Identification of miRNA expression profiles for diagnosis
and prognosis of prostate cancer

*To my grandfather*
*Curt "Cula" Carlsson*

*"Research is to see what everybody else has seen,*
*and to think what nobody else has thought"*

*Örebro Studies in Medicine 74*

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*Title*: Identification of miRNA expression profiles for diagnosis and prognosis of prostate cancer.

*Publisher*: Örebro University 2012
www.publications.oru.se
trycksaker@oru.se

*Print*: Ineko, Kållered 09/2012

ISSN 1652-4063
ISBN 978-91-7668-888-5

## Abstract

Jessica Carlsson (2012): Identification of miRNA expression profiles for diagnosis and prognosis of prostate cancer. Örebro Studies in Medicine 74, 55 pp.

Cancer of the prostate (CaP) is the most common malignancy diagnosed in men in the Western society. During the last years, prostate specific antigen (PSA) has been used as a biomarker for CaP, although a high PSA value is not specific for CaP. Thus, there is an urgent need for new and improved diagnostic markers for CaP.

In this thesis, the aim was to find a miRNA signature for diagnosis of CaP and to elucidate if differences in behavior between transition zone and peripheral zone tumors are reflected in miRNA expression. One of the major findings is an expression signature based on nine miRNAs that with high accuracy (85%) could classify normal and malignant tissues from the transition zone of the prostate. The results furthermore show that the major differences in miRNA expression are found between normal and malignant tissues, rather than between the different zones. In addition, tumors arising in the peripheral zone have fewer changes in miRNA expression compared to tumors in the transition zone, indicating that the peripheral zone is more prone to tumor development compared to the transition zone of the prostate.

A crucial step in pre-processing of expression data, in order to differentiate true biological changes, is the normalization step. Therefore, an additional aim of this thesis was to compare different normalization methods for qPCR array data in miRNA expression experiments. The results show that data-driven methods based on quantile normalization performs the best. The results also show that in smaller miRNA expression studies, only investigating a few miRNAs, RNU24 is the most suitable endogenous control gene for normalization.

Taken together, the results in this thesis show the importance of miRNAs and the possibility of their future use as biomarkers in the field of prostate cancer.

*Keywords*: Prostate cancer, microRNAs, prostate zones, normalization, endogenous controls.

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# List of abbreviations

| | |
|---|---|
| **BPH** | Benign Prostate Hyperplasia |
| **CaP** | Cancer of the Prostate |
| **cDNA** | Complementary Deoxyribonucleic Acid |
| **CV** | Coefficient of Variation |
| **CZ** | Central Zone |
| **DNA** | Deoxyribonucleic Acid |
| **FFPE** | Formalin Fixed Paraffin Embedded |
| **miRNA** | Micro Ribonucleic Acid |
| **mRNA** | Messenger Ribonucleic Acid |
| **PCA** | Principal Component Analysis |
| **PSA** | Prostate Specific Antigen |
| **PZ** | Peripheral Zone |
| **qPCR** | quantitative Polymerase Chain Reaction |
| **RNA** | Ribonucleic Acid |
| **RT-PCR** | Reverse Transcriptase Polymerase Chain Reaction |
| ***sd*** | Standard deviation |
| **TUR-P** | Transurethral Resection of the Prostate |
| **TZ** | Transition Zone |

# Original papers

The present thesis is based on four papers, which will be referred to in the text by their Roman numerals (paper I - IV):

I. **Carlsson, J.**, Helenius, G., Karlsson, M., Andrén, O., Lubovac, Z., Olsson, B. & Klinga-Levan, K. (2010) *Validation of endogenous control genes for miRNA expression studies in prostate tissues*. Cancer Genet Cytogenet; 202(2):71-75.

II. Deo, A., **Carlsson, J.**, Lindlöf, A. (2011) *How to choose a normalization strategy for miRNA quantitative real-time (QPCR) arrays*. Journal of Bioinformatics and Computational Biology; 9(6): 795–812.

III. **Carlsson, J.**, Davidsson, S., Helenius, G., Karlsson, M., Lubovac, Z., Andrén, O., Olsson, B. & Klinga-Levan, K. (2011) *A miRNA expression signature that separates between normal and malignant prostate tissues*. Cancer Cell International; 11(14).

IV. **Carlsson, J.**, Helenius, G., Karlsson, M., Andrén, O., Klinga-Levan, K. & Olsson, B. *Differences in microRNA expression during tumor development in the transition and peripheral zones of the prostate*. Manuscript.

# Introduction

## Cancer

Cancer can arise in almost all human tissues and it is believed that the basic processes that transform a normal cell into a cancer cell are essentially the same in all cancers arising in the human body. These basic properties of survival, proliferation and dissemination are called the hallmarks of cancer. Albeit these hallmarks are thought to be in common for all types of cancers, they are acquired through diverse distinct mechanisms during different times of the multistep tumorigenesis in different forms of cancer. The six original hallmarks (acquired traits) of cancer are: sustaining proliferative signalling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, angiogenesis and resisting cell death (1). Recently, two traits of cancer cells, important for tumorigenesis, have been proposed as new hallmarks of cancer: deregulation of cellular energetics and avoiding immune destruction (2). Crucial for the survival of cancer cells´ is the reprogramming of energy metabolism, which is necessary to support cell growth and proliferation during tumorigenesis at the same times as the cell is avoiding the immune system. Therefore, these two acquired properties of cancer cells have been suggested to be added as additional hallmarks of cancer (2).

Tumors are composed of multiple cell types, both normal and malignant, interacting with each other to create a so called tumor microenvironment. The normal cells, which have been recruited to the tumor, are called tumor-associated stroma. The tumor associated stroma is known to be active in tumorigenesis and assist the cancer cells in acquiring the different properties described as the hallmarks of cancer. Due to this, it is important to take the tumor microenvironment into consideration when investigating the features of cancer cells (2).

There are several models for how a tumor can arise in the human body. The most widespread model is that a single cell acquires a mutation, which gives the cell growth advantages compared to a normal cell. When this already mutated cell further divides, it gives rise to a clone in which further mutations can arise, thus providing the cell with even more growth advantages (Figure 1) (3). Tumors are known to be very heterogeneous, partly due to the fact that the individual tumor cells can harbour different mutations. Therefore, two tumors of the same cancer type could have

different sets of genes involved in the progression of the tumor even though they exhibit the same functional changes (4).

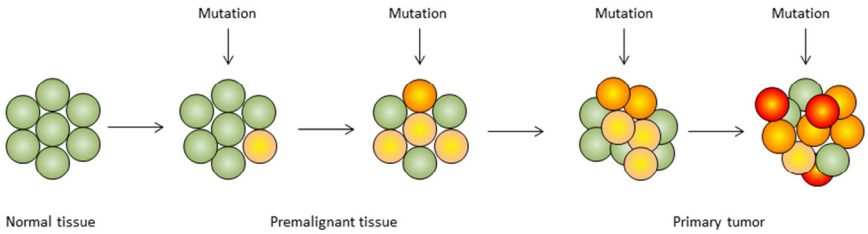


*Figure 1. A normal cell is transformed into a tumor cell by stepwise acquiring mutations, giving the cell advantages in e.g. growth.*

## Prostate cancer

The prostate is a conical shaped gland, located in front of the rectum, beneath the bladder and surrounding the upper part of the urethra, and the main function is to produce the seminal fluid (Figure 2). The gland consists of three glandular zones: the transition zone (TZ), the central zone (CZ) and the peripheral zone (PZ) and a fibromuscular stroma (Figure 3). All three zones have different glandular organization and propensities for diseases such as cancer. The TZ consists of two small lobes, accounting for 5 % of the prostatic volume, which surround the urethra. Most of the benign hyperplasias (BPH) arise in this zone, as well as 15-20 % of the tumors. The CZ is located outside the TZ and accounts for about 25 % of the prostatic volume. This zone is not a frequent position for tumor origin, only 10 % arise in the CZ, although peripheral tumors often invade this zone. Outside of the CZ, lining the prostate wall is the PZ, constituting about 70 % of the total prostatic volume. This is a common site of origin for prostate carcinomas, approximately 70-75 % of all tumors originate here (5).

