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The expression of miR-21 in prostate cancer measured by *in situ* hybridization and its relevance as a diagnostic biomarker

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Abbreviation

AP	Alkaline phosphatase
BCIP	5-bromo-4-chloro-3'-indolyphosphate
bp	Base pair
cISH	Chromogen *In situ* hybridization
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
dsRNA	Double stranded RNA
FFPE	Formalin-fixed, paraffin embedded
H&E	Hematoxylin and Eosin
ISH	*In situ* hybridization
LNA	Locked nucleic acid
miR/miRNA	MicroRNA
mRNA	Messenger RNA
NBT	4-nitro-blue tetrazolium
nt	Nucleotide
PSA	Prostate specific antigen
qPCR	Real time quantitative PCR
RISC	RNA Induced silencing complex
RNA	Ribonucleic acid
RNase	Ribonuclease
siRNA	Silencing RNA or small interfering RNA
ssRNA	Single stranded RNA
TMA	Tissue Microarray
UNN	University Hospital of Northern Norway
UTR	Untranslated region

Contents

Abstract

Background: Micro RNAs (miRNA) are a novel class of molecular regulators that have gained considerable attention in cancer research. Multiple studies in recent time have reported altered miRNA regulation in most cancers by deregulating of oncogenes and tumour suppressor genes. Additionally, certain tumours are represented by specific miRNA expression files. Prostate cancer is a markedly heterogeneous disease with considerable variation in clinical course ranging from asymptomatic disease to rapidly progressing fatal malignancy. The repertoire of prognostic markers is currently limited to preoperative serum-PSA, clinical or pathological stage, histological grade, and surgical margin.

Experimental design: In this study the expression of a well-known miRNA, miR-21, was examined whether it could have prognostic significance in prostate cancer. The expression was measured by chromogen *in situ* hybridization performed on tissue microarrays from prostate cancer, and the expression was compared to clinical and pathological information to determine its potential as a biomarker.

Results and conclusion: Univariate analyses indicate that miR-21 is an independent biomarker for biochemical failure and clinical failure but not cancer-specific death. To fully determine the value of miR-21 potential, it must be further analysed with multivariate analysis and be validated in an additional cohort to exclude local cofounders.

1 Introduction

1.1 Prostate cancer

Prostate cancer is a carcinoma that develops in the prostate gland. It advances slowly, and can lead to death if it is not treated. Prostate cancer is diagnosed almost exclusively in men above the age of 50, with only 35 new cases in patients under 50 for 2005-09 in Norway.[1] The most common treatment of prostate cancers is prostatectomy. Prostate cancer has a cumulative risk of 12.7% to develop before the age of 75, and 4299 in new cases were diagnosed in 2009 (in Norway).

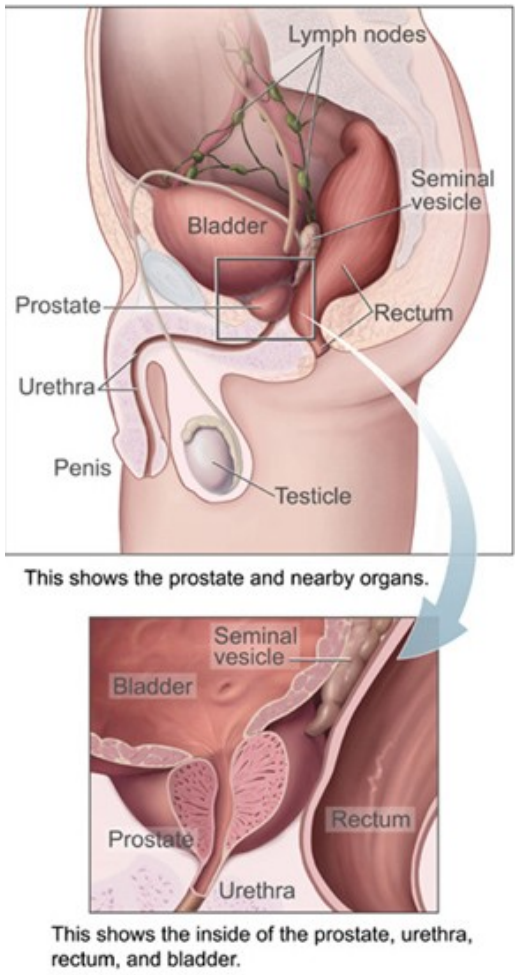

Figure 1: Location of the prostate.
(Figure is produced by National Cancer Institute, United States of America, www.cancer.gov)

1.1.1 Survival

Prostate cancer has a relative survival of 87%, but age is a factor (Figure 2).[1] More advanced tumours, Gleason 4+5 and 5+5, are not treated by surgery, but is treated with radiation, but as palliative treatment. A Swedish study found that from the years 1988 to 2008 there was a 25-fold

increase in surgical prostatectomies, but the reduction in five-year cancer-specific mortality was only by 3.9% for patients diagnosed between 1988 and 1992, and only 0.7% for those diagnosed between 1998 and 2002.[2]

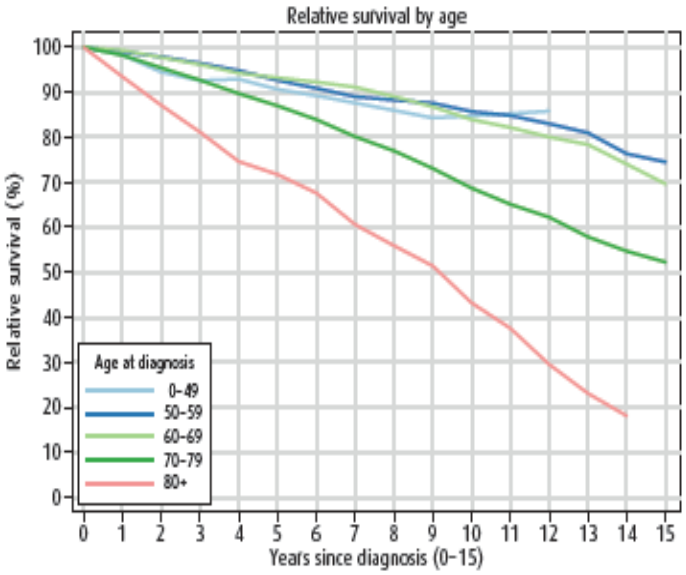

Figure 2: Relative survival for patients broken down by age.[1]

1.1.2 Classification

To determine the grade of differentiation of prostate tumours, the pathologists do histological grading according to The Gleason Scale.[3] This scale grades according to the degree of differentiation of the cancer cells (Figure 3). Grading of tumours using Gleason scale, gives the tumour a primary and often a secondary grade, where the primary grade accounts for >50% of the tumour and secondary represent 5-50% of the tumour. By adding the grades, it give a Gleason score with a value from 2-10, where higher value means a more aggressive tumour. For example Gleason grade 3+4 and 4+3 give the same score, 7, but with different primary and secondary grade. Due to this, a 4+3 is a more aggressive tumour than 3+4.[4] Today, normal practice is not to use Gleason grade of 1 or 2 in diagnosis, due too difficulty in distinguishing tumour tissue from normal tissue. This reduce the relevant Gleason score interval to 6-10 (3+3 up too 5+5).

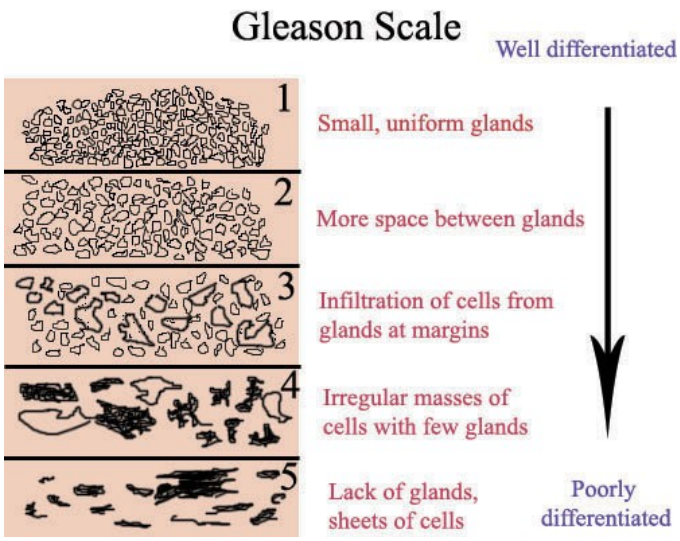

Figure 3: The Gleason scale. Used for grading the malignancy of prostate tumours. *Figure is retrieved from Wikipedia, and was released by creator to be used in freely by the public.*

As apparent from figure 4 there is an inverse correlation between the Gleason score and survival rate for prostate cancer, but for Gleason score 7, the survival is more dependent on the primary Gleason grade (Table 1).

