them are the diversity of miRNA targets and heterogeneity of the tumors in miRNA expression. Yet to my understanding, these small RNA molecules have a promising future to be used as diagnostic, therapeutic or prognostic biomarkers or even to be evaluated as targets for new treatment regimens. The findings in this thesis add further information on the significance of miRNAs as future biomarkers in NSCLC.

کاریگەریان تەنها لە سەر خانە شێرپەنجەییەکان هەیە نەوەك خانە ئاسایی یەکانی لەش. هەر بەو هۆیەشەوە بە باشتر دادەنرێت لە دەرمانە کیمیایی یەکان چونکە کاریگەری لاوەکی یان کەمتر بۆ نموونە قژ رووتانەوە, کەم بوونی بەرگری لەش ,رشانەوە ...هتد. ئەوەی لێرە جێ ی سەرنجە ئەوەیە کە ئەو جۆرە دەرمانە تایبەتانە تا ئێستا تەنها بۆ رێژەیەکی کەمی نەخۆشی یەکان دۆزراونەتەوە. بۆیە زۆربەی تویژینەوە زانستی یەکان تا ئێستا زۆر بە چڕی کار لە سەر دۆزینەوەی دەرمانی زیاتر دەکەن کە بەکاربێت بۆ هەموو جۆرەکانی ئەو نەخۆشی یه.

ئەو کتێبەش بەرهەمی سێ (3) ی تویژینەوەی منە کە لە چەند ساڵی رابردوودا ئەنجام دراون .

ئاما نجی سەرەکی تویژینەوەکانم بریتین لە چۆنیەتی بەرەو پێش بردنی چارەسەری نەخۆشی شێرپەنجەی سنگ بۆ بە دەستهێنانی ئەنجامی باشتر لە رێگەی دەرمانی کیمیایی و رێگەی تیشك . بۆ ئەم مەبەستەش تویژینەوەکانم بە کارهێناوە بۆ دۆزینەوەی پێکهاتەی نەناسراوی ناو خانە شێرپەنجەیی یەکان لە رێگەی بەکارهێنانی تەکنیکی زۆر تازوە پێشکەوتوو .ئاما نجیکی تری تویژینەوەکانم بریتی یەلە لێك جیاکردنەوەی ئەو نەخۆشانەی کە سوود وەردەگرن لەو نەخۆشانەی کە سوود وەرناگرن لە چارەسەری تیشك یان کیمیایی لە رێگەی دیاریکردنی جۆریکی تاییەت لە ترشی ناووکی بەمەش کاریگەری لاوەکی چارەسەر لە سەر نەخۆشەکە کەم دەبێتەوه .

لە ئەنجامی ئەو تویژینەوانە بۆمان دەرکەوت کە :

- یەکێك لەو پێکهاتە نەناسراوانەی ترشی ناوەکی کە پێ یان دەلێن (microRNA) کاریگەری هەیه لە سەر تەشەنەکردنی شێرپەنجەی سنگ . بۆیە ئەگەر ئەو پێکهاتەیە لە لەشی مرۆڤ لاببرێ ئەوا نەخۆشی یەکە تەشەنە ناکات و بەمەش ئەنجامی باشی نابێت .

- بوونی ئەو پێکهاتەیە کاریگەری لە سەر چارەسەری تیشکیش هەیه کە ئەوەش ئاماژەیە بۆ ئەوەی ئەو پێکهاتەیە لە لەشی مرۆڤ لاببرێ.

لە کۆتاییدا بۆمان دەرکەوت کە لە رێگەی تویژینەوەی زانستی یەوە دەتوانین رێگە چارەی نوێ تر بۆ نەخۆشی شێرپەنجەی بدۆزینەوە لە رێگەی بەکارهێنانی رێگەی نوێ بۆ پشکنینی خانە شێرپەنجەیی یەکان

6 POPULAR SCIENTIFIC SUMMARY IN KURDISH

پوختەی تویژینەوە :

شێر پەنجە بریتییە لە گەشەکردنێکی بێ کۆنترۆڵ لە هەندێك لە خانە ئاساییەکانی لەشی مرۆڤ خۆی , لە ئەنجامدا ئەو خانە نائاسایی یانە جێگەی خانە ئاسایی یەکان دەگرنەوەو فەرمانەکانی خۆی یان جێ بە جێ ناکەن , بۆ نمونە ئەگەر نەخۆشیەکە تووشی مێشکی مرۆڤ ببێت , ئەوا ناتوانێت کاروباری رۆژانەی بە ئاسایی بەرێوەببات لە کۆتاییدا ئەوەندە گەورە دەبێ کە دەبێتە هۆی تەشەنەکردن و وەستاندنی فەرمانی زۆربەی ئەندامە سەرەکییەکانی لەشی مرۆڤ . هەر چەندە هۆکارەکانی تووشبوون بە شێر پەنجە تا ئێستا بە روونی نازانرێت , بەڵام بوونی هۆکاری بۆماوەیی ,هەندێك هۆکاری دەرەکی وەك جگەرەکێشان لە ئەنجامی چەندەها تویژینەوەی زانستی دەرکەوتووە کە دەبێتە هۆی تووشبوون بە شێر پەنجە.

نەخۆشی شێر پەنجە بە یەکێك لە هەرە سەختترین نەخۆشیەکان دادەنرێت کە تووشی مرۆڤ دەبێت .

شێر پەنجەی سی یەکان (سنگ) بە باوترین و کوشندەترین جۆری شێر پەنجە دەژمێردرێت لە هەموو جیهان . رێژەی تووشبووان بە نەخۆشی شێر پەنجەی سنگ لە ساڵێکدا بە نزیکەی (1,600,000) یەك ملیۆن و شەش سەد هەزار کەس دەخەملێنرێت,وە لە هەمان کاتدا بە هۆکاری مردنی (1,300,000) یەك ملیۆن و سێ سەد هەزار کەس دادەنرێت لە هەموو جیهان لە ساڵێکدا. هەر چەندە لێکۆڵینەوەکان وا پیشان دەدەن کە نزیکەی 15./ی جگەرەکێشەکان تووشی شێر پەنجەی سی یەکان دەبن بەڵام 90 ./ی تووشبووان بە شێر پەنجەی سنگ جگەرەکێشن یان جگەرەیان کێشاوە لە رابردوودا .

بە شێوەیەکی گشتی چارەسەری شێر پەنجەی سنگ بریتی یە لە لابردنی گرێ یەکە لە رێگەی نەشتەرگەری ئەگەر نەخۆشی یەکە تەشەنەی نەکردبێ . بەڵام لە زۆربەی کاتەکان نەخۆشی یەکە تەشەنەی کردووە و لەو حاڵەتەشدا چارەسەری لە رێگەی دەرمانی کیمیایی یان لە رێگەی لێدانی تیشك (ئَشعه) دەبێت .

لە دە (10) ساڵی رابردوودا لە ئەنجامی چەندین تویژینەوەی زانستی دا هەندێك دەرمانی تازە دۆزراونەتەوە کە جیاوازن لە دەرمانە کیمیاییە باوەکان کە پێ یان ناڵێن دەرمانی کیمیایی بەڵکو دەرمانی تاییەتن (Targted therapy). یەکێك لە تایبەتمەندی یەکانی ئەو دەرمانە تازانە ئەوەیە کە

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