DOES EXPRESSION OF MIR-32 AFFECT PROLIFERATION ACTIVITY OF PROSTATE CELLS?

Susanna Haapanen
Syventävien opintojen kirjallinen työ
Tampereen yliopisto
Lääketieteen ja biotieteiden tiedekunta
Marraskuu 2017

University of Tampere
Faculty of Medicine and Life Sciences (MED)
Prostate Cancer Research Center (PCRC)
Molecular Biology of Prostate Cancer group (MBPCG)
HAAPANEN SUSANNA: DOES EXPRESSION OF MIR-32 AFFECT PROLIFERATION ACTIVITY OF PROSTATE CELLS?
Written raport, X pp.
Supervisors: dean, professor Tapio Visakorpi M.D., Ph.D. and docent Leena Latonen, Ph.D.
November 2017
Keywords: prostate cancer, micro-RNA, Ki-67

Prostate cancer is the second commonest cancer among men in the developed countries. The incidence has been in a steep rise since 1980s, partly due to better diagnostic methods, for instance the discovery of tumor indicating markers such as PSA. However, there has been no such marker to separate an indolent cancer from an aggressive form at the time of the diagnosis.

Many tumor-promoting factors have been found, one of which is micro-RNA 32 (miR-32) that has been associated with reduced apoptosis of cancer cells. Now, in this study, we explored whether miR-32 affects the proliferation activity of the prostate cancer cells, as well.

We had four different genotypes of murine prostates: wild type, transgenic miR-32, transgenic hiMyc and the combination of transgenic miR-32 and hiMyc. The prostate sample slides were HE stained and IHC stained. In IHC staining, anti-Ki-67 antibody was used to indicate proliferation activity and the results were analyzed by counting the positive and negative cells. As a result, we discovered that there was no statistically significant difference between miR-32 positive and negative sample groups. Nevertheless, this may be caused by our small sample count (18 pc) as a recent research has indicated the proliferation increasing effect of miR-32.

Tampereen yliopisto
Lääketieteen ja biotieteiden tiedekunta (MED)
Eturauhassyövän tutkimuskeskus (PCRC)
Eturauhassyövän molekyylibiologian ryhmä (MBPCG)
HAAPANEN SUSANNA: VAIKUTTAAKO MIR-32 ETURAUHASSYÖVÄN SOLUJEN PROLIFERAATIOAKTIIVISUUTEEN?
Kirjallinen työ, X s.
Ohjaajat: dekaani, professori Tapio Visakorpi ja dosentti Leena Latonen
Marraskuu 2017
Avainsanat: eturauhassyöpä, micro-RNA, Ki-67

Eturauhassyöpä on miesten toiseksi yleisin syöpä kehittyneissä maissa. Eturauhassyövän ilmaantuvuus on ollut jyrkässä kasvussa 1980-luvulta lähtien osin parantuneen diagnostisten menetelmien ja merkkiaineiden, kuten PSA:n, löytämisen ansiosta. Kuitenkaan yksikään keino ei ole kyennyt erottelemaan, mikä löydetty syöpä on latentti eikä aiheuta ongelmia miehen loppuelämän aikana ja mikä on aggressiivinen nopeasti kasvava ja leviävä.

Monia merkkiaineita on löydetty ja tutkittu niiden roolia eturauhassyövän kehittymisessä, etenemisessä sekä eri hoitokeinojen vaikuttavuudessa. Yksi näistä on mikro-RNA 32 (miR-32), joka on lyhyt proteiinia koodaamaton nukleotidijuoste. miR-32 on yhdistetty muun muassa syöpäsolujen vähentyneeseen apoptoosiin.

Tässä työssä on selvitetty, onko miR-32:lla vaikutusta syöpäsolujen proliferaatioaktiivisuuteen *in vivo* -hiirimallissa. Työssä käytettiin genotyypiltään neljänlaisia hiirten eturauhasia: villi tyyppi, transgeeninen miR-32, transgeeninen hiMyc sekä transgeeninen miR-32 ja hiMyc yhdessä. Eturauhasleikkeille tehtiin HE-värjäyksiä, ja näistä saatujen tulosten perusteella valittiin leikkeet IHC-värjäykseen. IHC-värjäyksessä käytettiin anti-Ki-67-vasta-ainetta osoittamaan jakautumisaktiiviset solut ja vastavärinä käytettiin hematoksyliiniä.

IHC-värjäyksen tuloksista laskettiin solumäärät, ja analyysin lopputuloksena saatiin, ettei miR-32 vaikuttanut solujen jakautumisaktiivisuuteen tilastollisesti merkitsevästi. Tässä näytesarjassa kuitenkin eturauhasnäytteiden alhainen määrä (18 kpl) saattoi vaikuttaa, etteivät mahdolliset erot tulleet esille. Hiljattain julkaistut tulokset miR-32-transgeenisillä hiirillä ovat osoittaneet, että miR-32 tosiaankin lisää eturauhasen rauhasepiteelin proliferaatioaktiivisuutta osoittaen olettamani liian pienen näytemäärän vaikutuksen syyksi tilastollisesti merkitsemättömille tuloksillemme.

PREFACE

This study is part of my studies in Licentiate Degree Programme in Medicine.

I had no previous experience in working in a research laboratory. Thus, I am most grateful to dean Tapio Visakorpi who was brave to give me the chance to do my Syventävä opinnäytetyö in his research group.

I would like to express my great gratitude to Päivi Martikainen, Marja Pirinen and Sari Toivola who acted as my guides to the secrets of tissue stainings and laboratory work.

I would also say a special thanks to my supervisor, docent Leena Latonen, Ph.D. She was always there to support me when needed and she patiently and clearly answered all my questions.

Tampere, November 19, 2017

Susanna Haapanen

TABLE OF CONTENTS

FIGURES AND TABLES

ABBREVIATIONS AND TERMS

ADT	androgen deprivation therapy
AR	androgen receptor
BMI	body mass index
BPH	benign prostate hyperplasia
BRCA1 and *2*	tumor suppressor genes that can have inherited mutations exposing to hereditary cancers, for example breast, ovarian and prostate cancer
BTG2	B-cell translocation gene 2
CG	coagulating gland aka anterior lobe/prostate
CRPC	castration resistant prostate cancer
CT	computed tomography
DHT	dihydrotestosterone
DP	dorsal lobe/prostate
FGF	fibroblast growth factor
HE	hematoxylin & eosin
HIFU	high intensity focused ultrasound
HGPIN	high grade PIN
HRE	hormone-response element
IHC	immunohistochemical
MRI	magnetic resonance imaging
LGPIN	low grade PIN
LP	lateral lobe/prostate
PCa	prostate cancer
PAP	prostatic acid phosphatase
PIN	prostatic intraepithelial neoplasia
PSA	prostate specific antigen
SD	standard deviation
PTEN	phosphatase and tensin homologue
VP	ventral lobe/prostate
Wnt	a signaling pathway
WT	wild type

1. INTRODUCTION

Prostate is the largest accessory sex gland and its diseases are a great cause of morbidity. Prostate cancer is one of the commonest health issues concerning prostate in addition to benign prostate hyperplasia and inflammation (Finnish Cancer Registry 2017, Kumar, Abbas & Aster 2015). Over 4,500 new cases are diagnosed in Finland every year so it affects many men (Finnish Cancer Registry 2017). However, its portion of the general mortality is relatively low compared to morbidity due to the old age of affected men and the diversity of the cancer (Finnish Cancer Registry 2017). Prostate cancer includes the whole spectrum from indolent, non-treatment-requiring cancer to aggressive metastatic form (Barbieri *et al*. 2013).

The risk factors of prostate cancer are not well known (Cassidy *et al*. 2006). Age is considered as the most important one and also hereditary factors have been considered. However, only about 5-10% of PCa cases have been connected to certain genetic mutations, for instance *BRCA1* and *2* (Cassidy *et al*. 2006).

2. BACKGROUND

Prostate is a tubuloalveolar accessory sex gland of the male reproductive system (Ross, Pawlina 2016). It has an important role in reproduction, but it is also the second commonest place for a cancer in men (Damber, Aus 2008).