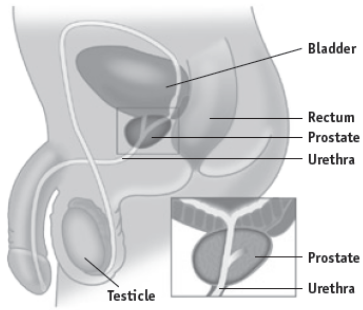


*Figure 2. The prostate is located in front of the rectum and below the bladder, surrounding the upper part of the urethra. Image adapted with permission from the U.S Department of Health & Human Services.*

Cancer of the prostate (CaP) is the most common form of male cancer in the Western society and in Sweden it accounted for 33.4 % (~10,000 cases) of all male cancers diagnosed during 2010. The incidence of CaP in Sweden has increased with an average of 2.4 % per year during the last 20 years. This increase could be due to the introduction of the prostate specific antigen (PSA) test during this time period, thus representing an increased amount of CaP diagnoses rather than an increased incidence (6). CaP is commonly diagnosed by PSA measurements followed by needle biopsies, although a high PSA level is not specific for CaP but can also be a sign of for example BPH or prostatitis. Thus, there are limitations when using PSA as a diagnostic tool, such as;

- PSA cannot distinguish between small, slow-growing tumors (indolent type of CaP), not requiring treatment, and tumors that have a more aggressive behaviour and need to be treated.
- PSA measurements give a high rate of false positives and only 25-30 % of all men with an elevated PSA level are diagnosed with CaP subsequent to a biopsy. This can in part be due to the fact that small tumors may not be detected in biopsies.
- PSA measurements also give false negatives, where the PSA level lies within a normal range even though the patient does suffer from CaP.

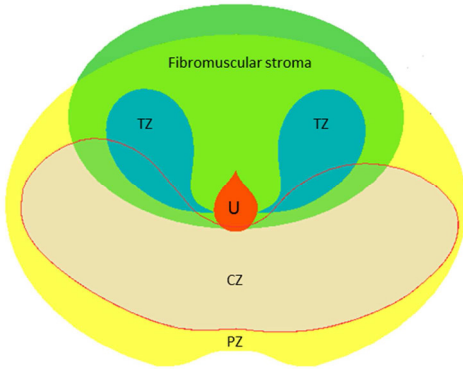


*Figure 3. The three glandular zones of the prostate. U= Urethra, TZ = Transition zone, CZ= Central zone and PZ = Peripheral zone*

Once diagnosed, CaP is graded by the use of Gleason grades and according to the staging system of the 2002 American Joint Committee on Cancer, called Classification of Malignant Tumors (TNM). The Gleason grading system, created by Gleason *et al*., in 1967, is based solely on the cellular patterns of the tumor. A cellular pattern with small and well-differentiated glands is assigned a Gleason grade of 1 while a cellular pattern with no recognizable glands is assigned a Gleason grade of 5. Instead of assigning the highest grade as the grade of the whole tumor, the grade is defined as the sum of the two most common patterns/grades and is subsequently called the Gleason score. A low Gleason score indicates a good prognosis for the patient while a higher Gleason score indicates a worse prognosis (7, 8). The TNM staging system is based on the size of the tumor (T), regional lymph node involvement (N) and metastasis at other sites than regional lymph nodes (M). The T-stage ranges from T0 to T4 where T0 is no primary tumor and T4 is when the tumor has invaded the adjacent organ structures, such as the bladder neck and rectum. The N category only has two stages, N0 when there is no regional lymph node involvement and N1 when there is a regional lymph node involvement. The M category also has two stages where M0 is no metastasis and M1 for metastasis. M1 is further divided into M1a-M1c, decoding where the metastasis is found (non-regional lymph node, bone or other) (9). Together, these two grading systems provide the best prognostic values known to date for CaP.

## MicroRNAs

MicroRNAs (miRNAs) are small non-coding RNAs of 18-24 nucleotides (nt), first discovered in 1993 by Ambros and colleagues (10). MiRNAs regulate gene expression post-transcriptionally in plants, animals and DNA viruses (11-16) and play a key role in a diverse range of biological processes including development, cell proliferation, differentiation and apoptosis (17, 18). To date, approximately 2,000 human miRNAs have been identified (miRBase release 19) (19) and it is believed that miRNAs regulate about 30 % of all protein coding human genes (20-22).

MiRNAs regulate gene expression in three main ways: 1) triggering an endonucleolytic cleavage of the mRNA target (23-25), 2) promoting translational repression or, 3) accelerating the deadenylation of the mRNA (26-29). The endonucleolytic cleavage of target mRNAs is generally favoured by a perfect match between the miRNA sequence and the target mRNA sequence, although some mismatches can occur (24, 30, 31). Translational repression is more common when there is a non-perfect match between the two sequences, and these non-perfect matches are commonly seen in humans.

## MicroRNAs and cancer

The first evidence of a differentially expressed miRNA in human cancer was found by Calin and colleagues in 2002, while investigating a chromosomal deletion in patients with chronic lymphatic leukaemia. This chromosomal deletion at 13q14 was found to result in a loss or a reduced expression of two miRNAs, miR-15 and miR-16, located within the deleted region (32). Since this discovery, close to 7,000 articles that describe the relationships between miRNAs and cancer have been published (based on a PubMed search on "cancer AND miRNAs").

An altered miRNA expression has been found in all human tumors investigated to date, which suggests that miRNAs are implicated in tumorigenesis and thus are potential candidates as biomarkers. Studies have shown that miRNAs can be used to distinguish between normal and malignant tissue, different tumors and subtypes of tumors and also be used to predict the clinical behaviour of the tumors (33). Lu *et al.* analysed 334 samples from human tumors and showed that a miRNA expression profile could be used to separate the tumor samples according to developmental origin of the tissue (34). This was also the conclusion by Volinia *et al.* where they showed that, based on miRNA expression, tumor samples from common solid tumors such as breast, colon and prostate could be

separated according to tissue origin. These results indicate that the miRNA expression pattern is tumor- and tissue- specific (35). In several other studies, the diagnostic and prognostic potential of miRNA expression signatures in cancer have been elucidated (36-39).

The advantages in using miRNAs as biomarkers have been reported in several studies. Lu *et al.* showed that when using a miRNA expression profile, 12 out of 17 poorly differentiated tissues were correctly classified, compared to only one out of 17 when using an mRNA expression profile. This indicates that miRNAs could be more specific for cancer classification compared to mRNAs (34). Another advantage with miRNAs is their small size, which makes them remain largely intact in formalin fixed paraffin embedded (FFPE) tissues. This is important since FFPE tissues are routinely archived in hospitals (34). Furthermore, miRNAs are present in a stable form in human plasma, where they are protected from endogenous RNase activity, making it possible to analyse miRNA expression in blood samples (40). Thus, the use of miRNA expression profiles in a clinical setting would be very beneficial since it is a less invasive and easier procedure taking a blood sample than a tissue sample. The miRNA expression profiles also have the potential to be more disease specific than mRNA expression profiles.

