4.1 Final publishable summary report

Executive summary

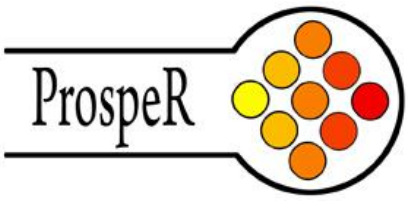
Annually, over 200 000 new prostate cancer cases are diagnosed in Europe. The widespread use of prostate specific antigen (PSA) tests on asymptomatic men and an aging population are likely to further increase this number. The rate of over-detection is high and most cases will never experience cancer symptoms during their lifetime. Hence, the current lack of means to predict prostate cancer outcome will have a devastating impact on both the life-quality of patients and public health care expenditure.

Molecular mechanisms of the development of prostate cancer are largely unknown apart from the recognised role of androgens. This has hampered development of efficacious prevention, specific diagnostics, prognostic, and therapeutic tools. Based on the emerging knowledge on the function of non-coding RNAs (ncRNAs) as regulators of key cellular mechanisms and our own data on expression of ncRNAs in prostate cancer, it is likely that ncRNAs are involved in the tumorigenesis and progression of prostate cancer.

In ProspeR, we have pursued two major clinical problems: 1) early identification of cases requiring aggressive curative treatment, and 2) development of efficient therapies against hormone-refractory prostate cancer. The project covered all phases of translational research, from discovery to validation and implementation.

We identified novel ncRNAs by deep-sequencing, profiled miRNAs by microarray analysis, investigated their expression by Q-RT-PCR, genetic/epigenetic alterations by array comparative genetic hybridisation (aCGH) and MeDiP-sequencing, and function by *in vitro* and *in vivo* experiments. We have developed diagnostic tools to discriminate between indolent and aggressive prostate cancer and identified putative therapeutic targets. Our results on ncRNAs could contribute to optimising individualised prostate cancer treatment by providing both novel biomarkers and drug targets.

The consortium, coordinated by the University of Tampere, Finland, consisted of six academic prostate cancer research centres and two companies committed to developing ncRNA detection technologies and novel drugs. The partners represented five countries.



Context and objectives

Prostate cancer is the most common male malignancy and the second most common cause of cancer related death in most European countries. It has been estimated that there were 225 000 new prostate cancer cases in Europe in 2002 (GLOBOCAN 2002). A special feature for prostate cancer is that a latent form of the disease is very common. Microscopic lesions of cancer have been found in autopsies from more than 50% of 70-80 year old men. Thus, not surprisingly, the use of prostate specific antigen (PSA) for screening of asymptomatic men has had a tremendous influence on the number of prostate cancers diagnosed, and subsequently led to over-treatment. Since there are no tools to reliably identify patients who really need to be treated, this major health care and economical problem is likely to get worse in the aging populations. Currently, the most commonly utilized prognostic factor, in addition to clinical stage, is the Gleason score, which reflects the histological differentiation of the tumor. The shortcoming of the Gleason score is the pure subjective nature of the parameter. None of the other suggested prognostic markers, such as proliferation index, aneuploidy, overexpression of EZH2 or hepsin, have become clinically widely used.

Although the majority of prostate cancer patients are diagnosed today with organ-confined disease, which is curable by prostatectomy or radiation therapy, 20-25% of the patients experience relapse within 5-years of treatment. Identification of these patients at the time of intent to cure would be important, since the patients could potentially benefit from adjuvant therapies and avoid the morbidity of unnecessary treatments. Unfortunately, there is no reliable way of predicting the outcome of treatment in individual cases.

The gold-standard treatment for advanced prostate cancer has for over half a century been androgen withdrawal. This is based on the understanding that the growth and progression of prostate cancer is dependent on androgens. Almost all patients initially show a response to androgen withdrawal therapy. However, inevitably, castration resistant tumour cells emerge and lead to clinical progression of the disease. Despite the recent findings of efficacy of docetaxel, abiraterone acetate, and enzalutamide, there are no definitively effective therapies available for such hormone-refractory tumours.

The lack of knowledge of the molecular mechanisms of the development and progression of prostate cancer has hampered the development of better prevention, diagnostics, prognostic, and therapeutic tools. In addition to the androgen signalling pathway, no major mechanisms of prostate cancer have been identified. A relatively recent breakthrough discovery in molecular biology has been the discovery of the importance of RNA as a regulatory molecule of many cellular processes. The importance of these ncRNAs (non-coding RNAs) has already been implicated in many malignancies. Therefore, the study of ncRNAs could lead to breakthroughs in treatment of prostate cancer by providing both novel diagnostic and predictive biomarkers as well as drug targets.

A special feature for prostate cancer is that a latent form of the disease is very common. Microscopic lesions of cancer have been found in autopsies from more than 50% of 70-80 year old men. The majority of these histological cancers would most probably never develop to a clinical cancer. Whether these incidentally found

small carcinomas represent the same disease entity as the clinically relevant, life-threatening tumors, is not known. While confined to the prostate gland, the cancer is curable by either prostatectomy or radiation therapy. The use of serum PSA testing has resulted in a dramatic stage migration of prostate cancer and today most men are diagnosed their tumour is confined to the gland. However, PSA testing has also lead to the diagnosis of many of incidental tumors, so called over-diagnosis. These tumors are unlikely to ever cause morbidity to the patient and thus their cure by surgery or radiotherapy represents over-treatment. Whilst more conservative observational treatments, such as 'active surveillance', are currently in clinical trials, there are no tools to accurately identify these indolent cancers. Once the cancer has invaded through prostate capsule, no curative treatments are available. The main palliative treatment option is androgen withdrawal. While the majority of the patients respond to this androgen withdrawal, eventually the disease will progress if the patient just lives long enough. The time of response may vary from months to many years. The use of chemotherapy in this setting is disappointing. For example, two recent phase III studies have shown that docetaxel with either estramustine or prednisone significantly prolongs survival of patients with hormone-refractory disease, but for only about 2 months.

Prostate cancer is a heterogeneous disease, where definitive risk factors include age, ethnic origin and family history. About 80% of prostate cancers occur in men over the age of 65 years, and of all cancers, the prevalence of prostate cancer increases most rapidly with age. Most human cancers have also a genetic form of the disease. Therefore, it is not surprising that there is a growing body of evidence that genetics is likely to play a major role also in the development of prostate cancer. This has been shown, for instance, in a large Scandinavian twin study that showed that ~40% of the risk of prostate cancer could be explained by heritable factors. Despite the strong evidence for genetic predisposition to prostate cancer, the findings from linkage studies have been disparate. Several susceptibility loci have been reported, but none of them have been definitively confirmed in independent studies. Several low-penetrance polymorphisms are also associated with the risk of prostate cancer at the population level.

