MicroRNAs as Predictive and Prognostic Biomarkers in Human Neoplasia: With Specific Focus on Colorectal Cancer, Giant Cell Tumor of Bone, and Leukemias

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ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

I **Mosakhani N**, Sarhadi VK, Borze I, Karjalainen-Lindsberg ML, Sundström J, Ristamäki R, Österlund P and Knuutila S. MicroRNA profiling differentiates colorectal cancer according to *KRAS* status. *Genes Chromosomes Cancer*. 2012; 51:1–9.

II **Mosakhani N**, Lahti L, Borze I, Karjalainen-Lindsberg ML, Sundström J, Ristamäki R, Österlund P, Knuutila S and Sarhadi VK. MicroRNA profiling predicts survival in anti-EGFR treated chemorefractory metastatic colorectal cancer patients with wild-type KRAS and BRAF. *Cancer Genet*. 2012;205:545-51.

III **Mosakhani N**, Pazzaglia L, Benassi MS, Borze I, Quattrini I, Picci P, Knuutila S. MicroRNA expression profiles in metastatic and non-metastatic giant cell tumor of bone. *Histol Histopathol*. *2012 Nov 21.* [Epub ahead of print].

IV **Mosakhani N***, Sarhadi VK*, Usvasalo A, Karjalainen-Lindsberg M-L, Lahti L, Tuononen K, Saarinen-Pihkala UM, and Knuutila S. MicroRNA profiling in pediatric acute lymphoblastic leukemia: novel prognostic tools. *Leuk Lymphoma.* 2012;53:2517-20 (Letter).

V **Mosakhani N**, Räty R, Tyybäkinoja A, Karjalainen-Lindsberg ML, Elonen E, Knuutila S. MicroRNA profiling in chemoresistant and chemosensitive acute myeloid leukemia.Submitted

* Equal contribution

ABBREVIATIONS

APC	adenomatous polyposis coli
ABL	c-abl oncogene 1, non-receptor tyrosine kinase
ACTB	actin, beta
AJCC	American Joint Committee on Cancer
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
ANKRD49	ankyrin repeat domain 49
ATN1	atrophin-1
ATP	Adenosine-5'-triphosphate
BAALC	brain and acute leukemia, cytoplasmic
B-ALL	B-cell ALL
BCL2	B-cell CLL/lymphoma 2
BCR	breakpoint cluster region
BMP	bone morphogenetic protein 4
BRAF	v-raf murine sarcoma viral oncogene homolog B1
CARD8	caspase recruitment domain family, member 8
CASP1	caspase 1, apoptosis-related cysteine peptidase
CDKN2A	cyclin-dependent kinase inhibitor 2A
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha
CGH	comparative genomic hybridization
CIMP	CpG island methylator phenotype
CIN	chromosomal instability
CLL	chronic lymphoblastic leukemia
CML	chronic myeloid leukemia
CNS	central nervous system
COX2	cytochrome c oxidase subunit II
CR	complete remission
CRC	colorectal cancer
DC	diasease control
DHFR	dihydrofolate reductase
dMMR	deficient mismatch repair
DNA	deoxyribonucleic acid
DNMT3A	DNA (cytosine-5-)-methyltransferase 3 alpha
DPC4	deleted in pancreatic carcinoma locus 4
EGFR	epidermal growth factor receptor
ELK1	ELK1, member of ETS oncogene family
EMT	epithelial–mesenchymal transition
ETV6	ets variant 6
FAB	French-American-British
FDA	Food and Drug Administration
FFPE	formalin-fixed, paraffin-embedded (tissue)
FISH	fluorescence in situ hybridization
FLRT2	fibronectin leucine rich transmembrane protein 2

FLT3	fms-related tyrosine kinase 3
FOXA1	forkhead box A1
FOXM1	forkhead box M1
GCTB	giant cell tumor of bone
GPCR	G protein coupled receptor
GSTM1	glutathione S-transferase mu 1
GSTT1	glutathione S-transferase theta 1
HIPK3	homeodomain interacting protein kinase 3
hMLH1	mutL homolog 1, colon cancer, nonpolyposis type 2
HNF1B	HNF1 homeobox B
HSC	hematopoietic stem cell
iAMP21	intra-chromosomal amplification of chromosome 21
IGF1	insulin-like growth factor 1
IL1B	interleukin 1, beta
IL-6	interleukin 6
ITD	internal tandem duplication
Ki67	antigen identified by monoclonal antibody Ki 67
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
MAPK	mitogen activated kinase-like protein
mCRC	metastatic colorectal cancer
mdr1	multidrug-resistance transporte 1
MDS	myelodysplastic
miRNA	micro-ribonucleic acid
MLL	myeloid/lymphoid or mixed-lineage leukemia
MMP-9	matrix metallopeptidase 9
MMR	mismatch repair
MPN	myeloproliferative neoplasms
mRNA	messenger RNA
MSI	microsatellite instability
MTHFR	methylenetetrahydrofolate reductase
MTR	5-methyltetrahydrofolate-homocysteine methyltransferase
NATs	N-acetyltransferase
ncRNA	non-coding RNAs
NET1	neuroepithelial cell transforming 1
NFIB	nuclear factor I/B
NGS	Next-generation sequencing
NOPHO	Nordic Society of Paediatric Haematology and Oncology
NPM1	nucleophosmin
NR2F1	nuclear receptor subfamily 2, group F, member 1
PAI1	phosphoribosylanthranilate isomerase
PD	progressive disease
PDPN	podoplanin
piRNA	piwi-interacting RNA
PKD1	polycystic kidney disease 1
PLP2	proteolipid protein 2
pMMR	proficient mismatch repair

pre-miRNA	precursors miRNA
pri-miRNA	primary miRNA
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
RANKL	receptor activator of NF-kappa-B ligand
RGS3,17	regulator of G-protein signaling 3, 17
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RUNX1, 3	runt-related transcription factor 1, 3
SCARF1	scavenger receptor class F, member 1
SETBP1	SET binding protein 1
siRNA	small interfering RNA
SLC26A3	solute carrier family 26, member 3
SMAD2,4,7	SMAD family member 2,4,7
snoRNA	small nucleolar RNA
snRNA	small nuclear RNA
T-ALL	T-cell ALL
TGF-B	transforming growth factor, beta
TLR4	toll-like receptor 4
TLX1	T-cell leukemia homeobox 1
TNC	tenascin C
TNM	tumor-node-metastasis
TOB1	transducer of ERBB2, 1
TP53	tumor protein p53
TPX2	TPX2, microtubule-associated, homolog
TS	thymidylate synthase
u-PA	plasminogen activator, urokinase
u-PAR	plasminogen activator, urokinase receptor
UTR	untranslated region
WBC	white blood cell count
VEGF	vascular endothelial growth factor
WHO	World Health Organization
ZAP-70	zeta-chain (TCR) associated protein kinase 70kDa

Gene names in the text are indicated in *italics* according to guidelines of the Human Genome Organization Nomenclature Committee (HGNC). Gene symbols not listed here can be found at http://www.ncbi.nlm.nih.gov/.

ABSTRACT

Recently, discovery of microRNA has provided new insights into cancer research, revealing the role of miRNAs in various biological processes, and evidence shows that their deregulation in many cancers has prognostic and predictive significance. Although specific miRNAs have been discovered in the malignancies studied in this thesis: colorectal cancer (CRC), giant cell tumor of bone (GCTB), acute lymphoblastic leukemia (ALL), and acute myeloid leukemia (AML), very little still is known about the association of miRNAs with progression and their response to treatment. By applying novel microarray techniques, we profiled miRNA expression in CRC, GCTB, ALL and, AML.

In the study focusing on primary tumors of 60 metastatic CRC (mCRC) patients, we detected *BRAF* mutations in 5 and *KRAS* mutations in 15 cases. In addition to 46 altered miRNAs in mCRC patients with and without *KRAS* mutation vs. normal colon tissue, we observed an miRNA signature associated with *KRAS* status when we compared 15 patients with mutant *KRAS* with 40 patients without this mutation. Four differentially expressed miRNAs, over-expressed miR-92a, miR-127-3p, miR-486-5p, and under-expressed miR-378, were evident in the mutated *KRAS* group vs. wild-type *KRAS* group.

In another study on CRC, miRNA profiling in primary tumors of 33 mCRC patients with wild-type *BRAF* and *KRAS* allowed identification of miRNAs related to their response to anti-EGFR monoclonal antibody treatment. We found up-regulated miR-31* and down-regulated miR-592 in progressive disease (PD) compared to that in disease control (DC). Evaluation of mRNA levels of *SLC26A3* and *ATN1,* drug-related genes and of miR-31* target genes showed their lower level of expression in PD vs. DC. Moreover, correlation between overall survival and miRNA expression assessed by two approaches, cluster analysis and the Cox proportional hazard regression model, revealed two common miRNAs, miR-140-3p and miR-1224-5p, to be related to survival in both analyses.

As for the study of the metastatic and non-metastatic GCTBs, we found 12 miRNAs as being differentially expressed (miR-136, miR-513a-5p, miR-494, miR-224, miR-542-5p). Expression levels of eight genes such as *NFIB*, *TNC*, and *FLRT2* correlated inversely with miRNA results. The expression levels of miR-136 and its target gene, *NFIB*, were verified by use of qRT-PCR. The level of NFIB protein also was higher in metastatic than in non-metastatic GCTBs. Further, we tested the protein expression level of NFIB in an independent validation cohort of 74 primary archival GCTB specimens to assess the power of

NFIB as a prognostic marker. Immunodetection showed a higher frequency of NFIB over-expression in primary tumors that developed metastases than in the disease-free group.

Moreover, we studied the miRNA expression profiles of primary tumors of 90 bone marrow core biopsies of ALL patients, including 11 patients with paired samples at diagnosis and at relapse. We found a set of miRNAs (miR-1281, miR-1225-3p, miR-877*, miR-423-5p, miR-29c) significantly related to survival ($q<0.05$). Further validation of miR-423-5p expression by qRT-PCR confirmed microarray analysis results and showed a direct correlation with survival. In comparisons between the diagnosis-relapse pairs, expression of miR-654-5p and miR-431 between the two groups significantly differed, and these miRNAs were down-regulated in relapse samples.

Comparison of miRNA profiling of 15 chemorefractory and 18 chemosensitive AML patients showed that the differentially expressed miRNAs were miR-363, miR-532-5p, and 342-3p, all of which were over-expressed in chemorefractory vs. chemosensitive patients. Verification by use of qRT-PCR of both miR-363 and miR-532-5p revealed similar results as with microarray. The miR-363 target genes *RGS17* and *HIPK3* both have been associated with drug response.

These studies provide new information about genomic changes involved in progression and resistance to treatment in various types of cancer, and also highlight the power of applying genomic-wide array screening techniques in malignancies. The novel findings in these studies may serve as a useful resource for future studies and aid in development of novel therapeutic targets to increase the survival rates of cancer patients.

INTRODUCTION

A recently discovered class of small RNA is microRNA with 20 to 24 nucleotides. MiRNAs are managers of gene expression and negatively regulate mRNA expression at the translational stage. They play a fundamental role in many cellular processes such as proliferation, apoptosis, survival, and tumorigenesis that impact on major biological systems such as cancer, and they have provided many new insights into cancer research (Mirnezami et al. 2009; Slaby et al. 2009).

The first evidence of a connection between miRNA and human cancers came from findings in chronic lymphocytic leukemia. Further, more than 50% of miRNA genes are located within regions of loss of heterozygosity, amplification, fragile sites, viral integration sites, and other cancer-related genomic regions (Mirnezami et al. 2009). MiRNAs are involved in the initiation, progression, and metastasis of human cancer (Calin and Croce 2006; Di Leva et al. 2006), and miRNA signatures that are related to diagnosis, staging, progression, and response to treatment are identifiable in human cancers (Setoyama et al. 2011). However, those miRNAs which can be associated with progression and response to treatment in many cancers remain unknown.

The microarray is one of the recent techniques that provide the possibility of profiling numerous miRNAs simultaneously in an experiment. With the microarray, miRNA profiling can reveal altered miRNAs, and novel alterations may serve as putative cancer markers which may aid in our understanding of molecular mechanisms underlying progression or those which are treatment-resistant.

This thesis is concentrated on miRNA profiling of four different cancers: colorectal cancer (CRC), giant cell tumor of bone (GCTB), acute lymphoblastic leukemia (ALL), and acute myeloid leukemia (AML), by use of an miRNA microarray. The main purpose is to improve knowledge of miRNAs involved in tumor progression and drug resistance that might open up new therapeutic applications.

REVIEW OF THE LITERATURE

1. Non-coding RNAs

A simple formula regarding biological information until recently was that DNA is transcribed into mRNA, and mRNA is translated into protein. Recent discoveries have, however, revealed a new subtype of non-coding RNAs (ncRNAs) such as small ncRNAs (less than 200 nucleotides) that subvert this formula by regulation of the transcription and translation of protein-coding RNAs (Figures 1A, and B) (Bernardao et al. 2012). These small RNAs include small interfering RNAs (siRNA), small nucleolar RNAs (snoRNA), small nuclear RNAs (snRNA), piwi-interacting RNA (piRNA), and microRNAs (miRNA) (Figure 1C). Another subtype of ncRNA comprises long ncRNAs, endogenous cellular RNAs of more than 200 nucleotides in length that have no open reading frame of significant length (<100 amino acids). Long ncRNAs act in different ways in the cell; for instance, they regulate gene expression and influence protein localization (Gutschner and Diederichs 2012).

2. MiRNAs

The first ncRNA, miRNA, was discovered in in the Ambros and Ruvkun labs in 1993 when a gene, lin-4, crucial for nematode *Caernohabditis elegans* development, was found to not encode a protein but to give rise to a small 22-nucleotide RNA. The RNA itself was responsible for silencing the lin-14 gene, via antisense complementarity to its 3' untranslated region (UTR) (Lee et al. 1993; Wightman et al. 1993). The second important miRNA was identified also in the nematode *Caernohabditi elegans* in 2000 which was let-7 (Reinhart et al. 2000). Let-7 was identified soon in human beings and in animals, as well (Pasquinelli et al. 2000; Basyuk et al. 2003). Subsequently, hundreds of miRNAs and their biological functions have been identified, and thus far (August 2012) 25,141 mature miRNAs in 193 species, including 2,042 mature human miRNAs, have been registered in the miRBase data base (http://microrna.sanger.ac.uk) (Ambros 2001; Vandenboom Ii et al. 2008). MiRNAs play the vital roles in basic biological functions such as growth, invasion, angiogenesis, proliferation, and differentiation via the negative regulation of over one-third of all human genes at the translational stage (Bartel 2004; Lee et al. 1993). The miRNAs may have either an oncogenic or a tumor-suppressive function (Croce 2009). MicroRNA genes represent 1 to 3% of the currently known genes in the human genome (Bartel 2004). Although the miRNA

genes are located in either introns or exons of protein-coding genes, a larger number of miRNA genes are found in intron regions (Negrini et al. 2009).