Currently, several different diagnostic test kits based on miRNA expression are available on the market. Three of these kits are used to differentiate between different subtypes of lung cancer such as small cell lung cancer, carcinoid, squamous non-small cell lung cancer and non-squamous cell lung cancer, but also between malignant pleural mesothelioma and carcinomas in the lungs and pleura (Rosetta Genomics, Philadelphia, USA). In addition, tests that differentiate between the four subtypes of kidney cancer and identify the primary origin of the tumor are available (Rosetta Genomics, Philadelphia, USA) as well as a test differentiating between pancreatic ductal adenocarcinoma and pancreatitis (Asuragen, Austin, USA). So far, there are no diagnostic tests based on miRNA expression for CaP.

## MicroRNAs and prostate cancer

Since the discovery of miRNAs, several attempts have been performed to find a miRNA expression signature which can be used for diagnosis and/or prognosis of CaP, although the results have been inconclusive with conflicting results as they often differ between different data sets. This could be due to different study designs, sample collection methods and the sensitivity and specificity of the platforms used in the studies. Albeit the results from previous studies are inconclusive, they still indicate that it is possible to find a miRNA expression signature which can be used to separate between normal and malignant prostate tissues (35, 41-45).

Collecting tissue samples by biopsies or by transurethral resection of the prostate (TUR-P) is considered to be an invasive method, therefore it would be of great clinical importance if a serum sample could be used to differentiate between a normal or malignant state of the prostate. This is now possible as recent studies demonstrated that miRNA expression signatures in serum samples was found to be able to separate between healthy individuals and individuals with CaP (40, 46-48), although more research is needed before this type of diagnostic test could be a clinical reality.

Even though several studies have been performed to elucidate the difference in miRNA expression between normal and malignant prostate tissues, there is still more knowledge to be gained regarding this matter. Many of the studies performed suffer from the fact that only a small number of miRNAs have been investigated and the overlap of the miRNAs investigated in the studies is also small. This could in part explain the differences in the miRNAs found to be differentially expressed between normal and malignant prostate tissues, since the same miRNAs were not included in all the studies. Investigating more miRNAs than in previous studies could increase the possibilities to find those miRNAs which differ most in expression between normal and malignant tissues and thus would give a more accurate miRNA expression signature for CaP diagnosis.

When this thesis work was started, no studies had been published on the use of endogenous control genes for miRNA expression studies in CaP and since the qPCR array technique was fairly new at this point, there had been no studies on how to best normalize the data obtained by using qPCR arrays.

# Aims

The aims of this thesis were to explore the miRNA expression patterns in normal and malignant prostate tissues and more specifically to:

- identify miRNAs that are differentially expressed between normal and malignant prostate tissues.
- investigate if the differentially expressed miRNAs could be used as diagnostic biomarkers for CaP.
- investigate if the differences in behaviour of tumors arising in the TZ and PZ are reflected in miRNA expression.
- investigate if using unique miRNA expression signatures for diagnosis of tumors arising in TZ or PZ would increase the classification accuracy compared to using a single miRNA expression signature for both TZ and PZ tumors.
- investigate the stability of endogenous control genes used for miRNA expression studies in prostate tissues.
- compare different normalization method for miRNA qPCR arrays and identify the most suitable normalization method of those investigated.

# Materials and methods

## Patient material

For paper I and III, patients were recruited from the population-based Swedish Watchful Waiting cohort (n=1,256). These men had symptoms of BPH (i.e. lower urinary tract symptoms) and were subsequently diagnosed with prostate cancer through TUR-P (49). All men in this cohort were at the time of diagnosis determined to have clinical stage T1a or T1b, Nx, and Mx. The prospective follow-up time of this cohort is now up to 30 years. The cohort includes samples from men who were diagnosed at the University Hospital in Örebro (1977–1991) and at four centres in the southeast region of Sweden: Kalmar, Norrköping, Linköping and Jönköping (1987–1999). The studies were approved by the ethical committee in the Uppsala-Örebro region (M58-05). The material in these studies consisted of malignant prostate FFPE material from 20 cases and adjacent normal tissue from each case, i.e. 40 paired samples in total. Cases were randomly collected within each category of Gleason score (6-10) to get an equal distribution of histological differentiation between low grade (6-7) and high grade (8-10) Gleason scores.

In paper II, a publically available data set was downloaded from GEO (accession number GSE19229). In this study, tissue had been collected from 20 patients (10 patients > 60 years, 10 patients < 30 years) with melanocytic neoplasms as well as three benign nevi from each patient group (50).

For paper IV, patients were recruited from the Cohort of Swedish Men (COSM), a cohort established during 1997 in the counties of Västmanland and Örebro in Sweden. The cohort includes 48,850 men born between 1918 and 1952. Up until December 2009, 3232 men in the cohort have been diagnosed with CaP, of which 300 have been subjected to radical prostatectomy. Complete follow up is available for all men with CaP until January 2011. From the 300 men subjected to radical prostatectomy, we selected 13 patients having a tumor with Gleason grade 3 in TZ (n=5), in PZ (n=5) or in both TZ and PZ (n=3). From the latter three patients, one sample of malignant tissue was taken from each zone. We also included normal prostate tissue from 10 patients diagnosed with bladder cancer, who had been subjected to radical cystoprostatectomy. A pathologist examined the prostate with the same routine procedure as after a radical prostatectomy and assessed the tissue for signs of prostate cancer without any histological findings. From each cystoprostatectomy patient, two

samples of normal prostate tissue were collected, one from the TZ and one from the PZ. The study was approved by the ethical committee in the Uppsala-Örebro region (2009/016).

## miRNA qPCR arrays

The TaqMan® MicroRNA Array Set v2.0 from Applied Biosystems was used in paper I-IV (Applied Biosystems, Foster City, CA, USA). It consists of two cards (Card A and Card B) containing 364 TaqMan® MicroRNA assays plus 20 control assays per card, enabling quantification of 667 unique human miRNAs in total. Card A contains miRNAs that tend to be functionally defined, and are commonly and/or highly expressed. The miRNAs in card B are infrequently expressed and/or expressed at low levels and most of them are usually not functionally defined.

## Endogenous control genes

The six endogenous controls investigated in paper I and II were MammU6 (small nuclear RNA), RNU48, RNU44, RNU43, RNU24 and RNU6B (small nucleolar RNAs) (51, 52). Three of these controls (MammU6, RNU48 and RNU44) appear on both cards, A and B, while the other three (RNU43, RNU24 and RNU6B) only appear on card B. On card A, only MammU6 appears in four replicates while the other two controls appear just once. On card B, all six controls appear in four replicates.