The acquired somatic genetic alterations, *i.e.* mechanisms of the prostate cancer, are also incompletely understood. Over the past 10 years, we and others have identified chromosomal aberrations in prostate cancer. The analyses have shown that losses of chromosomal regions at 6q, 8p, 10q, 13q, and 16q as well as gains at 7, 8q, and Xq are common in prostate cancer. Some of the losses seem to be early events and are already present in premalignant lesions called prostate intraepithelial neoplasia (PIN), while the gain or amplification of 8q seems to be associated with the aggressive phenotype of prostate cancer. Many of the target genes for the chromosomal aberrations are still unknown. The implicated genes include the genes for glutathione S-transferase π (GSTP1), phosphatase and tensin homolog (PTEN), tumor protein p53 (TP53) and androgen receptor (AR). Of these, hypermethylation of the promoter of GSTP1 seems to be an early event in the tumorigenesis, found in 70% of PIN lesions and in almost all carcinomas. On the other hand, mutations in PTEN and TP53 seem to be common in late, but rare in early stage of disease. Androgens and AR seem to be involved not just in early development but also in late progression of prostate cancer. It has been shown that the AR gene is amplified or mutated in about

30-40% of hormone-refractory prostate cancers implicating the gene in the emergence of androgen-independent form of the disease.

A major puzzle in the biology of prostate cancer is why so few mutations have been found, especially in the early disease. The most important reason for this could be that the right genes have not yet been studied. For example, genes encoding ncRNAs have been studied in any depth within the disease. During the past few years, thousands of RNA molecules that do not seem to contain any extensive open reading frame have been identified. These non-coding RNAs (ncRNAs), seem to be involved in many fundamental functions of the cell. For example, they have been implicated in regulation of gene transcription and translation, chromatin structure dynamics, DNA imprinting and methylation, and gametogenesis. MicroRNAs (miRNAs) are a class of small, non-coding RNAs that are endogenously expressed in animal and plant cells. They regulate expression of protein-coding genes at the translational level, either by triggering degradation or preventing translation of the target mRNAs. According to the current view, imperfect complementarity of nucleic acids leads to repression of translation of the target mRNA and is the main mechanism of miRNA regulation in animals, whereas perfect complementarity induces degradation of the target mRNA and is mainly detected in plants. The first piece of evidence of the connection of miRNAs and cancer was obtained, when Calin et al. showed that two miRNAs, miR-15 and miR-16, are the target genes of the 13q14 deletion that is common in chronic lymphatic leukaemias. After this initial finding, more than half of the known miRNAs have been reported to be located in cancer-associated genomic regions and to show copy number alterations in cancer. Furthermore, deregulation of several miRNAs has been detected in various different cancers. Functional studies on individual miRNAs have shown that they can act as oncogenes or tumor suppressor genes. Although targets of most miRNAs have not been identified, some have been shown to negatively regulate well-known oncogenes; miR-15 and miR-16 have been reported to repress the antiapoptotic factor BCL2 and the let-7 miRNA family to target the well known RAS oncogene.

Recently, several miRNA expression profiling studies have been performed in order to identify sets of miRNAs that are differentially expressed in cancer cells compared to their normal counterparts, i.e. the cancer-specific miRNA signatures. Although there are some miRNAs that have been reported to be aberrantly expressed in several different cancer types, most often the miRNAs showing up- or down-regulation in different cancers are not the same ones, indicating that the miRNA signatures of cancers of different cellular origin are likely to be unique. The role and significance of miRNAs in prostate cancer had not been thoroughly studied, although our earlier results on miRNA expression had suggested that the expression of some of the miRNAs might be regulated by androgens and indicated the potential of miRNA expression profiling in classifying tumors according to their clinical stage and subtype. The surprisingly high number of exclusively differentially expressed miRNAs indicated that miRNA expression has a fundamental significance in prostate tissue. Therefore, miRNA expression profiling could be utilised in developing new diagnostic tools for cancers that currently lack good molecular markers, such as prostate cancer.

Based on the emerging understanding of the importance of ncRNA in cancer, it is highly likely that ncRNAs are involved in the tumorigenesis of prostate cancer.

Furthermore, extrapolation of data from other malignancies suggests that they may be better biomarkers and drug targets than protein coding genes. The first expected outcome of this project was that novel ncRNA markers would identify both indolent prostate cancers that could be treated by active surveillance, and aggressive carcinomas that require early radical intervention.Taking into account the vast overdiagnosis of localised prostate cancer due to PSA screening, finding a marker that reliably identifies cancers that do not require treatment would have huge medical and economical benefits. On the other hand, there is no question that better therapies are needed for castration resistant prostate carcinoma. The second expected outcome of this program was the identification of novel drugable targets in advanced prostate cancer. These therapeutic clues and discoveries could also be licensed to the biopharmaceutical companies for further development.

The general aim of this project was to elucidate the role of ncRNAs in the development of prostate cancer, and evaluate the utility of ncRNAs as diagnostic/prognostic tools and therapy targets in clinical practice. The work followed a discovery-validation-implementation–pathway. Answers were sought for the two major clinical problems in the treatment of prostate cancer: 1) how to identify patients that need curative treatment for local disease, and 2) how to improve the treatment of castration resistant prostate cancer.

More specifically, the objectives of the project included: 1) identification of miRNAs and other ncRNAs that are aberrantly expressed in prostate cancer compared with non-malignant prostate tissue, *i.e.* the ncRNA signature of prostate cancer; 2) elucidating the possible genetic or epigenetic mechanisms of the aberrant expression of ncRNAs in prostate cancer; 3) studying whether the expression of ncRNAs is regulated by androgens and/or the androgen receptor; 4) identification of ncRNAs related to genetic predisposition of prostate cancer; 5) studying which ncRNAs are functionally involved in the development of prostate cancer, and thus, putative drug targets; 6) identification of the target proteins regulated by the ncRNAs involved in prostate tumorigenesis; 7) development and validation of diagnostic, prognostic and predictive tools based on ncRNAs or target proteins; and 8) pre-clinical testing of therapeutic strategies targeting ncRNAs found to be functionally important, as well as siRNA-based strategies against target proteins.

The ultimate goal was to obtain proof-of-principle on the usefulness of ncRNAs for clinical use in the diagnosis, prognosis and therapeutic targeting of prostate cancer. The project was subdivided into four R & D workpackages and a management work package.

Main S&T results/foregrounds

Methodological testing and validation

ncRNA research was rather new when the project commenced, and many methodological issues remained to be solved. This included validation of existing methods as well as developing new ones. It had to be taken into account that ncRNA, especially the miRNAs, are quite short in sequence, only about 20 nucleotides long, and that they are derived via sophisticated pathways from much longer primary transcripts that share sequence and may be degraded before any mature and hence active molecule is produced. Many miRNAs are also very much like each other in sequence and distinguishing them from each other could be challenging. ncRNA assay development for clinical purposes includes determining the best methods for extracting ncRNA from a variety of clinical specimens, including blood, urine and tumour tissue, developing a simple enough quantification method for use in clinical laboratories, and evaluating the utility of ncRNA analysis in prostate cancer diagnosis and prognosis. The novel assay must be robust and reliable.

Extraction protocols for retrieval of ncRNA from various types of clinical specimens were tested and optimised. In the case of liquid samples, blood and urine, the best yields were obtained from the cellular fraction of the sample after centrifugation. This is not surprising, as intact cells contain ncRNA in a more concentrated form than the serum/plasma from blood or supernatant of urine. The lower yields in the cell free fractions were reproducible, however, and this is important when considering their usefulness in a clinical setting. It was also noted that heparin-containing collection tubes for blood should not be used for analysis of miRNA by Q-RT-PCR.