2.1 Prostate

Prostate is located around the urethra under the urinary bladder (Ross, Pawlina 2016). Pubic symphysis is in front of the prostate and the posterior surface of the prostate is in contact with rectum (Drake *et al*. 2015). That is why the size of prostate can be palpated during rectal examination (Drake *et al*. 2015). The size of prostate can vary between men in correlation with their body size and age (Fowke *et al*. 2006). Usually older men have larger prostates than younger. In addition, the body mass index (BMI) associates with the size of prostate: the higher BMI the larger prostate (Fowke *et al*. 2006). The approximate weight of an adult prostate is 20 g (Joensuu *et al*. 2013, Kumar, Abbas & Aster 2015).

2.1.1 Structure

Human prostate

Prostate contains 30 to 40 individual glands which all have their own ducts (Drake *et al*. 2015). These ducts empty the contents of the glands into the prostatic sinuses which are enlargements on the posterior surface of the urethra (Drake *et al*. 2015). The glands are composed of a lumen which is surrounded by a bilayer of cells (Kumar, Abbas & Aster 2015). The basal layer is formed by low cuboidal cells and the second layer consisting of columnar cells is on top of the cuboidal cell layer (Kumar, Abbas & Aster 2015). The columnar cells are also called luminal cells (Shen, Abate-Shen 2010). They are polarized and have secretory function as they provide the secretion into the lumen of the gland. Luminal cells possess specific markers, for instance cytokeratins 8 and 18, which differentiate them from other cells. Cuboidal cells are basal cells which can be distinguished from other cell types by the expression of p63 and cytoceratins 5 and 14. There is also a third epithelial cell type: neuroendocrine cells. They are rare and their function is not yet established. Furthermore, the cells can be distinguished from each other by the expression of androgen receptor (AR); luminal cells express AR in excessive amounts but neither basal nor neuroendocrine cells do. (Shen, Abate-Shen 2010) Individual glands are separated from each other with fibromuscular stroma. (Kumar, Abbas & Aster 2015)

Prostate can be divided in different units anatomically and histologically. These zones are *central zone, peripheral zone, transitional zone* and *periurethral zone* (Drake *et al*. 2015, Kumar, Abbas & Aster 2015, Ross, Pawlina 2016).

Central zone contains approximately a quarter of the parenchyma (Ross, Pawlina 2016). It is located around the ejaculatory ducts and is unlikely to have inflammation or carcinoma. It differs histologically from other parts of prostate because the cells in this zone have more cytoplasm and their nuclei are larger. (Ross, Pawlina 2016)

Peripheral zone surrounds the central zone and constitutes 70% of the parenchyma (Ross, Pawlina 2016). Peripheral zone is the part of prostate which can be palpated during rectal examination. It is also the most common site for both carcinoma and inflammation. (Ross, Pawlina 2016)

Transitional zone contains only about 5% of prostate (Ross, Pawlina 2016). It surrounds the prostatic part of urethra. Transitional zone is the most common location for *benign prostatic hyperplasia (BPH)* because it is the site where the cells can start to divide vastly. Because of the location of the hyperplasia especially elderly men are more prone to have difficulties in urination. The glands in this zone are mucosal. (Ross, Pawlina 2016)

Periurethral zone is also near the urethra and it is the site for late BPH (Ross, Pawlina 2016). The hyperplasia of this zone complicates the difficulties in urination. It contains both mucosal and submucosal glands. Also, pathologic growth is possible mainly during hyperplasia. (Ross, Pawlina 2016)

The outermost layer of prostate is an anterior *fibromuscular stroma* which can contract during ejaculation to aid the secretion to flow into the urethra (Kumar, Abbas & Aster 2015, Ross, Pawlina 2016). The fibromuscular stroma is mostly dense irregular connective tissue and smooth muscle (Kumar, Abbas & Aster 2015, Ross, Pawlina 2016).

Murine prostate

Mouse has been used as a model organism in PCa for decades (Bhagavathi A. Narayanan *et al*. 2004, Foster *et al*. 1997, Y Sugimura, G R Cunha & A A Donjacour 1986). Also in this study, mouse was used as a model organism. However, murine prostate does differ from human prostate in some extent (Foster *et al*. 1997, Y Sugimura, G R Cunha & A A Donjacour 1986). The murine prostate is multilobular and its four lobes are more separated from each other than human prostate zones (Scudamore 2014). The lobes are *anterior, lateral, dorsal* and *ventral lobes*. The lobes are sometimes even referred as prostates to emphasize their separation from each other. In addition, their histological patterns are different, as dorsal lobe (DP) has small-diameter acini with extensive folding, on the contrary to large-diameter and poorly folded acini in lateral lobe (LP) and variable sized acini in ventral lobe (VP). Anterior lobe is also called coagulating gland (CG) and it consists

of a single papillary layer of epithelial cells (Scudamore 2014). Nonetheless, the basic functions are the same in mouse and human prostate as is their dependence on androgens (Y Sugimura, G R Cunha & A A Donjacour 1986).

2.1.2 Function

The main function of the prostate is to produce slightly alkaline fluid to semen (John E. Hall 2016, Ross, Pawlina 2016). The amount of protein is low in the secretion. However, prostate secretes many enzymes, for instance *fibrinolysin, prostatic acid phosphatase (PAP)* and *prostate-specific antigen (PSA),* as well as calcium, phosphate ion and citric acid. (John E. Hall 2016, Ross, Pawlina 2016)

Fibrinolysin mainly has only one purpose: to keep semen liquid, whereas, *PAP* has more voluminous operating field (Ross, Pawlina 2016). PAP acts as a cell growth and metabolism regulating enzyme of prostate and it can be used as a marker in PCa, especially in the metastatic form PAP-levels tend to rise. *PSA* is a serine protease which is mainly secreted into semen. (Ross, Pawlina 2016) PSA is a member of the tissue kallikrein family, and human kallikrein 3 (hK3), another name for PSA, indicates this connection (John R. Prensner *et al*., Steven P. Balk, Yoo-Joung Ko & Glenn J. Bubley 2003). PSA gene is situated on chromosome 19q13.4 (Steven P. Balk, Yoo-Joung Ko & Glenn J. Bubley 2003) and its transcription is induced by binding of AR (Ross, Pawlina 2016). PSA has a function in cleaving seminogelins to make semen more liquid (Steven P. Balk, Yoo-Joung Ko & Glenn J. Bubley 2003). In addition to its physiological function, PSA has a major therapeutic function as a tumor marker if its amount in blood is increased. PSA is also found in sweat glands, breast and placenta, for instance, but the function in other sites than prostate remain unclear. (Steven P. Balk, Yoo-Joung Ko & Glenn J. Bubley 2003)

The secretion of prostate is important in ejaculation (John E. Hall 2016). About 30% of semen is secreted by prostate (Ross, Pawlina 2016). Alkalinity may be important for the motility and fertility of the sperm because fluid from the vas deferens is acidic to keep sperm inactivated (John E. Hall 2016). Sperm activation requires pH 6.0 to 6.5 in contrast to the pH of 3.5 to 4.0 in the fluid from vas deferens. Therefore, the secretion from prostate also acts as a neutralizing factor. (John E. Hall 2016)

With the facilitation of 5α-reductase, prostate also converts testosterone to dihydrotestosterone (DHT) which is 30 to 50 times more potent androgen than testosterone (Boron, Boulpaep 2012).

2.1.3 Development

The prostate develops from pelvic urethra as endodermal evaginations which became surrounded by mesenchyme (Sadler, Langman 2012). The differentiation of human prostate

starts at the 10th week of fetal development and the secretory activity begins by the 13th to 15th weeks (Larsen, Schoenwolf 2009). Glandular epithelium differentiates from the endoderm and fibromuscular stroma in between the glands differentiates from the mesenchyme (Larsen, Schoenwolf 2009).