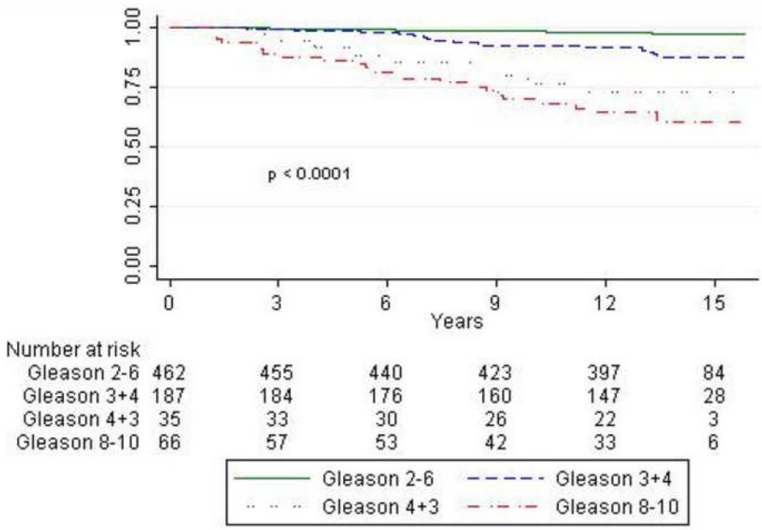

Figure 4: The survival rate based on Gleason score.[4]

Gleason Score	5-year	10-Year
2-6	99,1 (97,7 – 99,7)	98.4 (96.7 – 99.3)
3+4	98.9 (95.7 – 99.7)	92.1 (87.1 – 95.3)
4+3	88.6 (72.3 – 95.6)	76.5 (58.4 – 87.5)
8-10	86.2 (75.1 – 92.5)	69.9 (56.9 – 79.7)

Table 1: The survival rate based on Gleason Score (95% CI).[4]

1.2 MicroRNA and gene regulation

MiRNA are small non-coding RNAs that regulate gene expression by binding to the three prime untranslated region (3'-UTR) of mRNA.[5] The average miRNA is single stranded, 20-25nt long RNA molecule. They can originate from other transcribed RNA's UTR or introns that are released as the host gene matures, but miRNA are also transcribed and regulated like genes, with their own regulations and transcriptions sites. MiRNA can also be organized as clusters, where several miRNAs are expressed as a polycistronic message from a single promoter.

1.2.1 MicroRNA maturation

After transcription (Figure 5, step 1), the primary-miRNA (pri-miRNA)is matured by Drosha, an Rnase III superfamily endonuclease, which reduce the size to about 70bp double stranded pre-courser miRNA (pre-miRNA) (Figure 5, step 2). The pre-miRNA is then exported out of the nucleus, (Figure 5, step 3) where it is cut again by Dicer (Figure 5, step 4), and the fragment is reduced to 22bp long segment. The short double strand miRNA fragment is then disentangled by helicase (Figure 5, step 5) and the main strand bind to the RNA induced silencing complex (RISC) (Figure 5, step 6). The RISC then binds to the target site (Figure 5, step 7). RISC is a large complex of many RNA binding proteins. The Argonaute (Ago) RNase family proteins are important components of RISC, and are required for the final maturation into single stranded miRNA. Argonaute selects which strand that will be used as the guiding main strand. This selection is based on the thermodynamic stability of the 5' end.

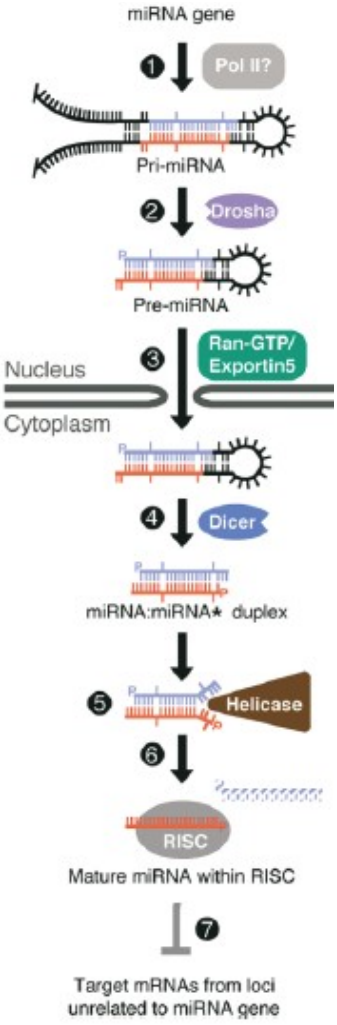

Figure 5: Overview of the maturation of miRNA.[5]
1-7: See 1.2.1 for details.

1.2.2 RNA interference

The degree of base-pairing to the target site of the mRNA determines the actual effect the miRNA have on the mRNA. The stronger the sequence specificity the miRNA has toward the target region, the stronger the RNA interference will be. The miRNA regulates mRNA expression by several methods (Figure 6).[6] The RISC will then cleave the mRNA if the miRNA have very strong interaction to the target sequence, which are caused by high sequence identity. A lower sequence identity will cause a lower binding affinity to the mRNA, and will lead to translational inhibition by interfering with the binding of ribosomal subunits to the mRNA, stopping the elongation, decapping of the mRNA , deadenylation and/or rapid degradation of the protein product. Translational repression is more common than mRNA degradation.

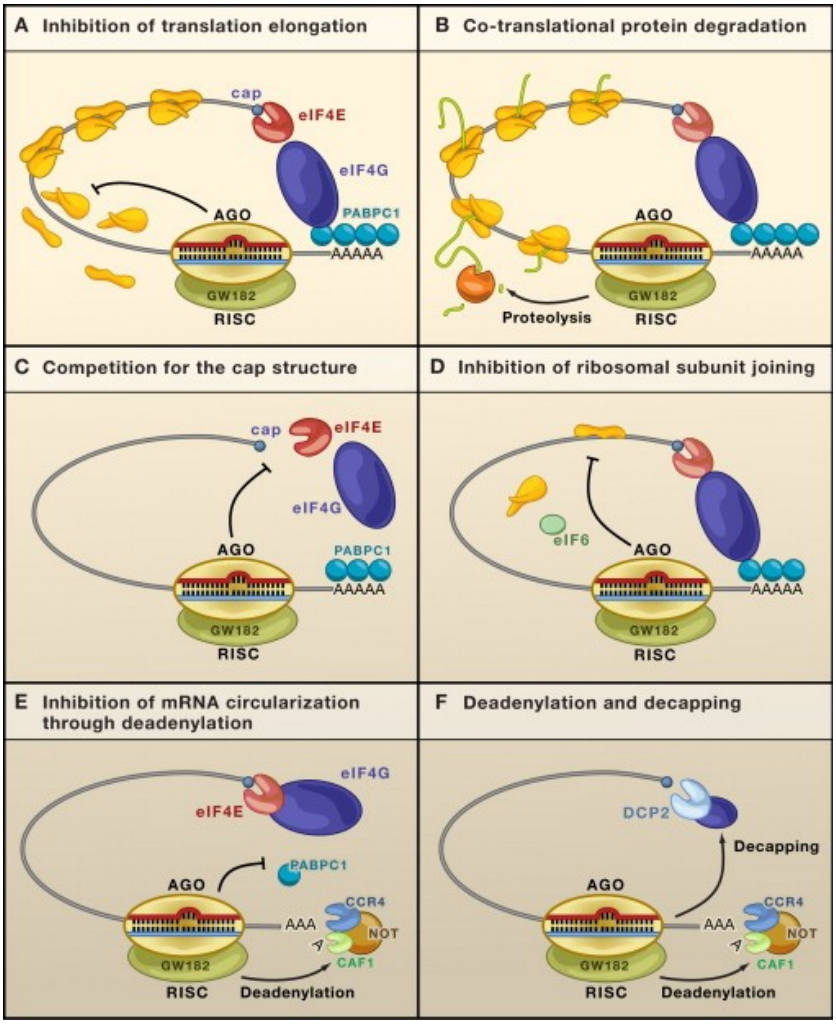

Figure 6: The mechanisms of miRNA mediated silencing.[6]

1.3 MiR-21

MiR-21 was first identified in glioblastoma,[7] as an anti-apoptoic factor, but when in large scale miRNA profiling over 540 tumour samples from six different tumours, miR-21 was the only miRNA detected to be dysregulated in all tumours. Mir-21 is located on chromosome 17q23.2, in the 10th intronic region of the protein-coding gene VMP1(also known as TMEM49), which is a human homologue of rat vacuole membrane protein. MiR-21 is transcribed as a part of the VMP1 gene, but have it's own transcriptional regulation independent of VMP1.[8] RNA polymerase II produce the primary-miR-21 transcript, which enters the normal miRNA maturation process (see section 1.2.1).