Pathological evaluations of biopsies and whole mount prostates after surgery are done mostly from FFPE samples and the leftover tissue blocks are an important resource of samples for research. miRNA yields extracted from FFPE material with a modified mirVANA miRNA Isolation Kit were low, yet reproducible and the snRNA yields from similar samples were more varied, but higher. However, very little RNA is actually needed for the newest Q-RT-PCR analysis methods.

ncRNA also turned out to be surprisingly stable, and only about 20% was lost during an experiment where the extracted RNA was store in room temperature for three days. Three freeze/thaw cycles destroyed about the same amount of the RNA studied (a snoRNA). Also rather surprisingly, the use of RNAse inhibitor did not increase the yields of miRNA extracted from freshly voided urine. Stability is also important in standardised clinical tests.

ProspeR started out with SOLEXA deep sequencing and Agilent microarray analyses of miRNA. In order to find out whether these two methodologies were reliable, a third method, namely quantitative reverse transcriptase polymerase chain reaction, Q-RT-PCR, was used to validate the results from the screening methods. The expression levels of several miRNA that had shown differential expression levels by microarray analysis were also analysed by TaqMan MicroaRNA Assays (Figure 1). Three experimental settings were each validated separately and the relative expression patterns were 79% concordant with the microarray analysis. The concordance with deep sequencing was not quite as good (63%), but satisfactory none the less. A question yet to be answered with regard to Q-RT-PCR, especially when looking at individual ncRNAs, is the method of normalisation. Many possible normaliser genes

and their combinations were tested but none was found to be completely optimal. This issue will continue to be addressed.

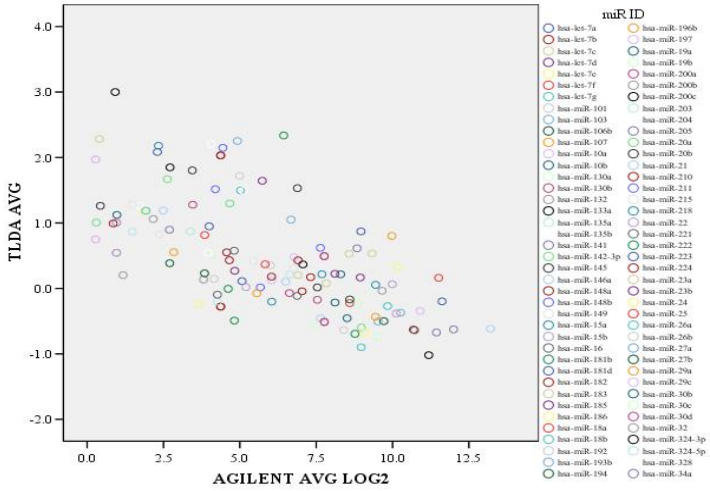


Figure 1. A representative Scatter plot comparing Q-RT- PCR delta-Ct values with microarray fluorescence scores produced from microRNA microarrays

Q-RT-PCR was also optimised for the detection of low quantity ncRNA from formalin fixed paraffin embedded (FFPE) prostate tissue and the sensitivity and reproducibility were suitable for further development of a clinical assay. Modern day Q-RT-PCR methods, which require a minute amount of sample material would most likely be the method of choice for clinical applications.

SNORDS

We were able to identify 28 novel miRNAs by massive parallel sequencing of different types of prostate samples (normal prostate, benign prostatic hyperplasia (BPH), hormone naïve local prostate cancer, hormone naïve lymph node metastases, locally recurrent castration resistant prostate cancer (CRPC)). All of the novel miRNAs were expressed at extremely low levels, however, and their further analysis was abandoned. Focus was therefore shifted to the interesting finding of numerous fragments that seemed to be derived of small nucleolar RNAs (snoRNAs) and transfer RNAs (tRNAs). In addition, several of these snoRNA and tRNA fragments showed differential expression and are up-regulated during disease progression.

Two bioinformatics programs, Short Sequence Location Mapper (SSLM) and ncRNA Mapper, were developed to aid in the analysis of short fragments derived from larger ncRNAs such as snoRNA and tRNAs. The software are freely available for use at www.gatcplatform.nl. SSLM is a stand-alone software program that aligns and visualizes fragments of 18-25 nucleotides onto the larger full length ncRNA. Using this program, one can determine whether fragments from longer ncRNAs are always derived from the same part of the ncRNA, or whether fragments are derived randomly (Figure 2). Using SSLM, we have shown that fragments from ncRNAs are generally derived from the same part of the ncRNA. ncRNA Mapper was developed in order to determine whether the fragments of ncRNAs are predominantly derived from the highest conserved parts of the ncRNA. This program takes the fragment

location from SSLM and links them to the conservation score of the ncRNA sequence. Using ncRNA Mapper, we confirmed that miRNAs are typically derived from the conserved parts of the pre-miRNA. This is as expected since the miRNA sequence is evolutionary important and conserved, while the loop and outer 5'and 3'sequences are allowed to have mutations without functional consequences and are therefore less conserved. Using these tools we found that, besides miRNA, downstream processing of many snoRNA and tRNA occurs, and that this is a non-random process that generates locus-specific smaller fragments with discrete size.