## RNA extraction and cDNA preparation

In paper I, III and IV, a pathologist marked normal and malignant tissue areas on H/E slides corresponding to the FFPE material prior to punching out 3-4 cores from the tissue blocks (ø 0.6 mm) using a Tissue Micro Array equipment (Pathology devices, Westminster, USA). The Recover All Total Nucleic Acid Isolation Kit optimized for FFPE samples (Ambion, Foster City, CA, USA) was used to extract total RNA. A reverse transcription reaction of 4-10 ng of total RNA was performed using the TaqMan® MicroRNA reverse transcription kit and Megaplex™ RT primers, human pool v2.0 (Applied Biosystems). Subsequently, the cDNA samples were pre-amplified using Megaplex™ PreAmp primers and TaqMan® Preamp master mix (Applied Biosystems).

## Quantitative PCR

The pre-amplified cDNA samples were diluted in a 0.1X TE Buffer (pH 8.0) before use in the qPCR reaction. The diluted pre-amplified cDNA was mixed with TaqMan® PCR master mix II No AmpErase UNG (Applied Biosystems) and run in a 40 cycle qPCR reaction on the TaqMan® MicroRNA A and B Cards. All reactions were performed on the Applied Biosystems 7900 HT system.

## Data analysis

For paper I, III and IV, raw Ct-values (Cycle threshold, i.e. the number of cycles where the amount of amplified cDNA crosses a defined threshold) were calculated using the SDS software (Applied Biosystems), applying manually selected thresholds for each miRNA.

In paper I, the stability of the endogenous controls was evaluated using NormFinder and BestKeeper (53, 54). In NormFinder, delta Ct values (raw $Ct_{normal}$ – raw $Ct_{malignant}$) were used as input values while in BestKeeper raw Ct values were used as input values. In order to investigate if there were differences between the replicates of each endogenous control, a statistical evaluation was performed by a one-way ANOVA. A Student's t-test was performed for comparisons of normal and malignant tissues together with a paired samples correlation (PASW Statistics 18, SPSS Inc, Chicago, USA). In both tests the null hypotheses were that there was no difference between replicates (ANOVA), and no difference between tissue types (t-test).

In paper II, raw data was normalized using five different approaches. In the first approach, NormFinder (53) and geNorm (55) was used to find the most suitable endogenous control gene. The geNorm analysis was performed in R (56) while NormFinder analysis was performed with an Excel add-in. After identifying the most suitable endogenous control gene, data was normalized using the ΔCt- method ($Ct_{miRNA} – Ct_{endogenous\ control}$). In the second approach, all Ct-values >35 were removed from the data before the array mean expression value was calculated and subtracted from each individual miRNA´s Ct-value. In the third approach, the mean expression value for each array was calculated without prior removal of Ct-values >35 and the mean value were subsequently divided with each individual miRNA´s Ct-value. In the fourth approach, quantile normalization was applied to the data using the *normQpcrQuantile* function available in the R package qpcrNorm, while in the fifth approach, quantile normalization was performed using the *normalizequantile* function available in the R

package Affy. The performance of the five different normalization methods was assessed by 1) boxplots to investigate the distribution patterns of Ct-values, 2) coefficient of variation (CV) and standard deviation (*sd*) values to investigate the dispersion of Ct-values; and 3) correlation coefficient (*r*). All assessments were performed in the programming software R (56).

In paper III, raw Ct values were normalized using qPCRNorm quantile normalization (57) before applying paired Student's t-tests to the normalized data. In order to investigate if the differentially expressed miRNAs ($p<0.0001$ and $p<0.00001$) could be used to classify normal and malignant tissues, they were used in a hierarchical clustering analysis using PermutMatrix (58). The miRNA expression signature consisting of differentially expressed miRNAs ($p<0.00001$) was also used in a principal component analysis (PCA) using Omics Explorer, version 2.0 beta (Qlucore AB, Lund, Sweden) in order to confirm the results from the hierarchical clustering. To analyse the generality of the miRNA expression signature and its potential as a diagnostic signature, a cross-validation test of generalization was performed. In this test, a set of 14 paired samples (both malignant and normal from the same individual) was randomly selected and a new expression signature was generated by identifying differentially expressed miRNAs within these 28 samples using a paired Student's t-test ($p<0.0001$). The remaining five paired samples (both malignant and normal from the same individual) were then clustered based on the expression values of the identified miRNAs and the separation of normal and malignant samples between clusters was noted. This procedure was repeated 15 times and the average classification accuracy was calculated.

In paper IV, raw data was normalized using the qPCRNorm quantile normalization method (57). A paired Student's t-test ($p<0.05$) was used to identify miRNAs that were differentially expressed between the TZ and PZ in normal tissues, whereas the corresponding unpaired t-test was used for identifying miRNAs that were differentially expressed between normal and malignant tissues in each zone, as well as for the comparison between malignant tissues from the different zones. Hierarchical clustering was performed on all samples and miRNAs investigated using the PermutMatrix clustering tool. Differentially expressed miRNAs were also clustered using the same method as well as used in a PCA using Omics Explorer, version 2.3 (Qlucore AB, Lund, Sweden). Experimentally validated target genes for the 15 miRNAs with the lowest *p*-values between normal PZ and TZ were extracted from TarBase (59) and miRecords (60) while predicted target genes for the same miRNAs were

extracted from MicroCosm targets (61). These target genes were then compared to genes previously identified as differentially expressed between normal TZ and PZ in prostate tissues, to investigate if there was an overlap. Experimentally validated target genes were also extracted for miRNAs identified as differentially expressed between normal and malignant TZ and PZ tissues using the same databases and pathway analysis was performed on these target genes using the DAVID functional annotation tool (62). A cross-validation test of classification accuracy was performed in the WEKA data mining tool (63) using the AD Tree algorithm (64, 65) to test the generality of the identified zone-specific signatures.

# Results

## Paper I

A one-way ANOVA was performed to identify differences between replicates of the endogenous controls. The results showed no significant differences between replicates ($p>0.05$) and therefore mean Ct-values were used in the following analyses. Two software applications, NormFinder and BestKeeper, were used in order to investigate the stability of the endogenous controls. Both tests identified RNU24 as the most stable control gene, followed by RNU44. A Student´s t-test revealed significant expression differences between normal and malignant samples for two of the control genes investigated, RNU43 and RNU44, making them unsuitable control genes in prostate tissues. Thus, these two genes were excluded from the list of potential control genes in this material. The expression of miR-130b, a previously identified stable control gene for miRNA expression studies in prostate tissues, showed a high variability between replicates and was only identified as stably expressed by BestKeeper and not by NormFinder.

## Paper II

RNU48 was suggested as a candidate endogenous control gene for both panel A and B and by both NormFinder and geNorm. Thus, this RNA was used in the subsequent normalization step using the ΔCt-method for each array individually. After normalization using RNU48, CV-values showed an increase compared to raw data although the *sd*-values had decreased for all miRNAs and the correlation was worse for most of the miRNAs. When looking at the distribution of the data, normalization had introduced more outliers and the distribution was less homogenous compared to raw data.

For normalization using mean values, two different approaches were used. In the first approach, all Ct-values >35 were excluded before the mean expression value was calculated and subsequently extracted from each individual miRNAs Ct-value. After normalization with this approach, CV-values had generally increased compared to raw data and for some miRNAs there was a dramatic increase in the dispersion. We also calculated the *sd*-values and correlation for the normalized data, although the *sd*-values had not improved compared to raw-data and the correlation was worse in the normalized data than in raw data. In the second approach, the mean expression value was calculated without prior removal

of Ct-values >35 and was subsequently divided with each individual miRNAs Ct-value. When data was normalized with this method, smaller CV- and *sd*-values was produced as well as a higher correlation compared to raw data, showing an improved consistency among arrays within each group.