Many signals are required for a successful development of the prostate (Shen, Abate-Shen 2010). Most of these signals affect the interaction between mesenchyme and epithelium. For example, Wnt, fibroblast growth factor (FGF) and Hedgehog are crucial for organogenesis of prostate tissue. Wnt has been connected to ductal development as FGF is essential for evagination. In addition, Hedgehog signaling pathway has been associated with both ductal development and evagination. Moreover, the development of the glandular structure is induced by the conversion of testosterone to DHT in the mesenchyme. The epithelium does not have ARs but the mesenchyme has and it channels effect of testosterone and DHT to the epithelium. (Shen, Abate-Shen 2010)

DHT is essential for development of mesenchyme but not the epithelium (Ross, Pawlina 2016, Shen, Abate-Shen 2010). However, it is crucial for differentiation and secretory function development of the epithelium. In addition, DHT maintains growth and function of normal prostate tissue. (Ross, Pawlina 2016, Shen, Abate-Shen 2010) DHT binds to AR which is in cytosol (Ross, Pawlina 2016, Isbarn *et al*. 2009). The DHT-AR-complex functions as a homodimer in the nucleus by binding to areas in DNA called hormone-response elements (HREs). Therefore, the transcriptions of genes in question are stimulated or inhibited. (Ross, Pawlina 2016, Isbarn *et al*. 2009)

2.2 Prostate cancer

Prostate cancer (PCa) is the second commonest cancer among men and the third commonest cancer-related cause of death in developed countries (Damber, Aus 2008). It has been estimated that about 161,360 new cases of PCa will be diagnosed and approximately 26,730 men will die of it in the United States in 2017 (American Cancer Society 2017). PCa is mostly diagnosed with men over 65 years old and lifetime risk of PCa is approximately 14% (American Cancer Society 2017). The incidence of PCa was 4,595 and PCa mortality was 856 in Finland in 2014 (Finnish Cancer Registry 2017). The incidence has been rising steeply since late 1980s (***Figure 1***.).

The commonest type of PCa is prostate adenocarcinoma (American Cancer Society 2017) and it is the type to which is referred in this study when discussed about PCa. Other forms of PCas are small-cell carcinoma, sarcomas, colloid carcinoma of the prostate, ductal adenocarcinoma and mesenchymal tumors (American Cancer Society 2017, Kumar, Abbas & Aster 2015). Also, secondary cancer is possible, and as the most common is urothelial cancer which spreads to prostate (Kumar, Abbas & Aster 2015).

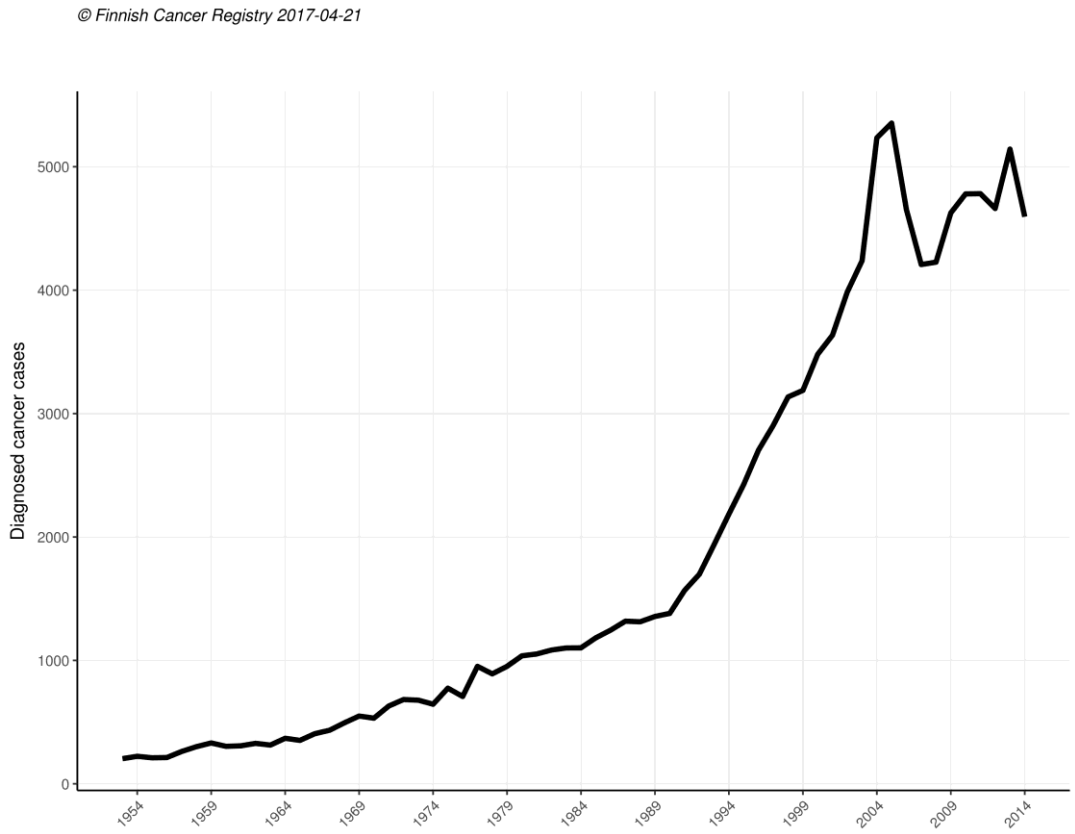

Figure 1. The number of diagnosed PCa cases annually. The steep rise in late 1980s was caused by discovering the PSA testing. The peak around the year 2004 was caused by generalized PSA screening. Data is from Finnish Cancer Registry (2017).

2.2.1 Development of cancer

Although PCa has been known as a disease for centuries, treatment options have been developed only during the past 100 years (Shen, Abate-Shen 2010). Usually, the development of prostate cancer starts with *prostatic intraepithelial neoplasia (PIN)* which continues to develop into *local PCa* and eventually metastatic *castration-resistant prostate cancer (CRPC)* (Abdel-Khalek, El-Baz, Ibrahiem 2004, Shen, Abate-Shen 2010).

PIN has two forms: low-grade PIN (LGPIN) and *high-grade PIN (HGPIN)* (Paavonen 2017). LGPIN is clinically considered insignificant and usually is not even written in pathologist's report (Paavonen 2017), but HGPIN has a significant risk to develop into PCa during the following 10 years (Bostwick 2000, Shen, Abate-Shen 2010). Fundamental difference between HGPIN and PCa is that the former has basal cells though their number is reduced but the latter does not have distinctive basal cells left (Abrahams *et al*. 2003). Many samples taken from prostate represent both HGPIN as well as PCa (Abdel-Khalek, El-Baz & Ibrahiem 2004). After development of local PCa, some cancers stay indolent but others progress and invade surrounding tissues and/or metastasize to other organs (Barbieri *et al.* 2013). The distinction between indolent and aggressive forms is not possible yet at the time of the diagnosis (John R. Prensner *et al.*) but some genetic

changes have been connected to aggressive form (Barbieri *et al*. 2013). For instance, retinoblastoma protein is usually inactivated in CRPC but not in the local PCa (Barbieri *et al.* 2013).

The commonest site for metastases are local lymph nodes (Shen, Abate-Shen 2010). Distant metastases usually spread through vascular system to bone. Bone tropism is especially common in PCa and the effect of cancer is generally osteoblastic changes. Other common sites for metastases are lung, liver and pleura. (Shen, Abate-Shen 2010)

2.2.2 Screening, diagnosis and treatment

PSA testing from blood has changed the diagnosing of PCa radically because PCa changes the structure of prostate so that PSA leaks into blood in excessive amounts (John R. Prensner *et al*., Ross, Pawlina 2016). PSA screening is not a direct indication of PCa because it can be elevated also in BPH, in inflammation and due to a trauma (John R. Prensner *et al*., Ross, Pawlina 2016). Furthermore, it does not differentiate whether the cancer is indolent or aggressive (Ross, Pawlina 2016). Both PSA-levels and the size of prostate is associated with the body mass index (BMI): the higher BMI, the higher PSA-levels, and the larger prostate, therefore standard points for a normal finding is difficult to determine (Fowke *et al*. 2006). In general, total PSA 4 ng/ml has been used as a threshold for normal result, although one third of PCas have PSA<4 ng/ml (Steven P. Balk, Yoo-Joung Ko & Glenn J. Bubley 2003). A better indication of PCa is the ratio of free and total PSA in blood. If this ratio is >25% when total PSA is elevated (4-10 ng/ml which is considered as “gray zone”), the risk of PCa is significantly lower than if ratio is <10% with the same total PSA value. (Steven P. Balk, Yoo-Joung Ko & Glenn J. Bubley 2003, John R. Prensner *et al.*) Even though using PSA as diagnostic marker is complex, it is useful in following a response to treatment or detecting a recurrence of the cancer (Steven P. Balk, Yoo-Joung Ko & Glenn J. Bubley 2003).

