IMMUNOHISTOCHEMICAL ANALYSIS OF UNC13B AND UBAP2 IN PROSTATE CANCER

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Kirjallinen työ, 18 s.

Ohjaajat: Professori Tapio Visakorpi ja FT Leena Latonen

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Avainsanat: geenimonistuma, immunohistokemia, karsinogeneesi, proteiiniekspressio

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Eturauhassyöpä on miesten yleisin syöpä Euroopassa, poislukien ihosyövät. Syövän geneettisiä mekanismeja tunnetaan puutteellisesti. Geneettisen materiaalin monistumia ja deleetioita on tunnistettu useita. Tässä tutkimuksessa selvitettiin proteiinien UNC13B ja UBAP2 ilmentymistä eturauhassyöpänäytteissä, sekä niiden mahdollista yhteyttä kliinispatologisiin muuttujiin.

Tutkittavat proteiinit värjättiin vasta-aineilla immunohistokemiallisesti. Yhteensä 477 prostatektomia-, sekä 230 hormoniriippumatonta eturauhassyöpänäytettä sisällytettiin analyysiin. Näytteet analysoitiin manuaalisesti virtuaalimikroskoopilla ja luokiteltiin neljään luokkaan värjäyksen intensiteetin mukaan.

Intensiteettiä verrattiin PSA:han, Gleason-luokkaan, kuolleisuuteen, diagnoosi-ikään, TNM-luokitukseen, Ki-67:n ekspressioon sekä androgeenireseptorin ekspressioon. UNC13B:n ekspressio oli merkittävästi suurempi hormoniriippumattomissa näytteissä. Molempien proteiinien ekspressio kasvoi prostatektomianäytteissä yhdessä Ki-67:n ja androgeenireseptorin ekspression kanssa.

Tulosten perusteella vaikuttaisi siltä, että UNC13B ja UBAP2-proteiinit ovat yhteydessä eturauhassyövän kehittymiseen. Niiden yhteyttä kliinispatologisiin muuttujiin ei kuitenkaan voitu osoittaa. Lisäksi näiden proteiinien vahva korrelaatio Ki-67:ään voisi auttaa ennakoimaan eturauhassyövän uusiutumista, mutta tämä tarvitsee lisätutkimuksia.

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Keywords: genetic amplification, immunohistochemistry, carcinogenesis, protein expression

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Prostate cancer is the most common non-skin cancer in men in Europe. Only a few genetic mechanisms of prostate cancer are known. Some amplifications and deletions of genetic material have been recognized. This study's purpose was to investigate protein expression of UNC13B and UBAP2 in prostate cancer specimen and their possible association to clincopathological variables.

Target proteins were immunohistochemically stained. A total of 477 primary prostatectomy samples and 230 hormone-refractory samples were included in the analysis. Specimens were analyzed manually with digital microscope. They were divided into 4 groups according to their staining intensity.

Intensity level scores were compared to PSA-levels, Gleason scores, progression free survival, survival, age at diagnosis, tumors T-stage, Ki-67 expression and androgen receptor expression. Expression of UNC13B was significantly higher in hormone-refractory samples. Expression of protein Ki-67 and androgen receptor increased when scoring intensities of UNC13B and UBAP2 increased in prostatectomy samples.

Findings suggest that expression of UNC13B and UBAP2 increase during prostate cancer development. However, associations between expression levels of these proteins and clinicopathological variables were not found. Strong correlation between Ki-67 and these two proteins in prostatectomy samples could mean that UNC13B and UBAP2 would predict cancer recurrence but this needs further research.

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1. INTRODUCTION

The molecular and genetic mechanisms of prostate cancer are still insufficiently defined. Prostate cancer, like other cancers, is formed when the cellular mechanisms of controlling proliferation and/or apoptosis break down. This is thought to happen partly at chromosomal level, where deletions, gains, amplifications or translocations of the genes result in their deregulation, that affect the previously mentioned cellular mechanisms (Saramaki, 2006). Prostate cancer cells usually have somatic genome alterations and some of them are genetic (alterations in DNA sequence) and some epigenetic (DNA sequence unaltered). Of gene copy number alterations, both gains and deletions are common in prostate cancer. For better understanding of carcinogenesis of prostate cancer, these chromosomal alterations should be further studied to identify the tumor suppressor genes and oncogenes driving the cancer (Damber & Aus, 2008; Gurel et al., 2008).

Researchers have access to a number of different xenografts, transplantations of human prostate cancer tissue to a different species, which are used to study the biology of prostate cancer. Using high resolution array-comparative genomic hybridization (aCGH), a novel amplification at chromosome 9p13.3 was recently identified in prostate cancer xenograft LuCaP35 (Saramaki et al., 2006).

Further research continued to study the amplification in 9p13.3 in prostate cancer xenografts and cell lines, aiming to identify genes whose amplified copy number increases their expression level. In clinical patient material, amplification of 9p13.3 area was slightly more common in hormone refractory prostate cancer specimens than in untreated prostatectomy tumors. A number of possible target genes were found, including UNC13B and UBAP2 (Leinonen, 2007).