Two different approaches were also used for the quantile normalization. In the first approach, the *normQpcrQuantile* method was applied to the data. The normalized data had very small CV- and *sd*-values as well as high correlation for all groups, which were better than raw data. In the second approach, the Affy quantile normalization method was applied to the data. This method produced highly similar results as the *normQpcrQuantile* method although slightly lower CV- and *sd*-values was seen as well as a slightly higher correlation. Albeit the mean expression value normalization produced the lowest CV- and *sd*-values, the quantile normalization methods produce better data distributions with more homogenous data compared to both raw data and mean expression normalized data.

## Paper III

By means of a Student's t-test, 30 miRNAs were identified as differentially expressed ($p<0.0001$) between the normal and malignant prostate tissues, while when using a more stringent *p*-value (0.00001), only nine miRNAs were identified as differentially expressed. These two sets of miRNAs were subsequently used in a hierarchical clustering and with the exception of one normal and one malignant sample, the miRNA expression signature consisting of nine miRNAs could be used to separate between the normal and malignant samples (Figure 4). The miRNA signature including 30 miRNAs however performed worse with three misplaced samples (one malignant and two normal).

The expression signature including nine miRNAs was also used in a PCA analysis, which confirmed the results of the hierarchical clustering, where one group with normal samples and one group with malignant samples were seen. A PCA analysis was also performed on all 667 miRNAs in order to find subgroups within the malignant samples, revealing that 16 miRNAs ($p<0.017$) could be used to divide the samples into four groups. With the exception of three samples, these four groups showed concordance with the four Gleason scores included in the study.

A cross-validation test of generalization was performed in order to test the generality of the expression signature including nine miRNAs. The results of the cross-validation test showed an overall error rate of 15 % of

the expression signature, meaning that 85 % of all samples are correctly classified using this miRNA expression signature.

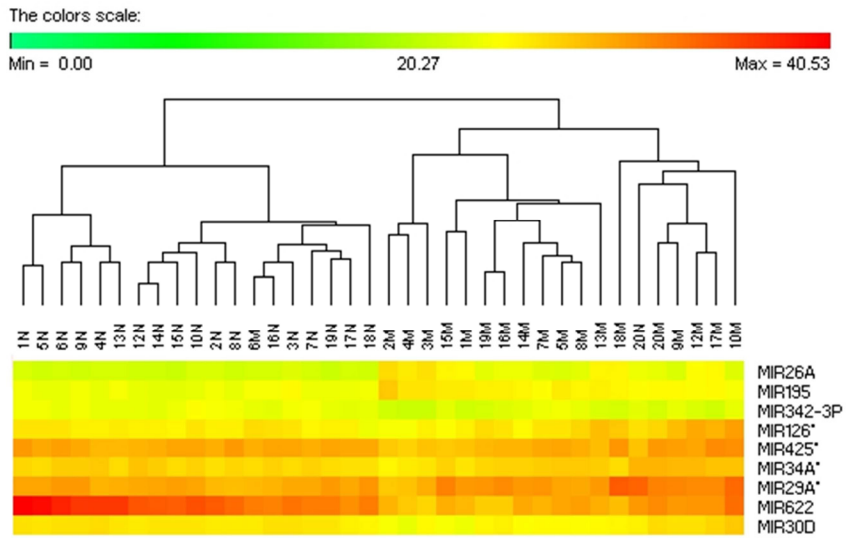


*Figure 4. miRNA expression signature including nine miRNAs. Differentially expressed miRNAs (p <0.00001) were clustered and the results show that the expression profiles of these nine miRNAs could be used to separate between the normal (N) and malignant (M) tissue samples with the exception of one normal sample (20N) and one malignant sample (6M).*

## Paper IV

The results of the hierarchical clustering on all samples and miRNAs investigated revealed a separation between normal and malignant tissues into two major clusters, with the exception for two normal samples. This indicate that the expression profiles of all 667 miRNAs investigated can be used to separate between these two types of tissues (normal and malignant). There is also a tendency for PZ tissues to cluster together and TZ tissues clustering together, although this is not as obvious as for the normal vs. malignant state.

Student's t-tests were performed, with and without correction for multiple testing, on all combinations of sample groupings. Between normal and malignant tissues from the TZ, 149 miRNAs were found to be significantly differentially expressed (231 before applying correction for multiple testing). The same comparison in PZ identified 65 significantly differentially expressed miRNAs (150 before correction). In contrast, only a single miRNA was significantly differentially expressed between the TZ and PZ in normal tissue (51 before correction) and none between the TZ and PZ in malignant tissue (50 before correction). The differentially expressed miRNAs were subsequently used in a hierarchical clustering and a PCA. Overall, the clusterings based on miRNAs differentially expressed between TZ and PZ showed several misplaced samples, whereas the clusterings based on miRNAs differentially expressed between normal and malignant samples showed perfect separations of the samples groups into two major clusters (Figure 5 shows separation of normal and malignant samples).

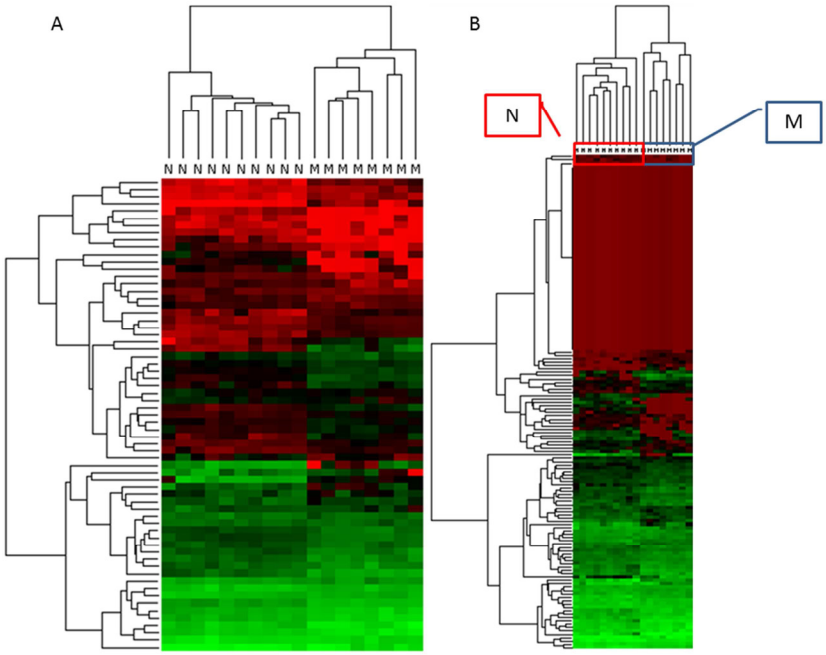


*Figure 5. Hierarchical clustering results of differentially expressed miRNAs between A) normal (N) and malignant (M) PZ tissues B) normal (N) and malignant (M) TZ tissues.*

The 65 miRNAs identified as differentially expressed between normal and malignant PZ tissues were compared to the 149 miRNAs differentially expressed miRNAs between normal and malignant TZ tissues. The comparison revealed that 38 miRNAs (22 %) of the differentially expressed miRNAs were common for both groups, while 111 (63 %) and 27 (15 %) miRNAs were uniquely differentially expressed between normal and malignant tissues in TZ and PZ, respectively. A comparison of the target genes for miRNAs differentially expressed in PZ and TZ showed that these tumors had 124 target genes in common (59 %), while only 61 (29%) and 24 (12 %) target genes where specific for the TZ and PZ tumors, respectively. A pathway analysis was also performed on the validated target genes which resulted in 100 different pathways of which 75 (75 %) were common for both the TZ and PZ, 17 (17 %) were specific for the TZ and 8 (8 %) were specific for the PZ. Specific pathways for the TZ includes pathways for infection and inflammation responses and PTEN dependent cell cycle arrest while for PZ the specific pathways include cell

cycle control, Dicer pathway, TGF-beta signalling pathway and Wnt signalling pathway.