Biopsy is usually taken when PSAlevels are high and then the tissue sample is histopathologically graded using *Gleason scoring* (Damber, Aus 2008, Epstein 2010, Ross, Pawlina 2016, Shen, Abate-Shen 2010). Gleason score grades the tumors from 2 to 10, where 2 is most differentiated and 10 is least. The score is a sum of two commonest histological patterns of the sample which are graded from 1 to 5 (most and least differentiated, respectively) and then summed together. (Damber, Aus 2008, Epstein 2010, Ross, Pawlina 2016, Shen, Abate-Shen 2010) The result is presented as two numbers and their sum. The first number represents grade pattern which is the commonest tissue pattern in the sample and the second number the least differentiated component or the second commonest pattern. (Sandeman *et al*. 2016)

Digital rectal examination is always performed to examine the size of prostate and ultrasound, magnetic resonance imaging (MRI) and computed tomography (CT) are also used

to determine the size and possible invasion to other organs (Damber, Aus 2008, Joensuu *et al.* 2013). Bone metastases can be searched with bone scan (Bill-Axelson *et al.* 2015, Damber, Aus 2008, Joensuu *et al*. 2013). None of these is still able to determine whether PCa needs to be treated or if "watchful waiting" is the best option for maintaining the quality of life (Shen, Abate-Shen 2010). In localized low risk (Gleason <7) PCa, it has been noted that watchful waiting has the equal mortality as prostatectomy or radiation but quality of life (measured with urinary incontinence and sexual function, for instance) remain better with observation compared to radical treatment options (Barocas *et al.* 2017).

Radical prostatectomy has been performed only for little over 100 years (Shen, Abate-Shen 2010). It has been shown to reduce the PCa related deaths and risk of metastases in contrast to watchful waiting during the first 10 years after surgery especially patients younger than 65 years (Bill-Axelson *et al*. 2008). However, after 10 years from the surgery there is no evidence of the benefit of prostatectomy in local PCa (Bill-Axelson *et al*. 2008). In addition, overall mortality does not reduce significantly with radical prostatectomy in contrast to watchful waiting in local PCa (Bill-Axelson *et al*. 2015). As a substitute to radical prostatectomy, *radiation* can be used (Shen, Abate-Shen 2010). It can be performed as either external beam or implantation of radioactive pieces to the prostate (Shen, Abate-Shen 2010).

There are some experimental treatments as well. One of them is *high intensity focused ultrasound (HIFU)* (Blanco Sequeiros *et al*. 2017). HIFU has been successfully used to treat leiomyomas but there is constant research on its benefits for treating diseases of prostate, bone and brains. There have been trials on treating PCa with HIFU but the outcomes have been versatile. Thus, future research will show the opportunities of HIFU for treating PCa. (Blanco Sequeiros *et al*. 2017)

The androgen deprivation therapy (ADT) has been performed from the 1940s and it can be combined with prostatectomy or radiation (Shen, Abate-Shen 2010). ADT is especially combined to the treatment if metastases or recurrence is detected (Bill-Axelson *et al*. 2015). However, none of these have been showed to be enough for curing the disease and in the aggressive forms of PCa the cancer shall relapse eventually (John R. Prensner *et al*., Shen, Abate-Shen 2010). PCa is depended on androgens and in androgen deprivation therapy this dependency is utilized (Damber, Aus 2008). Physiological concentration of testosterone already saturates ARs so an increase in testosterone concentration to supraphysiologic levels does not increase the volume of prostate or the risk for PCa in healthy men (Isbarn *et al*. 2009, Morgentaler, Traish 2009). However, in PCa, and especially in CRPC, the amount of ARs has amplified through various genetic changes and signaling pathway stays active despite ADT (Jalava *et al*. 2012). Hence, the castration resistance usually develops within 2 years from the beginning of ADT (Damber, Aus 2008).

2.2.3 MicroRNAs and cancer

Micro-RNAs (miRNA) are small about 19-25 nucleotides long noncoding RNAs which are part of the posttranscriptional regulation of messenger-RNAs (mRNA) (Ambs *et al*. 2008, Wang *et al*. 2015, Wu *et al*. 2013). Hence, they have an influence on apoptosis, cell cycle and androgen receptor pathway, for instance (Ambs *et al*. 2008, Wang *et al*. 2015, Wu *et al*. 2013). The dysfunction of miRNAs can cause problems in tissue as they regulate the mRNAs. In prostate, the dysfunction of miRNAs is associated with the development pathway of PCa from PIN through local PCa to metastatic CRPC (Lo, Yang & Hsieh 2013, Shen, Abate-Shen 2010).

Especially in CRPC, miRNAs are expressed in a manner which differs from the normal prostate tissue (Jalava *et al*. 2012). For example, the expression of miR-573 is suppressed in metastatic PCa compared with the localized form (Wang *et al.* 2015). miRNAs can also act as tumor suppressors and as in the case of miR-340 it is underexpressed in PCa (HUANG *et al*. 2016). The use of miRNAs as prognostic markers has also been suggested (Xu *et al*. 2015). For instance, miR-129 is expressed in peripheral blood mononuclear cells and its amount is decreased in PCa. It has also tumor suppressive features, and it may be a target for therapeutic action in the future. (Xu *et al*. 2015) miRNAs do have tumor-promoting members, too (Jalava *et al*. 2012, Shen *et al.* 2016, Wu *et al*. 2013). One example of these is miR-32 which has been associated with decreased amount of apoptosis in cultured cells improving the survival of tumor cells (Jalava *et al*. 2012).

Micro-RNA 32 (miR-32) is a 22 nucleotides long miRNA which is located on the long arm of chromosome 9, exactly at 9q31.3 (Fang, Gao 2014). miR-32 is androgen regulated (Jalava *et al.* 2012). Yet, the overexpression of miR-32 has been connected to CRPC and some of the samples showed moderate overexpression in PCa, too. miR-32 has been connected to the underexpression of *BTG2* in CRPC although the role of other mechanisms could not be eliminated. (Jalava *et al*. 2012) *BTG2* is a tumor suppressor and its down-regulation has been associated to breast cancer, PCa and laryngeal cancer, for example (Jalava *et al*. 2012, Liu *et al*. 2009, Rouault *et al*. 1996). miR-32 also has an influence on the phosphatase and tensin homologue (PTEN) which is known as a tumor suppressor, at least in colorectal carcinoma (Wu *et al*. 2013). miR-32 decreases the expression of PTEN, and, thus, the tumor may become more aggressive and invasive (Wu *et al*. 2013). Although it has been shown that miR-32 suppresses apoptosis there, is no evidence whether it affects the proliferation activity of PCa cells (Jalava *et al.* 2012)

2.3 Objective

The aim of this research is to discover if the expression of miR-32 affects the proliferation activity of prostate cancer cells *in vivo*.

3. MATERIALS AND METHODS

3.1 Mouse models and sample preparation

In this study, 18 prostates from mice (age of six months) with four different genotypes which are wild type (WT), transgenic miR-32, transgenic hiMyc and the combination of transgenic miR-32 and hiMyc were used. miR-32 is the investigated miRNA and hiMyc is added into the genotype to provoke PCa (Ellwood-Yen *et al*. 2003).