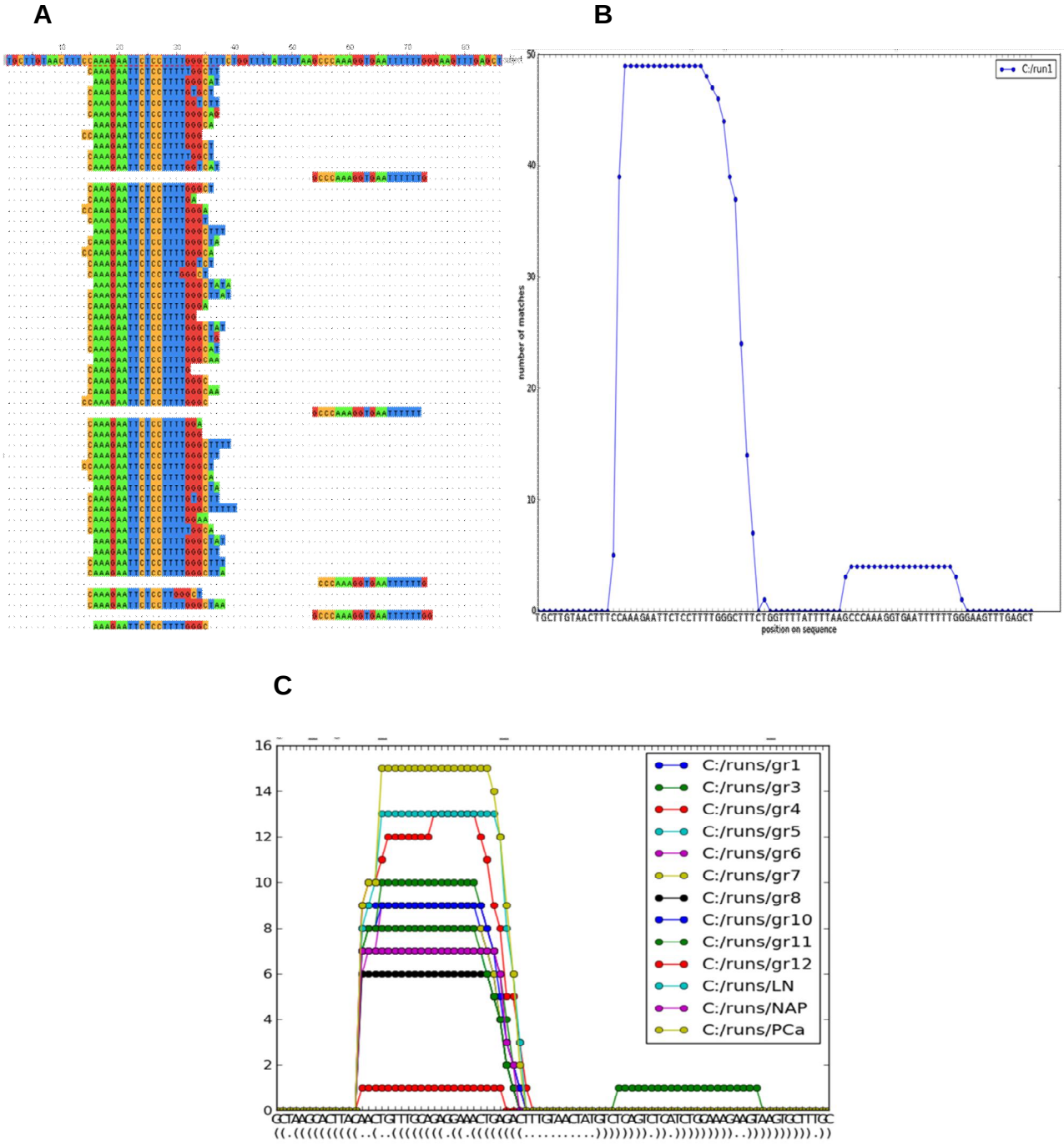


Figure 2. A) Solexa sequencing reads from one of our prostate cancer samples are aligned onto one particular full length ncRNA by SSLM. An algorithm than calculates and visualizes the frequency and location of the reads as a line-graph **(B).** For each Solexa run, these graphs are produced and compared **(C).**

The fragments of four snoRNA with potential importance in prostate cancer were selected for further investigation. Due to the challenges in accurately detecting the full length snoRNAs, custom LNA-based Q-PCR primer sets were designed to determine their expression levels and the expression levels of the fragments in different sample types and validate the results of the sequencing analysis. Statistically significant results concordant with the data obtained from deep-sequencing were obtained for one of them (SNORD78). As expected, its expression levels were elevated in organ-confined prostate cancer specimens compared to normal adjacent prostate. More importantly, further increase of these snoRNA fragments was observed in patients with recurrent disease after radical prostatectomy and in metastatic samples. The same could be seen from the sequence reads of three other snoRNAs transcribed from the same locus.

As androgen regulation of ncRNAs was one of the key questions asked within the project, the newly developed Q-RT-PCR assays for the snoRNAs were also used to determine the androgen regulation of the genes. Unfortunately the experiments on androgen stimulated and androgen depleted cell lines could not unequivocally show androgen regulation of the four snoRNAs tested. Chromatin-immunoprecipitation-sequencing to find androgen receptor binding sites at the loci was also futile.

Diagnostic and prognostic miRNA panels

To profile a larger sample set of prostate cancers we performed microarray hybridisation on prostate cancer cell lines, xenografts and clinical samples. Unsupervised clustering analysis of the initial sample set (cell lines and xenografts) placed all cell lines in one arm of the dendrogram and the xenografts in another. In addition, since the xenografts were derived from two different series, they mostly clustered in their own series. Another important finding was that within the main clusters, androgen independent samples clustered separately from the androgen sensitive and androgen dependent samples. These results indicate that in addition to growth conditions (in vitro vs. in vivo) the sensitivity to androgens plays an important role in regulation of miRNA expression.

One hundred and two clinical prostate tissue specimens were also analysed by microarray. After extensive statistical testing, the 80 miRNAs that varied most significantly between normal and cancer tissues were used for unsupervised hierarchical clustering analysis. The analysis clearly separated the normal samples from the aggressive cancer samples. In addition, the organ confined cancer samples were separated in to two groups: Group I clustered with the normal samples and Group II with the aggressive cases. The prognostic characteristics of the two groups differed significantly with Group II patients at a greater risk of metastases after radical prostatectomy and cancer related death, as well as higher Gleason score.

To predict the group (Group I = good or Group II = bad) of future samples a predictor panel was constructed by training a Bayesian Covariate Compound Predictor (BCCP). The best predictor consisted of 25 miRNAs significantly differentially expressed between the groups. Similarly, a diagnostic classifier was constructed to classify samples into a normal group or organ confined cancer group. It consisted of 54 miRNAs significantly differentially expressed between the normal cases and the

organ confined cancer samples. Cross validation showed excellent accuracy of diagnostic prediction. The diagnostic classifier includes 14/25 miRNAs from the predictor panel. These panels together can distinguish between normal and cancer, and further classify the cancer samples into a good prognosis group and a bad prognosis group. Yet another panel was constructed by comparing the expression levels of miRNAs in organ confined samples and lymph node metastases to identify miRNAs whose expression is altered during (metastatic) progression of the disease. Many of the miRNAs identified were again the same as in the other two panels, indicating that these genes may be important in the development and progression of prostate cancer. The panels have been described in more detail by Martens-Uzunova & Jalava et al., (2012)

miRNAs extracted from frozen plasma concentrate (77 prostate cancer patients and 28 controls) were profiled by Exiqon RT-PCR analysis kit of 750 miRNAs. Ten miRs were found to be significantly increased in the sera of prostate cancer patients compared with normal individuals. Q-RT-PCR analysis confirmed two (miR-107 and miR-574-3p) of them that could be associated with prostate cancer. miRNAs that may be associated with non-metastatic (organ confined) prostate cancer were found by comparing the expression profiles of 53 prostate cancer patients without metastasis and 28 normal controls. Nine miRNAs came up in this analysis and one (miR-107) was validated by Q-RT-PCR. Finally, a comparison of miRNA expression profiles from fourteen patients with metastatic PCa and 53 patients with non-metastatic PCa identified nine miRNAs that were differentially expressed between the two groups and four (miR-141, miR-375, miR-200b and miR-574-3p) of these were validated by Q-RT-PCR. In an analysis of paired plasma samples taken from eight patients before and after androgen deprivation therapy, the expression of two miRNAs (miR-375 and miR-141) had the same pattern as the PSA level.

The above mentioned differential expression patterns were validated in serum (84 prostate cancer patients and 28 healthy controls) and urine (135 prostate cancer patients) samples. The expression differences for miR-107 and miR-574-3p in cancer vs. healthy, and miR-200b and miR-375 in the metastatic vs. non-metastatic analyses were confirmed also in these sample types. miR-141 was also shown to be 10-fold more expressed in the plasma of metastatic prostate cancer cases than in healthy controls, indicating that this miRNA could be useful in detecting metastatic prostate cancer. A more detailed description of these results can be found in the paper by Bryant et al., (2012).

A comparison between free urinary miRNA and miRNA derived from the cellular fraction obtained after centrifugation of urine revealed that free urinary miRNA are more consistent in their expression patterns than cellular miRNA, although their concentrations are much lower. The free urinary miRNAs were also able to discriminate between patients with cancer and those without, and further separate patients with early (T1/T2) and late (T3/T4) stages of the disease. The free urinary miRNAs may be more representative of the miRNAs in serum/plasma.

The miRNA index quote - miQ

The original microarray data of the clinical samples was also used as primary discovery set for another diagnostic and prognostic marker. Thirteen miRNAs were selected for validation in FFPE material by Q-RT-PCR. The selection criteria included

prognostic potential based on the panels and previously reported deregulation in cancer. Nine of the selected miRNAs were confirmed as differentially expressed in a validation cohort of 49 prostate cancer patients and 25 healthy controls. The expression of some of the individual miRNAs was correlated to diagnosis, aggressiveness, metastasis, survival, and/or treatment response.