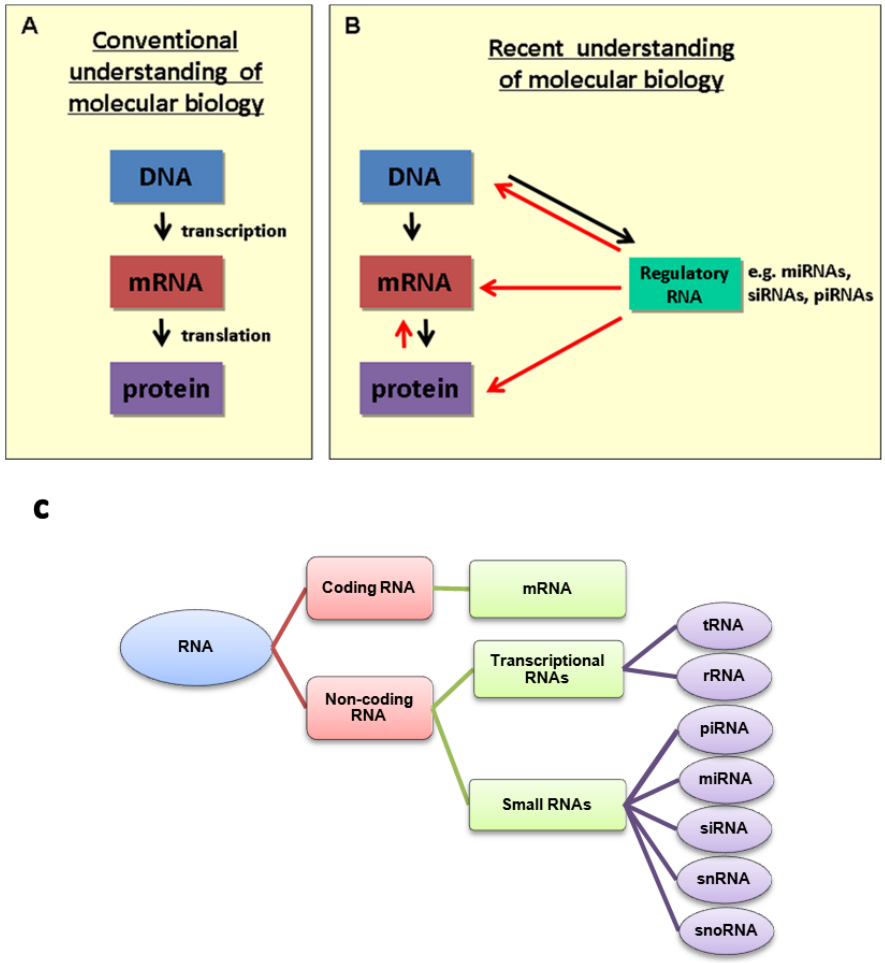

Figure 1. Molecular biology. (A) Schematic overview of molecular biology before and (B) after discovery of non-coding RNA transcripts. (C) RNA family. (Figure 1(A) & (B) are reprinted from *Heart, Lung and Circulation,* Vol. 21(3), Bernardo BC, et al. A microRNA guide for clinicians and basic scientists: background and experimental techniques, pp. 131-142, Copyright (2012), with permission from Elsevier. Figure 1 (C) has been modified from Buckingham S., 2003).

2.1. MiRNA biology

The miRNA genes are initially transcribed in the nucleus and are called primary miRNAs (pri-miRNAs) (>1000 bases) which contain multiple stem loop/hairpin structures. The enzyme Drosha then cleaves pri-miRNAs into precursors (pre-miRNAs) with 60 to 100 nucleotides. The pre-miRNAs are transported to cytoplasm by Exportin 5 and cleaved by Dicer to form an miRNA: miRNA* duplex about 15 to 22 nucleotides in length. This duplex then unwinds into mature miRNA and passenger miRNA (miRNA*). Recently, deep sequencing data have shown that some miRNAs* are not degraded; they even play a functional role in the regulation of miRNA homeostasis and exert downstream effects on transcription and translation of RNA and DNA (Suzuki and Miyazono 2011). The mature RNA is incorporated into an RNA-induced silencing complex (RISC) where it binds to a complementary sequence in the 3'UTR of target mRNA. The mechanism of inhibition of translation depends on the degree of miRNA-mRNA complementarity (imperfect or perfect) that results in inhibition of protein synthesis or mRNA degradation (Figure 2) (Bernardo et al. 2012).

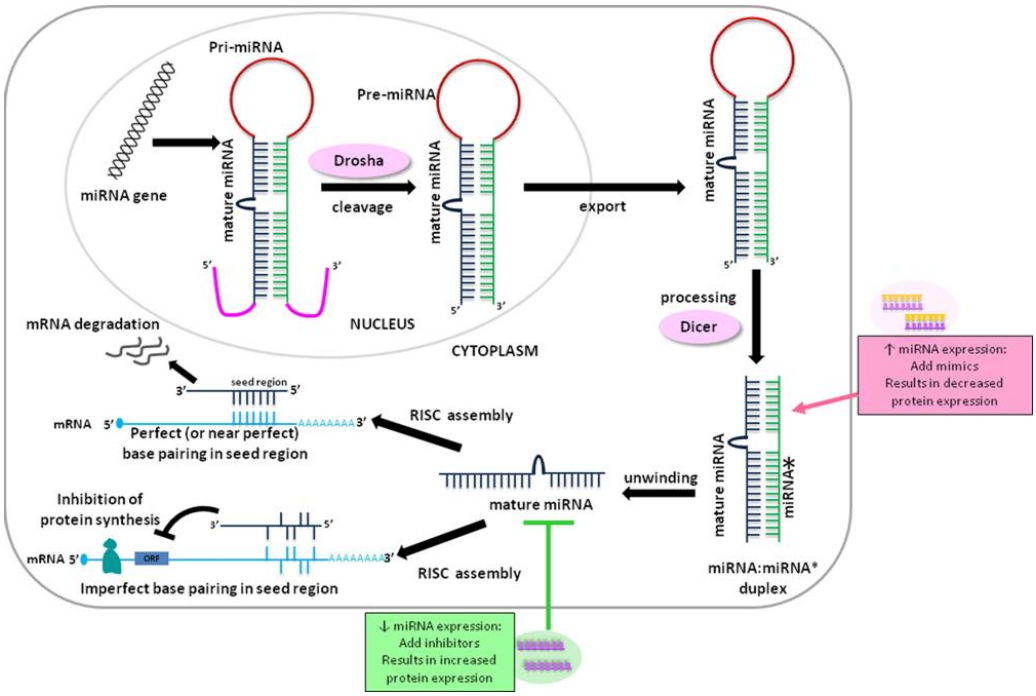

Figure 2. Biogenesis of microRNA. Reprinted from *Heart, Lung and Circulation*, Vol. 21(3), Bernardo BC, et al. A microRNA guide for clinicians and basic scientists: background and experimental techniques, pp. 131-142, Copyright (2012), with permission from Elsevier.

2.2. MiRNAs in human diseases

Since the year 2000, over 2000 studies have been conducted regarding miRNAs (Figure 3A) (van Rooij et al. 2012) and their correlations with various diseases such as cancers, cardiovascular disease, schizophrenia, renal function disorders, psoriasis, primary muscular disorders, Fragile-X mental retardation syndrome, diabetes, chronic hepatitis, AIDS, and obesity (http://cmbi.bjmu.edu.cn/hmdd) (Figure 3B).

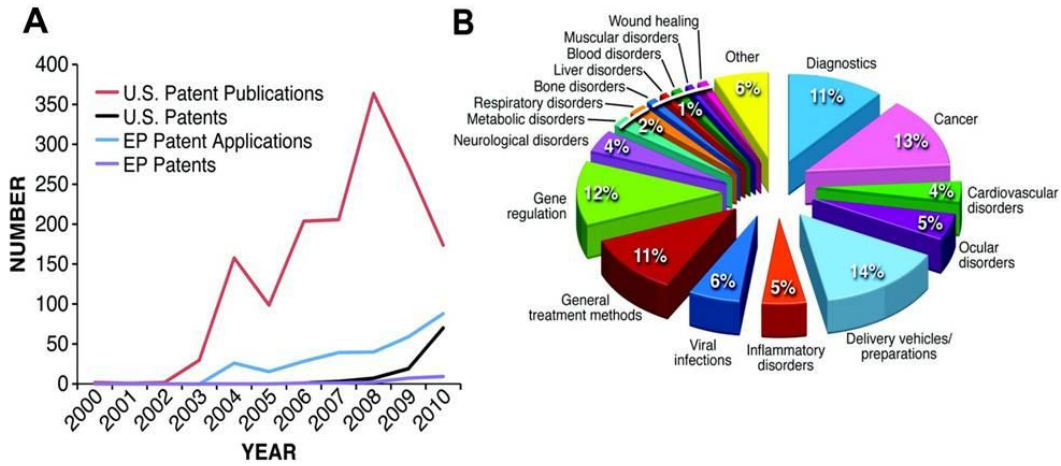

Figure 3. (A) Number of yearly published patent applications and issued patent associated with miRNAs and their applications in US and Europe. (B) State of distribution of miRNAs' documents in medicinal preparations. Reprinted from Circulation Research, Vol. 110(3), van Rooij E., et al. Developing microRNA therapeutics, pp. 496-507, copyright (2012), with permission from Wolters Kluwer Health

2.3. MiRNA in cancer

The first investigation that indicated the relation between miRNA and human cancers was in 2000. This study found miR-15 and miR-16-1 in the most commonly deleted region, 13q14, in CLL; subsequently, their frequent deletion or down-regulation has been detected in the majority of CLL cases (Calin et al. 2002). MiR-15 and miR-16-1 function as tumor suppressors, and their expression inversely correlates with anti-apoptotic BCL2 expression where inhibition of BCL2 by miR-15 and miR-16-1 enhance apoptosis in leukemic cells. Thus, somatic deletion of miR-15 and miR-16-1 facilitates leukaemogenesis as bypass apoptosis (Cimmino et al. 2005).

Another early finding that indicates a link between miRNA and cancer formation is the detection of over 50% of miRNA genes in regions of loss of heterozygosity,

amplification, fragile sites, viral integration sites, and other cancer-associated genomic regions (Calin et al. 2004; Mirnezami et al. 2009). Deregulation of miRNA expression has been evident in various types of cancers including colorectal cancer and in leukemia, lung, and breast cancers. MiRNA profiling compared to mRNA expression profiling seems to be a more accurate method of classifying tumor subtypes (Lu et al. 2005).

MiRNAs can function as either oncogene- or tumor-suppressors. Over-expression of oncogenic miRNAs and under-expression of tumor-suppressive miRNAs can contribute to tumorgenesis by affecting pathways promoting acquisition of the hallmarks of cancer. These hallmarks include sustaining proliferative signaling, resisting cell death, evading growth suppressors, inducing angiogenesis, enabling replicative immortality, and activating invasion and metastasis (Hanahan and Weinberg 2000).

2.4. MiRNAs as prognostic markers

Prognostic markers provide informaion on the likely course of cancer disease in an untreated individual that are objectively measurable (Italiano 2011). The possible applications of miRNAs not only in molecular diagnostics but also in molecular prognostics, particularly in cancer, are provided by discovery of the role of miRNA in numerous pathological processes, and for cancer prognosis, miRNA can be complementary to other genomic and proteomic biomarkers (Cho 2007).

The role of miRNA in prediction of outcome and prognosis is evident in several cancer studies. In pancreatic cancer, over-expressed miR-21 correlates with the presence of liver metastasis, and with the Ki67 proliferation index that suggests changes in miRNA expression as being associated with cancer progression (Roldo et al. 2006). Family members of let-7 and miR-155 were identified as related to survival, and a lower level of let-7 and a higher level of miR-155 correlate with poor post-operative survival in lung cancer (Takamizawa et al. 2004; Yanaihara et al. 2006). Over-expression of miR-155, which also associates with the poor-prognosis phenotype in B cell lymphomas, suggests a general predictor role for miR-155 in aggressive tumor phenotypes (Calin et al. 2005; Mirnezami et al. 2009).

Expression of miR-200c is associated with overall survival after surgery in colorectal cancer (Xi et al. 2006). In CLL, miRNA profiling independently predicts the prognosis of disease and expression of poor prognostic markers such as ZAP-70 and status of the immunoglobulin variable-region heavy-chain gene. Thirteen miRNAs discriminate

between patients with good and poor prognosis. Two of these are miR-16-1 and miR-15a, which show lower levels in patients with good prognosis. According to reports, loss at 13q14.3, harboring these genes, is a favorable prognostic feature (Calin et al. 2005).

2.5. MiRNA as predictive markers

Predictive biomarkers provide information on likely benefit from a particular treatment type (Italiano 2011). Despite significant advances in cancer therapy, drug resistance is still a major obstacle in its treatment. Thus, better understanding of mechanisms underlying drug resistance is needed to improve treatment results. The main mechanisms of resistance are as follows: 1) altered expression of the ATP-binding cassette family of transporters on cell membrane transporters, 2) alterations in DNA repair pathways, 3) resistance to apoptosis, and 4) target modifications (Rodrigues et al. 2012). Recent studies have indicated an emerging role for miRNAs, in addition to genetic and epigenetic changes (methylation/acetylation), in the anticancer-drug-resistant phenotype (Giovannetti et al. 2012), which opens up the possible application of miRNAs in evaluation of outcome and modification of response in known anti-tumor therapies (Hummel et al. 2010). MiRNA can change cellular response to a specific drug or class of drugs not only through survival or apoptotic signaling but also by interfering with drug targets and DNA repair (Giovannetti et al. 2012). To restore drug sensitivity via miRNAs, potential approaches include activation of tumor suppressor miRNAs or inactivation of oncogenic miRNAs and modulation of miRNA target genes, oncogenes, and tumor suppressor genes, through up- or down-regulation of miRNAs (Sarkar et al. 2010; Giovannetti et al. 2012). For example, Miller et al. (2008) found significant up-regulation of eight miRNAs and down-regulation of seven miRNAs in a tamoxifen-resistant cell line, relative to a sensitive cell line.

Another study on breast cancer reveals that miR-451 regulates multi-drug-resistance 1 (*mdr1*) which is an important factor in drug resistance. A set of differentially expressed miRNAs also exists in a multi-drug-resistant human gastric-cancer cell line (Kovalchuk et al. 2008). Up-regulation of miR-214 causes increased resistance to cisplatin in ovarian cancer (Yang et al. 2008). Furthermore, the crucial role of miRNAs also has been demonstrated in drug resistance in other types of cancers such as prostate and non-small cell lung cancers (Fujita et al. 2008; Garofalo et al. 2008).

Altogether, recent evidence suggests that a drug-resistant-associated miRNA signature could serve for stratifying patients and choosing treatment options that most likely

will show successful results for a particular individual. In addition, this aids in discovery of new drugs and biomarkers that may enhance management and outcome in patients with cancer (Gorenchtein et al. 2012).

3. MiRNA detection

To investigate the cellular roles of miRNAs, a key approach is to profile the mature miRNAs in specific tissue types at various disease stages (Wark et al. 2008). Considerable effort has been devoted to developing new methods for high-throughput detection of miRNAs. A time line for miRNA discovery and detection is in Figure 4 (Wark et al. 2008). For several reasons, however, detection of miRNAs is technically challenging. Due to the short length of mature miRNAs, very little sequence is available to design complementary microarray and perform reliable amplification or labeling of each miRNA without introducing signal bias. MiRNAs show sequence similarity in the same family, and only one nucleotide can differentiate them from each other. As the target sequence is present in both pri- and pre-miRNAs, it is also important to make certain that they do not contribute to the array detection signal (Wark et al. 2008; Bernardo et al. 2012).

3.1. Microarray and deep sequencing

The miRNA microarray is one of the recent techniques that allow profiling of numerous miRNAs (~1000 miRNAs) simultaneously, while requiring only small amounts of total miRNAs. The relative expression level of miRNA is detectable by microarray. In brief, the sample's dephosphorylation and direct-labeling take place in the same tube. Then, hybridization occurs between RNA samples and microarrays containing probes for each identified miRNA from the Sanger miRBASE public database. Agilent's SurePrint inkjet technology synthesizes 40- to 60-mer oligonucleotide probes directly on the array, and this leads to high-purity, high-fidelity probes. The Agilent miRNA microarray probe is represented in Figure 5A. Agilent's microarray contains ~15,000 features printed in an 8-plex format. Probe design requires prior sequence information; this is a limitation of microarrays (Bernardo et al. 2012). Capture probes are in one of two forms, synthetic oligonucleotides or cDNA fragments (Yin et al. 2008). In addition, exogenous and endogenous positive and negative control probes are essential to aid normalization and provide absolute reference

points for quality control and quantitative comparison of different microarrays (Yin et al. 2008). For a schematic overview of miRNA microarray see Figure 5B.

Recently, next-generation sequencing has overcome some of the microarray limitations. The advantages of this technique are as follows: 1) having prior sequence information is unnecessary, 2) information about all RNA classes is supplied by this method, and 3) it provides the possibility to discover novel miRNAs or other types of small RNAs. The limitation is the size of the data output, which is large and complex, hence requiring extensive computational power, data storage, bioinformatics analysis for interpretation of data, and further functional analysis to test the data (Bernardo et al. 2012).

The validation of data obtained by microarray or deep sequencing is essential with quantitative real time PCR, Northern blotting, or in situ hybridization (Bernardo et al. 2012).