Whole prostates of mice were embedded into paraffin to make a paraffin block. Prostate samples were cut in a way that three slices were on a same glass and every tenth cut was segregated to RNA analysis. The samples were attached to glasses by heating them in 60˚C for two hours. From the sample glasses, every third was separated into *hematoxylin & eosin staining (HE staining)* and the rest were left for *immunohistochemical staining (IHC staining).*

3.2 Staining

First, HE staining was executed. Second, based on tissue histology visible on the HE staining, there were samples selected for IHC staining. IHC staining was executed with anti-Ki-67 antibody. Both HE and IHC stained sample glasses were imaged with slide scanner microscope after staining and coverslipping.

3.2.1 HE staining

HE staining is the basic staining in histology (Cardiff, Miller & Munn 2014, Fischer *et al.* 2008). Hematoxylin is not strictly a basic dye but its properties are alike with basic dyes. Therefore, it stains nuclei purple or even black-blue. Eosin is acidic dye and thus stains protein components, for example collagen and elastic fibers, pink and usually cytoplasm is stained with different degrees of pink. (Cardiff, Miller & Munn 2014, Fischer *et al.* 2008)

Samples were stained with KEDEE KD-RS3 Slide Stainer. The HE staining protocol is in Appendix 1.

3.2.2 Immunohistochemical staining

Ki-67 is a proliferation marker (Questdiagnosis 2017). There are only small amounts of Ki-67 in the nucleus of non-proliferating cells, but large amounts in proliferating cells (Questdiagnosis 2017). The large amount of Ki-67 in the nucleus of a cancer cell is associated with poorer prognosis of the cancer (Hamilton *et al*. 2012). Thus, ICH staining with anti-Ki-67 antibody shows the Ki-67 positive and negative nuclei and consequently, shows the most actively proliferating cells.

IHC staining was executed with rabbit (SP6) anti-Ki-67 antibody (Leica Biosystems) and counterstained with hematoxylin. Ki-67 positive nuclei appear brown whereas negative ones are stained blue. The staining procedure was automated with a LAB VISION Autostainer 480 and the staining protocol is in detail in Appendix 2.

3.3 Quantitative analysis

The analysis from the proliferation activity is executed as quantitative analysis. Stained sample slides were whole slide imaged. Then, snapshots were taken of regions of interest (ROI) which were analyzed with Java-based image processing program called ImageJ. The Ki-67 positive nuclei were calculated from images and compared to hematoxylin counterstain for total nuclear count. Based on these results, the proliferation activity could be estimated.

Means of Ki-67 positivity for each different genotype sample group were calculated as well as standard deviation. These means were compared to each other with two-tailed t-tests using Microsoft Office Excel 2016.

3.4 Ethical aspects

All animal experimentation and care procedures were carried out in accordance with guidelines and regulations of the national Animal Experiment Board of Finland, and were approved by the board of laboratory animal work of the State Provincial Offices of South Finland (licence number ESAVI/5147/04.10.07/2015)

4. RESULTS

The Ki-67 positive and Ki-67 negative cells were counted from each ROI by eye. At least total of 500 cells from each ROI were counted. There are large differences in Ki-67 positivity percentages, at least between hiMyc positive and hiMyc negative samples as expected. There are also some differences in Ki-67 positivity within a sample group (***Table 1.***). In addition, samples with transgenic hiMyc outnumber the ones without as samples without hiMyc are used only as controls. WT samples without transgenic hiMyc expression represent normal epithelium as oncogenic hiMyc-expressing represent cancerous tumors.

Table 1 The sample specific Ki-67 positive and negative cell counts as well as Ki-67 positivity percentage for each sample are shown.

	miR-32- , hiMyc-			miR-32+, hiMyc-			miR-32-, hiMyc+						miR-32+, hiMyc+					
	221	223	1387	43	20	235	1392	25	42	21	224	1390	212	213	214	253	252	1391
Ki-67 + cells	46	69	37	155	5	87	779	831	1126	1040	1312	621	1830	1601	879	1548	1257	1859
Ki-67 – cells	1306	1204	1252	1253	617	1278	1138	363	805	932	978	730	1897	1580	1064	997	754	1407
Total cell count	1352	1273	1289	1408	622	1365	1917	1194	1931	1972	2290	1351	3727	3181	1943	2545	2011	3266
Ki-67 + percentage (%)	3.4	5.4	2.9	11.0	0.8	6.4	40.6	69.6	58.3	52.7	57.3	46.0	49.1	50.3	45.2	60.8	62.5	56.9

Pictures from IHC staining samples are shown in ***Figure 2***. As predicted, hiMyc negative samples (***Fig. 2 A, B, E and F***) have fairly low number of Ki-67 positive cells on the contrary to hiMyc positive samples (***Fig. 2 C, D, G and H***) that are more Ki-67 positive as well as has a larger cell count in general. When looking at the pictures, it seems that there might be slightly more Ki-67 positive cells in miR-32 positive samples than in miR-32 negative samples.

Therefore, mean and standard deviation (SD) of Ki-67 positivity percentage for each sample group was calculated with Excel. The calculated values were: WT 3.9 % (SD: 1,3 %), transgenic miR-32 6.1 % (SD: 5.1 %), transgenic hiMyc 54.1 % (SD: 10.2 %) and the combination of transgenic miR-32 and transgenic hiMyc 54.2 % (SD: 7.0 %) Ki-67 positivity in IHC in the studied tissues is shown in ***Figure 3***.

As can be seen in ***Figure 3.***, it appears that miR-32 might have an effect to the proliferation of the cells. Therefore, there were done testing between different sample groups with two-sample t-tests and the results from these tests are presented in ***Table 2***. However, as shown in the table, there were no statistical difference between miR-32 negative and miR-32 positive sample groups. This indicates that miR-32 does not affect the proliferation activity of PCa cells. ***Table 2 C*** was used as a control to demonstrate the cancer provoking function of hiMyc.