MiR-21 have been shown to be significantly dysregulated in breast cancer, glioblastoma, hepatocellular carcinoma, lung cancer, oesophageal cancer, tongue squamous cell carcinoma, colorectal cancer, cervix cancer, prostate cancer and others.[9]

1.3.1 MiR-21 function

After transcription, miR-21 interferes with many cellular mechanisms that are related to both cancer and heart disease.[10] MiR-21 plays a crucial role in tumour cell proliferation, apoptosis and invasion and others (Figure 7). Common protein targets are the p53 network,[11] Cell division cycle 25 homolog A (Cdc25A),[12] Trypomyosin alpha-1[13] chain and Trypomyosin alpha-3 chain[14] (TPM1 and TPM3), Reversion-inducing-cystein-rich protein (RECK),[14,15] Phosphatase and tensin homolog (PTEN)[16,17], Programmed cell death protein 4 (PDCD4),[18,19] Fas ligand (FasL),[20] B cell translocation gene 2 (BTG2)[21], Sprouty homolog 1 and 2 (SPR1/SPR2)[22] and Nuclear factor I/B (NFIB).[8] RECK, p53, PDCD4, PTEN, Cdc25A and FasL are all well-known tumour suppressor genes. Increased expression of miR-21 will speed up cell proliferation, reduce cell adhesion and promote cell migration, and suppress apoptosis signals from killing cells. This will in the end promote both the growth and the migration of the tumour. The specific effect of miR-21 varies between tissues.

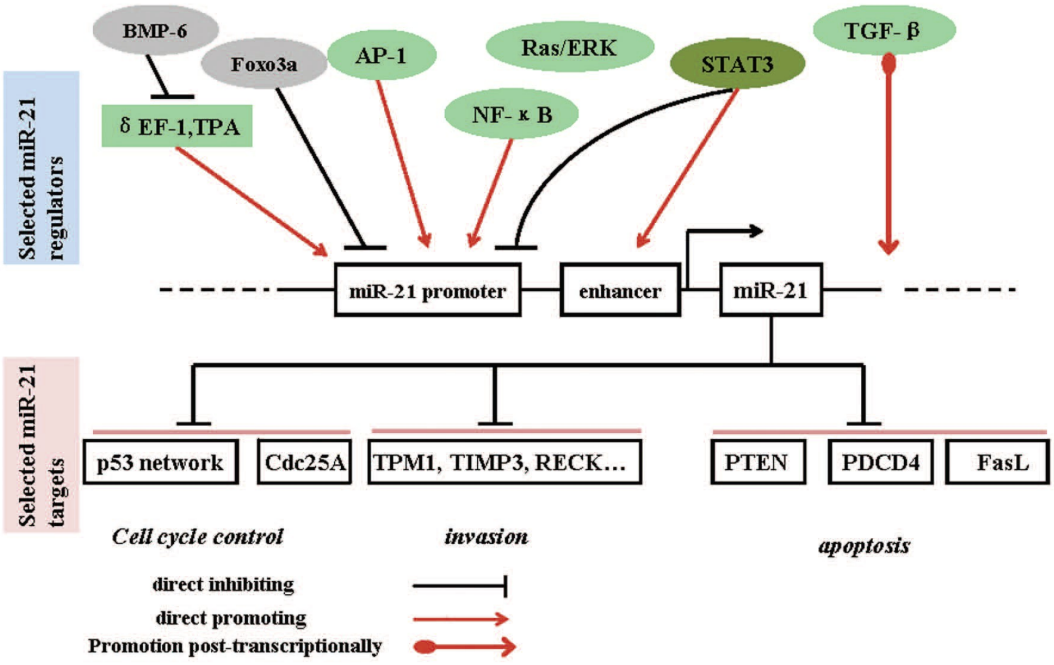

Figure 7: The transcriptions factors and the validated targets of miR-21 that influence apoptosis, cell cycle and invasion..[9]

Several of these targets are branching points in the tumour suppressor functions of cells. The p53

network can stop the cell cycle, start DNA repair processes, and when needed initiate apoptosis. RECK is a membrane-anchored inhibitor of matrix metalloproteinases (MMP), mainly MMP-2 and MMP-9,[15] which degrades the extracellular matrix outside the cells, which release matrix associated growth factors and reduce cell adhesion.

1.3.2 MiR-21 regulation

Part of the regulation that controls miR-21 transcription are shown in Figure 7, and Figure 8 shows an example of the self-promoting function of miR-21 exhibits, where miR-21 inhibits its own suppressors. The promotors that are connected to miR-21 are not active at the same time, as their effect is tissue specific. For example STAT3 role in miR-21 regulation varies between tissues. In some instances, constitutively active STAT3 induce miR-21 expression,[7,23,24] but in others IFNβ activated STAT3 negatively regulated the function of miR-21.[25]

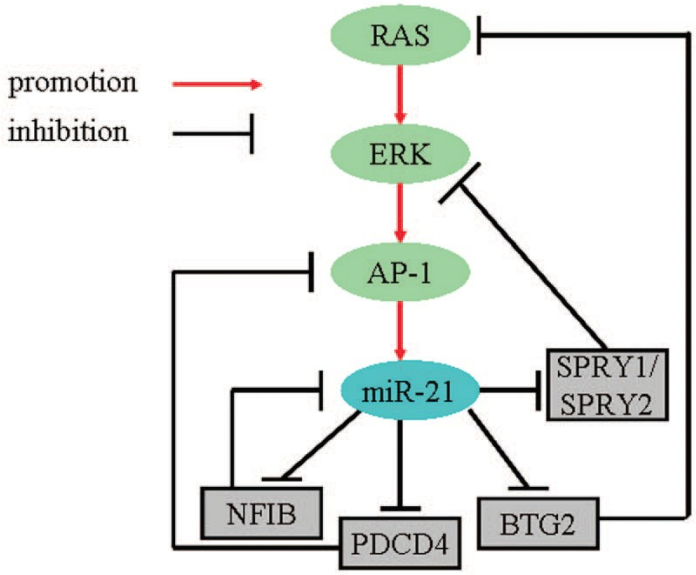

Figure 8: Showing the positive feedback effect of miR-21 regulation.[9]

1.4 Chromogen in situ Hybridization

In chromogen *in situ* hybridization a labeled Locked Nucleic Acid (LNA) probe, containing the complementary sequence to the miRNA, RNA or DNA that is being measured, is added to a tissue sample. This probe hybridize to the target sequence, and an antibody fused with an alkaline-phosphatase (AP) domain at the end will recognize the label on the LNA probe. The label used in this experiment is digoxigenin (DIG), which is attached to the 5' and 3' ends of the probe. The

antibody then binds the DIG at the ends of the probe, by adding substrate for the AP domain, the AP will convert soluble substrates 4-nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3'-indolylphosphate (BCIP) into a water and alcohol insoluble dark-blue NBT-BCIP precipitate which can be used to detect the amount of the bound probe (Figure 9).

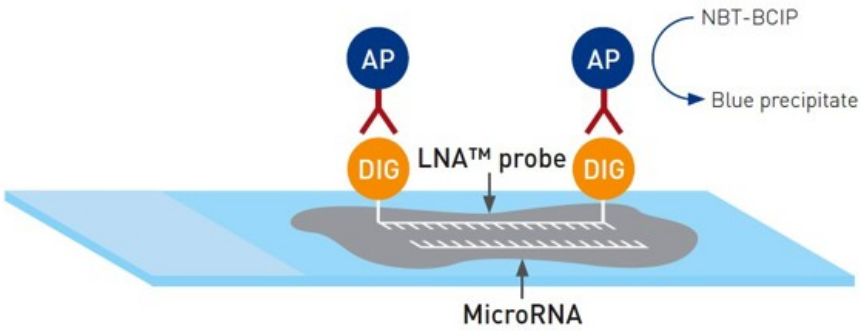

Figure 9: A overview of the cISH principle. Retrieved from Exiqon miRCURY LNA™ microRNA ISH Optimization Kit (FFPE) protocol.