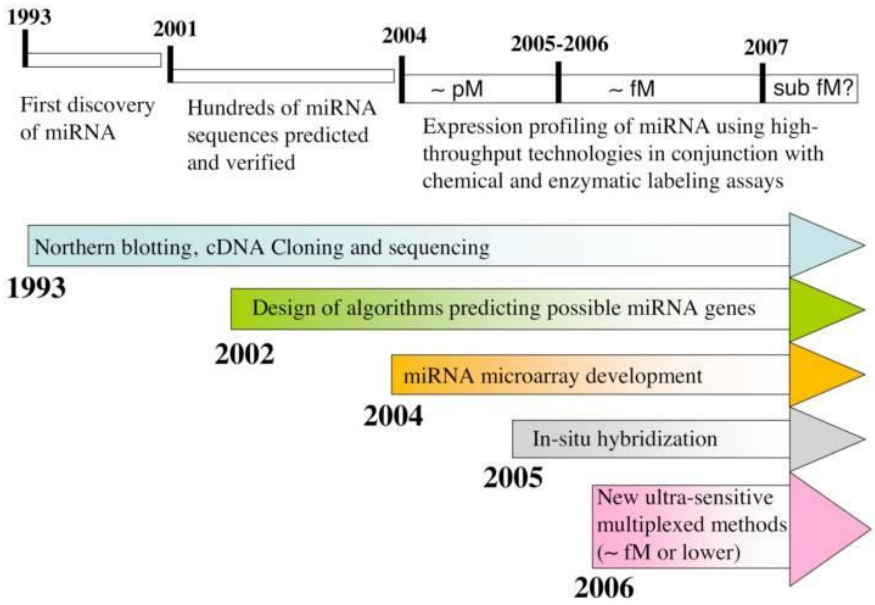

Figure 4. Discovery and detection of miRNA during the time. Reprinted from Angewandte Chemie International Edition,Vol. 47(4), Wark AW., et al, Multiplexed detection methods for profiling microRNA expression in biological samples, pp. 644-652, copyright (2008), with permission from John Wiley and Sons.

3.2. Quantitative real time PCR (qRT-PCR)

The most-used method to verify data from genome-wide expression profiling is qRT-PCR. Two different approaches allow detection of specific miRNAs by qRT-PCR; one utilizes miRNA-specific reverse transcription primers and initially uses stem–loop miRNA-specific

primers, and another adds a common sequence (poly(A) tail) to the 3′-end of all miRNAs and utilizes universal reverse transcription primers (Bernardo et al. 2012). Among the number of existing fluorescent-based technologies for qPCR, only SYBR Green and TaqMan® have successfully served to detect miRNA. It is also important to normalize miRNA expression by use of suitable reference or housekeeper gene following qPCR (Bernardo et al. 2012).

3.3. Northern blotting

One of the earliest methods to detect single miRNA molecules directly is northern blotting, without any need for chemical or enzymatic modification of the target molecules prior to analysis (Wark et al. 2008). Compared to array and qRT-PCR, this method has the advantage of detection of both miRNA and pre-miRNA. However, because of small size and low abundance of miRNA molecules, northern blotting can be technically challenging. This method also is time consuming, and requiring a large amount of RNA (10-15µg) (Bernardo et al. 2012). This method is described by Varallyay et al. (2008).

3.4. MiRNA in situ hybridization

For visualization and localization of genes in a cell or in tissue, in situ hybridization is the method most commonly used. Since the stringency and specificity of this method decrease with shortening of the probes, its use in miRNA detection is limited. Conventional in situ hybridization works for highly expressed miRNAs, but it shows inconsistent or negative results for ones with low expression (Bernardo et al. 2012).

3.5. MiRNA in formalin-fixed and paraffin-embedded (FFPE) samples

FFPE tissue is a widely used archive material for biomarker discovery and validation (Lewis et al. 2001). These types of samples represent a challenge for mRNA profiling due to RNA degradation during fixation (Macabeo-Ong et al. 2002), and storage (Cronin et al. 2004). In contrast, due to their stability and small size, miRNAs are better preserved, and their RNA can be readily extracted from FFPE samples (Hui et al. 2009). This therefore greatly enhances our ability to assess miRNAs as cancer biomarkers. In addition, the reliability of bone marrow core biopsies, which undergo decalcification besides the formalin fixation, has been studied for miRNA profiling, because decalcification also influences the integrity of

nucleic acids, especially RNA, and results have confirmed that use of core biopsies is feasible for miRNA profiling (Borze et al. 2011).

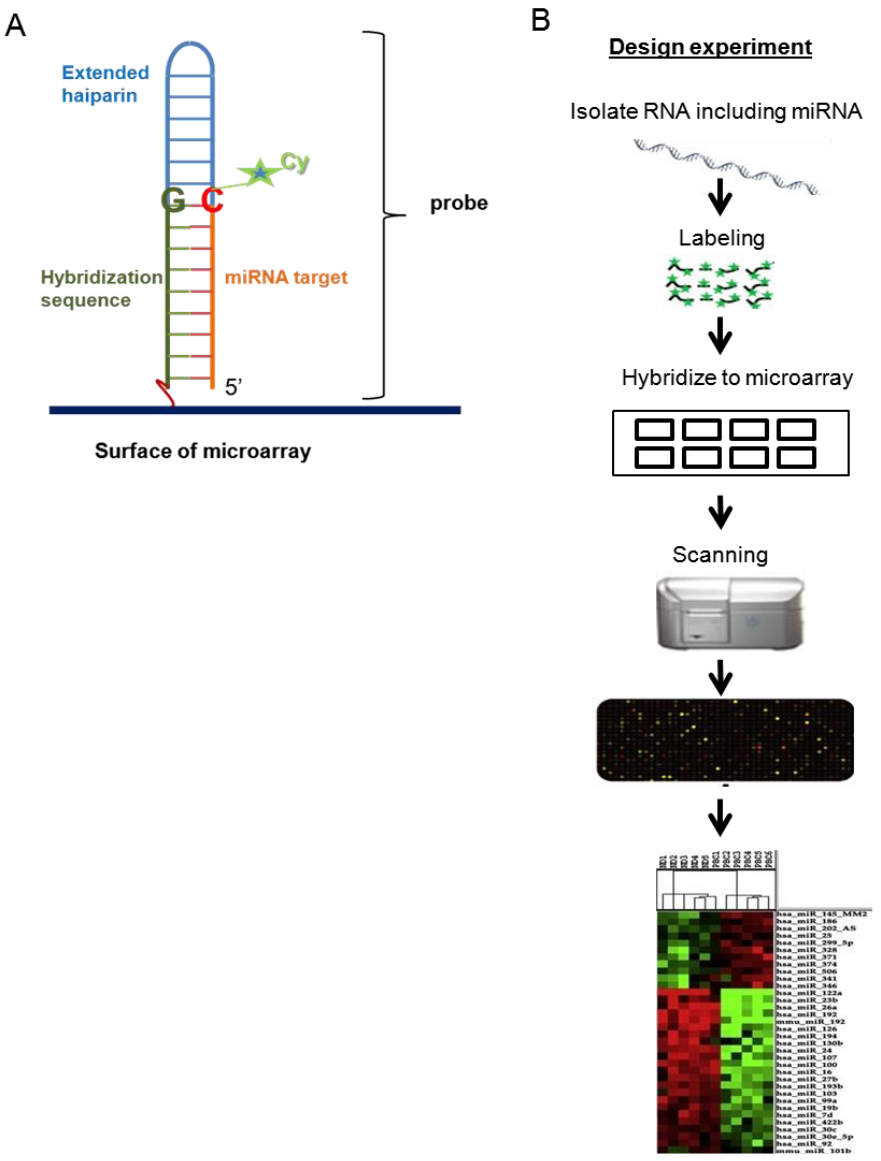

Figure 5. (A) MiRNA probe. (B) Schematic overview of miRNA microarray.

4. MiRNA target genes

MicroRNAs cover 1 to 3% of the genomes and regulate 30% of all human genes, and each miRNA regulates up to several hundred target genes; each target gene may also be regulated by multiple miRNAs (Bartel 2004; Lewis et al. 2005). Several databases are available to predict the miRNA target genes (Table 1). Each database has its own defined criteria based on combination of target features such as seed sequence matching and the evolutionary conversation of 3' UTR sequences of candidate genes (Takada and Asahara 2012). For this reason, these databases often create different gene target lists. However, combination use of multiple databases reduces false positivity, and the results can be more specific (Bernardo et al. 2012).

Table 1. Databases for prediction of miRNA target genes

Data base	Web address
miRBase	http://microrna.sanger.ac.uk
TargetScan	http://www.targetscan.org
miRanda	www.microRNA.org
mirTarget2	http://mirdb.org/miRDB
Tarbase	http://diana.cslab.ece.ntua.gr/tarbase
PICTAR	http://pictar.mdc-berlin.de/

4.1. Verification of targets

Despite the power of computational databases to predict miRNA target genes, however, verification of target genes is essential, because interaction between miRNA and the target is complex, with poor overlap between databases (Bernardo et al. 2012). Reporter assay is one method for verification of the target of miRNA, because alteration in luciferase expression indicates whether a miRNA can bind to a target mRNA. Using miRNA mimics and inhibitors, and then measuring predicted targets of miRNAs by qPCR and Western blotting is another method, but one less direct.

5. Survey of neoplasia studied in this thesis

Four different types of neoplasia, colorectal cancer (CRC), giant cell tumor of bone (GCTB), acute lymphoblastic leukemia (ALL), and acute myeloid leukemia (AML), were studied here because identification of their prognostic and predictive biomarkers still is crucial. On the other hand, these cancers have been studied for years in our labrotary in collaboration with clinicians and physicians who can provide to us materials and relevant clinical data.

5.1. Colorectal cancer (CRC)

5.1.1. Clinical characteristics and treatment of colorectal cancer

CRC is a disease arising in the epithelial cells lining the colon or rectum of the gastrointestinal tract. Overall annual detection of CRC is over one million cases, which include 9.4% of all cancer diagnoses, and it is the fourth deadliest cancer worldwide (Courtney et al. 2012). Both rates of incidence and death increase with age. Overall, 90% of new cases and 94% of deaths occur among persons aged 50 and older. CRC incidence is more common in men (American Cancer Society, 2011). In Finland, CRC is the third most common cancer type, and its incidence is about 2700 new cases annually (www.cancer.fi/). CRC at an early stage has often no symptoms. The symptoms and signs depend on tumor site. In general, symptoms include rectal bleeding and anemia which are sometimes associated with weight loss and changes in bowel habits. The signs of CRC are as follows: worsening constipation, blood in the stool, weight loss, fever, loss of appetite, and nausea or vomiting.

Several factors related to increasing or decreasing risk for CRC are divided into modifiable and non-modifiable risk factors. Non-modifiable risk factors include personal or family history of CRC or adenomatous polyps, and a personal history of chronic inflammatory bowel disease. Environmental factors (physical inactivity, obesity, high consumption of red or processed meats, smoking, moderate-to-heavy alcohol consumption) are known as modifiable risk factors (American Cancer Society, 2011). However, environmental factors are suggested as the risk factor series which play a major role in the etiology of this disease (Boyle and Leon 2002). CRC cases most commonly present sporadically, with family history of CRC in 25% of patients (Migliore et al. 2011). On the other hand, inherited mutations in major CRC genes occur in 5 to 6% and in the rest of the familial forms, gene-environment interactions lead to disease (Jasperson et al. 2010). Some inherited conditions which predispose an individual to development of colorectal cancer are

familial adenomatous polyposis (FAP), attenuated FAP (AFAP), MUTYH-associated polyposis (MAP), and Lynch syndrome (hereditary nonpolyposis colorectal cancer); rare syndromes include hamartomatous polyposis conditions (Peutz-Jeghers syndrome, juvenile polyposis syndrome, and others) and hyperplastic polyposis (Aaltonen et al. 1993; Hemminki et al. 1997; Migliore et al. 2011).

Human colon carcinogenesis progresses by dysplasia/adenoma to carcinoma. Stages are defined according to a tumor-node-metastasis (TNM) classification, as defined by the American Joint Committee on Cancer (AJCC) (Wolpin and Mayer 2008). The TNM definition is based on three variables: size and extent of the primary tumor (T), nodal involvement (N), and presence or absence of metastases (M). The individual T, N, and M variables are then combined into a grouped TNM stage, ranging from I to IV (Table 2) (Walters et al. 2012). Another system for classifiying tumors of the digestive system is based on the WHO classification which is used in Finland. TNM staging has nowadays replaced Duke´s classification or its Astler-Coller modification in which classifications the staging is based on the evaluation of the depth of invasion in the bowel wall and presence or absence of metastases. In Duke´s stage A, the growth is confined to mucosa, in stage B, the growth extends into the muscularis and serosa, in stage C it has spread to regional lymph nodes, and in stage D, distant metastases occur (Labianca et al. 2010).

CRC treatment commonly is based on surgery, radiation, and chemotherapy. Moreover, two targeted monoclonal antibody therapies directed against the extracellular of EGFR (cetuximab, and panitumumab) have been approved by the US Food and Drug Administration (FDA) to treat metastatic CRC by inhibiting the function of this transmembrane. EGFR interacts with signaling pathways affecting cellular growth, proliferation, and programmed cell death. EGFR is overexpressed in up to 80% of CRC. Autonomous activation of EGFR can occur through mutation or amplification. *EGFR* mutation, however, is very rare, in CRC but over-expression of EGFR through amplification or increased copy number is detectable in 10 to15% of CRC (Shia et al. 2005). Anti-EGFR monoclonal antibodies have shown efficacy in 10 to 20% of mCRC (Wolpin and Mayer 2008). In addition to *KRAS* mutation, other genetic alterations may also be predictive biomarkers of response to EGFR-targeted monoclonal antibodies such as over-expression of EGFR ligands (amphiregulin and epiregulin), *BRAF* and *PIK3CA* mutations, and loss of PTEN (Heinemann et al. 2013). Data suggest that combination of wild-type *KRAS*, *BRAF*, and *PIK3CA* and expression of PTEN protein in patients leads to the best response to cetuximab and panitumumab (Merla and Goel 2012). Among them, the *KRAS* mutation is a major predictive

marker and the only one with its status determined in clinical practice before the start of treatment with anti-EGFR monoclonal antibodies based on a recommendation of the European Medicines Agency and the Food and Drug Administration (Lievre et al., 2010 In Finland, patients who are potential candidates for anti-EGFR monoclonal antibody treatment for metastatic disease all have their *KRAS* status checked before treatment initiation. *BRAF* mutations occur in 10 to 15% of CRC; they serve, however, better as aprognostic marker than as a predictive marker (Merla and Goel 2012).

Resistance to an anti-cancer drug is the major hurdle for the success of chemotherapy (Longley et al. 2006). Recent studies have demonstrated the impact of miRNAs on the response of CRC cells to chemotherapeutic drugs. Over-expression of miR-192 in CRC cells and of miR-22 in *TP53*-mutated CRC cells leads to increased chemosensitivity to methotrexate and to paclitaxel, respectively (Song and Ju 2010; Li et al. 2011). The mechanism of anticancer activity of paclitaxel is via arresting microtubular polymerization and inducing apoptosis in cancer cells via binding to and inhibiting an apoptosis-stopping protein called Bcl-2 (Li et al. 2011).

In CRC cell lines, over-expression of miR-143 also causes increased chemosensitivity to 5-fluorouracil (Borralho et al. 2009). The inhibition of miR-31 in CRC cells results in increasing chemosensitivity to 5-fluorouracil (Wang et al. 2010). On the other hand, over-expression of miR-215 reduces chemosensitivity to methotrexate and tomudex (Song and Ju 2010), with a lower level of miR-34a in 5-fluorouracil-resistant DLD-1 CRC cells than in parental cells (Zhai and Ju 2011). Methotrexate and tomudex are inhibitors of dihydrofolate reductase (DHFR) and thymidylate synthase (TYMS, TS), respectively. DHFR and TS have been the main anti-cancer targets for the last 50 years, because of their critical functions (Song et al. 2010). The basis of systemic treatment for CRC is fluorouracil, a fluorinated pyrimidine that functions principally via inhibition of thymidylate synthetase, the rate-limiting enzyme in pyrimidine nucleotide synthesis (Wolpin et al. 2008).

Treatment choices for CRC are based on stage and site of the tumor, as well as risks and advantages and vary from one patient to another. Survival is directly related to detection and the type of cancer involved, but overall is poor for symptomatic cancers, as they are typically quite advanced (Elizabeth et al. 2008).