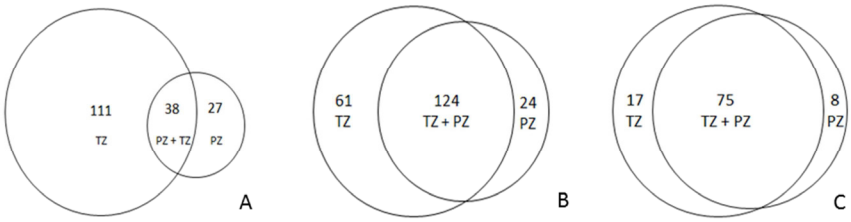


*Figure 6. Venn diagram showing the overlap between A) differentially expressed miRNAs in normal and malignant tissues in TZ and PZ, B) overlap of validated target genes for those miRNAs and, C) overlap of pathways for the validated target genes. The overlaps were 22 %, 59 % and 75 %, respectively.*

# Discussion

## Importance of choosing the correct normalization method

During the last years, qPCR has become a powerful technique in miRNA expression profiling as it combines high sensitivity, specificity and high signal detection. There are several variables in a qPCR experiment that need to be controlled for, both technical such as differences in sample collection, RNA extraction and target quantification, and biological variables such as sample-to-sample inconsistency. Normalization is performed with the purpose to remove experimentally induced variation and to differentiate true biological changes. An inappropriate normalization could induce misleading effects and thus affect the conclusions drawn from the results and therefore, the choice of normalization method is a crucial step in data analysis.

Previously, the number of normalization methods available for qPCR data has been limited due to the small number of genes investigated in each experiment. With new techniques emerging, it is now possible to investigate a few hundred genes on a qPCR array, which makes data-driven methods for normalization possible also in qPCR experiments. Albeit the qPCR arrays make it possible to investigate larger numbers of genes, it usually has to be performed in multiple panels, which introduces new normalization challenges, not seen in microarray studies, where typically only one array is used for all genes investigated. Therefore, data-driven normalization methods that have been developed for microarrays could be inappropriate for data generated by qPCR arrays. Even though the use of qPCR arrays makes it possible to normalize the generated data using data-driven methods, the usage of endogenous control genes is still necessary in those studies where only a few number of targets are investigated. In paper I and II, we investigated the most suitable endogenous control gene for miRNA expression studies in CaP and which normalization method that performs the best for miRNA qPCR arrays.

In paper I, the stability of six different endogenous control genes (RNU48, RNU44, RNU43, RNU24, RNU6B and MammU6) was tested in normal and malignant prostate tissues. In several studies, miRNAs considered to be stably expressed have also been used as endogenous controls for qPCR normalization, for example miR-130b has been used for normalization in prostate tissue studies (45). Therefore we also included this miRNA in the stability analysis. The prerequisite for a stable control was in this study considered to be a stable expression independent of

software application used. Both software applications used (NormFinder and BestKeeper) identified RNU24 as the most stably expressed endogenous control gene in this tissue material and a Student's t-test showed no variance in expression between normal and malignant tissues for this control gene. NormFinder did not identify miR-130b as stably expressed although, BestKeeper identified it as the third most stable of the seven genes included in the test. Since miR-130b did not meet the prerequisite for a stably expressed gene, it was not considered as a candidate endogenous control gene in this material.

In paper II, we compared the usage of endogenous control genes for normalization to the usage of data-driven normalization methods based on mean and quantile normalization. RNU48 was identified as a candidate endogenous control gene for both panel A and B by both software applications (NormFinder and geNorm). When using this RNA for normalization, *sd*-values decreased although CV-values increased for all groups, as did the correlation between groups with the same background. Compared to the other normalization methods tested in this study, the endogenous controls performed the poorest.

Two approaches were used for mean value expression normalization. In the first approach all Ct-values >35 were removed prior to calculating the mean value for each array, which were subtracted from each individual miRNA´s Ct-value. When normalizing the data using this approach, CV-values generally increased and *sd*-values did not improve compared to raw data, indicating a poor normalization. In the second approach, the mean expression for each array was calculated without prior removal of Ct >35 and subsequently divided with each individual miRNAs expression value. This method produced normalized data with very small CV- and *sd*-values and high *r*-values, all better than for the previous methods and raw data.

When applying the *normQpcrQuantile* normalization method to our data, small CV- and *sd*-values were produced as well as high *r*-values for all groups, all of which are better than raw data. However, the results were not as good as when using mean expression values since the *sd*-values had not decreased to the same extent. In the second approach, Affy quantile normalization, developed for microarray data, was used for normalization. When this algorithm was applied to our data, the results were very similar to the results using *normQpcrQuantile*, although slightly lower CV- and *sd*-values were produced as well as better *r*-values. Even though the *sd*-values did decrease, it was not the same extent as when using mean expression values.

In order to investigate the data distribution, box plots were generated for normalized data, which showed that the quantile-based normalization methods generated the most homogenous distributions of the data and also an improved distribution compared to raw data. Even though the array mean expression normalization methods produced the lowest CV- and *sd*-values, the distribution of the normalized data is less homogenous than those of quantile normalized data. When looking at the distribution of data normalized using endogenous controls, the normalization had introduced more outliers, indicating that this is not a good normalization method.

To conclude, of the different methods compared in this study, the mean and quantile-based methods performed the best, since both methods showed a decrease in CV- and/or *sd*-values and an increase in correlation between samples compared to raw data. The mean normalization did produce the smallest *sd*-values, however the distribution patterns of the data showed that the quantile-based methods are to prefer over the mean normalization, since the quantile-based methods produced a more homogenous data.

The results from paper I and II clearly shows how important it is to consider the normalization method used in each study. Endogenous control genes for normalization performed the poorest in our comparison, although when only investigating the expression of a few targets, data-driven normalization methods cannot be performed and thus endogenous control genes are crucial for normalization in these studies. When that is the case, it is instead very important to thoroughly investigate which endogenous control gene that is the most suitable one in that specific study.

## MicroRNAs as biomarkers for prostate cancer

Several miRNA profiling studies have been performed on prostate cancer, but the results are inconclusive and no single miRNA expression signature for prostate cancer has yet been found. In paper III we aimed to acquire more knowledge about the role of miRNAs in prostate cancer by analysing 667 unique miRNAs in 20 prostate cancer samples and 20 normal prostate tissue controls. We found that 30 miRNAs were differentially expressed at the 0.0001 significance level although a miRNA expression signature used in a diagnostic purpose should include only a limited number of miRNAs due to practical and economic reasons. Thus, a more stringent *p*-value (0.00001) was applied to the data, revealing a subset of nine differentially expressed miRNAs. Both miRNA expression signatures were used in a hierarchical clustering analysis in order to find the most accurate CaP signature. The results showed that the expression signature consisting of

nine miRNAs is better at separating between normal and malignant prostate tissues, only two samples were misplaced (one normal and one malignant).