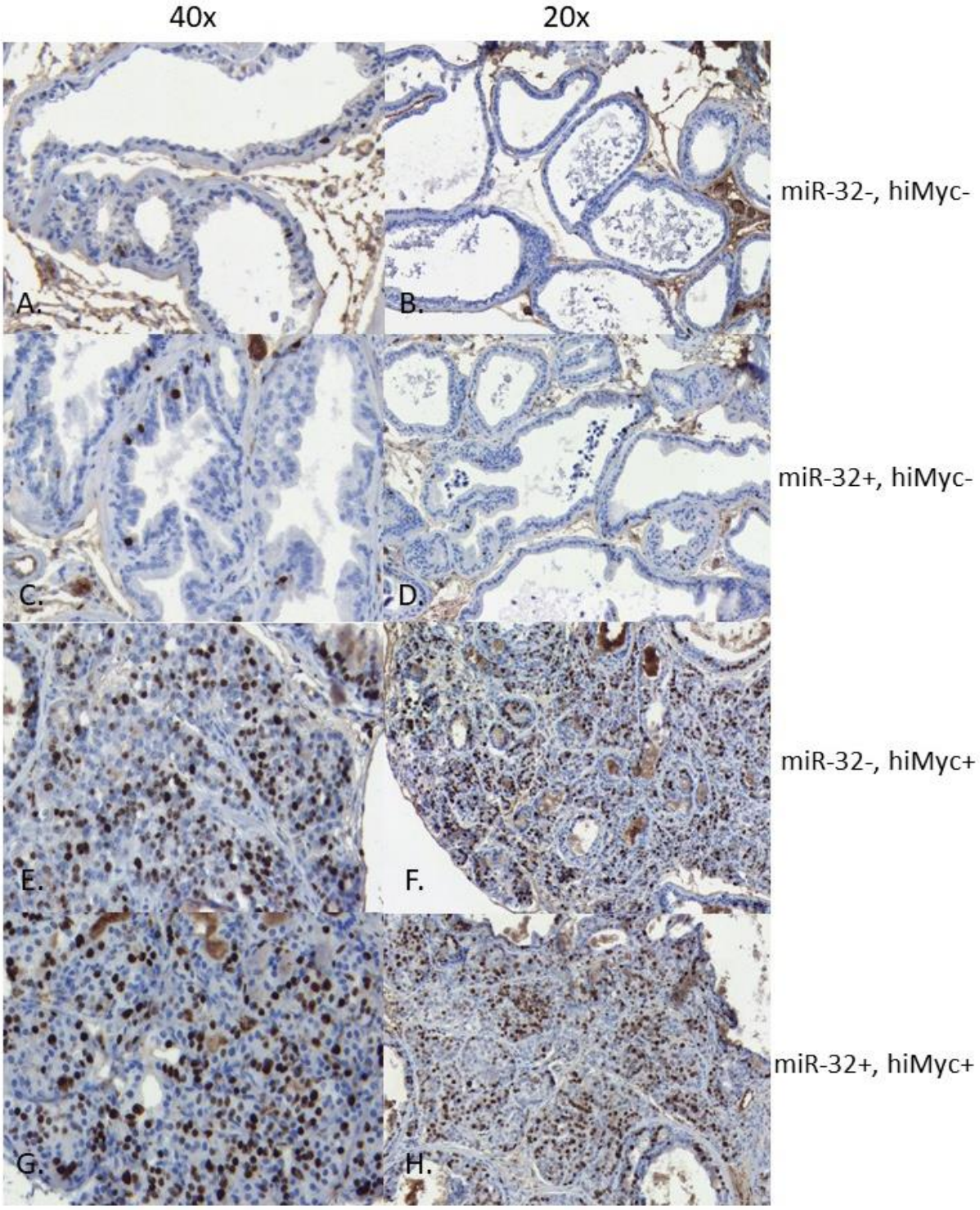

Figure 2. IHC stainings of the samples. 40x magnification and 20x magnification are shown for detailed tissue structure and the general view of the tissue, respectively.

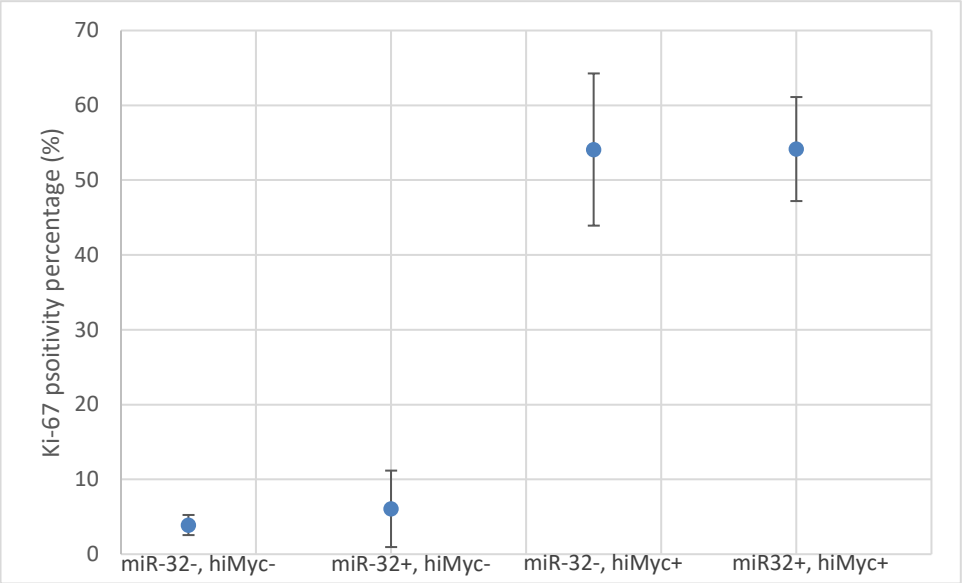

Figure 3. The means and SDs of Ki-67 positivity percentage of different sample groups are presented in a comparable form. The difference between transgenic hiMyc and without hiMyc is clear.

Table 2. There are three separate tables from three separate t-tests. Table A. compares transgenic miR-32 to WT. Table B. compares transgenic hiMyc without miR-32 to the combination of transgenic hiMyc and transgenic miR-32. Table C. is used as a control to express the cancer provoking function of hiMyc.

A. hiMyc -

	miR-32 -	miR-32 +	P-value (two-tailed)
Mean	3.9	6.1	0.517

B. hiMyc +

	miR-32 -	miR-32 +	P-value (two-tailed)
Mean	54.1	54.2	0.990

C. miR-32 -

	hiMyc -	hiMyc +	P-value (two-tailed)
Mean	3.9	54.1	<0.001

5. DISCUSSION

In this study, we investigated whether miR-32 affects the proliferation activity of PCa cells. It had already been shown to affect the apoptosis activity *in vitro* (Jalava *et al*. 2012). Unfortunately, the results from this study did not confirm an association between proliferation of the cells and the presence miR-32 as the results were statistically insignificant. We only had 18 mice so, perhaps, the sample number was not large enough. Thus, a larger number of samples might have emphasized the differences better than our number of samples.

Jalava *et al*. showed that miR-32 could be affecting the emergence of CRPC as well as miR-148a (Jalava *et al*. 2012). They stated that miR-32 affects cells by reducing their apoptosis with a mechanism that remained partly unclear. At least, they found a putative target gene *BTG2* (B-cell translocation gene 2). *BTG2* suppresses cell proliferation through unclear mechanisms (Karve, Rosen 2012) and is downregulated by miR-32 (Jalava *et al*. 2012). In addition, miR-32 was excessively overexpressed in CRPC. In our study, we investigated the effect of miR-32 to proliferation activity of the PCa cells. Jalava *et al*. indicated that miR-148a affects the proliferation activity but they did not know the same effect for miR-32 (Jalava *et al*. 2012). A recent research shows that expression of miR-32 increases the proliferation activity of prostate epithelium (Latonen *et al.* 2017). Therefore, the proposal that miR-32 might affect the proliferation from our study is true although the results were not statistically significant.

The mouse model could be considered more reliable as it is *in vivo* model on the contrary to the *in vitro* model of LNCaP cells of Jalava *et al*. (Jalava *et al*. 2012). Mouse model can emphasize PCa better in its natural environment than cell line model because, in mouse, the PCa is interacting with other parts of the organism. In cell line model, the humoral and other signals that may have an effect to the cells are absent but, then in cell culture, the conditions are more controlled and the causality can be established more reliably than *in vivo* models. Additionally, LNCaP cells are human cells so their physiology is the same as with other human cells in comparison to mouse cells that do have at least a few differences to human cells.

Nevertheless, there were many strengths in this study. First, the Ki-67 positive and negative nuclei were counted by eye which could be a weakness, too, because it is not an objective method as some application or software would have been. However, a software might not be able to deal with artefacts in the images appropriately and it could change the cell count whereas the counting person can assess which spot is a nucleus and which is not. Although, everyone has a little different how they see and evaluate colors which could have caused bias especially with the nuclei that are not distinctly either blue or

brown but somewhere in the between. Nonetheless, this can be controlled by using the same person to count all the pictures and after analyzing the last one, counting the first ROI again and checking whether the cell counts change. It was done in this study with the result of no change in the cell count. In addition, two people do the counting separately and their results are combined to decrease the possibility of a subjective error.

Automated staining is a strength, too. A staining machine or an autostainer does the same staining program the same way every time. Thus, human error can be minimized especially when conducting large batches of samples with the same staining program. We used automated staining for both HE and IHC staining to minimize human error. In addition, as I had no previous experience in doing stainings, I was well familiarized by the laboratory technicians. During working I learned the procedures and, eventually, I was able to perform the staining by myself.

BIBLIOGRAPHY

Abdel-Khalek, M., El-Baz, M. & Ibrahiem, E. 2004, "Predictors of prostate cancer on extended biopsy in patients with high-grade prostatic intraepithelial neoplasia: a multivariate analysis model", *BJU International*, vol. 94, no. 4, pp. 528-533.

Abrahams, N.A., Bostwick, D.G., Ormsby, A.H., Qian, J. & Brainard, J.A. 2003, "Distinguishing atrophy and high-grade prostatic intraepithelial neoplasia from prostatic adenocarcinoma with and without previous adjuvant hormone therapy with the aid of cytokeratin 5/6", *American journal of clinical pathology*, vol. 120, no. 3, pp. 368-376.