Table 2. TNM classification (American Joint Commission on Cancer)

Stage	T	N	M
I	T1	N0	M0
	T2	N0	M0
II	T3	N0	M0
	T4	N0	M0
III	T1, T2	N1 or N2	M0
	T3, T4	N1 or N2	M0
IV	Any T	Any N	M1

T=size and extent of the primary tumor, N=nodal involvement, M=presence or absence of metastases.T1=Tumor invades submucosa, T2=Tumor invades muscularis propria, T3=Tumor invades through the muscularis propria into pericolorectal tissues, T4= Tumor directly invades other organs or structures and/or perforates visceral peritoneum, N0= No regional lymph node metastasis, N1= Metastases in 1–3 regional lymph nodes, N2= Metastases in 4 or more regional lymph nodes, M0= No distant metastasis, M1=distant metastasis

5.1.2. Genetic alterations in colorectal cancer

The process of carcinogenesis of CRC is multistep (from normal epithelium to metastasis) with involvement of an accumulation of mutations in tumor suppressor genes and oncogenes (Figure 6). A number of genes and cytogenetic changes are associated with either increased or decreased risk for CRC. Several loci identified by genome-wide association studies as possible common low-risk susceptibility alleles are polymorphisms at 8q23.3, 8q24, 10p14, 11q23, 15q13, 14q22.2, 16q22.1, 18q21, 19q13.1, and 20p12.3 (Migliore et al. 2011). Polymorphisms of *GSTT1*, *GSTM1*, *COX2*, *MTHFR*, *NATs*, *MTR*, *SMAD7*, *APC*, and *IGF1* associate with increased risk for CRC (Migliore et al. 2011).

The inactivation of the tumor suppressor gene *APC* and activation of the oncogene *KRAS* are the among earliest trigger genetic events (Bellacosa 2003). The *KRAS* mutation is detectable in over 40% of CRCs. The RAS signaling affects various cellular functions such as cell proliferation, growth differentiation, cell survival, apoptosis, cytoskeleton organization and function, inflammation, and cell transformation.

Loss of function of *SMAD2,4,* and *TP53* are indications of malignant transformation (Bellacosa 2003). Mutation of *TP53* is found in 29% of all CRCs, with higher frequency in advanced-stage tumors and in tumors with poor prognostic features (Migliore et al. 2011). The frequency of *TP53* mutation in CRC cases, however, based on WHO 2010, is 70% (Bosman et al. 2010).

The achievement of genomic instability is a key hallmark of CRC which is classified into three subtypes: microsatellite instability (MSI), chromosomal instability (CIN), and the CpG island methylator phenotype (CIMP) (Migliore et al. 2011).

The first report of MSI was in 1993 as the presence of thousands of somatic alterations in the length of DNA in sporadic (Ionov et al. 1993; Thibodeau et al. 1993) and familial CRC (Aaltonen et al. 1993). There are at least 500,000 microsatellites in the human genome (de la Chapelle and Hampel 2010). MSI is detectable in 15 to 20% of sporadic CRC and is due to a loss of DNA mismatch repair function, secondary to inactivation of MMR genes (Migliore et al. 2011). The most common mechanism of MMR inactivation is via an acquired methylation of the h*MLH1* gene promoter (Goel et al. 2007). In monomorphic microsatellites the same number of repeat units is shared in all individual and in polymorphic microsatellites, a varied number of repeats among individuals is shared (de la Chapelle and Hampel 2010).

In 80 to 85% of CRCs, CIN is the most common type of genomic instability and is characterized mainly by chromosomal rearrangements and numerical abnormalities (Migliore et al. 2011). The most frequent aberration detectable by CGH in primary CRC involved gains at 3/3q, 5/5p/5q, 7, 8q, 20/20q, 13, and the X, and losses at 8p and 18/18q (Migliore et al. 2011). However, cytogenetic changes at 18q (including tumor suppressor genes *SMAD4/DPC4)* is the most recurrent aberration which is found by all studies in either primary tumors or in colon cancer cell lines or in fixed colorectal cancer tissue blocks (Migliore et al. 2011). Both microsatellite instability and loss of heterozygosity at 18q are identifiable prognostic markers (Lurje et al. 2007; Migliore et al. 2011). In the first published paper regarding dysregulated miRNAs in CRC, down-regulation of miR-145 and miR-143 in CRC compared to that in the normal controls was reported in 2003 (Michael et al. 2003). Subsequently, several studies have focused on miRNA profiling in either CRC cell lines or tumor samples in which numerous miRNAs are consistently dysregulated (Table 3) (Zhai and Ju 2011).

A number of miRNAs have been described as related to epithelial–mesenchymal transition (EMT), an evolutionarily conserved developmental process (Table 4). The miRNAs, detected as diagnostic markers in plasma or stool samples from CRC patients, are presented in Table 4. Moreover, the great potential of miRNAs as prognostic biomarkers is identified for CRC (Table 4), with miR-200c as the first reported miRNA related to colon cancer prognosis (Zhai and Ju 2011).

Table 3. Common miRNAs deregulated in CRC

Regulation	miRNA
over-expressed	miR-20,miR-21,miR-31, miR-99b
under-expressed	miR-143,miR-145, miR-192

Data based on Zhai & Ju (2011)

Table 4. The miRNAs identified related to metastasis, diagnosis, and prognosis of CRC

Category	miRNA
miRNAs associated with EMT	miR-200 family, miR-192, miR-215, miR-194, miR-21, miR-31, miR-9, miR-335
miRNAs as diagnostic markers	miR-106a, miR-141, miR-17-3p, miR-21, miR-29a, miR-92
miRNAs as prognostic markers	miR-106a, miR-141, miR-143, miR-200c, miR-21, miR-215, Let-7 and LIN28B

Data based on Zhai & Ju (2011)

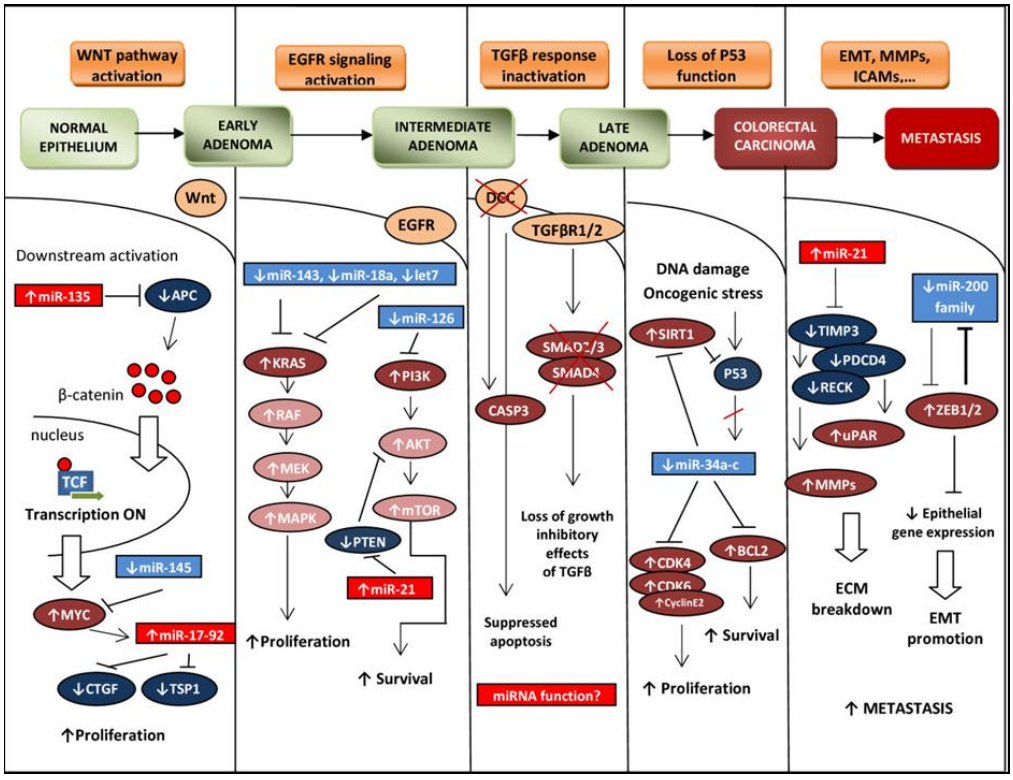

Figure 6. Pathways, target genes and miRNAs involved in carcinogenesis of CRC. Reprinted from *Molecular Cancer*, Vol. 8, Slaby O et al., the MicroRNAs in colorectal cancer: translation of molecular biology into clinical application, pp. 102, Copyright (2009), under the terms of Creative Commons Attribution License.

5.2. Giant cell tumor of bone (GCTB)

5.2.1. Clinical characteristics and treatment of giant cell tumor of bone

GCTB is a benign tumor of the skeleton that is locally aggressive and may rarely undergo malignant transformation into giant cell rich sarcoma and metastazise (McDonald et al. 1986; Katz et al. 1987; Fletcher et al. 2013). GCTB represents 4 to 5% of all primary bone tumors and 20% of all benign primary bone tumors (Fletcher et al. 2013). The incidence varies among races; for instance, its incidence is up to 20% of all primary bone tumors in China (Sung et al. 1982). The range of local relapse in patients is from 34 to 50%, and up to 6% of GCTB cases experience metastasis which occurs exclusively in the lung (McDonald et al. 1986; Katz et al. 1987). This disease occurs in adults at ages 20 to 45.

GCTB is characterized by a distinctive, multinucleated osteoclast-like population (Figure 7). Giant cells are not regarded as neoplastic, and the mononuclear cells of the tumor form the neoplastic tumor population. In addition to GCTB, infiltration of giant cell is obvious in various type of diseases such as aneurysmal bone cysts, chondroblastoma, giant cell-rich sarcomas and carcinomas, and primary giant cell sarcomas of bone (Domovitov and Healey 2010; Won et al. 2011). In GCTBs, involvement of the ends of long bones is predominant, around the knee (distal femur, proximal tibia) and wrist (distal radius) (Turcotte 2006). The most common symptoms are pain and deformity at the disease site with increased risk for fracture (Turcotte 2006). According to the Enneking staging system, GCTB patients are classified into three stages. Tumors are limited to bone in stage I and extend into the surrounding soft tissue in stage 3 (Enneking 1986). The patients' radiographic grading is from 1 to 3 according to the Campanacci system: grade I with well-defined margins of lesions and no penetration into the cortex, and grade III with irregular margins and cortical destruction (Campanacci et al. 1987).

Rare familial syndromes are associated with giant cell-rich tumors. There is a familial clustering of Paget's disease and GCTB (Rendina et al. 2004).

The macroscopic appearance of GCTB is as a hemorrhagic soft mass that erodes bone. Microscopically, the tumor's characterization is a minor stromal cell population and a second population of monocytes and eponymous multinucleated giant cells (Fletcher et al. 2002). The proteins expressed in GCTB are expressed by osteoclasts.

The treatment option for resectable GCTB is surgery including curettage, en bloc resection, and amputation. The adjuvant therapies may reduce recurrence rate. Because

the heat of polymerization of methylmetharcrylate cement may sterilize the margins of surgery, risk for recurrence decreases after curettage (Klenke et al. 2011). En bloc resection is usually reserved for patients with stage III, and the recurrence rate is less than 20% (Campanacci et al. 1987). The site of the disease is clinically important, because the curative surgery of tumors which involve the sacrum, base of the skull, and the axial skeleton are accompanied by high intraoperative complication rates and by risk for incontinence and impotence (Thomas 2012). Radiotherapy is effective when surgery is contraindicated, such as by unacceptable morbidity or difficult achievement of adequate margins (Caudell et al. 2003). Control rates with only radiotherapy are 60 to 84%, and this may be less in pretreated patients or for recurrence (Caudell et al. 2003). Radiofrequency ablation and chemoembolization are other local ablative therapies (Santiago et al. 2009; Onishi et al. 2010). Chemotherapy serves usually for advanced GCTB, for symptomatic and progressive disease (Stewart et al. 1995; Faisham et al. 2006).

Treatment of recurrent or unresectable GCTB with the fully human monoclonal antibody against RANKL called denosumab, which works by inhibiting osteoclast-mediated bone destruction, shows a positive response in approximately 90% of patients and reduced pain or improved functional status or both in about 85% of cases. This suggests that denosumab is a viable treatment option for advanced or metastatic GCTB cases not amenable to surgery (Beebe-Dimmer et al. 2009; Thomas et al. 2010).

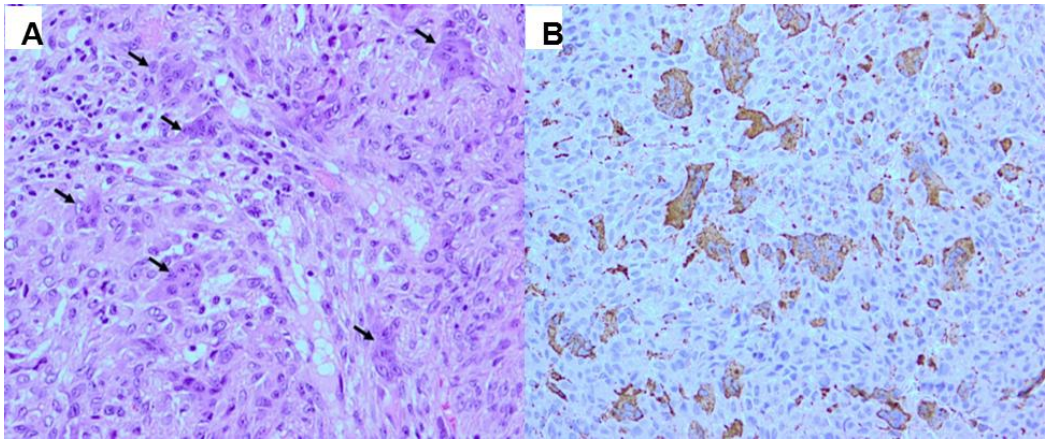

Figure 7. Histopathologic features of giant cell tumor of bone (GCTB). (A) GCTB cells with Hematoxylin-eosin staining (20x). (B) Monocyte/macrophage marker stained, CD68 (20x). This picture was kindly provided by Dr. Helena Autio-Harmainen

5.2.2. Genetic alteration in giant cell tumor of bone

Based on numerous investigations, size, anatomic site, radiological appearance, presence of pathological fractures, histological grading, and DNA content do not associate with rate of relapse and are not predictor markers for aggressive behavior or metastasis of the GCTB (Zheng et al. 1995). To identify predictors of the biological behavior of GCTB and prognostic indicators, several studies have therefore been conducted.

Over-expression of c-myc is found in most of GCTB samples which develop metastases. A highly significant correlation is observable between over-expression of c-myc and rate of metastasis (Gamberi et al. 1998). C-myc is involved in control of cell growth, differentiation, and apoptosis in various cell systems. Amplification or over-expression of the c-myc oncogene occurs also in various sarcomas and in Ewing's and osteosarcoma cell lines (Barrios et al. 1993, 1994a, 1994b).

An essential process for tumor growth and metastases is neoangiogenesis (Keck et al. 1989; Leung et al. 1989). On the other hand, production of VEGF is critical for the formation of the osteoclast (Niida et al. 1999). Evaluation of the expression level of VEGF in GCTB reveals its presence in spindle-shaped stromal-like tumor cells, round macrophage-like cells, and osteoclast-like multinucleate giant cells. A correlation exists between levels of VEGF expression and Enneking's clinical stage of GCTB, with higher levels of VEGF gene expression at stage III than at stages I/II. An increase in vascular density correlates with a high occurrence of metastases and poor prognosis in numerous neoplasms. Thus, VEGF has prognostic value and may be associated with an advanced stage of GCTB (Zheng et al. 2000). Another study has shown, in addition to VEGF, a correlation between clinical stage of GCTB and expression level of MMP-9, with advanced stages showing a higher expression level of both VEGF and MMP-9 (Kumta et al. 2003). Their expression levels differ also between recurrent and stage II and III lesions; in recurrent lesions, their levels of expression are higher (Kumta et al. 2003).

Amplified *IL-6*, *u-PA*, *u-PAR*, and *PAI1* genes are apparent in GCTB, and the percentage of samples with these amplifications increases with metastases, thus suggesting a possible association of these factors with a higher biological aggressiveness of GCTB (Gamberi et al. 2004). Cytogenetic analysis of GCTB reveals a gain at the 20q11.1-containing *TPX2* gene in a series of relapsed samples, and *TPX2* is proposed as a prognostic candidate oncogene (Smith et al. 2006). A correlation has been apparent between high expression of *TNC* and poor prognosis of GCTB. The over-expression of *TNC* is related to

risk for local recurrence and metastasis (Pazzaglia et al. 2010). Involvement of TNC in tumor growth, metastasis, angiogenesis, and in the inhibition of immunosurveillance is reported (Orend and Chiquet-Ehrismann 2006). In another study, mutation of p53, a tumor suppressor gene, is suggested as a prognostic marker (Papanastassiou et al. 2010).