Determining the expression levels of individual miRNA (and other genes) requires some kind of normalisation. To circumvent this issue, different combinations of the nine miRNAs were tested and a ratio with an equal number of denominators and numerators was chosen as the arrangement. The best combination of miRNAs for diagnostic discrimination was (miR-96 x miR-183) / (miR-145 x miR221), which was denoted miRNA index quote (miQ). miQ was a better discriminator than any of the individual miRNAs alone. The value of miQ was approximately 17 times higher in the group with prostate cancer than in the group without. miQ was not dependent on the percentage of tumour cells from the original sample, which is a good sign considering that biopsies from which it would be measured in a clinical test sometimes have very little cancer. The diagnostic potential of miQ was validated in another cohort of patients consisting of 52 prostate cancer samples and 19 normal samples, as well as one internal and two external validation sets (Figure 3).

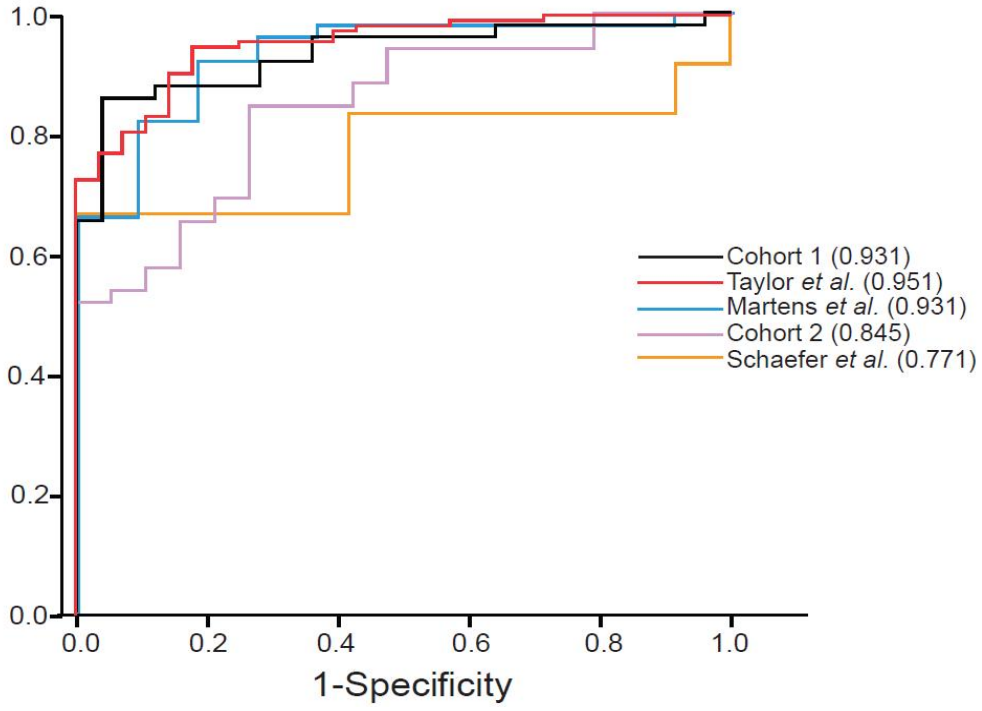


Figure 3. Receiver operator characteristic curves for differentiation of patients with prostate cancer from those without based on miQ levels in 5 patient cohorts. Area under the curve (AUC) are indicated within brackets for the individual cohorts

The accuracy of miQ was compared to the accuracy of PSA, and was found to be much higher. It was also shown to be an independent predictor by logistic regression analysis. In addition to being able to discriminate between non-cancerous samples

and cancerous samples, miQ was also found to be an independent predictor of aggressiveness, including the likelihood of metastasis. Kaplan-Meier analysis of survival showed that patients with miQ values in the upper third of the scale had a significantly shorter survival time after transurethral resection of the prostate (TURP) and a significantly shorter biochemical recurrence free time after prostatectomy. In fact, the median survival time of patients with low miQ after TURP was three years longer than patients with high miQ.

This is the first time miR-96, -183, -205 and -221 are combined into a ratio, but these four individual miRNAs have previously been reported to have diagnostic and/or prognostic properties in prostate cancer in at least two independent studies. The advantage of using this type of ratio is to circumvent the dependency of normalisation factors stable enough for clinical demand, making miQ more attractive in a clinical setting, but also increased discrimination, specificity, robustness, and may be an advantage considering the heterogeneity of prostate cancer. Prostate cancer is a multifocal and heterogeneous disease were different molecules may be deregulated by different mechanisms at different foci. By including several independent factors the risk of missing a malignant event is reduced.

miR-32

One of the miRNA that came up in the microarray analyses as significantly overexpressed in cancer and in particular in CRPC compared to the non-malignant controls was miR-32. It was chosen for further experiments. Functional studies with miR-32 transfected prostate cancer cell lines showed that overexpression of the miRNA reduced apoptosis. miR-32 was also shown to be androgen regulated by stimulating untransfected androgen-sensitive LNCaP prostate cancer cells. This result verified our earlier studies on the androgen receptor overexpression cell line models LNCaP-ARmo and LNCaP-ARhi. Androgen regulation was also implied by chromatin immunoprecipitation (ChIP) analysis coupled with deep sequencing that showed an androgen receptor binding site (ARBS) about 14 kb upstream of the miR-32 gene.

Putative targets for miR-32 were identified by microarray analysis of the miR-32 transfected LNCaP cells. Down-regulated genes included six genes that were predicted as targets by three different miRNA target prediction programs. BTG2 was the most interesting of these, and was selected for further validation. Its downregulation in the miR-32 transfected cells was confirmed by Q-RT-PCR and Western blotting. Direct evidence of an interaction was obtained by co-transfecting LNCaP cells with the miRNA and a reporter gene coupled to the BTG2 3'-UTR. Rescue experiments verified these interactions.

BTG2 protein expression was studied by immunohistochemistry on tissue microarrays of clinical prostate cancer specimens. In the CRPC, where the expression of miR-32 was generally higher, the staining intensity and hence the protein expression was much lower than in the hormone naïve samples. In none of the CRPC samples was the BTG2 staining intensity regarded as high, whereas in the hormone naïve samples almost 10 % showed high expression. The percentage of completely negative CRPC samples was almost 30% as opposed to about 10% in the hormone naïve samples. The patients with negative staining had a significantly shorter progression free time after prostatectomy compared to the patients with high

expression. These results have been published in further detail by Jalava et al., (2012).

miR-32 was also selected for further experiments to evaluate its suitability for therapeutic targeting. Its expression level in normal prostate was low, but somewhat higher in some important tissues such as white brain matter and liver. Within the prostate tissue, the miRNA seemed to be expressed equally in the epithelial cells and the stromal cells. The expression seemed to be equal also in the healthy and cancerous epithelial cells from the same sample. More samples need to be studied to confirm this.