1.4.1 LNA probes

LNA probes are RNA molecules that have a bridge bond between the 2' and the 4' in the ribose ring (Figure 10). This additional bond increase the rigidity of the molecular arrangements, which will increase the specificity of the probe by reducing the hairpin structures and secondary structures that the LNA molecule can form. This also increase the specificity since the probe is not able to have many mismatches when binding to the complementary sequence.[26,27]

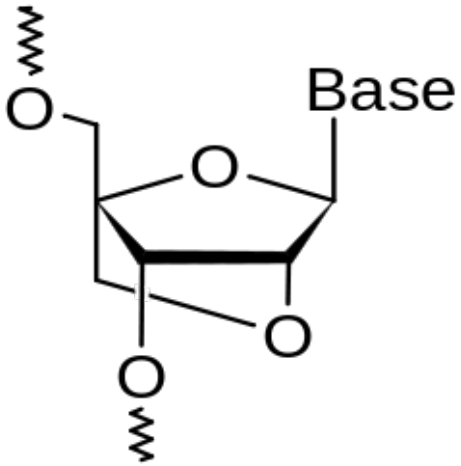

Figure 10: The chemical structure of an LNA monomer.

1.5 Experimental background

Gleason grade is at the moment the only diagnostic criteria used to determine the malignancy of the tumours in prostate. As seen in Figure 2, 4 and Table 2, there is a good correlation between the Gleason score and survival, but there is no molecular biomarker or other criteria that can be used to determine the malignancy of a tumour. Because of this lack of established and validated biomarkers, there is a need to find new biomarkers for grading in prostate cancer. The addition of a new biomarkers should increase both patient survival and life quality, as they would help clinicians determine the best treatment.

2 Method and materials

2.1 Screening miRNAs

The present study was based on a miRNA microarray screening completed spring 2012. The microarray hybridization and data collectection was outsourced to Exiqon, Denmark. In brief this screening project took 30 samples from patients with prostate cancer, scored as Gleason score 7 (both 3+4 and 4+3) divided into two separate groups; rapid biochemical failure and no biochemical failure. From theses samples total RNA was isolated and samples were sent to Exiqon in Denmark, where microarray screening was performed. From this primary screening there was found approximately 650 miRNAs with detectable expression. The relative expression of the detected miRNAs was then compared between the two groups. From these results several miRNAs (miR-21, miR-23a, miR-141, miR-143, miR-145, miR-205 and miR-222) were chosen to be validated using quantitative polymerase chain reaction (qPCR).

From the qPCR validation, there was one miRNA that showed statistical significant difference between the two groups (Table 2). Based on this qPCR validation and on research showing the function of miR-21,[9] this miRNA was chosen to be the best candidate for investigation.

miRNA	Mean rapid BF Group	Mean No BF group	Difference (RapidBF – NoBF)	p-value
hsa-miR-21	-0,1069	-0,9998	0,8929	0,0495
hsa-miR-23a	-1,1510	-1,2706	0,1197	0,3444
hsa-miR-141	-2,6779	-3,7654	1,0876	0,0831
hsa-miR-143	1,4310	1,3294	0,1016	0,7391
hsa-miR-145	3.2624	3,4119	-0,1495	0,5746
hsa-miR-205	-0,9374	-0,4547	-0,4828	0,6830
hsa-miR-222	0,7817	-1,5475	-0,2201	0,3522

Table 2: The results from the qPCR validation. The expression values are given in two-fold change normalized values based on the expression levels miR-23b. The p-values were determined using a student's t-test. The two groups are rapid biochemical failure (BF) and no biochemical failure.

2.2 Tissue Microarray

Tissue microarray (TMA) is a method to investigate molecular targets on tissue samples from several sources at the same time.[28] The TMA can have samples from several different organ systems, several patients and/or several samples from the same patient. These samples are harvested

from paraffin-embedded formalin-fixed (PEFF) tissue samples by using a needle (0.6mm) inserted into an area marked by a pathologist to be representative of the tumour tissue, stroma tissue and normal tissue. This sample is then inserted into the TMA recipient paraffin block. The TMA is then sliced (4 μm) and affixed to glass slides (Figure 11).

After the TMA was affixed to glass slides, they were sealed in paraffin, to preserve the slides until use. After slicing and sealing the TMAs can be stored for approximately six months before the quality of the slides will be too uncertain to use.

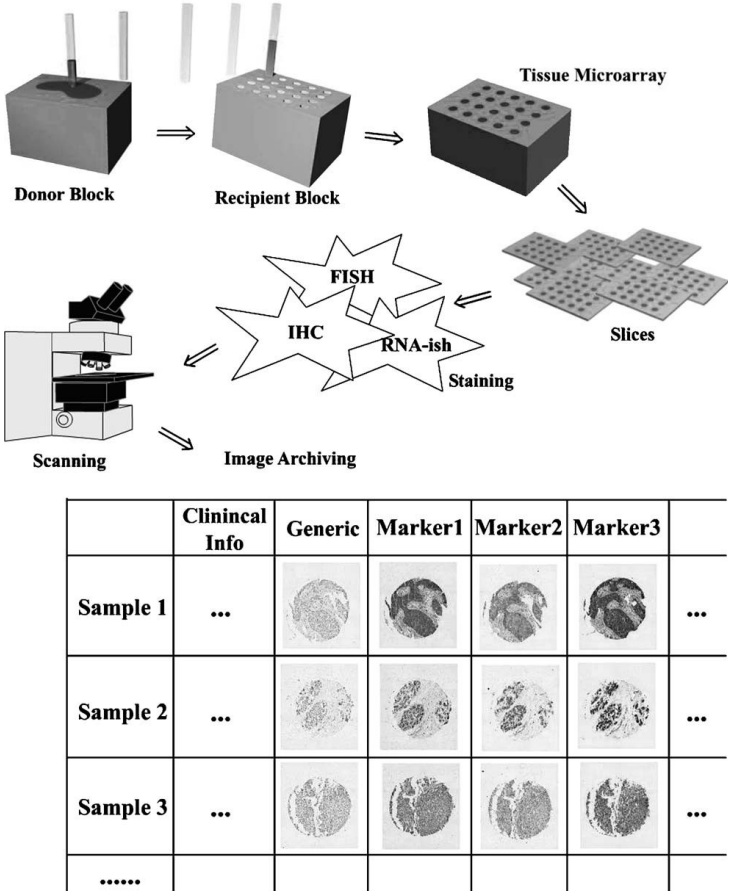

Figure 11: Showing the full procedure from TMA production, too analysis.[28]

2.3 Patient cohort

The patient included in this study was diagnosed with prostate cancer at the University Hospital of Northern Norway (UNN), Nordland Hospital Trust (NLSH) and St Olav's University Hospital.

The inclusion criteria were patient who had their prostate resected between 1995 and 2005. Patients were then excluded if they met any of the following criteria;

- Removal of prostate, but not with radical intent
- Other pelvic surgery with prostate in specimen
- Radio- or chemotherapy prior to surgery
- Other malignancy within five years prior to prostate cancer diagnosis
- Inadequate paraffin-embedded formalin fixed tissue blocks

After the preliminary exclusion, the cohort had N=671, 116 patients was excluded (Figure 12). The remaining tissue samples, N=555, was used to create tissue microarray.