To identify predictive markers of aggressive behavior, it is useful to compare patients with and without developing lung metastasis. Results reveal higher frequency of thioredoxin peroxidase, allograft inflammatory factor 1, ubiquitin E2N, and glutathione peroxidase 1 over-expression in primary tumors of lung metastases or locally relapsed compared to frequency in a disease-free group (Conti et al. 2011). Moreover, Kaplan–Meier analysis shows a relation between high expression of glutathione peroxidase 1 and local recurrence and metastasis (Conti et al. 2011). The role of tumor suppressor gene RUNX3, which plays an important role in carcinogenesis and in the progression of malignancies, has been under study in GCTB, indicating that loss of RUNX3 may associate with carcinogenesis but not with aggravation (Han and Liang 2012).

EGFR is implicated in bone remodeling (Zhu et al. 2007). Evaluation of the contribution of protein expression to aggressiveness and recurrence of GCTB demonstrates that EGFR expression is more frequent in recurrent than in non-recurrent cases, and in clinicoradiologically aggressive than in latent cases (Balla et al. 2011).

Despite numerous studies on GCTB, miRNAs and their role in GCTB progression still remain unstudied. Work on miRNAs may lead to identification of new biological markers to predict the tumor's clinical behavior.

5.3. Leukemias

In normal hematopoiesis, immature pluripotent hematopoietic stem cells (HSC) self-renew or differentiate into any of the mature blood cell lineages (Figure 8). In leukemia, the normal developmental program of the hematopoietic system is disordered, and malignant hematopoietic precursors accumulate (Izraeli 2004). The type of leukemia classification is based on the malignant cell lineage as being either myeloid or lymphoid. Additionally, some rarer leukemia types express no lineage-specific antigen and show a mixed phenotype of neoplastic cells. Leukemia is classified also into two types, chronic and acute, based on the maturity of the leukemic cells. In the chronic type, symptoms are mild, disease develops slowly, and the neoplastic cells are more mature; in the acute type, symptoms are more severe, disease develops rapidly, and the neoplastic cells are immature different types of progenitor forms. Some general features for leukemia are shared in common with other diseases, ones such as fatigue, malaise, abnormal bleeding, excessive bruising, weakness, weight loss, bone or joint pain, infection and fever, abdominal pain, and enlarged spleen, lymph nodes, and liver. Thus to confirm the diagnosis, several tests are essential.

There are four main types of leukemia: ALL, AML, CLL and CML. The types studied in this thesis are ALL and AML, because in our laboratory, several studies have been conducted on these two types of cancer but not on the biological and clinical aspects of miRNAs.

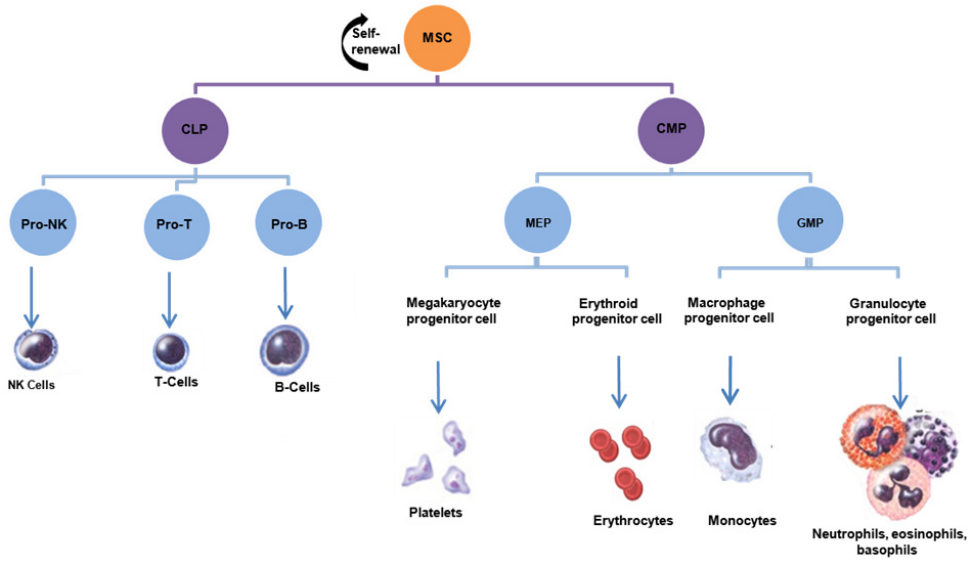

Figure 8. Normal hematopoiesis. MSC, Multipotent stem cell; CLP, Common lymphoid progenitor; CMP, Common myeloid progenitor; Pro-NK, Natural killer progenitor cell; Pro-T, T-cell progenitor cell; Pro-B, B-cell progenitor cell; MEP, Megakaryocyte erythroid precursor cell; GMP, Granulocyte macrophage precursor cell. Figure 8 has been modified from Wadhwa & Thorpe 2008.

5.3.1. Acute lymphoblastic leukemia (ALL)

5.3.1.1. Clinical characteristics and treatment of acute lymphoblastic leukemia

ALL is characterized by continuous multiplication of malignant and immature lymphoid cells. It is the most common malignant disease in children and includes 25% of all pediatric malignancies. Two peaks of incidence occur at ages 2 to 4 years (4-5/100 000) and at over age 50 (1/100 000) (Faderl et al. 2003). In Finland, the incidence of acute leukemias is approximately 240 new cases every year (Engholm et al. 2009, NORDCAN: cancer Incidence, Mortality, Prevalence and Prediction in the Nordic Countries, Version 3.5 http://www.ancr.nu).

Age and WBC count are prognostic indicators of outcome (Gustafsson et al. 2000; Seibel et al. 2008). Children at ages 1 to 9 have a better outcome than do either infants or adolescents (Gustafsson et al. 2000). In B-cell precursor ALL, WBC>$30x10^9$/l is associated with poor prognosis and in T-ALL, WBC>$100x10^9$ raises the risk for relapse (Rowe et al. 2005).

Pediatric ALL patients with B-cell precursor, ones aged 1 to 9 years with initial WBC<$50x10^9$/l, are categorized as a standard-risk group, and the rest of the patients as at high risk, according to the National Cancer Institute (Smith et al. 1996). Immunophenotype, cytogenetic abnormalities, and response to induction therapy are other commonly used factors (Smith et al. 1996). Risk classification has improved treatment results. Thus, refining of the risk classification by use of any new factors identified is necessary. Detection of the miRNAs involved may open new insight into the mechanism of ALL and also into classification of ALL risk groups.

The treatment commonly used for ALL includes remission-induction, consolidation, and maintenance therapy (Faderl et al. 2003). The goal of treatment is restoration of normal hematopoiesis, prevention of drug-resistant subclones of blast cells, central nervous system (CNS) prophylaxis, and elimination of minimal residual disease via post-remission consolidation (Faderl et al. 2003). The treatment protocol for pediatric ALL has changed from national protocols to uniform protocols in Nordic countries during the 1980s to 1992. Common Nordic protocols were established for all risk groups in 1992 (Gustafsson et al. 1998). Since 1990, the Finnish Leukemia group has introduced in Finland three consecutive clinical trials (each trial including six treatment blocks and maintenance

therapy during three years). Regarding treatment for infants, a large international collaborative trial, Interfant-99, began in 22 countries in 1999 (Pieters et al. 2007).

Although the outcome of childhood ALL has significantly improved, 25% of the patients still experience relapses. A considerable proportion, two-thirds of the failures, occurs among patients in standard risk or intermediate risk groups who have no unfavorable prognostic features at diagnosis (Gustafsson et al. 1998). Identification of new prognostic markers in ALL patients to refine more precisely risk groups is therefore necessary. However, both relapse and mortality due to treatment is higher in adults than in children. Poor outcome in adults is probably due to poorer treatment tolerance and changes in biology of the disease with age (Pui and Evans 2006).

5.3.1.2. Genetic alteration in acute lymphoblastic leukemia

Hyperdiploidy (>50 chromosomes), chromosomal number change, is common in pediatric ALL (25%) but rare in adults (Forestier et al. 2006). The outcome in pediatric ALL with hyperdiploidy is excellent, with event-free survival for 5 years being more than 80%. Gains in chromosomes X, 4, 6, 10, 14, 17, 18, and 21 are the most common alterations in the hyperdiploidy cases (Mertens et al. 1996). Hypodiploidy, however, is uncommon in all age groups and is related to very poor prognosis (Kantarjian et al. 2000). Chromosomal translocation is a characteristic feature in ALL. The most common translocation in a pediatric B-cell precursor, but rare in adults, is t(12;21)(p13;q22) that leads to formation of the *ETV6-RUNX1* (*TEL-AML1*). An association between this translocation and good prognosis has emerged (Rubnitz et al. 1997; Uckun et al. 2001; Jabber Al-Obaidi et al. 2002). In adults, B-cell precursor ALL t(9;22)(q34;q11) is the most common translocation, resulting in formation of *BCR-ABL* fusion and the Philadelphia chromosome. This translocation is marker for poor prognosis in ALL, independent of age (Ribera et al. 2005). Two common forms of rearrangements of 11q23 involving *MLL*, ones occurring frequently in infant ALL, are t(4;11)(q21;q23) and t(11;19)(q23;p13) (Meyer et al. 2009). The prognosis is very poor in infants with *MLL* rearrangements and event-free survival, at about 45 to 50% (Pieters et al. 2007).

Despite some similarity in cytogenetic changes between B-cell precursor and T-ALL, differences do exist. Those genes which are important in T-cell development are involved also in T-ALL. Over 30% of T-ALL cases have T-cell receptor gene rearrangements in 14q11, 7q34, and 7p14 (Cauwelier et al. 2006). In pediatric t(11;14)(p13;q11) and in adult

t(10;14)(q24;q11) translocating *TLX1*(*HOX11*) is more common (Karrman et al. 2009; Marks et al. 2009), causing over-expression of the affected genes. Over-expression of *TLX1* is associated with good prognosis in T-ALL (Baak et al. 2008).

In addition to chromosomal translocations, other genetic lesions essential to induce overt leukemia include deletion or epigenetic silencing of *CDKN2A* at 9p21.3 and intra-chromosomal amplification of chromosome 21 (iAMP21) (Knudson 1971; Harewood et al. 2003). *CDKN2A* encodes $p16^{INK4A}$ and $p14^{ARF}$, which are tumor suppressors, and inactivation of *CDKN2A* deactivates TP53 and retinoblastoma pathways. Deletions of *CDKN2A* occur in approximately 70% of T-ALL and 30% of B-cell precursor ALL (Bertin et al. 2003). iAMP21 is associated with poor prognosis. *RUNX1* is located in this amplified area at chromosome 21, and multiple copies of this gene are identified as a recurrent change in ALL (Robinson et al. 2003; Robinson et al. 2007).

MiRNAs harbor prognostic implications in ALL. The association between miRNA expression and prognostic parameters such CNS relapse, specific risk group, and disease recurrence is evident (Zhang et al. 2009). Pediatric ALL patients who develop CNS relapse compared to patients with non-CNS relapse show over-expression of miR-7, miR-198, and miR-633 and under-expression of miR-126, miR-345, miR-222, and miR-551a (Zhang et al. 2009). Eighteen deregulated miRNAs are related to specific risk groups: standard risk, intermediate risk, and high risk (Zhang et al. 2009). In those patients with WBC<50 000 mm^3 at diagnosis, over-expression of miR-100 is detectable (de Oliveira et al. 2012).

Hyper-leukocytosis and cytogenetic groups with unfavorable markers associate with an increased level of miR-16 (Kaddar et al. 2009). Higher level of miR-16 expression correlates with shorter disease-free survival in both B- and T-ALL and with shorter overall survival in T-ALL (Kaddar et al. 2009). Another study found expression of 31 miRNAs to be relative to the likelihood of disease-free survival. Of these, 14 miRNAs are considered independent prognostic markers (Schotte et al. 2011) and can discriminate between two groups of patients, one with a favorable expression profile and a 5-year disease-free survival of 89.4±7% and one with a less favorable profile and a 5-year disease-free survival of 60.8±12% (Schotte et al. 2011). The other miRNAs associated with shorter overall survival are miR-146a, miR-181a/c, and miR-92a; an miRNA associated with longer overall survival is miR-221 in ALL patients (Ohyashiki et al. 2010; Wang et al. 2010).

Several studies show an association between miRNAs and response to treatment in ALL cases. MiRNAs identified in predicting prednisone response and also early

prednisone responses are miR-18a, miR-193a, miR-218, miR-532, miR-550, miR-625, miR-633, and miR-638 (Zhang et al. 2009; Xu et al. 2011). In addition, a low level of miR-454 expression is associated with L-asparaginase resistance, and expression of 20 miRNAs with vincristine and/or daunorubicin resistance (Schotte et al. 2011). Combination of the drugs allows rapidly eradication of most tumor cells and induce remission, which means presence of leukemic blasts <5% in the bone marrow, normal blood cells, and no tumor cells in blood, and absence of other signs and symptoms of the disease.

5.3.2. Acute myeloid leukemia (AML)

5.3.2.1. Clinical characteristics and treatment of acute myeloid leukemia

In AML, immature leukemic blasts accumulate in the bone marrow, peripheral blood, and sometimes in soft tissue. The most common acute leukemia in adults is AML, which accounts for about 80% of acute adult leukemias. Its annual incidence is 2.7/100 000, and it is slightly male predominant (Cornell and Palmer 2012). Median age at diagnosis is about age 72. Approximately 120 cases are diagnosed each year in Finland (Elonen 2007). Although the etiology of AML is mainly unknown, some associated risk factors are exposure to ionizing radiation, petroleum, benzene, and benzene-containing compounds, and smoking (Estey and Dohner 2006; Cornell and Palmer 2012). Germline *Runx-1* mutations and some autosomal–recessive disorders such as Bloom syndrome, fanconi anemia, and ataxia telangiectasia elevate AML risk (Cornell and Palmer 2012).

Definitions of subtypes of AML were updated by the World Health Organization (WHO) based on genetic and clinical factors in 2008 (Table 5) (Cornell and Palmer 2012). This system has mainly replaced the French-American-British (FAB) classification which is based on cell morphology and maturity and defines AML as subtypes M0 through M7.

WBC counts in AML patients may be high, normal, or low. The risk for infection is high, because of lack of WBCs or poorly functioning WBCs, or both (Cornell and Palmer 2012). When the blast count is more than 50, 000, leukostasis that is due to leukemic blasts' blocking of capillary blood flow is more common. This phenomenon is an ontological emergency and needs to be treated quickly with leukopheresis. The symptoms are shortness of breath, cardiac dysfunction, severe muscle aches or cramping, and ocular, neurological, or cognitive dysfunction.

Diagnosis is based on findings with flow cytometry, and with cytogenetic and molecular genetics by use of bone marrow biopsy and aspirate. To diagnose AML, detection of a 20% blast count in bone marrow aspirate or peripheral blood is necessary (Vardiman et al. 2002). AML risk groups are favorable, with a 5-year survival rate of 55 to 65%, intermediate at 38 to 40%, and unfavorable at 11 to 15% (Slovak et al. 2000; Grimwade et al. 2004). Risk-group classifications aid in anticipating chemotheraphy response, relapse risk, and overall survival. Features related to poor prognosis in AML include advanced age, WBC>30 000, antecedent hematological disorders such as MDS (myelodysplasia),

myeloproliferative neoplasms (MPN), and therapy–related myeloid neoplasms (Estey 2001; Bello et al. 2011). In patients over age 60, occurrence of unfavorable cytogenetics is 23% compared to 15% in those less than 60 (Grimwade et al. 2010).

Treatments used in AML are induction and post-remission therapy by use of chemotherapy and allogeneic bone marrow stem cell transplantation (Estey and Dohner 2006; Cornell and Palmer 2012). After achievement of an undetectable level of leukemic cells by induction chemotherapy, consolidation is given to maintain remission (Cornell and Palmer 2012). The great number of cases who receive no consolidation therapy will experience relapse within 4 to 6 months of initial treatment (Cassileth et al. 1988). Despite initial remission, relapse is common (Derolf et al. 2009). Although AML treatment has improved, 5-year survival for patients diagnosed in 2006-2010 was 21.4% (Pulte et al. 2010). Higher mortality has been seen in elderly patients and in those with poor performance status with intensive induction chemotherapy (Appelbaum et al. 2006a; Koreth et al. 2009). Finding novel therapeutic approaches is therefore necessary, particularly in this group of patients.