Ambs, S., Prueitt, R.L., Yi, M., Hudson, R.S., Howe, T.M., Petrocca, F., Wallace, T.A., Liu, C., Volinia, S., Calin, G.A., Yfantis, H.G., Stephens, R.M. & Croce, C.M. 2008, "Genomic profiling of microRNA and mRNA reveals deregulated microRNA expression in prostate cancer", *Cancer research*, vol. 68, no. 15, pp. 6162.

American Cancer Society. 2016. Prostate Cancer. cited 31.10.2016. updated 11.3.2016

Barbieri, C.E., Bangma, C.H., Bjartell, A., Catto, J.W.F., Culig, Z., Grönberg, H., Luo, J., Visakorpi, T. & Rubin, M.A. 2013, "The mutational landscape of prostate cancer", *European urology*, vol. 64, no. 4, pp. 567-576.

Barocas, D.A., Alvarez, J., Resnick, M.J., Koyama, T., Hoffman, K.E., Tyson, M.D., Conwill, R., McCollum, D., Cooperberg, M.R., Goodman, M., Greenfield, S., Hamilton, A.S., Hashibe, M., Kaplan, S.H., Paddock, L.E., Stroup, A.M., Wu, X. & Penson, D.F. 2017, "Association Between Radiation Therapy, Surgery, or Observation for Localized Prostate Cancer and Patient-Reported Outcomes After 3 Years", *JAMA*, vol. 317, no. 11, pp. 1126-1140.

Bhagavathi A. Narayanan, Narayanan K. Narayanan, Brian Pittman & Bandaru S. Reddy 2004, "Regression of Mouse Prostatic Intraepithelial Neoplasia by Nonsteroidal Anti-inflammatory Drugs in the Transgenic Adenocarcinoma Mouse Prostate Model", *Clinical Cancer Research*, vol. 10, no. 22, pp. 7727-7737.

Bill-Axelson, A., Holmberg, L., Filén, F., Ruutu, M., Garmo, H., Busch, C., Nordling, S., Häggman, M., Andersson, S., Bratell, S., Spångberg, A., Palmgren, J., Adami, H. & Johansson, J. 2008, "Radical Prostatectomy Versus Watchful Waiting in Localized Prostate Cancer: the Scandinavian Prostate Cancer Group-4 Randomized Trial", *JNCI: Journal of the National Cancer Institute*, vol. 100, no. 16, pp. 1144-1154.

Bill-Axelson, A., Holmberg, L., Johansson, J., Norlén, B.J., Adami, H., Palmgren, J., Garmo, H., Nordling, S., Busch, C., Spångberg, A., Bratell, S., Andersson, S., Häggman, M. & Ruutu, M. 2015, "Radical Prostatectomy versus Watchful Waiting in Early Prostate Cancer", *The New England Journal of Medicine,* vol. 352, no. 19, pp. 1977-1984.

Blanco Sequeiros, R., Joronen, K., Komar, G. & Koskinen, S.K. 2017, "Suurienergiainen kohdennettu ultraääni (HIFU) kasvainten hoidossa", vol. 2, pp. 143-9.

Boron, W.F. & Boulpaep, E.L. 2012, *Medical physiology,* Updated 2. ed. edn, Saunders, Elsevier, Philadelphia, Pa.

Bostwick, D.G. 2000, "Prostatic intraepithelial neoplasia", *Current Urology Reports,* vol. 1, no. 1, pp. 65-70.

Cardiff, R.D., Miller, C.H. & Munn, R.J. 2014, "Manual Hematoxylin and Eosin Staining of Mouse Tissue Sections", *Cold Spring Harbor Protocols,* vol. 2014, no. 6, pp. pdb.prot073411.

Cassidy, J., Bissett, D., Spence, R.A.J. & Payne, M. 2006, *Oxford handbook of oncology,* Second edition edn, Oxford University Press, Oxford.

Damber, J. & Aus, G. 2008, "Prostate cancer", *The Lancet,* vol. 371, no. 9625, pp. 1710-1721.

Drake, R.L., Vogl, W., Mitchell, A.W.M. & Gray, H. 2015, *Gray's anatomy for students,* 3. ed. edn, Churchill Livingstone Elsevier, Philadelphia, Pa. [u.a.].

Ellwood-Yen, K., Graeber, T.G., Wongvipat, J., Iruela-Arispe, M.L., Zhang, J., Matusik, R., Thomas, G.V. & Sawyers, C.L. 2003, "Myc-driven murine prostate cancer shares molecular features with human prostate tumors", *Cancer Cell,* vol. 4, no. 3, pp. 223-238.

Fang, Y.-. & Gao, W.-. 2014, "Roles of microRNAs during prostatic tumorigenesis and tumor progression", *Oncogene,* vol. 33, no. 2, pp. 135-147.

Finnish Cancer Registry. 2017. Prostate Cancer. cited 20.4.2017.

Fischer, A.H., Jacobson, K.A., Rose, J. & Zeller, R. 2008, "Hematoxylin and Eosin Staining of Tissue and Cell Sections", *Cold Spring Harbor Protocols,* vol. 2008, no. 5, pp. pdb.prot4986.

Foster, B.A., Gingrich, J.R., Kwon, E.D., Madias, C. & Greenberg, N.M. 1997, "Characterization of Prostatic Epithelial Cell Lines Derived from Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) Model", *Cancer Research,* vol. 57, no. 16, pp. 3325.

Fowke, J.H., Motley, S.S., Cookson, M.S., Concepcion, R., Chang, S.S., Wills, M.L. & Smith, J.A. 2006, "The association between body size, prostate volume and prostate-specific antigen", *Prostate Cancer and Prostatic Diseases,* vol. 10, no. 2, pp. 137-142.

Hamilton, N.A., Liu, T., Cavatiao, A., Mawad, K., Chen, L., Strasberg, S.S., Linehan, D.C. & Cao, D. 2012, "Ki-67 Predicts Disease Recurrence and Poor Prognosis in Pancreatic Neuroendocrine Tumors", *Surgery,* vol. 152, no. 1, pp. 107-113.

HUANG, K., TANG, Y., HE, L. & DAI, Y. 2016, "MicroRNA-340 inhibits prostate cancer cell proliferation and metastasis by targeting the MDM2-p53 pathway", *Oncology Reports,* vol. 35, no. 2, pp. 887-895.

Isbarn, H., Pinthus, J.H., Marks, L.S., Montorsi, F., Morales, A., Morgentaler, A. & Schulman, C. 2009, "Testosterone and Prostate Cancer: Revisiting Old Paradigms", *European Urology,* vol. 56, no. 1, pp. 48-56.

Jalava, S.E., Urbanucci, A., Latonen, L., Waltering, K.K., Sahu, B., Jaenne, O.A., Seppaelae, J., Laehdesmaeki, H., Tammela, T.L.J. & Visakorpi, T. 2012, "Androgen-regulated miR-32 targets BTG2 and is overexpressed in castration-resistant prostate cancer", *Oncogene,* vol. 31, no. 41, pp. 4460-4471.

Joensuu, H., Roberts, P.J., Kellokumpu-Lehtinen, P., Jyrkkiö, S., Kouri, M. & Teppo, L. 2013, *Syöpätaudit,* 5th edn, Duodecim.

John E. Hall 2016, *Guyton and Hall Textbook of Medical Physiology,* Thirteenth edition. edn, Saunders, US.

John R. Prensner, Mark A. Rubin, John T. Wei & Arul M. Chinnaiyan *Beyond PSA: The Next Generation of Prostate Cancer Biomarkers*.

Karve, T.M. & Rosen, E.M. 2012, "B-cell translocation gene 2 (BTG2) stimulates cellular antioxidant defenses through the antioxidant transcription factor NFE2L2 in human mammary epithelial cells", *The Journal of biological chemistry,* vol. 287, no. 37, pp. 31503-31514.