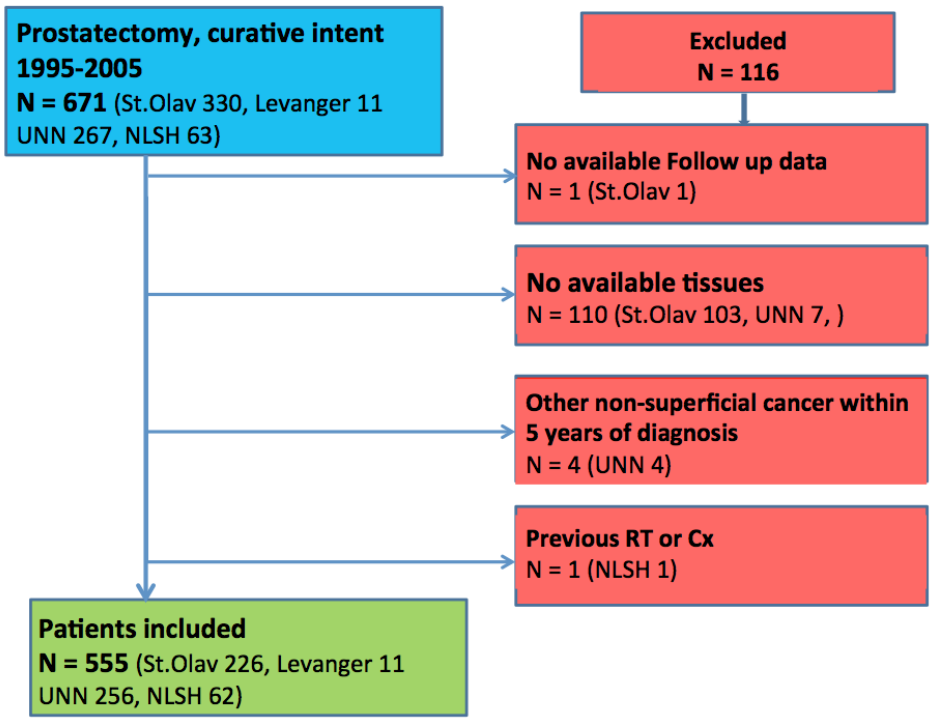

Figure 12: The secondary exclusion of patients from the study cohort.

The clinical and pathological data that have been added to the database contain information about;

- Operating clinic
- Type of surgery
- Pre-operative Prostate specific antigen (PSA)
- Post-operative PSA measurements
- Primary and secondary Gleason grade
- Gleason score
- Post-operative treatment
- Age at surgery
- Other malignancies
- Tumour size
- Tumour infiltration
- TNM pT-stage, Sub pT-stage and N-stage
- If the tumour had free margins
- Perineural infiltration
- Vascular infiltration

In this study, three distinct end-points are used. Each of these three end-points have a matching time variable, which is either time from operation to the event or last follow-up, as appropriate;

- Biochemical failure – a PSA $\geq$ 0.4 ng/ml post-surgery. Signifies disease progression.
- Clinical failure – disease cause detectable effects in patient.
- Prostate specific death – death from prostate cancer.

2.4 Chromogen In Situ Hybridization protocol

The protocol used in this study is based on Exiqon's miRCURY LNA™ microRNA ISH one day protocol.[29] The volumes and concentrations used are optimized for 12 slides. (10 sample slides, 1 U6 – positive control and 1 Scrambled negative control). The protocol can be completed over two days.

2.4.1 Protocol Optimization

To optimize the cISH protocol, hybridization runs were performed using the temperatures 50 ºC, 53 ºC and 55 ºC, accoring to the T_M of the probe. During these first runs, it was discovered that the tissue cores on the TMA slides fell off during the protocol. The loss of tissue cores varied from 50-80%.

Test runs were needed to reduce the core loss. Trying to use using different glasses; Thermo Scientific Menzel-Gläser Superfrost® Plus Gold and Superfrost® Plus, and different pre-treatment,

incubation time prior to deparafinization.

After these optimization test's were performed, a final hybridization temperature was set at 50 ºC and with a 48 hour pre-treatment in an incubator to affix the cores better to the slides. To conclude, the gold slides did not perform better then the normal Thermo Scientific Menzel-Gläser Superfrost® Plus glass slides.

2.4.2 Preparations

Before starting the protocol, the slides were incubated at 59 ºC to affix the cores to the glass slides, and to remove paraffin. Important, all buffers ware made in the lab and autoclaved to remove RNase contamination (Appendix).

2.4.3 Protocol

Deparaffinization

Deparaffinization was performed in choplin jars.

Xylene	5 min
Xylene	5 min
Xylene	5 min
Absolute Ethanol	5 min (Ethanol is changed thrice)
96% Ethanol	5 min (Ethanol is changed twice)
70% Ethanol	5 min (Ethanol is changed twice)
PBS	2-5 min

Proteinase-K treatment

Create a hydrophobic barrier around the cores with either a DAKO-pen or by using sterile paper to wipe off the PBS left on the slide. Moreover, 1000 µl of the Proteinase-K solution was added on to each slide, and incubate the slides at 37 ºC for 20 min using the Abbott Molecular StatSpin® ThermoBrite™, (Table 3).

Reagent	Stock concentration	Amount
Proteinase-K	20mg/ml	15 µl
Proteinase-K buffer		15 ml

Table 3: Proteinase-K solution. The buffer is in the appendix.

Wash in PBS at room temperature twice after Proteinase-K treatment.

Dehydration

Dehydrate the slides in new ethanol solutions.

70% Ethanol	1 min (change ethanol twice)
96% Ethanol	1 min (change ethanol twice)
Absolute Ethanol	1 min (change ethanol twice)

The slides were air dried on clean, sterile paper and then locked up in an air-tight and dark container overnight.

Denaturation

Start and set a Grant QBT heatblock to 90 ºC. 1x microRNA ISH buffer was prepared from stock 2x microRNA ISH buffer and DEPC water.

Needed amount of probes were retrieved from storage freezer, and thawed. The probes was denatured by heating it with the heat block for 4 minutes, and spun down, and the required amount of 1x micrRNA hybridization buffer was added (Table 4, 5 and 6).

Reagent	Stock concentration	Target concentration	Amount
Scrambled miRNA probe	25 µM	50 nM (1:500)	1 µl
1x microRNA ISH buffer			500 µl

Table 4: Dilution scheme for the scrambled probe.

Reagent	Stock concentration	Target concentration	Amount
U6 miRNA probe	0.5 µM	1 nM (1:500)	2 µl
1x microRNA ISH buffer			1000 µl

Table 5: Dilution scheme for the U6 probe.

Reagent	Stock concentration	Target concentration	Amount
LNA mir-21 probe	25 µM	50 nM (1:500)	2 µl
1x microRNA ISH buffer			1000 µl

Table 6: Dilution scheme for the miR-21 probe.

Hybridization

In the hybridization procedure, 70 µl diluted probe was added to each slide. The slides were covered with Hybrislip™ hybridization covers, sealed with Rubber Cement and then hybridized for 60 minutes at the determined hybridization temperature (see section 2.4.1).

After hybridization, the Hybrislips was removed with knife or tweezers and put in a choplin jar with room temperature 5x SSC.

Stringency wash

The slides were washed in SSC buffers heated to the hybridization temperature. The choplin jar was kept in the incubator during the washing, keeping the temperature stable.

5x SSC	5 min
1x SSC	5 min
1x SSC	5 min
0.2x SSC	5 min
0.2x SSC	5 min
0.2x SSC, RT	5 min
PBS, RT	–

Blocking

Maleric acid and blocking solution were mixed (Table 7 and 8) to produce a final blocking solution. 800 µl blocking solution was added to each slide and incubated in humidity chamber for 15 min.

Reagent	Amount
10x Maleric Acid	2 ml
DEPC water	18 ml

Table 7: 20 ml 1x Maleric acid solution.

Reagent	Amount
10x Blocking solution	2 ml
1x Maleric Acid	17.6 ml
Sheep serum	400 µl

Table 8: 20 ml 1x Blocking solution.

Antibody reaction

The blocking solution was removed from the slides, and a hydrophobic barrier was created with a sterile paper. 500 µl antibody solution (Table 9) was added to each slide and incubated at 30 °C for 30 min. While incubating, one NBT/BCIP tablet was added into 10 ml DEPC water, and stored in dark.

Reagent	Amount
1x Blocking solution	8 ml
Sheep anti-DIG-AP AP150 U antibody	10 µl

Table 9: Dilution scheme for 1:800 anti-DIG-AP antibody.

Wash the slides in PBS-Tween after antibody reaction.

PBS-T 3 min
PBS-T 3 min
PBS-T 3 min

Alkaline-phosphatase substrate reaction

When the NBT/BCIP tablet was fully dissolved in DECP water, Levamisole was added (Table 10). 400 µl of the substrate solution was added onto each slide and then incubated in the ThermoBrite™ at 30 °C for 120 minutes. Of importance was the protection against light during the reaction.

Reagent	Amount
NBT/BCIP	One tablet
DEPC water	10 ml
Levamisole 100 mM	20 µl

Table 10: AP substrate solution.

Stop of substrate reaction

To stop the alkaline phosphatase reaction the slides was washed in KTBT buffer (Appendix).

KTBT	5 min
KTBT	5 min

Washing

The slides was washed in double distilled water to remove all buffer.