Table 5. 2008 WHO classification of AML

AML with recurrent genetic abnormalities
a. AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*
b. AML with inv(16)(p13;1q22) or t(16;16) (p13.1;q22)
c. APL with t(15;17)(q22;q12); *PML-RARA*
d. AML with t(9;11) (p22;q23); *MLLT3-MLL*
e. AML with t(6;9)(p23;q34); *DEK-NUP214*
f. AML with inv3(q21q26.2) or t(3;3) (q21;q26.2); *RPN1-EVI1*
g. AML (megakaryoblastic) with t(1;22) (p12;q13); *RBM15-MKL1*
h. Provisional entity:AML with muated *NPM1*
j. Provisional entity: AML with mutaed *CEBPA*
AML with MDS (myelodysplasia)-related changes
Therapy-related myeloid neoplasms
AML, not otherwise specified
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
Blastic plasmacytoid dendritic cell neoplasm

5.3.2.2. Genetic alteration in acute myeloid leukemia

Cytogenetic and genetic alterations are regarded as important prognostic factors in AML. By the WHO system, AML patients are classified into three risk groups (poor, intermediate, and favorable) based on cytogenetic changes (Grimwade et al. 1998; Byrd et al. 2002). Balanced translocations, t(15;17), t(8;21), t(16;16), and inv(16), are associated with favorable prognosis (Appelbaum et al. 2006b). Those patients with a normal karyotype are classified in an intermediate group risk and comprise 40% of all patients (Grimwade et al. 2004). *NPM1* and *CEBPA* mutations are associated with favorable outcome, and likelihood of achieving complete remission is higher in those patients with mutated *NPM1* or *CEBPA* in the absence of the *FLT3* receptor; overall survival has also improved in this group (Schlenk et al. 2008). *FLT3* mutations are associated with poor prognosis and lead to internal transmembrane mutation duplications (*FLT3*/*ITD*) with activation of the *FLT3* receptor tyrosine kinase. *NPM1* mutations occur in 50 to 60% and FLT3 mutations in 30% of cases (Falini et al. 2007; Kottaridis et al. 2001). Study of the effect of *DNMT3A* mutation status on prognosis of AML patients indicates that overall survival and relapse-free survival is worse in patients with mutation than in those with the wild type (Ribeiro et al. 2012).

Comparing miRNA profiling of AMLs carrying *FLT3-ITD* with *FLT3* wild-type patients reveals three up-regulated miRNAs (miR-10a, miR-10b, and miR-155) in *FLT3-ITD*. Over-expressed miR-124, miR-128-1, miR-194, miR-219-5p, miR-220a, and miR-320 positively correlate with the risk for an event such as failed remission, relapse, or death (Seca et al. 2010). However, a correlation exists between higher miR-181a expression and higher complete remission rate, longer overall survival, and a trend toward longer disease-free survival. Elevated level of this miRNA also is associated with higher complete remission rate and longer disease-free survival in patients with poor molecular risk such as those of the *FLT3-ITD* and *NPM1* wild type (Schwind et al. 2010). Up-regulation of miR-181a and miR-335 and down-regulation of miR-194 and miR-34a are associated with the *GEBPA* mutation (Marcucci et al. 2008). Some of the miR-181 family target genes (*TLR4*, *CARD8, CASP1,* and *IL1B*) show an inverse correlation with expression of members of this family that suggests a link between down-regulation of the miR-181 family and AML aggressiveness (Seca et al. 2010).

In patients with poor- and intermediate-prognosis karyotypes, the over-expressed miR-191 and miR-199 are associated with shorter overall survival and event-free

survival (Garzon et al. 2008). Level of miR-9 and let-7d is low in good-prognosis patients, while being high in intermediate and adverse AML (Dixon-McIver et al. 2008).

Evidence shows the role of miRNAs in the mechanism of resistance to anti-leukemia chemotherapies, and drug-resistant subtypes present specific miRNA signatures (Zhao et al. 2010; Zimmerman et al. 2010; Schotte et al. 2011). MiR-136 is found at a lower level in an AML HL-60-resistant cell line than in parental cells and indicates a link between this miRNA and multidrug resistance of leukemic cells (Zhao et al. 2010). Cytarabine is the backbone of chemotherapy used mainly in treatment of AML. It kills cancer cells by interfering with DNA synthesis. When the cell line is pretreated with an anti-miR-21, the potency of cytarabine increase in an AML-derived cell line (Li et al. 2010). Thus, identification and targeting of miRNAs involved in drug resistance may aid us in developing anti-cancer drugs with lower side effects and mortality rates.

AIMS OF THE STUDY

The general aim of the study was to identify miRNAs related to prognosis and drug response by means of the latest versions of miRNA microarrays.

The specific aims of this thesis were to identify

- MiRNA signatures associated with colorectal cancer and with *KRAS* status
- MiRNA signatures associated with resistance to anti-EGFR monoclonal antibody treatment in chemorefractory metastatic colorectal cancer patients with wild-type *KRAS/BRAF* and with third- to sixth-line anti-EGFR monoclonal antibody treatment.
- MiRNAs associated with metastasis and progression in primary tumors of giant cell tumor of bone.
- Any significant association between expression of miRNAs and overall survival, and differences in miRNA expression in matched diagnosis-relapse samples in acute lymphoblastic leukemia.
- Any miRNA signature associated with drug response in acute myeloid leukemia.

MATERIALS AND METHODS

1. Patient specimens (I-V)

Table 6 is a summary of the patients included in the studies.

1.1. Study I: FFPE tissue sections were collected from the primary tumors of 60 metastatic CRC (mCRC) patients at Helsinki and Turku University Central Hospital before any treatment was initiated. Of these 60, 30 were female and 30 male, mean age 61 years. In 44 patients, the site of the primary tumor was the colon and in 16 patients was the rectum. The percentage of tumor cells was provided by pathologists at the Helsinki and Turku University Central Hospitals. Tumor content ranged from 30 to 90% in both mutated and non-mutated *KRAS* sample groups.

1.2. Study II: Included were 33 FFPE sections from primary tumors of 33 patients with mCRC treated at Helsinki University Central Hospital and at Turku University Central Hospital. All the primary tumors were from the diagnosis period, prior to the start of any treatment with wild-type *KRAS* and *BRAF* status. The patients experienced no other malignancy and had third- to sixth-line treatment with cetuximab or panitumab and with or without irinotecan, and were chemorefractory or intolerant to irinotecan, oxaliplatin, and 5-fluorouracil. Based on their responses to anti-EGFR monoclonal antibody treatment, the patients were divided into two groups; a disease control and a progressive disease group.

1.3. Study III: The primary tumors from 10 GCTB patients were collected at the Rizzoli Orthopaedic Institute, Bologna, Italy. No primary tumor had been subjected to treatment. Both fresh frozen sections and FFPE sections obtained from each patient were used in our study. Of ten patients, two were at stage I, two at stage II, and the remaining six patients at stage III. Five patients developed lung metastases, but the other five developed none. The percentage of tumor cells equal to or more than 90% was estimated for each sample. In addition, NFIB protein expression was evaluated in 74 FFPE samples of primary GCTB including 45 disease-free and 29 metastatic.

1.4. Study IV: A total of 90 archived bone marrow core biopsy samples came from 79 pediatric patients diagnosed at Helsinki University Hospital, Finland, during 2000-2006. The

clinical data of these patients were extracted from the Nordic Society of Paediatric Haematology and Oncology (NOPHO) ALL database. The patients were treated according to the NOPHO-2000 and Interfant treatment protocols for ALL, with 11 pairs being available from the same patients, one sample at diagnosis and one at relapse. We categorized the ALL patients into 10 groups based on their cytogenetic features. Screening of the genomic aberrations was done by either one or more of these methods: interphase fluorescence in situ hybridization (FISH), array CGH, and PCR. Key characteristics of the ALL patients are in Table 1 in Study II. Eight bone marrow samples from healthy donors, obtained from an archived May–Grünwald–Geimsa-stained bone marrow aspirate smear, served as controls.

1.5. Study V: From 33 AML patients, 33 bone marrow core biopsy specimens at the time of diagnosis (before initiation of any treatment) were included in our study. Of these patients, 15 were chemorefractory and 18 chemosensitive (Table 1 in V). All patients were diagnosed and treated at Helsinki University Central Hospital, Finland, during 1993-2009, with treatment according to national AML protocols (AML-92 or AML-04). Patients without complete remission or with early relapse were defined as chemorefractory, and those patients with complete remission by the end of their first induction with following consolidation were defined as chemosensitive. None of the chemosensitive patients received allogeneic stem-cell transplantation.

Table 6. Specimens and studies

Study	Type of cancer	Total no.sample	Method used in study			
			miRNA microarray	qRT-PCR	IHC	Affymetrix array
Study I	CRC	60	✓	✓		
Study II	CRC	33	✓	✓		
Study III	GCTB	84	✓	✓	✓	✓
Study IV	ALL	90	✓	✓		
Study V	AML	33	✓	✓		

IHC, immunohistochemistry

2. Ethical permission

Studies I and II were approved by the HUS ethics committee as no. 173/13/03/02/09. The research protocol was approved for Study III by the ethics committee of the Rizzoli Institute, Bologna, Italy. The appropriate Institutional Review Boards and the National Authority for

Medico-legal Affairs approved Study IV. The samples for Study V were collected with approval from the HUS Ethics committee, Department of Medicine, Helsinki, Finland, and Valvira in Finland.

3. Nucleic acid extraction (I-V)

To extract DNA from tumor tissue samples, the QIAamp DNA FFPE Mini Kit (Qiagen, Valencia, CA) was used for Studies I and II. Total RNA was extracted in Study III with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and in Studies I, II, IV, and V with the miRNeasy FFPE Mini Kit (Qiagen) according to the manufacturer's protocols. The quality of RNA was assessed with the RNA 6000 chip, and miRNA with the small RNA chip (Agilent Technologies, Santa Clara, CA, USA). The control RNA sample from colon tissue used in Studies I and II was commercially available (FirstChoice® Human total RNA, Applied Biosystems/Ambion, Austin, TX, USA).

4. Mutation analysis (I & II)

To screen *KRAS* and *BRAF* mutations in CRC patients (Studies I and IV), we used the TheraScreen *KRAS* mutation kit (Qiagen, DxS Ltd, Manchester, UK) and the *BRAF* Mutation Test Kit (Qiagen, DxS Ltd), using a real-time PCR assay. The patients with wild-type *KRAS* were included in the *BRAF* mutation test, because *KRAS* and *BRAF* mutations are nearly mutually exclusive in CRC (Frattini et al., 2004).

5. MiRNA microarray (I-V)

We performed an miRNA expression profile with Agilent's miRNA Microarray system V2 (comprising 723 human and 76 human viral miRNAs, Sanger database v.10.1) for Studies I and II and Agilent's miRNA Microarray system V3 (comprising 866 human and 89 human viral miRNAs catalogued in the Sanger miRNA database v12) for Studies III, IV, and V. The RNA was labeled and hybridized for 20 hr according to the miRNA complete labeling and hybridization kit protocol version 2.0. The microarrays were then washed with prepared washing buffers and scanned with Agilent's scanner (G2505B, Agilent Technologies). Preprocessing of Agilent's scanner images was performed with Agilent's feature extraction

software with default parameters. Data were analyzed with GeneSpring Software Version 11.0.2.

6. Quantitative real time RT- PCR (qRT-PCR) (I-V)

In order to confirm the microarray results, we performed qRT-PCR for selected miRNAs in Studies I to V. In brief, cDNA was generated from total RNA with the miScript Reverse Transcription Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Then qRT-PCR was done by use of the SYBR Green miScript PCR system (Qiagen) on a Light-cycler, software v.3.5 (Roche Applied Science, Mannheim, Germany). The primer of selected miRNAs and endogenous control U6 came from Qiagen.

In Study II, we measured the level of two predicted target genes of differentially expressed miRNA by use of the RT² SYBR Green PCR Master Mix (Qiagen) with a Light-cycler, software v.3.5 (Roche Applied Science), following the manufacturer's guidelines. cDNA was synthesized from total RNA by use of the RT^2 First Strand kit (Qiagen). Housekeeping gene human *18SrRNA*, *SLC26A3*, and *ATN1* primers came from Qiagen.

In Study III, to verify identified differentially expressed genes by array affymetrix, the High Capacity cDNA Archive kit (Applied Biosystems) and TaqMan Expression Assays (Applied Biosystems) were our choice, used according to manufacturer's instructions. The *ACTB* gene as a housekeeping gene was commercially available (Applied Biosystems). The relative quantification (RQ) of both miRNA expression and gene expression were calculated with formula $2^{-\Delta\Delta CT}$.

7. Immunohistochemistry (III)

In Study III, the expression level of NFIB protein was evaluated by immunohistochemistry (IHC) with Streptavidin-biotin peroxidase DAB rabbit/mouse (Dako, Glostrup, Denmark) detection systems utilizing mouse monoclonal anti-NFIB antibody (1:1000, Abnova). Those samples with less than 10% NFIB-positive cells were considered negative, those with ≤ 25% positive cells considered weakly positive, with 25 to 49% moderately positive, and with more than 50% positive cells strongly positive. When the NFIB immune reactivity was moderate to strong, NFIB was over-expressed, but in those negative to weakly immune, reactivity was under-expressed.

8. Gene expression analysis with Affymetrix microarrays (III)

The mRNA data used in Study III were previously obtained by use of Affymetrix Human Genome U133 Plus 2.0 (Affymetrix, Santa Clara, CA, USA) for the same samples in study by Pazzaglia et al. (2010). In brief, the cDNA is converted into a double-stranded state and then transcribed into cRNA labeled with biotin in an in vitro trascription reaction (IVT). The IVT reaction is a linear amplification (usually 20-100 fold) of the original mRNA. The biotinylated cRNA is then hybridized on array and afterwards a streptavidin-phycoeytrin conjugate is added that binds biotin and emits fluorescent light after laser excitation. After appropriate washings, the chips were analyzed with Affymetrix® GeneChip® Command Console® Software (AGCC).

RESULTS AND DISCUSSION

1. MiRNA expression profiling in colorectal cancer and association of miRNA expression with *KRAS* status (I)

In Study I, we compared miRNA profiling of all primary tumors of mCRC (55 samples with and without *KRAS* mutation) with normal colon tissue. We removed five samples from our further analysis, because they carried the *BRAF* mutation. The analysis revealed 46 altered miRNAs ($q<0.05$), in which 39 were significantly under-expressed and 7 significantly over-expressed (Table 1 in Study I). Our finding is accordance with Lu et al. (2005) that expression level of miRNA is lower in cancer than in normal tissue. Our study revealed 19 identified miRNAs as novel. The miRNAs with the highest fold changes were under-expressed miR-1 (FC=60.2), miR-195 (FC=35.7), and miR-143 (FC=26.7), and over-expressed miR-494 (FC=25.2). High-throughput sequencing also found a lower expression level of miR-1 and miR-195 in CRC than in paired normal mucosa (Hamfjord et al. 2012). MiR-1 functions as a tumor suppressor by down-regulating the MET oncogene, and its down-regulation may enhance CRC progression (Reid et al. 2012).

Next we investigated the impact of *KRAS* status on miRNA expression. *KRAS* is an important gene in the EGFR signaling pathway which can predict resistance to anti-EFGR treatment; treatment success depends on the presence of the *KRAS* wild type. On the other hand, 35 to 40% of CRC patients carry the *KRAS* mutation. Thus, identification of novel markers which can improve the strategy of treatment in this group of patents is essential.

The expression level of four miRNAs significantly discriminated between 15 CRC patients with and 40 without the *KRAS* mutation ($q<0.05$). MiR-92a, miR-127-3p, and miR-486-5p were over-expressed, and miR-378 was under-expressed in the mutated *KRAS* group. Higher expression of miR-127-3p and miR-92a were significantly confirmed with qRT-PCR. In accordance with our finding, up-regulation of miR-92 is detected in several cancers such as CRC (Ng et al. 2009). This miRNA, along with other miRNAs in the miR-17-92 cluster, is involved in promoting cell proliferation and anti-apoptotic processes of cancer cells, and in sustaining angiogenesis (Mendell 2008). Thus, the role of elevated miR-92 in mutated *KRAS* patients may in part clarify this group's inferior outcome (Andreyev et al. 1998).