The earlier miR-32 transfection experiments had been transient, and the effects of the miRNA overexpression in cells needed to be studied also over a longer period of time to ensure that the cells would at least be viable for in vivo studies. Stably transduced LNCaP cells grew much faster than the control cells just like the transiently transfected cells and therefore the miRNA could be studied further in vivo. The in vivo experiments, however, are still ongoing.

miR-34c

miR-34c was found to be down regulated in prostate cancer by the microarray analysis described earlier. Its expression was studied by Q-RT-PCR in 49 TURP samples and 25 BPH samples and found to inversely correlate with the aggressiveness of the disease and emergence of metastasis. Kaplan-Meier analysis also showed that patients with low miR-34c expression had significantly shorter survival. Ectopic expression of miR-34c in prostate cancer cell lines decreased cell growth both by decreasing proliferation and by increasing apoptosis. Overexpressing miR-34c also suppressed migration and invasion, indicating that this miRNA could be a tumour suppressor in prostate cancer. The levels of known cancer proteins p53, E2F3 and c-Met, which have previously been verified as targets of miR-34c, decreased upon miR-34c induction over a period of four days.

miR-34c expression was also studied by Q-RT-PCR in a panel of human tissues and was found to be almost exclusive to the male reproductive tract. This was an interesting expression pattern and makes the miRNA potentially targetable. The in vivo studies to confirm this are still ongoing.

miR-15a – miR-16-1

Gross genetic alterations, deletions, gains and amplifications of chromosomal regions were screened for by array comparative genomic hybridisation (aCGH), a method that allows the inspection of the relative copy number of the whole genome at a sufficient resolution to identify single genes. In addition, we used our pre-existing SNP hybridisation data on some samples. We concentrated our efforts on homozygous (both copies) deletions and high level amplifications of miRNAs. Only one gross genetic alteration was found in more than one of the six prostate cancer cell lines and thirty prostate cancer xenografts studied. The locus containing miR-15a and miR-16-1 was homozygously deleted in two of the xenografts. These deletions were confirmed by fluorescence in situ hybridisation (FISH) analysis. It was also noted from the screening that this locus is hemizygously (one copy) deleted in the majority of the samples.

The expression levels of the two miRNAs were studied by Q-RT-PCR and were found to be generally quite high in the samples, but in the cases with the deletion, the expression of miR-15a was abolished to background level. miR-16-1, however, is also transcribed from another locus and therefore the deletion did not affect it's expression as dramatically. The expression levels of the miRNAs were investigated also from the microarray data of 102 clinical samples. There was no significant difference between the expression levels in non-malignant and malignant samples. Since Hemizygous mutations of the miR-15a/miR-16-1 locus are common, mutation analysis by direct sequencing was performed on the cell lines and xenografts, as well as fifty clinical prostate cancers to see whether the remaining allele would be altered as the "second hit" of inactivation. Only one mutation was found and it had arisen in laboratory conditions, which means that mutations in miR-15a are rare or non-existent in clinical prostate tumours. These results have been published by Porkka et al., (2010).

miR-193b

In order to find epigenetically regulated miRNAs in prostate cancer, microarray analysis of prostate cancer cell lines treated with DNA demethylating and histone deacetylation inhibiting agents was performed. Six of the 38 miRNAs with increased expression in any one of the cell lines also had decreased expression in nine clinical prostate cancer cases compared to four BPH samples. The decreased expression in cancer was later shown also in the microarray data set of 108 prostate samples. miR-193b was heavily methylated in 22Rv1 at a CpG island approximately 1 kb upstream of the locus.

Functional studies on 22Rv1 showed that returning the expression of miR-193b to the cell line significantly reduced the growth rate, and that anchorage independent growth was partially inhibited. Although no mutations were detected in the cell lines, and the methylation in the other cell lines and the clinical samples studied was modest at the most, the results indicate that miR-193b is a putative epigenetically silenced tumour suppressor miRNA in prostate cancer. More detailed description of the work performed has been published by Rauhala et al. (2010).

Androgen regulation of miRNAs in AR overexpressing cell lines

Androgen regulation of miRNAs was screened for by performing microarray analysis on androgen receptor overexpressing cell lines LNCaP-ARhi and VCaP after androgen stimulation, as well as thirteen pairs of intact and castrated xenografts. Seventeen miRNAs were more than 1.5 fold up-regulated upon androgen treatment in the cell lines and 42 were were up-regulated in the in the AR positive xenografts after castration. Only four of these miRNAs were shared between the cell lines and xenografts. Of these, miR-141 was overexpressed in prostate cancer clinical samples than in BPH. Functional studies by overexpressing miR-141 in parental LNCaP cells resulted in enhanced growth and conversely, anti-miR-141 expression suppressed the growth of LNCaP-ARhi. These results, which have been published by Waltering et al. (2011), indicate that miR-141 may contribute to the progression of prostate cancer.

miRNA in lymphoblastoid cell lines

We searched for ncRNA that predispose to cancer by studying the miRNA expression in lymphoblastoid cell lines derived from prostate cancer patients and

their healthy family members. The idea was to see whether miRNA profiles in blood leukocytes of diseased and healthy individuals were different. The samples were divided into three categories: healthy, aggressive disease, and “moderate” disease based on the clinical characteristics of the subjects studied. Unsupervised clustering analysis of the microarray hybridisation results placed healthy controls in a different arm of the dendrogram than the diseased cases. The microarray expression levels were validated for five miRNAs with commercial Q-RT-PCR assays.

miRNAs affecting prostate cancer phenotypes

To identify prostate cancer specific miRNAs, functional screens on several high-throughput platforms were carried out. A considerable number of miRNAs were found to affect prostate cancer cell growth, apoptosis, and migration, as well as other phenotypes. The most effective miRNAs were chosen for validation experiments. Emphasis was put on miRNAs that inhibit proliferation in AR positive prostate cancer cell lines, since the proliferation effects were more pronounced in these cells. 26 miRNAs were found to induce apoptosis and 34 miRNAs inhibited proliferation in five AR positive cell lines. 71 unique mature miRNA sequences were found to influence the levels of AR in the five cell lines. 21 of these were selected for validation analyses. All of them decreased AR protein levels in LNCaP and 22Rv1 cells, but only about half of them affected the mRNA levels in both cell lines, suggesting that the main mechanism of AR inhibition is translational. Reporter assays for the 21 miRNAs revealed that thirteen of them, including miR-34c and miR-135b, inhibited the expression of the reporter gene coupled with the predicted target sequence. Most miRNAs (15/21) reduced viability to at least to the same extent as the AR siRNAs when transfected in toMDA-Pca-2b cells. These results, which have been published by Östling & Leivonen et al. (2011) demonstrate that miRNAs binding to the long 3’UTR of the *AR* gene need to be considered as one mechanism for how PCa cells regulate the levels of AR. This provides future opportunities and starting points to explore the applications of miRNAs and their derivatives in PCa therapy.

miRNAs affecting prostate cancer migratory and invasive potential

Efforts in the identification of miRNAs responsible for the migration properties of prostate cancer cell lines were focused on the assessment of individual miRNAs that have been identified as deregulated in prostate cancer in WP1. After an initial screen and cross reference with published data our analyses focused on miR-141 and miR-200c as potential suppressors of migration. Their expression levels were manipulated in the two migratory cell lines DU145 and PC-3 and two non-migratory cell lines VCaP and PC346C.