Core counter-staining

Counter staining of the cell nucleus was done by washing the slides in Nuclear Fast Red for 1 minute, which gave a red nuclear contrast. The slides were then washed in water and then stored in water.

Dehydration and mounting

The slides were dehydrated in ethanol. Of importance is that the blue precipitate is solvable by ethanol, so only a few of the slides were dehydrated at the same time. This reduce the amount of time each slide is in the ethanol.

70%	10 dips
96%	10 dips
96%	10 dips
Absolute Ethanol	10 dips
Absolute Ethanol	10 dips

When mounting with HistoKit, it is important to use enough HistoKit, so that no air bubbles can be trapped under the cover glass. HistoKit was added with a glass rod to the slide and cover-slips were placed on the slide. If air bubbles were trapped, they were removed by gently pressing the coverslip with a tweezer.

The slides were left in a hood to defume overnight before inspection in a microscope.

2.5 Image and statistical analysis

Scoring was done by using the ARIOL imaging system (Genetix, San Jose, USA)composing of a microscope (Olympus BX 61) equipped with an automatic stage and slide loader, together with a

camera. Each slide was photographed at 1.25x magnification. A core-map was then created before the slides were registered with a barcode, and a pre-scan was performed. The core-map was then matched too the pre-scanned cores to assign each core a coordinate. Several focus points was added to cores. Focus points should be added to cores that contain clear contrasts, which will help focus the camera. After matching the map and adding focus points, the main scan was performed.

Most cores contain both tumour cells and stromal cells (non-neoplastic cells). Stroma cells are connective tissue cells that support the epithelial cells of the prostate with their original function. Tumour-associated stroma can be both tumour promoting or suppressing. Each core was then scored by two pathologist blinded to each other and to the clinical data using the definitions in Table 11. After scoring, the information was connected to clinical and pathological information in a SPSS file.

Score	**Definitions**
0	No staining
1	Weak staining
2	Moderate staining
3	Strong staining
4	Missing core or missing relevant cells

Table 11: The scores definitions given by the pathologists.

Statistical analysis was performed based on the average score compared with clinical and pathological information using IBM® SPSS® Version 20. Interclass correlation (ICC) analysis was used to determine the inter-rater agreement. Univariate analysis was performed to investigate the relationship between the miR-21 expression and the existing clinical and pathological information**.**

3 Results

The *in situ* hybridization procedure was performed without any significant problems. The scrambled negative control tests the specificity of the experiment, and in both series it showed that there was very little nonspecific staining (Figure 13). The U6 positive control tests if the nucleic acid in the sample have degraded to such a degree that the tissue can not be used in miRNA analysis. The strength of the staining indicated that the tissue has not degraded to an extent that invalidate the experiment. Examples of the different scores assigned to slides based on staining intensity are shown in figure 14. Several slides were randomly selected to be redone to assess the reproducibility of the experiment (Figure 15). The reproduced slides were found to be of sufficient quality and similarity.

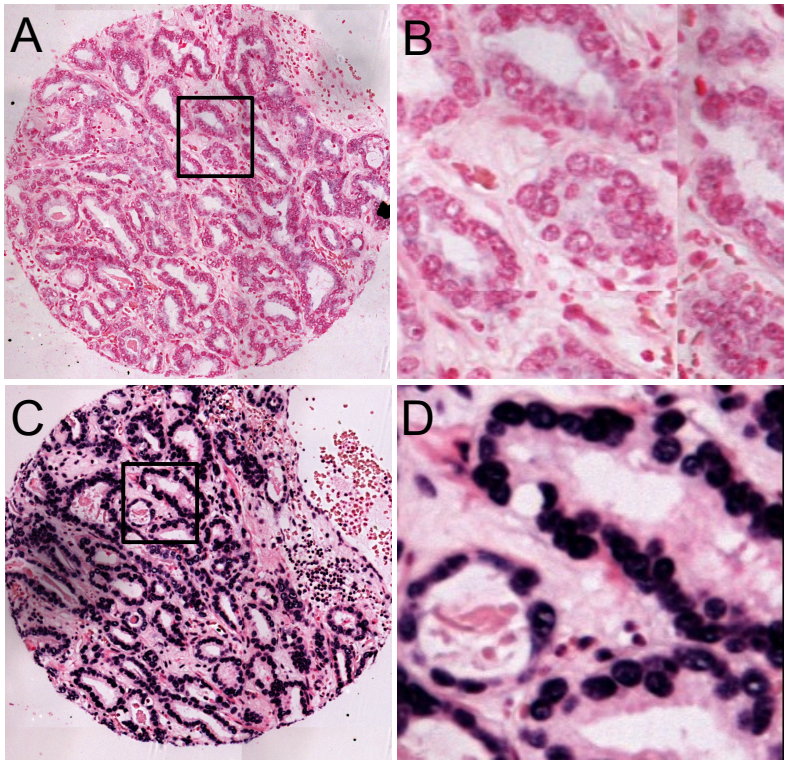

Figure 13: Cores and magnification from TMA slides. A and C: 20x magnification. B and D: 40x magnification.
***A and B;** Scrambled probe (negative control). No blue precipitate from Alkali Phosphatase (AP) reaction.*
***C and D;** U6 probe (positive control). Strong nuclear staining.*

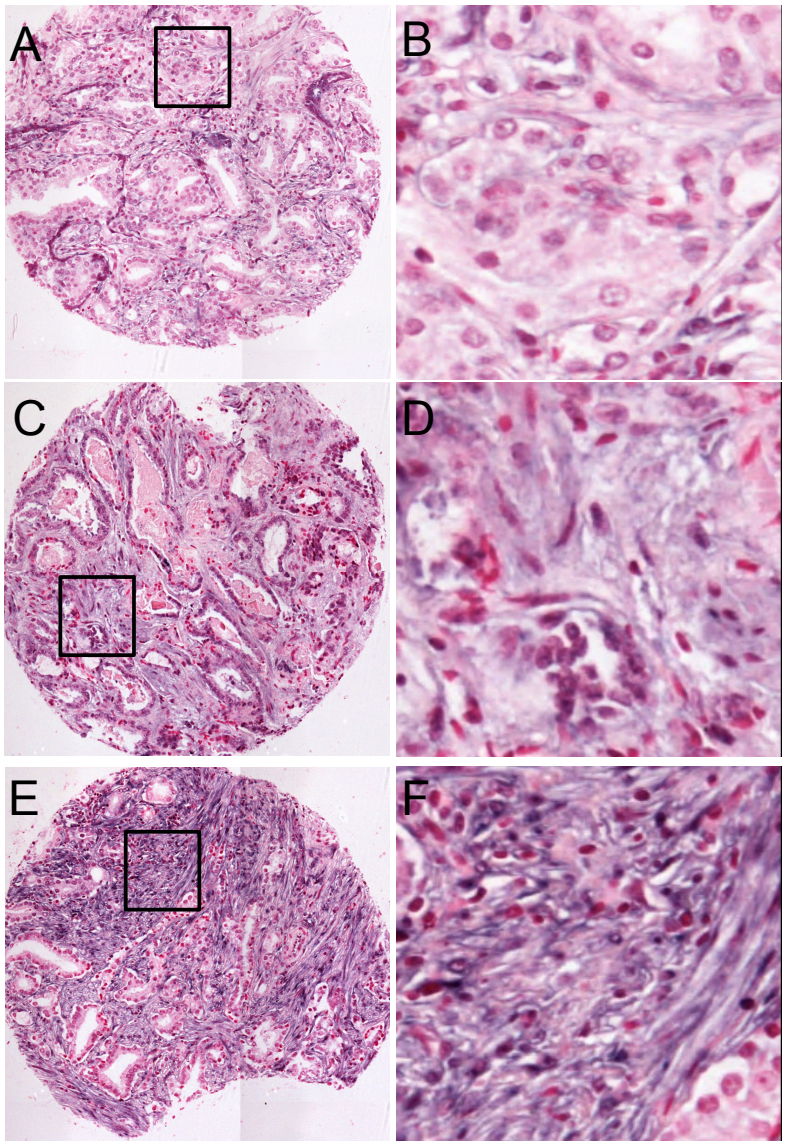

Figure 14: Cores and magnification from TMA slides showing different intensity levels of the substrate, which indicate the presence of miR-21 probe. A,C,and D: 20x magnification. B, D and F: 40x magnification.
A and B; *Example of score 1.*
B and C; *Example of score 2.*
E and F; *Example of score 3.*