An elevated level of miR-486-5p in mutated *KRAS* samples was also detected by Ragusa et al. (2010), a level which may be due to activation of *KRAS* signaling. MiR-486-

5p is one of the miRNA discriminators between squamous cell carcinoma and normal lung tissue (Tan et al. 2011).

MiR-127-3p is up-regulated in enriched lung adenocarcinoma tumor-initiating cells from the A549 cells, and its expression trend is validated in A549 cell line- and primary lung- adenocarcinoma samples, with suggestions that miR-127-3p may play an important role in regulating the bio-behavior of tumor-initiating cells (Lin et al. 2012).The level of miR-127-3p as a potential serum biomarker in esophageal squamous cell carcinoma is also higher in patients' serum than in control individuals' serum (Zhang et al. 2010).

Target genes predicted by at least four of six databases—to minimize the false positivity—for differentially expressed miRNAs in CRC vs. normal were 828, and in mutated vs. wild-type *KRAS* were 65 common genes. Then, predicted target genes were screened to seek their significant involvement in biological networks. Among the pathways, the G protein-coupled receptor (GPCR) -signaling pathway was one of the most significant. GPCRs regulate cells (Fichter et al. 2010), and effects of activated GPCR on growth-promoting are mediated via activation of receptor tyrosine kinases such as EGFR (Shah and Catt 2004; Wetzker and Bohmer 2003). Some target genes of discriminator miRNAs between CRC, with and without *KRAS* mutation, were *RGS3*, *TOB1*, and *HNF1B*. These target genes are involved in apoptosis, dedifferentiation, and anti-proliferative processes (Nishiura et al. 2009; Buchner et al. 2010). Thus, our observations suggest that miRNAs and their target genes may play an important role in mechanisms of resistance to anti-EGFR monoclonal antibody treatment in mutated *KRAS* tumors and may prove useful for developing treatment strategies in this group of patients.

2. MiRNA expression in anti-EGFR monoclonal antibody-treated metastatic colorectal cancer (II)

Half the mCRC patients with no mutation in *KRAS* and *BRAF* fail to respond to anti-EGFR monoclonal antibody treatment. To see whether any association exists between miRNA profile and treatment response in this group of patients, first the cases were screened for *KRAS* and *BRAF* mutations, and those with either *KRAS* or *BRAF* mutations we removed from analysis. We examined only the primary untreated tumors of mCRC that had undergone third-to sixth-line treatment with cetuximab or panitumab. The patients' division into two groups: DC (including responders and stable disease) and PD—evaluation of response to

anti-EGFR monoclonal antibody treatment—was based on the Response Evaluation Criteria for solid tumors 1.0 (Therasse et al. 2000).

We found two differentially expressed miRNAs: up-regulated miR-31* and down-regulated miR-592, between PD and DC. Their expression trend was verified by qRT-PCR. The function of miR-31* is not well known in CRC, but its higher expression is detectable in malignantly transformed oral leukoplakia when compared to oral leukoplakia and oral squamous cell carcinoma tissues (Chang et al. 2012; Xiao et al. 2012). Evidence shows that miR-31* also plays a biological function in cancer development (Xiao et al. 2012).

MiR-592 is associated with the transition of normal colon to carcinoma (Oberg et al. 2011), being down-regulated in hepatocellular carcinoma (Wang et al. 2012). The level of miR-592 is also lower in deficient mismatch repair (dMMR) than in proficient mismatch repair (pMMR) CRC (Sarver et al. 2009). dMMR cells show resistance to anti-metabolites and to platinum drugs such as 5-fluorouracil and cisplatin (Aebi et al. 1996; Drummond et al. 1996).

By applying strict criteria to screen potential target genes of miR-31* and miR-592, we found 592 common genes in four databases. *SLC26A3* and *ATN1,* miR-31* target genes that are drug-related genes in CRC, were verified by qRT-PCR to assess their expression level in DC and PD groups. In the PD group, expression levels of *SLC26A3* and *ATN1* detected were lower than for the DC group. A lower expression level of *ATN1* occurs in chemoradiation-resistant colorectal cell lines (Spitzner et al. 2010). *SLC26A3* is under-expressed in the early neoplastic process and in CRC compared to normal samples (Schweinfest et al. 1993; Byeon et al. 1996; Antalis et al. 1998). SLC26A3 inhibits cell growth and colony formation in numerous cancers such as CRC (Chapman et al. 2002). In mucinous CRC, a subtype of CRC, the level of *SLC26A3* is lower than for the non-mucinous form, clinically; 5-year overall survival is worse in mucinous CRC, and lymph nodes are involved more extensively (Kim et al. 2011). The function of this gene is related to tumorigenesis (Kim et al. 2011). Its expression level is associated with disease control and progression-free survival in *KRAS* wild-type CRCs, and it is one of the four genes involved in the prediction classification model of cetuximab efficacy (Baker et al. 2011).

When we divided the patients into two groups based on their overall miRNA expression by unsupervised clustering and then by applying Kaplan-Meier survival analysis, the overall survival with mCRC between these groups significantly differed ($p=0.03$). Seventy-five miRNAs were differentially expressed between the two groups (Table 1 in

Study II). Among miRNAs, up-regulated members of the let-7 family (let-7f, let-7g, let-7d, let-7i, let-7a, let-7e, and let-7b) appeared in the group of patients with poorer prognosis. Let-7f was verified by qRT-PCR ($p<0.005$). Pearson correlation analysis was unable to establish any significant negative correlation between PCR data as to let-7f and overall survival, but it showed a trend toward poorer survival for CRC patients who showed higher expression of let-7f (R=-0.3, P=0.08). Members of the let-7 family act as potential tumor suppressors, and they are down-regulated in various types of cancers compared to normal tissue. Their higher levels are, however, associated with poorer survival, which is in accordance with our results (Ali et al. 2010; Silva et al. 2011; Zuo et al. 2011). Higher expression of let-7 in lung cancer and in myelodysplastic and pancreatic cancers correlates with poor overall survival (Ali et al. 2010; Silva et al. 2011; Zuo et al. 2011). We studied also the correlation between each miRNA and overall survival with the Cox proportional hazards regression model, and 11 miRNAs were significantly correlated with overall survival (Table 2 in Study II). This analysis showed two miRNAs to be associated with poor overall survival (up-regulated miR-140-3p and down-regulated miR-1224-5p), as did cluster analysis.

An association exists between epigenetic silencing of miR-1224 and progression of bladder cancer. Moreover, up-regulated miR-1224-5p correlates with complete pathological response to neo-adjuvant chemo-radiotherapy in locally advanced rectal cancer (Della Vittoria Scarpati et al. 2012). The level of miR-140 is high in colon cancer stem-like and its blocking in resistant cells sensitizes them to 5-fluorouracil treatment (Song et al. 2009). The miRNAs identified in our study may play an important role in CRC biology and may serve as prognostic predictors.

3. Prognostic value of miRNA in giant cell tumor of bone (III)

Despite numerous studies on GCTB, information and knowledge are lacking regarding the role of miRNA in pathogenesis of GCTB and also in tumor progression. To evaluate whether any miRNA is involved in GCTB progression and metastasis, first we compared the miRNA profiling, and then we integrated mRNA and miRNA expression data.

Regarding 12 differentially expressed miRNAs identified between metastatic and non-metastatic tumors: 6 miRNAs were absent from all non-metastatic but detectable in metastatic tumors (miR-513a-5p and miR-let-7a* in 60%, and miR-224, miR-10b*, miR-934, and miR-876-5p in 40%), and 5 miRNAs were absent from all metastatic but expressed in

non-metastatic tumors (miR-136 in 60%, and miR-542-5p, miR-505*, miR-542-3p, and miR-1 in 40%); expression level of miR-494 was higher in metastatic tumors.

Verification of miR-1, miR-494, miR-136, and miR-513a-5p by qRT-PCR showed an expression trend similar to that shown by microarray analysis. However, only miR-136 was significantly differentially expressed between metastatic and non-metastatic tumors, but its function related to cancer is still unknown. MiR-1, miR-542-5p, and miR-542-3p as tumor suppressors have been identified in numerous cancers such as thyroid carcinogenesis, head and neck squamous cell carcinoma, and neuroblastoma (Bray et al. 2011; Leone et al. 2011; Nohata et al. 2011; Zhao et al. 2011). MiR-542-3p regulates the cell cycle and is a cell-proliferation inhibitor. A higher level of miR-224 occurs in various human tumor types including colorectal, hepatocellular, and renal cancers (Kang et al. 2003; Mees et al. 2009; Mencia et al. 2011). Similarly, over-expression of miR-224 is associated with breast cancer invasion and CRC progression, as well as with invasion and metastasis in pancreatic ductal adenocarcinoma; this suggests that miR-224 is an onco-miR (Nguyen and Massague 2007; Arndt et al. 2009; Huang et al. 2012).

When mRNA and miRNA array data were combined, eight target genes (*PDPN*, *BAALC*, *NR2F1, TNC*, *NET1*, *SETBP1*, *NFIB*, and *FLRT2*) showed an inverse correlation with miRNA identified in GCTB, and these eight were concordant with the list of miRNA-predicted targets genes in databases (Figure 9 and Table 3 in Study III). Further, the higher expression level of *NFIB*, the miR-136 target gene, in metastatic GCTB, was verified by use of qRT-PCR, which we found by analysis of array data. A similar pattern of NFIB expression at protein level by IHC was confirmed not only in the same samples but also in a larger series of GCTB (Figure 10). The primary tumor of metastatic GCTB produced moderate to strong immunostaining (>25% positive cells), but non-metastatic tumors produced weak immunostaining. The expression level of NIFB significantly correlated with rate of metastasis but not with confounding factors such age, sex, size of tumor, or outcome. The role of over-expressed *NFIB* has been evident in tumor progression. Moreover, NFIB leads to increased cell growth and reduces apoptosis in a murine hematopoietic cell line and in triple-negative breast cancer, and it acts as an oncogene in small-cell lung cancer (Dooley et al. 2011; Moon et al. 2011; Rice et al. 2011).

TNC over-expression inversely correlated with undetectable miR-1 and miR-542-5p in metastatic GCTB. A high level of *TNC* is a metastasis risk factor and associates with poor prognosis in GCTB (Pazzaglia et al. 2010).

To examine the biological and functional networks, we performed pathway analysis only with the differentially expressed mRNAs, the target genes of differentially expressed miRNAs that also inversely correlated with miRNA expression. As a result, we identified the FOXA1 pathway as related to miRNAs' target genes. FOXA1 as a central component of this pathway is the first class of forkhead-type proteins identified in mammals. *NFIB*, the miR-136 target gene, and *NR2F2*, the miR-513a-5p target gene, belong to FOXA1. Because FOX proteins are important in many biological processes such as proliferation, migration, and invasion (Myatt and Lam 2007), the induction or repression of their function can result in tumorigenesis and cancer progression (Myatt and Lam 2007); this may, in part, explain the trend of some primary GCTB cases toward development of metastases.

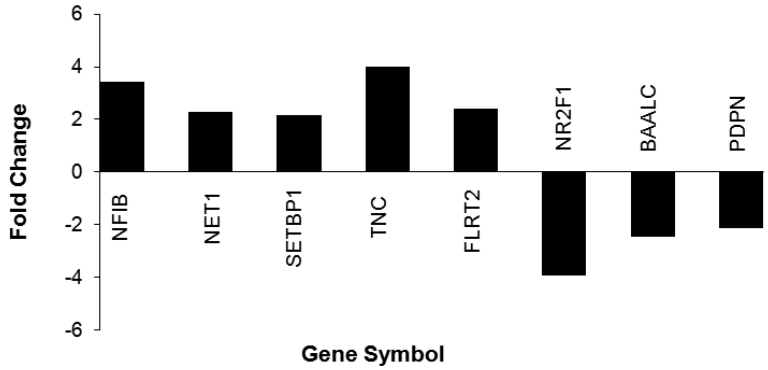

Figure 9. Over-expressed and under-expressed miRNA target genes in metastatic vs. non-metastatic GCTB.

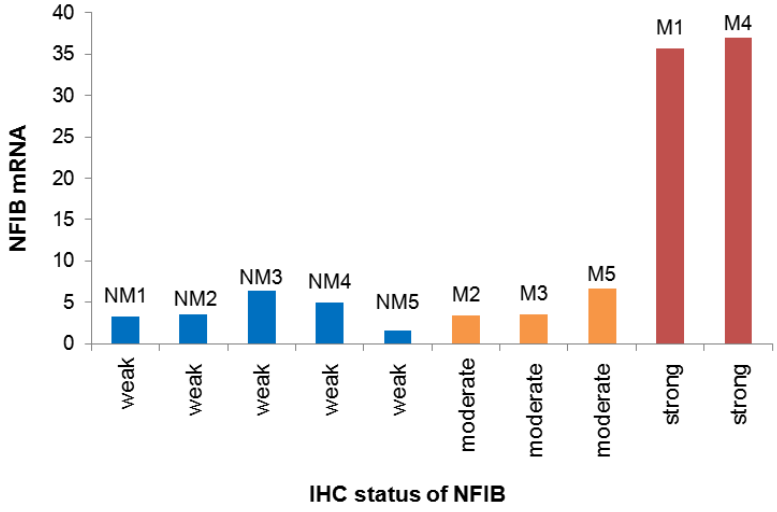

Figure 10. NFIB immunostaining and mRNA level in GCTBs, NM=non-metastatic; M=metastatic

4. Detection of novel prognostic markers in acute lymphoblastic leukemia by miRNA profiling (IV)

Our analysis showed 93 differentially expressed miRNAs in pediatric ALL vs. normal bone marrow samples, including 37 up-regulated and 56 down-regulated miRNAs (Tables 7 & 8). The differences in miRNA expression between ALL and normal samples were distinguishable by hierarchical clustering as well (Figure 11). Although some earlier studies demonstrate miRNA profiling in ALL (Mi et al. 2007; Zanette et al. 2007; Schotte et al. 2009; Zhang et al. 2009), only two studies evaluate miRNA profiling in pediatric ALL, and the up-regulation of miR-181a/b, miR-146a, miR-155, miR-34a, and miR-130b seen in our ALL patients is similar to that reported in those two pediatric ALL studies: Schotte et al. 2009 and Zhang et al. 2009. A high level of miR-130b and low levels of miR-223, miR-451, and miR-22 appeared in our study, as they did during comparison of ALL and AML in another study (Mi et al. 2007). However, let-7e and miR-19a have shown expression patterns in other ALL studies that differ from the pattern in ours (Zanette et al. 2007; Schotte et al. 2009) (unpublished data).