There are overlaps in the results between our study and the studies previously performed, for example are our results consistent with the data from Volinia *et al*. where miR-195 was upregulated and miR-26 down-regulated in cancer, although in Porkka *et al*., the opposite results were found (35, 41). The platforms used in these two studies were the same thus, a possible reason for the difference in results can be that different patient materials were used in the studies. In two other studies, miR-126* (now named miR-126-5p) and miR-622 were shown to be upregulated in malignant prostate tissues compared to normal prostate tissues, which is also consistent with our results (44, 47).

As previously mentioned, several studies have been performed in order to find a miRNA expression signature for CaP diagnosis although the results have been inconsistent. One of our hypotheses is that these inconsistencies between studies could be due to different methods of collecting the tissue samples such as TUR-P, radical prostatectomy or biopsies. The main difference between these three methods for collecting tissue samples is that the samples are taken from different prostatic zones. TUR-P samples are mainly taken from the TZ, while radical prostatectomy allows samples to be taken from both TZ and PZ, and biopsy samples are mainly taken from the PZ. Also, TUR-P is performed using electrical resection, and it is not clear how this affects the gene expression in the tissue.

In order to test the hypothesis that sample collection affects the results, and to validate the miRNA expression signature found in paper III, the expression values of the nine miRNAs included in the signature were extracted from the data set used in paper IV. The tissue material used in paper III was collected through TUR-P, while the tissue material in paper IV was collected through radical prostatectomy. When the expression values of the nine miRNA signature had been extracted from the data set in paper IV, they were subsequently used in a hierarchical clustering in order to see if they could be used to separate between normal and malignant samples. The results show one cluster containing only normal samples and one cluster containing all malignant samples and five normal samples (Figure 7). Based on this, the signature performed slightly poorer in the data set from paper IV compared to the data set in paper II, which could be due to the different methods of tissue collection used or that the miRNA biomarkers have been selected in one data set and tested in a second one. Another reason for this difference could be the fact that the

original data set (paper III) only includes tissue material from the TZ, while the new material used in paper IV includes tissue material from both TZ and PZ (see discussion about zonal differences in miRNA expression). Therefore we also performed hierarchical clusterings on the samples from the TZ and PZ individually, to see how the signature performed on tumors arising in the different prostatic zones. When using the expression signature for TZ tissues individually, two clusters were formed; one containing only normal samples and one containing all malignant samples and two normal samples (Figure 8A). When instead using the expression signature for only PZ tissues, two clusters were produced, one containing only normal samples and one containing all malignant samples and three normal samples (Figure 8B) (Table 1). The results indicate that the expression signature works better for tumors arising in the TZ compared to the PZ, although this cannot be validated until the signature has been tested in a larger study.

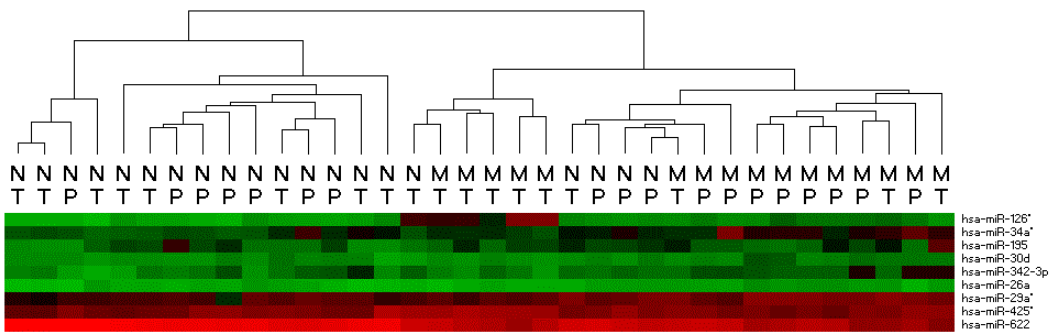


*Figure 7. Results of hierarchical clustering of the nine miRNAs included in expression signature on both TZ and PZ samples. Two major clusters are produced, one with normal samples and one with malignant samples, although the malignant cluster also contains five misplaced normal samples. NT= Normal transition zone, NP = Normal peripheral zone, MT = Malignant transition zone, MP = Malignant peripheral zone.*

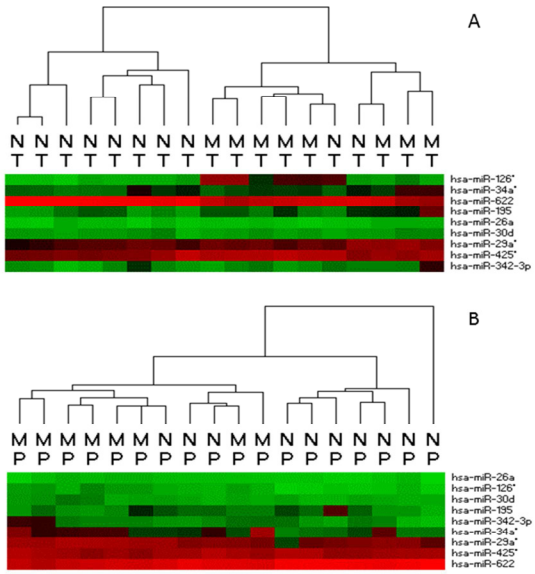


*Figure 8. Results of hierarchical clustering of the nine miRNAs included in the expression signature on A) TZ, and B) PZ samples separately. NT = Normal transition zone, MT = Malignant transition zone, NP = Normal peripheral zone and MP = malignant peripheral zone.*

Table 1. Results using the nine miRNA expression signature on four different data sets.

| Dataset | No. of samples | Correctly Classified (%) | Incorrectly Classified (%) |
|---|---|---|---|
| Paper III | 38 | 95 | 5 (1N, 1M) |
| Paper IV (TZ+PZ) | 36 | 86 | 14 (5N) |
| Paper IV (TZ) | 18 | 89 | 11 (2N) |
| Paper IV (PZ) | 18 | 83 | 17 (3N) |

Albeit the expression signature has been tested in a new data set, this second data set is still too small (n=36) to draw any conclusions about the clinical performance of the signature. Thus, the miRNA expression signature found (paper III) still needs to be tested in a new and larger patient cohort in order to estimate its potential as a future diagnostic tool for CaP. Also, if the signature should be used in a clinical setting, it would be beneficial if the expression signature could be evaluated in blood samples instead of tissues, since a blood sample is a less invasive procedure

which is easier to perform and also easier to perform in larger quantities than tissue samples. Thus, the miRNA expression signature has to be validated in blood as well.

## Zonal differences in miRNA expression

The incidence of CaP differs markedly between the prostatic zones, with most tumors arising in the PZ (70-75 %) followed by the TZ (20-25 %) and the CZ (10 %). Although little is known about the molecular mechanisms behind this phenomenon, it is reasonable to assume that it is caused by pre-existing transcriptome differences in the normal tissue between the three prostatic zones. A few studies has been performed in order to investigate these assumed transcriptome differences between normal TZ and PZ prostate tissues, showing that there indeed are mRNA genes which are differentially expressed between the zones in the normal prostate. No corresponding studies have although been performed on the miRNA expression and therefore the aim in paper IV was to elucidate if miRNAs are differentially expressed between TZ and PZ, both in normal and malignant prostate tissues. Unique miRNA signatures for tumors arising in the TZ and PZ could be beneficial in diagnosis of CaP if these signatures reflect significant differences between tumors of different zonal origin. Separate signatures for tumors of different zonal origin could also help to predict an accurate prognosis, since tumors arising in PZ are suggested to be more aggressive and are associated with a worse outcome compared to tumors arising in TZ.