Overall, the obtained initial results demonstrated that overexpression of miR-200c suppresses the migratory capabilities of DU145 and PC-3 cells, while down-regulation of MiR-141 promotes migration in VCaP cells. By measuring the expression level of *ZEB1*, *ZEB2* and *TGFβ2* in PCa cell lines before the manipulation of miR-141 and miR-200c expression, we showed a converse correlation between the expression level of these genes and migratory ability of cell lines. Consistent with other findings, we also found an inverse correlation between the expression level of these genes and the expression level of miR-141 and miR-200c.

Overexpression of miR-141 and miR-200c in DU145 cells resulted in decreased expression of *ZEB1*, *ZEB2* and *TGFβ2*. However, in PC-3 cells overexpression of

miR-141 resulted in decreased expression of only *TGFβ2*, which can be due to the regulation of those genes at translational level. Taken all together, although miR-141 and miR-200c have almost the same seed sequences, they might bind and repress different target mRNAs. In combination with the likelihood that each PCa cell line has different cascade to regulate migration, miRNAs can have different unique effects on the complex traits of migration.

Potential impact, dissemination, and exploitation

ProspeR has deepened our understanding of the role of ncRNA in prostate cancer development and progression. Novel biomarkers or their combinations have been identified and validated for clinical use. Due to the increasing incidence of prostate cancer and aging European population, these developments will have a significant impact on the society. Although the project concentrated on prostate cancer, it is possible that at least some of the ncRNAs that were implicated in this particular disease are important also for the development of other malignancies. Thus, the project may have wider impact than just for the diagnosis and treatment of prostate cancer.

The project produced results at different levels: 1) scientific publications (eleven original research papers and two reviews already published, seven papers submitted or in preparation, more foreseen); 2) PhD theses (six finished, at least three more coming) and other forms of researcher training (including four Bachelor's theses and six Masters' theses), 3) innovations for diagnostics to be exploited further by industry, 4) diagnostic kits to be directly commercialized, and 5) innovations for drug development to be exploited further by industry.

Dissemination of the research findings

The research findings of ProspeR have (eleven original research papers and two reviews) and will be (seven currently submitted or in preparation and more to follow) published in high quality peer-reviewed scientific journals. Some of the 6-9 PhD theses produced with ProspeR derived data will also publicly available at the respective institutions' web sites. Thus far, almost 70 oral presentations and almost 60 poster presentations including ProspeR derived data have been given at scientific conferences. It is likely that for the next few years the numbers will climb. The audiences at the meetings have mostly comprised of scientists, from PhD students and post-docs to professors and medical doctors. Some presentations have been given at smaller workshops with a few dozen participants and some at large international congresses with thousands of delegates.

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Since prostate cancer is at the moment a hot topic, there is great need for information and therefore the public has to be able to reach the consortium as well. The web site (http://www.uta.fi/ibt/institute/research/visakorpi/prosper.html) that was constructed for data transmission within the consortium also contains an open-access site, not just for researchers and health professionals, but also for the general public.

Societal and humane impact

Since the incidence of prostate cancer has rapidly increased in Western countries and is projected to keep doing so, the impact of the disease to societies and health care systems in these countries has been and will be devastating. Thus, overcoming this humane and social problem is of utmost importance. The increased incidence together with poorly selective biomarkers has led to tremendous pressure on urological clinics in Europe and vast over-treatment of the disease. This has, amongst other things, reduced the possibilities to treat other urological problems, since clinics are fully employed in treatment of prostate cancer. On the other hand, despite the fact that the majority of prostate cancer patients do not died of the disease, prostate cancer is the second most common cancer cause of death after lung cancer in men.

Understanding the molecular mechanisms of the development and progression of prostate cancer forms a basis for the development of new tools for prevention, diagnostics, prognostic evaluation, and treatment of prostate cancer. Identification of

genetic alterations underlying the progression of prostate cancer is the first step towards the development of improved prognostic markers allowing clinicians to tailor the best possible therapy for each patient. Unfortunately, there are currently no good markers that would reliably predict whether clinically organ-confined prostate carcinomas have or will metastasise during the lifetime of the patient and thus result in life threatening disease.

The results of this project have given some clues to answer many of the above mentioned problems. Novel biomarkers (or combinations of biomarkers), such as the diagnostic and prognostic panels, and the miQ, will enable better prognostic evaluation of a patient's tumour. They will also prevent unnecessary treatments that are potentially both harmful for the patients and expensive for the society.

We identified an index quote profile of only four miRNAs, miQ, which is increased in prostate cancer tissues and shows potential as an objective tool for prostate cancer diagnosis and prognosis. Our findings suggest that miQ could represent an alternative or complementary clinical test for several aspects of prostate cancer management. Our finding that ncRNA can also be measured reliably from easily extractable biological material, such as blood and urine, and that analysis of their expression levels may be able to distinguish healthy men from patients with prostate cancer, could also reduce the need for invasive biopsy procedures. Again, this would not only have a direct impact on the quality of life of men who may or may not have prostate cancer, but also on the public health care systems across Europe.

Unfortunately, there have been no major advances in development of new therapies for advanced prostate cancer for decades. The gold-standard treatment against advanced prostate cancer, androgen withdrawal, is more than half a century old, and although new drugs that target the androgen pathway have been developed, the benefit in added lifetime has been modest. Thus, there is an urgent need for novel targets for therapies. ProspeR has miR-32 as such a target. Another promising new target for therapy is miR-34c, which targets multiple cancer related genes.

Impact on researcher training

The objectives of ProspeR were translational, covering aspects of prostate cancer from molecular mechanisms to clinics. Therefore, ProspeR formed a strong platform for education. Six PhD theses including data derived from the project have already been completed (three in Tampere, two in Sheffield, and one in Malmö), and at least three more (one from Tampere, Malmö, and Turku each) will be finished within the next two years. In addition, four Bachelor's theses (Erasmus MC) and six Master's theses (five from Erasmus MC and one from Tampere) have been produced with ProspeR data.

Similarly to PhD, Master's and Bachelor's training, ProspeR provided a good platform for post-doctoral training. Numerous post-doctoral scientists contributed to ProspeR as supervisors of theses and as researchers themselves. In addition, several undergraduates (first and second year students) have taken their laboratory placements in the ProspeR labs, done ProspeR related research and written essays/reports towards their degrees.

An added value in training in ProspeR was the involvement of industry, which exposed researchers trained in academic surroundings to issues related to exploitation of the research findings, including IPR and R&D (drug development *etc*.) in the pharmaceutical field.

ProspeR has provided its students with skills in modern molecular genetics, molecular biology, statistics, bioinformatics, array technologies, a wide range of analyses using clinical sample material, and in utilisation of patient materials in general, drug design and development, and quality control. In addition to the typical intradisciplinary training (*i.e.* either biomedical or clinical), ProspeR provided an excellent platform for laboratory training for resident physicians in urology and on the other hand for biologists to learn about clinical questions. Such translational education is very important since the treatment of cancer, including prostate cancer, will be increasingly based on molecular mechanisms. For some of the younger students ProspeR has also provided their first opportunity to present their reseach results either orally or in poster format, and the Annual project meetings have enabled networking with scientists, insluding the senior ones, from the partner institutions.

Impact on European industry and technology

The world market in prostate cancer diagnostics is huge. Since PSA or other markers do not reveal the behaviour of a given tumour, novel biomarkers that would reliably identify early prostate cancer requiring active treatment would be vastly profitable. So far, there are no ncRNA targeting therapies available, and the drugability of ncRNAs is not known. ProspeR may have a direct impact on both diagnostic and pharmaceutical industry. Novel diagnostic tools and proof-of-principle level concepts for treatment targets have been produced, although they are not ready for the market yet.