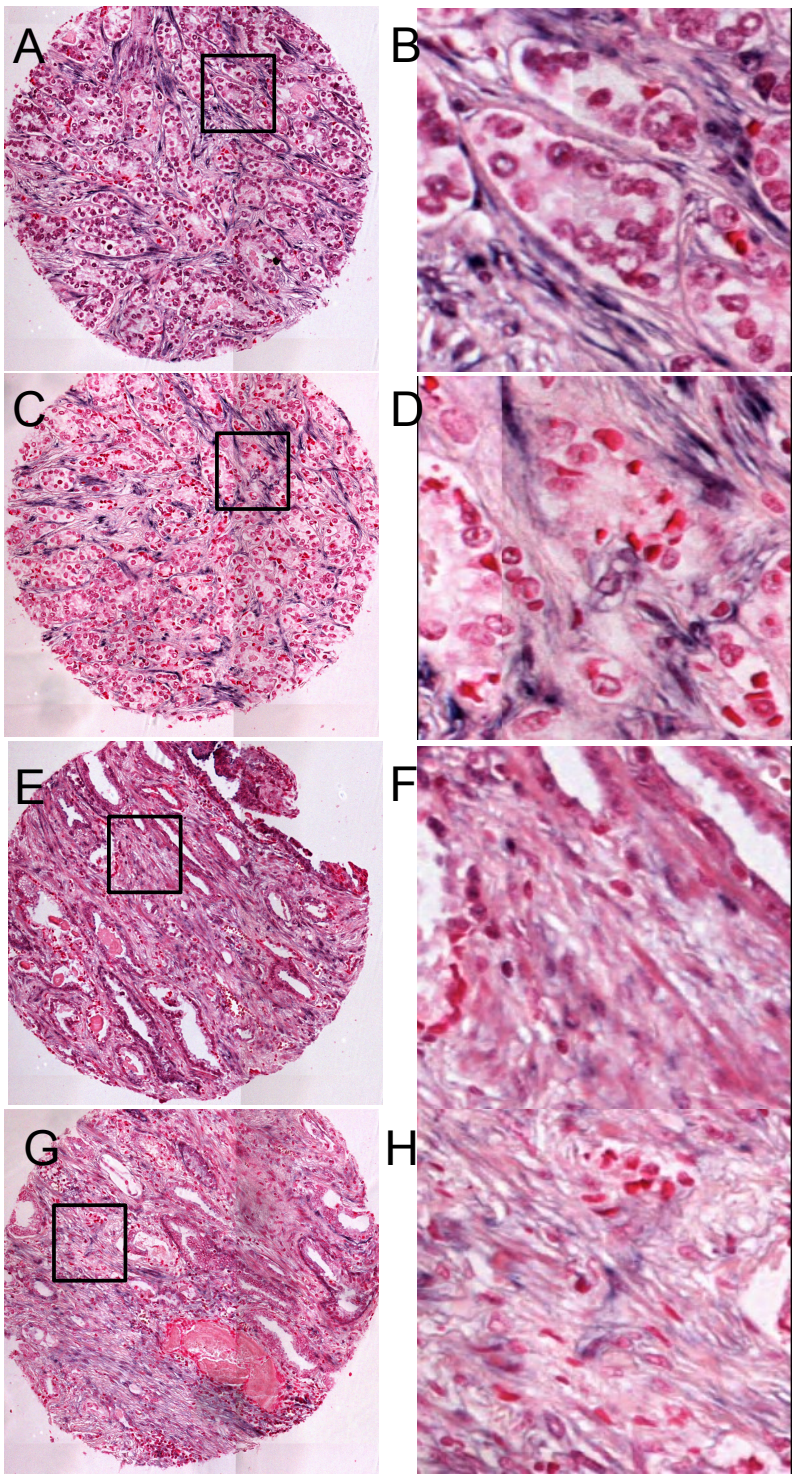

Figure 15: Two randomly selected cores from patients that are compared across the different series to determine the reproducibility of the experiment. A,C, E and G: 20x magnification. B,D, F and H: 40x magnification.
***A, B, C and D;** Core from one patient. Shows similar pattern and staining intensity. A and B is from the first series. C and D from the second.*
***E, F, G and H;** Secondary core from a new patient. Also show similar pattern and intensity. E and F are from the first series. G and H from the second.*

The scores from the two pathologist were collected for statistical interclass correlation analysis (ICC). The method used was two-way random analysis that tests for absolute agreement. The ICC was calculated, and the results are shown in Table 12.

	Intraclass correlation	**95% Confidence Interval**		**p-value**
		Lower bound	**Upper Bound**	
Single Measure	0.734	0.709	0.757	<0.001
Average Measure	0.847	0.830	0.862	<0.001

Table 12: The interclass correlation values found when the scores were analysed.

Descriptive statistics were performed on the collected data (Table 13).

		miR-21 in tumour cells	miR-21 in stroma cells
N	Valid	435	439
	Missing	120	116
Mean		1.2784	0.9808
Median		1.2500	1.0000
Percentiles	25	0.7500	0.5000
	50	1.2500	1.0000
	75	1.7500	1.2500

Table 13: Showing basic descriptive statistics of the data.

Univariate analysis was performed using the value 2 as cutoff (<2=Low, >2=High). Statistical significance was calculated by using Log Rank/Mantel-Cox test, and results are considered significant when $p<0.05$.

The expressions of miR-21 in stroma and tumour cells were compared to the frequencies of the three different end-points (Figure 16). The significance of the results are shown in table 14.