Kumar, V., Abbas, A.K. & Aster, J.C. 2015, *Robbins and Cotran Pathologic Basis of Disease,* 9th edn, Elsevier Saunders.

Larsen, W.J. & Schoenwolf, G.C. 2009, *Larsen's human embryology*, 4., rev. ed. edn, Elsevier Churchill Livingstone, Philadelphia, Pa.

Latonen, L., Scaravilli, M., Gillen, A., Hartikainen, S., Zhang, F., Ruusuvuori, P., Kujala, P., Poutanen, M. & Visakorpi, T. 2017, "In Vivo Expression of miR-32 Induces Proliferation in Prostate Epithelium", The American Journal of Pathology, vol. 187, no. 11, pp. 2546-2557.

Liu, M., Wu, H., Liu, T., Li, Y., Wang, F., Wan, H., Li, X. & Tang, H. 2009, "Regulation of the cell cycle gene, BTG2, by miR-21 in human laryngeal carcinoma", *Cell Research*, vol. 19, no. 7, pp. 828-837.

Lo, U., Yang, D. & Hsieh, J. 2013, "The role of microRNAs in prostate cancer progression", *Translational Andrology and Urology*, vol. 2, no. 3, pp. 228-241.

Morgentaler, A. & Traish, A.M. 2009, "Shifting the Paradigm of Testosterone and Prostate Cancer: The Saturation Model and the Limits of Androgen-Dependent Growth", *European Urology*, vol. 55, no. 2, pp. 310-321.

Paavonen, Timo. Tumor pathology I-IV -lecture series (Kasvainpatologiaa I-IV -luentosarja). Spring 2017.

Questdiagnosis. 2017. Ki-67, IHC with interpretation. cited 8.1.2017. reviewed 12/2016

Ross, M.H. & Pawlina, W. 2016, *Histology*, Seventh edition, international edition edn, Wolters Kluwer, Philadelphia.

Rouault, J., Falette, N., Guéhenneux, F., Guillot, C., Rimokh, R., Wang, Q., Berthet, C., Moyret-Lalle, C., Savatier, P., Pain, B., Shaw, P., Berger, R., Samarut, J., Magaud, J., Ozturk, M., Samarut, C. & Puisieux, A. 1996, "Identification of BTG2, an antiproliferative p53-dependent component of the DNA damage cellular response pathway", *Nature Genetics*, vol. 14, pp. 482-486.

Sadler, T.W. & Langman, J. 2012, *Langman's medical embryology*, 12. ed. edn, Wolters Kluwer, Lippincott Williams & Wilkins, Philadelphia, Pa. [u.a.].

Sandeman, K., Rannikko, A., Kenttämies, A. & Mirtti, T. 2016, "Eturauhassyövän diagnostiikka murroksessa", *Duodecim*, vol. 24, pp. 2351-8.

Scudamore, C.L. 2014, *A Practical Guide to the Histology of the Mouse*, 1st edn, Wiley-Blackwell, GB.

Shen, J., Siegel, A.B., Remotti, H., Wang, Q. & Santella, R.M. 2016, "Identifying microRNA panels specifically associated with hepatocellular carcinoma and its different etiologies", *Hepatoma Research*, vol. 2, pp. 151-162.

Shen, M.M. & Abate-Shen, C. 2010, "Molecular genetics of prostate cancer: new prospects for old challenges", *Genes & Development,* vol. 24, no. 18, pp. 1967-2000.

Steven P. Balk, Yoo-Joung Ko & Glenn J. Bubley 2003, "Biology of Prostate-Specific Antigen", *Journal of Clinical Oncology,* vol. 21, no. 2, pp. 383-391.

Wang, L., Song, G., Tan, W., Qi, M., Zhang, L., Chan, J., Yu, J., Han, J. & Han, B. 2015, "miR-573 inhibits prostate cancer metastasis by regulating epithelial-mesenchymal transition", *Oncotarget,* vol. 6, no. 34, pp. 35978.

Wu, W., Yang, J., Feng, X., Wang, H., Ye, S., Yang, P., Tan, W., Wei, G. & Zhou, Y. 2013, "MicroRNA-32 (miR-32) regulates phosphatase and tensin homologue (PTEN) expression and promotes growth, migration, and invasion in colorectal carcinoma cells", *Molecular Cancer,* vol. 12, pp. 30.

Xu, S., Yi, X., Zhou, W., Cheng, W., Ge, J. & Zhang, Z. 2015, "Downregulation of miR-129 in peripheral blood mononuclear cells is a diagnostic and prognostic biomarker in prostate cancer", *International Journal of Clinical and Experimental Pathology,* vol. 8, no. 11, pp. 14335.

Y Sugimura, G R Cunha & A A Donjacour 1986, "Morphological and histological study of castration-induced degeneration and androgen-induced regeneration in the mouse prostate", *Biology of Reproduction,* vol. 34, no. 5, pp. 973.

APPENDIX 1. HE STAINING PROTOCOL

Table 3. *HE staining program performed with KEDEE KD-RS3 Slide Stainer.*

STEP	**SUBSTANCE**	**DURATION**
STEP 01	Hexane	3 min
STEP 02	Hexane	3 min
STEP 03	ABS	2 min
STEP 04	ABS	2 min
STEP 05	94% Ethanol	2 min
STEP 06	70% Ethanol	1 min
STEP 07	dH_2O	30 s
STEP 08	Hematoxylin	4 min
STEP 09	H_2O	5 min
STEP 10	dH_2O	40 s
STEP 11	Eosin	30 s
STEP 12	H_2O	1 min
STEP 13	dH_2O	30 s
STEP 14	94% Ethanol	2 min
STEP 15	ABS	2 min
STEP 16	ABS	2 min
STEP 17	ABS	2 min

Staining was performed with KEDEE KD-RS3 Slide Stainer (***Table 3.***). After the staining, the slides were placed in xylene for three minutes. Then, coverslips were applied with Dako Coverslipper, with a DPX mounting medium. When the solutions had vaporized, the glasses were ready for imaging with slide scanner microscope.

APPENDIX 2. ANTI-KI-67 IHC STAINING PROTOCOL

The used antibody was SP6, a rabbit anti-Ki67 antibody (Leica Biosystems).

Sample glasses were deparaffinised by submerging in n-hexane twice for 3 minutes, then 99.9% EtOH twice for two minutes. Then they were air dried. Using a Lab Vision^{TM} PT Module, we preheated Tris-EDTA buffer containing 0.05% Tween-20 d to 65°C and the added the slides in. The slides were buffered to 98°C for 15min followed by cooling back to 65°C. We removed the slides and stored them in 1x Tris-Buffered Saline (TBS) -Tween solution until staining to prevent them from drying out.

Staining was performed with a LAB VISION Autostainer 480. The program is presented in ***Table 4.***

Table 4. *IHC staining program executed with a LAB VISION Autostainer 480.*

Step	**Solution / Reagent**	**Time**
1	TBS-Tween	Wash
2	**Blocking Agent** - 3% H_2O_2	5 Min
3	TBS-Tween	Wash
4	**1º Antibody** – SP6 1:100 dilution (Leica Biosystems)	30 min
5	TBS-Tween	Wash x2
6	**2º Antibody –** N-Histofine® Simple Stain MAX PO (Multi) (Nichirei Biosciences)	30 min
7	TBS-Tween	Wash
8	TBS-Tween	Wash

9	**Reaction Substrate** - ImmPact DAB Peroxidase Substrate (Vector Laboratories)	5 min
10	TBS-Tween	Wash x2
11	TBS-Tween	Wash
12	**Auxiliary Stain** - Mayer's Haematoxylin	2 min
13	TBS-Tween	Wash

First after the staining, the samples were transferred to dH_20 for 2 min. Second, they were dehydrated by immersing the slides in a series of 70% EtOH – 96% EtOH – 99.9% EtOH for 2min each. Third, the slides were submersed in xylene twice for two minutes, to prepare slides for coverslipping. Coverslipping was performed with Dako Coverslipper, with a DPX mounting medium. After the fumes have vaporized, the slides were imaged with slide scanner microscope.