Since the ncRNA field is new, analytical methods to investigate and measure ncRNAs are incomplete. ProspeR results can provide new tools for ncRNA research that will help the field in general. This would also give a competition advantage for European research. The diagnostic and therapeutic tools to be developed in the wake of the project may also turn out ot be useful in the diagnosis and treatment of other malignancies enhancing the impact on pharmaceutical industry even further.

One of the ProspeR consortium members, Exiqon A/S, is a leading European SME in the field of miRNA research as a leading supplier of high-value gene expression analysis products for the life sciences, research and drug discovery industries. Exiqon's rapidly growing product offerings integrate innovative chemistries with web-based software tools to help scientists achieve rapid and reliable results. Exiqon is a market leader in the miRNA research products field and is currently developing novel miRNA-based diagnostic tools for diagnosis and improved treatment selection in cancer. This project strenghtens Exiqon’s R&D towards clinical diagnostic kits. Exiqon’s products are already widely used in academia, biotechnological industry and pharmaceutical indistry. It has a long history of collaboration with pharmaceutical and diagnostic companies in promoting the use of miRNAs as biomarkers. This should facilitate the line of translation and this project could have impact on promoting European biotechnological and pharmaceutical industry also more broadly in the field of cancer diagnostics via ncRNA research. The ncRNA field is relatively new, and

thus there are not many companies involved in the field. Therefore, European industry has a good chance to become World leaders within this field. This is a very good opportunity especially for SMEs working in diagnostic and novel therapy strategies.

Exiqon A/S has a close contact with customers in the life science field and knowledge of the issues that are relevant to life science researchers. The company has its own sales and marketing organisation in the U.S. as well as R&D and production facilities in Denmark. It is therefore very well prepared for the exploitation, development, production and marketing of any research products resulting from the ProspeR application. Furthermore, Exiqon A/S holds proprietary techniques for miRNA detection which are being applied in development of miRNA-based diagnostics for cancer and therefore clinical developments which are relevant for diagnostic applications and which fit within Exiqon's strategic focus can easily be transitioned into commercial exploitation.

Orion Corporation, on the other hand, is a globally operating pharmaceutical company, which develops, manufactures and markets pharmaceuticals and active pharmaceutical ingredients for global markets. It is about the 70th largest pharmaceutical company in the world, medium sized in Europe. The core therapy areas of the company's product and research strategy are diseases of the central nervous system, cardiovascular diseases and critical care, as well as well as hormonal and urological therapies. Orion has a large R&D unit with the personnel of ~700 people. The company's research expenditure in 2011 was about 10% of its turnover. Thus, Orion has an excellent capability to exploit further any putative drug targets identified by ProspeR. Discovery research at Orion has already yielded *e.g.* many potential androgen antagonists. One of these has entered clinical trials in 2011 and another's pre-clinical trials have been completed. The company has a strong interest in exploitation of any novel drug targets for treatment of prostate cancer.

An important strategic point at Orion is partnering. Partnering with other companies and research entitites allows for more efficient use of resources and mutually benefits both parties. Partnering may also be seen as a shortcut to other market areas, when the collaborator is either an expert in a given field or located on another continent.

The discoveriees produced in this project will naturally first be exploited by the ProspeR partners Exiqon A/S and Orion Corporation. SMEs or small bigger companies, are the likely first choice beneficiaries of developments from projects such as ProspeR. As more flexible than truly big market leaders, SMEs and small-big pharma will have better possibilities to take advantage of the innovation in this novel field. However, it is likely that other European companies will be needed to fully utilise and commercialise the innovations. Thus, ProspeR will have industrial impact beyond Exiqon and Orion.

We discovered a set of novel microRNAs by next generation sequencing of prostate cancer specimens. Exiqon has designed locked nucleic acid (LNA)-based capture probes for a set of these novel microRNAs and has offered these to the research community as an integral part of the company's microarray product platform. The LNA-based Q-RT-PCR assays developed for snoRNA detection may also become

useful for other researchers outside the consortium, especially if the importance of snoRNA in cancer is validated.

Over the years, Exiqon has made a virtue of offering microarray products with a wider coverage than publicly available databases such as miRBASE. This wider offering, termed the miRPLUS program, has been an important part of the competitive advantage that Exiqon microRNA microarrays have enjoyed over recent years. The validity of the miRPLUS program is underscored by the inclusion of a large number of miRPLUS sequences in recent miRBASE updates, and thanks to sequencing efforts such as the one described here in the Prosper project, Exiqon customers have been able to access this larger microRNAs diversity space prior to miRBASE inclusion.

Website and contact details

Further information may be obtained from Professor Tapio Visakorpi, (tapio.visakorpi@uta.fi) or Dr. Outi Saramäki (outi.saramaki@uta.fi). The project website can be found at http://www.uta.fi/ibt/institute/research/visakorpi/prosper.html.

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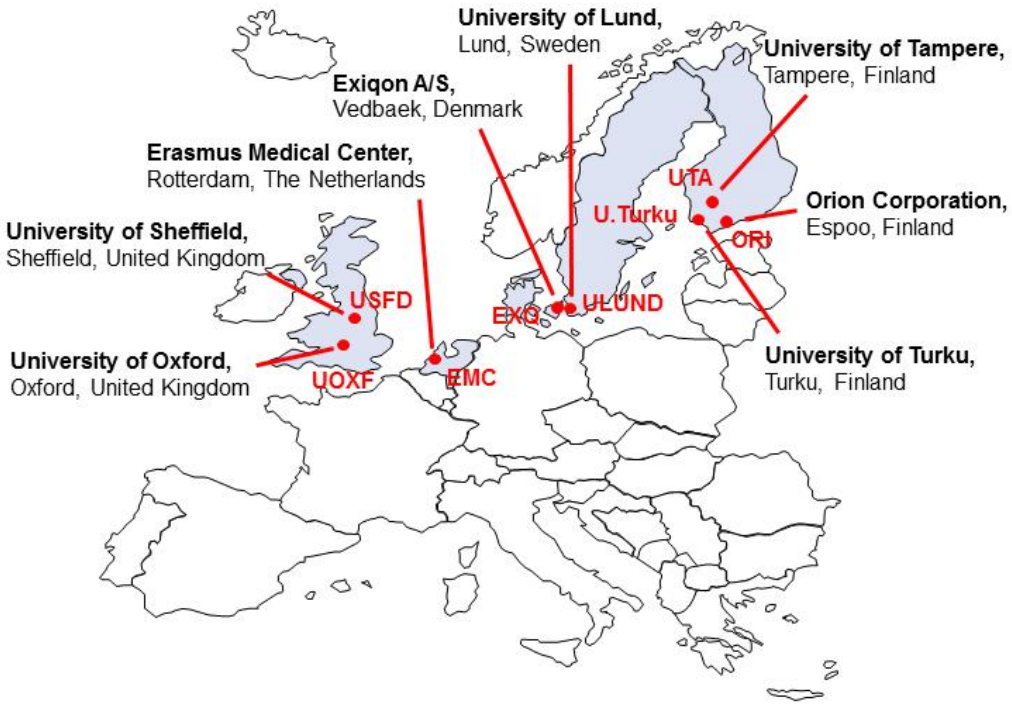

Map of ProspeR consortium