Table 7. Up-regulated miRNAs in pediatric ALL as compared to normal bone marrow

MiRNA	q-value	FC	miRNA	q-value	FC
hsa-miR-181b	<0.0001	12.19	hsa-miR-125b	0.002	3.03
hsa-miR-128	<0.0001	9.91	hsa-miR-574-3p	<0.0001	3.03
hsa-miR-181a	<0.0001	7.53	hsa-let-7e	<0.001	3.02
hsa-miR-892b	<0.0001	6.15	hsa-miR-625	0.001	2.99
hsa-miR-1290	<0.0001	5.21	hsa-miR-221	0.003	2.92
hsa-miR-1288	<0.0001	5.13	hsa-miR-130b	<0.001	2.72
hsa-miR-1305	<0.0001	5.03	hsa-miR-198	<0.0001	2.54
hsa-miR-146a	0.003	4.55	hsa-miR-320d	<0.001	2.46
hsa-miR-501-5p	<0.0001	4.53	hsa-miR-877*	0.009	2.46
hsa-miR-500	<0.0001	4.25	hsa-miR-1914*	<0.0001	2.36
hsa-miR-222	0.007	4.03	hsa-miR-34a	0.006	2.29
hsa-miR-155	<0.0001	4.01	hsa-miR-766	0.001	2.21
hsa-miR-513b	<0.0001	3.96	hsa-let-7f-1*	0.033	2.21
hsa-miR-1246	<0.0001	3.8	hsa-miR-720	0.001	2.09
hsa-miR-513a-5p	<0.0001	3.79	hsa-miR-760	0.001	2.07
hsa-miR-513c	<0.0001	3.78	hsa-miR-630	0.001	2.06
hsa-miR-23b	0.001	3.29	hsa-miR-320b	0.002	2.03
hsa-miR-199a-3p	0.003	3.17	hsa-miR-1268	0.003	2.01
hsa-miR-331-3p	<0.0001	3.09			

Table 8. Down-regulated miRNAs in pediatric ALL as compared to normal bone marrow

miRNA	q-value	FC	miRNA	q-value	FC
hsa-miR-144	<0.0001	95.95	hsa-miR-1306	<0.0001	3.27
hsa-miR-144*	<0.0001	62.16	hsa-miR-106b	<0.0001	3.18
hsa-miR-451	<0.0001	50.76	hsa-miR-7	<0.001	3.09
hsa-miR-96	<0.0001	24.54	hsa-miR-19a	<0.0001	3.07
hsa-miR-486-5p	<0.0001	18.53	hsa-miR-29c	<0.001	2.99
hsa-miR-185	<0.0001	16.28	hsa-miR-18b	0.005	2.98
hsa-miR-194	<0.0001	11.43	hsa-miR-424*	<0.001	2.84
hsa-miR-183	<0.0001	10.82	hsa-miR-19b	<0.001	2.82
hsa-miR-223	<0.0001	9.31	hsa-miR-374a	0.005	2.76
hsa-miR-223*	<0.0001	8.39	hsa-miR-15a	<0.0001	2.74
hsa-miR-101	<0.0001	8.38	hsa-miR-107	<0.0001	2.73
hsa-miR-192	<0.0001	8.37	hsa-miR-29b	0.001	2.61
hsa-miR-486-3p	<0.0001	8.3	hsa-miR-1202	<0.0001	2.6
hsa-miR-595	<0.0001	7.59	hsa-miR-29a	0.001	2.51
hsa-miR-1228*	<0.0001	6.04	hsa-miR-148b	0.006	2.5
hsa-miR-148a	<0.001	5.9	hsa-miR-30e	<0.001	2.45
hsa-miR-32*	<0.0001	5.84	hsa-miR-25	<0.001	2.44
hsa-miR-16	<0.0001	5.62	hsa-miR-16-2*	0.002	2.43
hsa-miR-362-5p	<0.0001	5.56	hsa-miR-652	0.024	2.36
hsa-miR-215	<0.0001	5.49	hsa-miR-103	<0.0001	2.24
hsa-miR-18a	<0.0001	5.25	hsa-miR-1287	0.011	2.19
hsa-miR-660	<0.0001	5.23	hsa-miR-26b	0.003	2.18
hsa-miR-22	<0.0001	5.15	hsa-miR-212	<0.001	2.12
hsa-miR-1180	<0.0001	4.85	hsa-miR-338-5p	0.005	2.1
hsa-miR-532-5p	<0.0001	4.75	hsa-let-7g	0.004	2.06
hsa-miR-187*	<0.0001	4.69	hsa-miR-590-5p	0.007	2.03
hsa-miR-15b	<0.0001	3.91	hsa-miR-1285	0.001	2.03
hsa-miR-769-3p	<0.0001	3.40	hsa-miR-27a	0.011	2.01

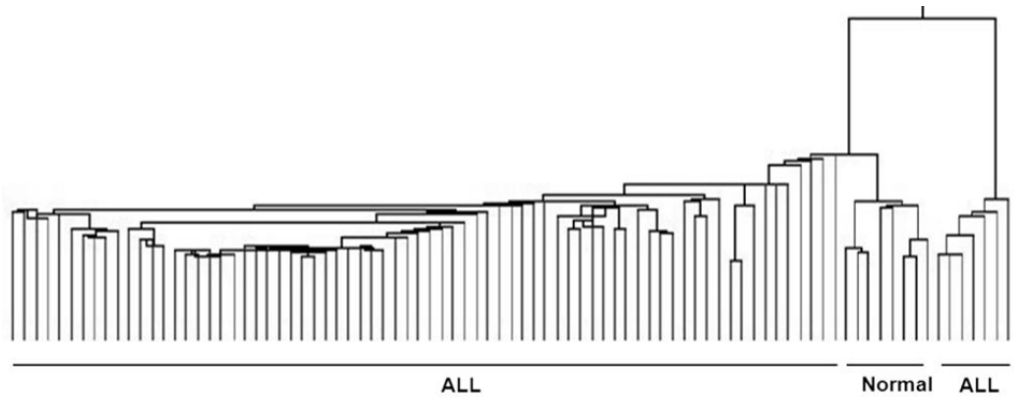

Figure 11. Hierarchical clustering of samples based on miRNA expression. The ALL patients have a distinct expression profile separating them from normal bone marrow control samples.

To see the correlation between miRNA expression and survival, we applied two approaches for data analysis. First we compared groups with and without events (relapse or death). In the first comparison, patients with an event showed down-regulation of miR-150 versus that of patients without any event. MiR-150 is a lymphoid-specific and very important miRNA in hematopoiesis (Xiao et al. 2007; Vasilatou et al. 2010). Its deregulation occurs in several hematological malignancies including ALL (Zanette et al. 2007). MiR-150 functions as a tumor suppressor (Watanabe et al. 2011) and is reported to inhibit cell proliferation and to induce cell apoptosis in T-ALL (Ghisi et al. 2011). Moreover, our target analysis revealed *ELK1* and *PLP2* as being targets of miRNA-150, and of these, *ELK1* is involved in the MAPK pathway and *PLP2* in the metastatic process (Cohen-Armon 2007; Sonoda et al. 2010). Duplication of the *ELK1* oncogene at Xp11.23 occurs in ALL patients, and these patients show very poor prognosis (Yasar et al. 2010). Thus, down-regulated miR-150 in ALL cases with an event (relapse or death) may lead to over-expression of *ELK1* and *PLP2,* and this may explain why these patients have an unfavorable prognosis.

In our second approach, we performed unsupervised clustering of miRNA data from all patients and obtained two groups of patients who differed clearly in their miRNA expression pattern (Figure 1a in Study IV). Survival analysis and event-free survival by the Kaplan-Meier method showed that these two groups had significantly differing overall survival and event-free survival, independent of certain cytogenetic changes, of risk group, of immunophenotype, and of age (Figures 1b & 1c in Study IV). Among 28 significantly up-regulated and 2 down-regulated miRNAs ($q<0.05$) in group 1 compared to group 2 (Table 1 in Study IV), miR-423-5p ($q=0.001$), which was exclusively expressed in one group and totally absent from the other group, was selected, and we confirmed its expression pattern with qRT-PCR ($p=0.005$). Kaplan-Meier analysis of the qRT-PCR results from this miRNA revealed that higher expression of miR-423-5p was associated with better overall survival ($p=0.04$) but not with event-free survival. This association was independent of confounding factors: immunophenotype, age, risk group, cytogenetics. The expression of miR-423-5p was undetectable in the group of patients with poor prognosis.

Although no correlation between miR-423-5p and progression exists thus far, two miR-423-5p target genes, *SRA1*/*SCARF1* and *FOXM1,* showed an association with overall survival and prognosis in numerous cancers (Carter et al. 2006; Leoutsakou et al. 2006; Ma et al. 2009). An inverse correlation appears between expression of SRA1 and prognosis and overall survival in ovarian cancer, and its higher expression is found in relation to aggressive ovarian cancer phenotypes (Scorilas et al. 2001; Leoutsakou et al. 2006).

FOXM1 is a survival-predictor gene in numerous cancers (Carter et al. 2006), and due to the inhibitory role of a reduced level of *FOXM1* in leukemia-cell proliferation, this gene could prove useful in targeted therapy regimens for leukemia patients (Nakamura et al. 2010). Together, these findings may reveal the importance of the miR-423-5p role in survival by its negative regulation of these genes.

Our analysis was unable to find any differences in miRNA expression between clinical risk groups (standard, intermediate, and high risk) which are clustered based on clinical and cytogenetic evaluation.

Differentially expressed miRNAs, miR-654-5p and miR-431, were also identified in matched diagnosis-relapse samples. The role of the miR-431 target gene, *PKD1*, is known in endothelial cells for mediating signal transduction in association with angiogenesis by VEGF (Ha and Jin 2009). Angiogenesis is an essential factor for tumor growth (Ferrara and Davis-Smyth 1997), and on the other hand, based on the role of PKD1 in angiogenesis, it is a suitable target in order to develop targeted therapy in angiogenesis-related diseases (Altschmied and Haendeler 2008). In considering this point, however, we must remember that changes in miRNA expression may be due more to treatment than to the original tumor cells.

5. MiRNAs and the drug resistance of acute myeloid leukemia (V)

The role of miRNAs in drug resistance of AML patients was not well known when we designed this study. We used core biopsy samples because their reliability has been investigated (Borze et al. 2011), and also because in all biopsies, tumor percentage was more than 80%. We compared the miRNA profile of core biopsy samples of AML, ones resistant to and sensitive to chemotherapy, to see whether any difference existed in their miRNA expression. MiRNA was considered as differentially expressed when the difference was more than a two-fold change, and the q-value was less than 0.05. After these filtrations, three miRNAs (miR-363, miR-532-5p, and miR-342-3p) remained. All were over-expressed in chemoresistant compared to chemosensitive patients.

The most significant miRNAs selected for verification by qRT-PCR showed an expression similar to that with microarray (Figure 12). Only the increased level of miR-363 in chemoresistant versus chemosensitive patients was, however, significant (P=0.03).

MiR-363 belongs to the oncogenic miR-17-92 cluster, leading to suggestions that members of this family may function similarly (Wald et al. 2011). Another miRNA,

miR-532-5p, is involved in tumor progression in melanoma, and its higher level is detectable in metastatic tumors but not in primary tumors (Kitago et al. 2009). The level of miR-532-5p is also high in ovarian carcinoma vs. borderline tissues (Lee et al. 2012). Involvement of miR-342-3p in different types of cancer has also revealed that while this miRNA is over-expressed in primary multiple myeloma and acute promyelocytic leukemia, its expression is lower in polycythemia vera, or in essential thrombocythemia. Another study suggests that miR-342-3p may be involved in the B-cell transformation in CLL (Li et al. 2011). Its deregulation occurs in two esophageal carcinoma cell lines after 24- or 72-h treatment with cisplatin or 5-fluorouracil (Hummel et al. 2011).

When the potential miRNA target genes were predicted by at least four of six databases, 81 candidate genes were identified; further pathway analysis showed involvement of these genes in eight biological networks such as the TGF-B pathway and BMP signaling. Interestingly, the role of both *RGS17* and *HIPK3*—miR-363 target genes—has been investigated in drug response in numerous cancers like those of the prostate and ovary. RGS17 plays a critical role in T-cell proliferation and IL-2 production; lack of RGS17 results in impaired T-cell activation (Oliveira-Dos-Santos et al. 2000). The role of these genes in relation to their miRNA regulators should, however, be verified also in AML patients who have received chemotherapy.

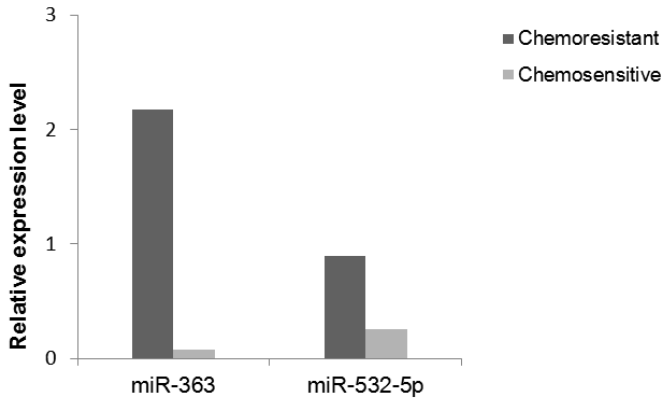

Figure 12. Relative expression levels of miR-363 and miR-532-5p in chemoresistant and chemosensitive AML patients.

CONCLUSIONS

The work reported in this thesis seeks to identify miRNAs that can serve as prognostic and predictive markers in human cancers (CRC, GCTB, ALL, AML) by use of miRNA microarray. This microarray discriminated between cancer patients with differing responses to treatment and differing prognoses. To understand the pathogenesis behind miRNA changes, prediction of miRNA target genes and pathway analysis provided clues to the various biological processes underlying their progression and resistance to treatment. The results presented here confirm that miRNA profiling is an accurate method of discriminating between tumor subtypes.

In addition to earlier recognized CRC- and *KRAS*-related miRNAs, we identified changes not previously described as CRC- and *KRAS*-associated. Four miRNAs (miR-127-3p, miR-92a, miR-486-5p, and miR-378) distinguished CRC patients who had the *KRAS* mutation from those with no mutation. The *KRAS*-related miRNAs and their target genes play a role in cell proliferation and apoptosis (Study I). Moreover, miRNAs (miR-31* and miR-592) associated with a response to anti-EGFR monoclonal antibody therapy were evident when the primary tumors were compared between anti-EGFR monoclonal antibodytreated chemorefractory mCRC patients with no mutation in *KRAS* and *BRAF*. Although the roles in drug response of up-regulated miR-31* and down-regulated miR-592, differentially expressed between patients with PD and DC, are not clear thus far, their potential target genes, *SLC26A3* and *ATN1*, have shown some associations with drug response. Verification of *SLC26A3* and *ATN1* showed their levels to be lower in PD than in DC patients. Besides these findings, we evaluated the correlation of miRNA expression with overall survival, and this resulted in detection of 84 differentially expressed miRNAs between patients with poor and good prognosis by clustering and Cox proportional hazards regression model analyses in which two miRNAs, miR-140-5p and miR-1224-5p, were included in both analyses (Study II).

In Study III, we aimed to recognize miRNAs which may be involved in progression and metastasis in GCTB, and miRNA profiling could distinguish primary tumors of metastatic tumors from those that were non-metastatic. By integration of the mRNA and miRNA expression data, we assessed whether any of the predicted miRNA target genes was in the list of mRNAs differentially expressed between metastatic and non-metastatic tumors; this resulted in identification of eight target genes.

In addition to doing verification of a higher level of *NFIB* by qRT-PCR in metastatic tumors, we also assessed its protein level by IHC in the same samples as well as in an independent validation cohort of GCTB samples. The expression of miR-136 and of both NFIB mRNA and protein was independent of other variables such as age, sex, size of tumor, or outcome, but a significant correlation appeared between NFIB expression and rate of metastasis.

Our study on ALL (Study IV) did reveal miRNAs related to progression and survival for the first time. Comparison of the miRNA profiling of paired diagnosis-relapse samples showed miR-431 and miR-654-5p as differentially expressed miRNAs between them. Through the use of two different approaches, 31 miRNAs related to overall survival emerged. One of them, miR-423-5p, underwent further study, showing that those patients with no expression of miR-423-5p had a poorer prognosis independent of age, risk group, cytogenetic changes, or immunophenotype.

To identify mechanisms underlying drug resistance in AML, the miRNA profiles of chemorefractory and chemosensitive diagnosed AML sample were compared which showed a response and were resistant to chemotherapy. Results indicated that three miRNAs (miR-363, miR-532-5p, and 342-3p) were differentially expressed between samples which were chemoresistant versus chemosensitive. The association of their potential target genes, ones such as *RGS17*, *HIPK3,* and *ANKRD49,* with treatment response and invasion was apparent. Some of the pathways affected by differentially miRNAs were the TGF-B pathway and BMP signaling (Study V).

MiRNA microarray succeeded in assessing miRNA expression on a global scale and is an effective screening method to detect altered miRNA. This method allows assaying the expression level of hundreds of miRNAs in a single experiment. Moreover, miRNA profiling of FFPE samples by use of miRNA microarray provided reliable results. Therefore, miRNA microarrays can make an important contribution to both basic and applied research and have the potential to change the practice of medicine by providing the means for personalized diagnosis, cancer detection, and prognostic assessment (Yin et al. 2008). Genome-wide analysis of miRNA expression is, however, now largely sequencing based. Next generation sequencing (NGS) allows discovery of novel miRNAs in addition to known miRNAs and may replace some of the current microarray applications.

In future, the prognostic and predictive biomarkers identified for the cancers studied in this thesis need to be validated in a larger set of samples using alternative approaches, and their roles in the mechanism of drug resistance and progression should be

explored by means of functional experiments. Identification of novel biomarkers provides an opportunity to develop cancer-treatment strategies to prevent unwanted drug side-effects and unnecessary medical costs.

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