Student's t-test revealed that the major difference in miRNA expression were found in the comparisons between normal and malignant tissues (149 differentially expressed miRNAs for TZ and 65 for PZ), while only a single miRNA was significantly differentially expressed between TZ and PZ in normal tissues and none between TZ and PZ in malignant tissues. This was also confirmed by hierarchical clusterings and PCA analyses where the results for normal vs. malignant tissues showed a separation into two major clusters (one with normal samples and one with malignant samples), while for the clusterings between TZ and PZ, several misplaced samples were seen. A cross-validation was also performed which confirmed that the main differences in miRNA expression occur between normal and malignant tissues rather than between the tissues from the different prostatic zones. Albeit, the individual miRNA expression changes between TZ and PZ are statistically non-significant, they can be significant when considered in combination, which is indicated by the clusterings and PCA

where the non-significant changes in miRNA expression between TZ and PZ are large enough for detection of zonal origin of the tissue.

The fact that more miRNAs are uniquely differentially expressed between normal and malignant TZ tissues than in PZ tissues indicate that the changes during tumor development are more extensive in the TZ compared to the PZ. These indications lead to the hypothesis that the PZ is more prone to tumor development, since fewer changes are needed in order for a tumor to arise in this zone. Results from a target gene and pathway analysis show a large overlap between both target genes and pathways involved in tumor development of TZ and PZ tumors, although there are still unique genes and pathways for each zone. This further strengthens the hypothesis that the steps in tumor development are unique in the two zones.

One miRNA, miR-433, was identified as significantly differentially expressed between normal TZ and PZ. This miRNA have two validated target genes, *HDAC6* and *FGF20*, which in previous studies have been shown to be upregulated in tumor tissues (66-68). Up-regulation of *HDAC6* results in increased proliferation and angiogenesis and decreased apoptosis (69). *FGF20* is under normal circumstances only expressed in the adult central nervous system although it is expressed in malignant tissue (70), where it stimulate proliferation and differentiation. miR-433 is over-expressed in normal TZ tissues compared to normal PZ tissues and therefore we hypothesize that this extra suppression of its target genes (*HDAC6* and *FGF20*) in the TZ results in an extra protection against tumor development, which could be a possible explanation for the difference of tumor occurrence between the prostatic zones.

The results from paper III together with the results from paper IV indicate that two separate biomarker sets for diagnosis of CaP, based on the zonal origin of the tumors, might be needed. The difference in molecular patterns between TZ and PZ could also be a possible explanation to why no biomarkers (e.g. mRNA expression or mutation statuses) have been found for diagnosis of CaP, since the two zones probably exhibit different mutations as well as different mRNA and miRNA expression patterns. Thus, e.g. a mutation found in a TZ tumor might not be present in a PZ tumor, and therefore a diagnostic biomarker set for CaP based on TZ tumors would not be able to diagnose PZ tumors.

Albeit this study has shed some light on the differences in miRNA expression between normal and malignant TZ and PZ tissues, we still have to further investigate if these differences have an impact on the tumor incidence and behaviour of the prostatic zones.

## Concluding remarks

- **A miRNA expression signature consisting of only nine miRNAs is enough for classification of normal and malignant prostate tissues.** This signature was after a cross-validation estimated to classify samples with an accuracy of 85 %.
- **The major difference between TZ and PZ in the prostate arise during tumor development.** 149 miRNAs were identified as differentially expressed between normal and malignant TZ tissues and 65 miRNAs were identified as differentially expressed between normal and malignant PZ tissues. Only one miRNA, miR-433, was found to be differentially expressed between normal TZ and PZ while none was found to be differentially expressed between malignant TZ and PZ tissues.
- **A higher expression of miR-433 in normal TZ compared to normal PZ is hypothesized to have a protective effect against tumor development in the TZ.** This miRNA have two validated target genes, *HDAC6* and *FGF20*, which has been shown to be expressed at higher levels in tumors compared to normal tissues. Since miR-433 is overexpressed in normal TZ compared to normal PZ this would indicate that *HDAC6* and *FGF20* are less expressed in TZ compared to PZ, thus serving as protective factors against tumor development.
- **More miRNAs are differentially expressed in TZ tumors than PZ tumors**. This suggests that more changes are needed in TZ in order for a tumor to develop, which could explain why TZ tumors are less common than PZ tumors.
- **Global normalization methods for qPCR arrays perform better than endogenous control genes**. Quantile normalization methods performed the best while endogenous control genes performed worst in the comparison of normalization methods for qPCR arrays, showing that endogenous control genes should, if possible, be avoided in normalization of qPCR data.

- **For studies of single miRNAs, RNU24 is proposed to be the most suitable endogenous control gene for miRNA expression studies in prostate tissues.** Despite these results, every miRNA expression study should begin with an investigation of the most suitable endogenous control gene for the specific experimental conditions in that study.

## Grants

This work has been possible by the kind support of the Swedish Knowledge Foundation through the Industrial PhD program in Medical Bioinformatics at Corporate Alliances (Karolinska Institute), Lions cancer research foundation, Nyckelfonden, Örebro county council research committee, Wilhelm and Martina Lundgrens research foundation, the Nilsson-Ehle foundation, Längmanska culture foundation, Hillevi Fries research foundation and Assar-Gabrielsson foundation.

# Acknowledgements

I would like to express my sincere gratitude to;

My main supervisor, **Karin Klinga Levan**, for giving me this great opportunity to work within this field and for all help during my work with this thesis.

My co-supervisor **Björn Olsson**, for teaching my all about bioinformatics and English grammars and for always finding the time to proof-read my papers and this thesis.

My co-supervisors in Örebro, **Gisela Helenius**, for always helping and supporting me and teaching me new techniques! **Ove Andrén**, for teaching me all about prostate cancer and finally, **Mats Karlsson** for always finding the time for helping me with the pathology part of this thesis.

**Zelmina** and **Angelica**, thanks for all the help, support and answering all my endless questions when Björn was away on parental leave. I would never have done it without you!

All my colleagues in Skövde, especially **Sanja**, **Benjamin**, **Kitti**, **Eva**, **Jasmine** and **Kajsa**. Thanks for all the help and all long discussions. Also, I would like to thank **Sofia**, **Jenny** and **Malin** for all the great AW nights.

All my colleagues in Örebro, especially **Sabina**, **Anna-Lena**, **Johanna S**, **Johanna L**, **Kristin**, **Berolla** and **Bella**. Thanks for all your support, but mostly for all the fun times we have shared.

**Maria**, thanks for all the times you let me stay at your place (both in Sweden and NYC), for all the fun times and for all the help and support. I'm guessing its payback time soon!

**Sandra**, for being such an inspiration, always believing in me and supporting me.

**Dennis**, for being such a great person and always supporting me.

**Aditi**, **Oskar**, **Jonathan** and all other great friends I have met during the years in Skövde. Thanks for all the fun times, hopefully we will have more of those in the coming years!

And last but definitely not least, my beloved family; **Mum**, **Dad**, **Thomas**, **Ulla-Britt** and **Grandma**. Thanks for all your support during this time, I love you all!

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