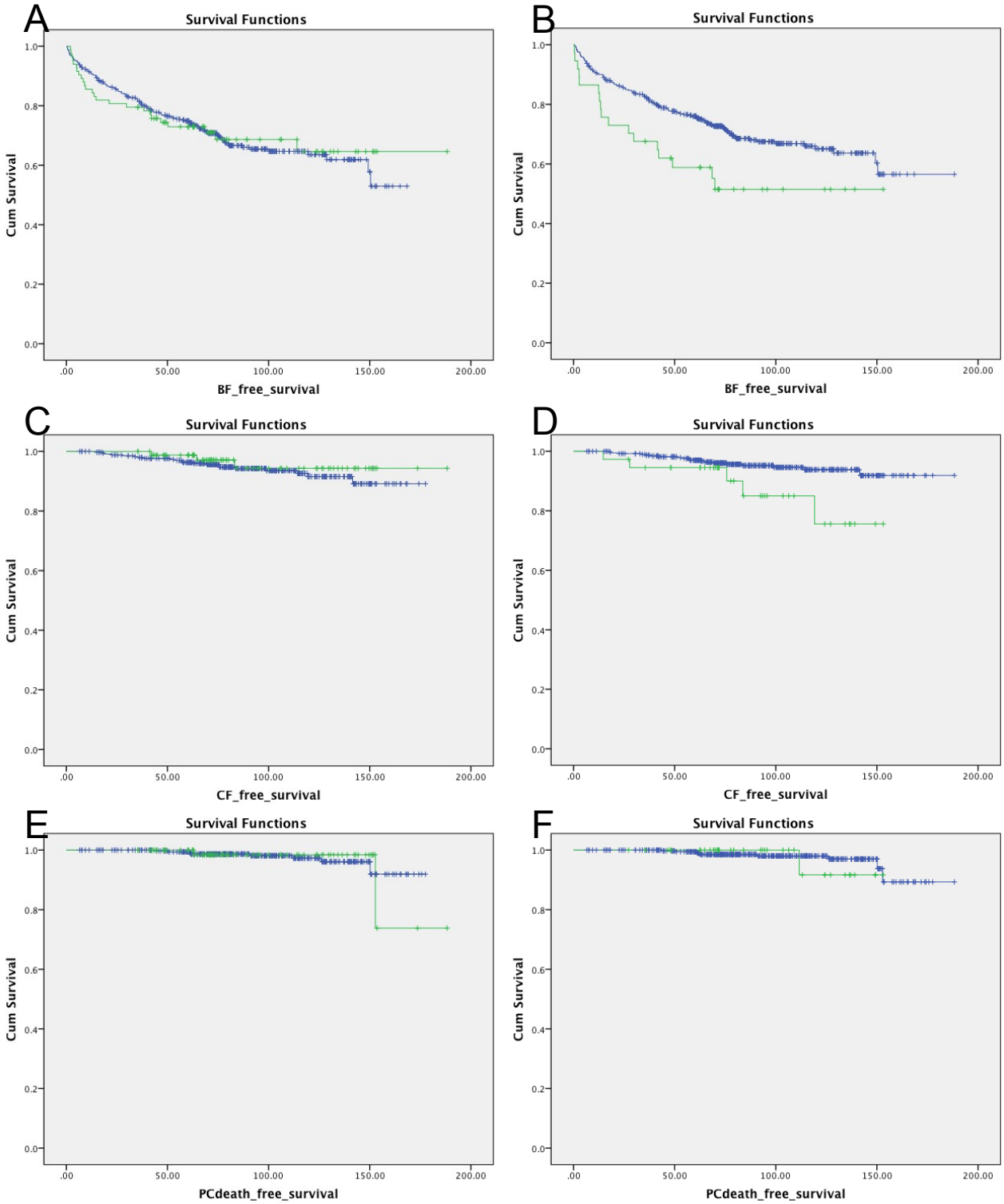

Figure 16: The expression of miR-21 in prostate tumours. The green curve is high expression and blue is low expression. ***A:*** *The effect of miR-21 expression in tumour cells compared to biochemical failure.* ***B:*** *miR-21 expression in stroma and biochemical failure.* ***C:*** *miR-21 expression in tumour cells and clinical failure.* ***D:*** *miR-21 expression in stroma and clinical failure.* ***E:*** *miR-21 expression in tumour cells and prostate cancer-specific death.* ***F:*** *miR-21 expression in stroma cells and prostate cancer-specific death.*

Expressing cells	End-point	Chi-Square	Degrees of freedom	p-value
Stromal cells	Biochemical Failure	5.248	1	0.022
	Clinical Failure	5.285	1	0.020
	Prostate cancer-specific death	0.083	1	0.773
Tumour cells	Biochemical Failure	0.008	1	0.929
	Clinical Failure	0.638	1	0.425
	Prostate cancer-specific death	0.004	1	0.951

Table 14: The significance from the miR-21 expression based on cell type and end-point. Only the expression of miR-21 in stroma cells are significant and only when used on the end-points biochemical failure and clinical failure.

4 Discussion

There were a few practical problems during the production of the TMA blocks. A main problem was that the tissue was very dense and hard. This caused significant problems in the TMA production, as several needles were destroyed.

Another problem was the size of some of the donor blocks. The blocks were larger than the apparatus could contain, so to accommodate the large blocks, a few pieces had to be removed. With the size of the blocks and the pieces removed, the tissue from the donor block could not be inserted into the main blocks directly. Instead of inserting the tissue directly, an intermediate block was made, and from this intermediate block the final TMA blocks were produced.

After the TMA slides were scanned and scored by the pathologists, some patients were found to be missing from the scored slides. A search of the TMA maps revealed that 54 patients that had been included in the patient cohort were missing from the TMA slides. Further investigation revealed that the majority of the missing patients (N=36) had been excluded from the TMA production due to error in the production or had viscous tissue (N=10) that could not be included into the TMA. The remaining patients were missing (N=8).

The advantage of using TMA, is that samples from several patients can be investigated at the same time. The problems with this approach is that tumour profiling by using second generation sequencing systems have showed that tumours are not homogeneous.[30] The tumour sequencing projects have showed that different locations in the tumour have differences both in the genome and the transcriptome[31]. These differences are easily explained by the differences in the environment in the area. The central zone of the tumour might be hypoxic and nutrient poor, while the borders of the tumour can be less hypoxic and nutrient rich, in comparison. These differences in the local environment of the tumour area will promote different mutations, as the cells need to survive under different conditions.

Using TMA based approach to cancer research has limitations, mainly if the samples can be considered representative for the tumour. TMAs have also been very effective in screening and validating molecular markers[32-34]. TMA identified biomarkers have been showed to help with clinical practice, and been helpful in finding new therapeutic targets for several malignancies[35,36].

In situ hybridization measuring miRNA is a confirmed and established method for detecting miRNAs.[37] When the sequence of the miRNA is validated and the probe is validated, the produced results are specific when the controls are valid. The problem is usually not specificity, but sensitivity. Due to this, the protocol often need to be adjusted too every probe individually. Several probe concentrations, Proteinase-K pretreatments and hybridization temperatures might be needed to gain a detectable signal. A common problem is temperature differences between the SSC stringency wash after the hybridization and the hybridization temperature, which will increase unspecific bindings. As the signal obtained in this study are repeatable it can be concluded that the signal is the real effect and not a random noise.

As cISH is a semi-quantitative method of measuring miRNA, there are other methods that are more quantitative, such as quantitative PCR (qPCR). qPCR is a method that can give quite good quantitative estimates of original copy count. The drawback are that qPCR uses isolated DNA or RNA, the method can not show the localisation of the expression, and that is very difficult and time consuming to perform qPCR on a large set of patients. Due to these reason, cISH is a much better method when using large number of samples. cISH also allow for differentiating between expression in tumour associated stroma cells and tumour cells. Another possibility when using cISH is that immune histochemistry (IHC) can be performed in tandem, allowing for double staining. When considering the goal of the study, finding new molecular biomarkers to use in prognosis of prostate cancer, cISH is preferred since the findings are easier to translate into a clinical setting, since the results from a staining can be visually inspected in a microscope by a pathologist, and directly compared to IHC analysis that have been done in parallel.

In this study the cutoff was that if the samples had moderate to high staining intensity of miR-21 they were considered to be high expression. If the sample had less then moderate intensity it was considered low expression. The reason for this was to create a group that had considerable higher expression than the remaining group, where the effect of miR-21 expression might be considered the strongest, even though that using the median or mean value is the more common approach.

5 Conclusion

Chromogen *in situ* hybridization is an effective and powerful method when used to analyse miRNA expression on tissue microarray.

MiR-21s role in prostate cancer is that of an oncogene, and it's expression in stroma can be used as a prognostic factor for two of the three end points used in this study; biochemical failure and clinical failure. This study and the study by Li *et al.* [38] both show that miR-21 can be used as an independent prognostic biomarker.

6 Reference

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7 Appendix

miRCURY LNA Detection probes

Probe	**Sequence**
3'-DIG, 5'-DIG labeled hsa-miR-21 probe	5'-NTC AAC ATC AGT CTG ATA AGC TAN-3'
3'-DIG, 5'-DIG labeled U6 probe	5'-CAC GAA TTT GCG TGT CAT CCT T-3'
3'-DIG, 5'-DIG labeled scrambled probe	5'-GTG TAA CAC GTC TAT ACG CCC A-3'

0.1% DEPC water

Regents	**Amount**
Diethylpolycarbonat	1 ml
milliQ water	1000 ml
Leave in 37 °C incubator overnight. Autoclave after incubation.	

Tris-HCl, 10 mM

Regents	**Amount**
1 M Tris-HCl	10 µl
DEPC water	990 µl

Proteinase-K, 20mg/ml

Regents	**Amount**
Proteinase-K, 12 mg stock powder	12 mg
10 mM Tris-HCl	600 µl
Spilt into 10 µl tubes and stored in freezer.	

10x Blocking solution

Regents	**Amount**
10x Blocking buffer from DIG Wash and Block Buffer Set	1 ml aliquots stored in freezer.

100 mM Levamisole

Regents	**Amount**
Levamisole, 240.75 g/mol	250 mg
DEPC water	10.4 ml

Sheep-serum, 60 mg/ml

Regents	**Amount**
Sheep-serum, dried stock	600 mg
DEPC water	10 ml
250 µl aliquots stored in freezer	

Proteinase-K buffer

Regents	**Amount**
DEPC water	900 ml
1 M Tris-HCl, pH 7.4	5 ml
0.5 M EDTA	2 ml
5 M NaCl	0.2 ml
Adjust Amount to 1000 ml with DEPC water and autoclave.	

SSC salt buffers

Regents	**Amount**
5x SSC	250 ml 20xSSC + 750 ml DEPC water
1x SSC	100 ml 20xSSC + 900 ml DEPC water
0.2 SSC	10 ml 20x SSC + 990 ml DEPC water
Autoclave.	

PBS pH 7.2-7.6

Regents	**Amount**
PBS tablets	5 tablets
DEPC water	1000 ml
Autoclave.	

PBS-T 0.1%

Regents	**Amount**
PBS pH 7.2-7-6	300 ml
Tween 20	300 µl

KTBT-buffer

Regents	**Amount**
Tris-HCl	7.9 g
NaCl	8.7 g
KCl	0.75 g
DEPC water	1000 ml
Autoclave.	