UNC13B is a cytosolic diacylglycerol -binding protein that belongs to the UNC13/munc13 family of proteins. It is involved in vesicle maturation during exocytosis as part of neurotransmitter release. Hyperglycemia is known to up-regulate UNC13B. Nevertheless, relevance of UNC13B in both epithelial cells and prostate cancer cells remains unknown. UBAP2 (ubiquitin associated protein 2) has a UBA domain and its function is also unknown (National Center for Biotechnology Information,).

The purpose of this study was to analyze UNC13B and UBAP2 protein expression levels in clinical prostatectomy and hormone-refractory tumor samples. One aim was also to discover possible associations between protein expression levels and clinical and pathological parameters. Ultimate goal

is, eventually, to determine if these two genes are potential oncogenes. This research project was conducted in Molecular Biology of Prostate Cancer –Group at University of Tampere. Supervision was performed by Professor Tapio Visakorpi and Leena Latonen, PhD. Collecting and analyzing the data were done during winter 2012-2013.

2. REVIEW OF THE LITERATURE

The data for this literature review was collected from Ovid MEDLINE(R) Daily Update - and Ovid MEDLINE(R) In-Process & Other Non-Indexed Citations and Ovid MEDLINE(R) 1946 to Present -databases in June 2013.

2.1. Prostate cancer

In Europe, prostate cancer is the most common non-skin cancer in men with an estimated 382,000 new cases in 2008. It is also the third most common cause of death from cancer in men with almost 90,000 deaths. Incidence has been increasing, except latest trends show that in Sweden, Finland and The Netherlands incidence is not growing or has even started to decrease. In Finland, mortality has declined significantly by 2,9% annually during 1998—2007 (Bray et al., 2010).

There are only a few established risk factors for prostate cancer. These include positive family history, age and ethnicity. Prostate cancer risk is highest at the age of 70—74 and risk begins to increase after 55 years of age. African Americans have 60% higher risk than Caucasians for developing prostate cancer and their mortality is approximately double that of Caucasians (Gann, 2002). The true incidence of prostate cancer is higher than epidemiological studies show because not everyone develops symptoms, which is confirmed by autopsy findings (Damber & Aus, 2008).

Prostate glands main function is to produce and secrete seminal fluid from its epithelial glands. In addition to the glands, which may give rise to adenocarcinoma, prostate has a fibromuscular stroma. Glands consist of three types of cells: basal, luminal and neuroendocrine. The luminal cells secrete

prostate specific antigen (PSA), express androgen receptor (AR) and secrete components of prostatic fluid (Feldman & Feldman, 2001).

The final diagnosis of prostate cancer is made by examining a biopsy of patients prostate. However, a lot of unanswered questions lie for the indication of taking a biopsy. Prostate-specific antigen (PSA) testing was introduced in the early 1980s. It is an organ-specific substance, levels of which rises in blood in prostate cancer but also in benign prostate diseases, such as benign prostate hypertrophy (BPH). One major problem is overdiagnosing, which has caused anxiety and loss of quality of life in many cases (Lilja et al., 2008). In order to screen for a disease, the method for screening needs to be minimally invasive and its benefits must beat its disadvantages. PSA-testing is probably the most studied method for screening of prostate cancer. Recently, two large population-based studies have been performed. PLCO trial in the USA randomized 76,693 men and the ERSPC trial in Europe randomized 162,387 men for screening with PSA and digital rectal examination (DRE) -group and control group. Neither of them found significant differences in mortality but introduced ethical issues such as previously mentioned overdiagnosing and possible overtreatment (Eckersberger et al., 2009). Digital rectal examination is also a powerful diagnostic method as an indicator for taking a biopsy. Actually, abnormal DRE is an absolute indication for taking a biopsy even if PSA-levels are normal (Issa et al., 2006).

The architectural pattern of the tumor is graded by Gleason-scale, which was introduced in the 1960s. A few needle biopsies are taken from prostate gland and they are graded on a scale of 1 to 5, that is, from most differentiated to the least differentiated pattern. The two most representative pattern scores are then summed and referred to as the Gleason score, ranging from 2 to 10 (Epstein, 2010). When diagnosis of prostate cancer has been made, surgery, radiation therapy, hormonal therapy and combinations of these are used in treatment. When choosing between treatments, tumor characteristics and patients life expectancy are taken into account (van den Bergh et al., 2013).

2.2. Biology of prostate cancer

For better understanding of molecular biology of any neoplasia, a few distinguishing charasteristics or hallmarks of cancer have been introduced. With great simplification, they are eight mechanisms and/or

capabilites of tumor cells. In addition, clinical tumors have numerous types of genome instability and inflammation processes, which is why cancer is nowadays taken as an evolving and dynamic system. Probably the most important hallmark of cancer is its ability to sustain proliferation by deregulating growth factors that regulate normal cell cycle homeostasis. Hallmarks also include evasion of growth suppressor signals, ability to resist programmed cell death (also referred to as apoptosis), development of unlimited replicative potential, angiogenesis induction, ability to invade and metastasize, metabolic reprogramming and evasion of the immune system. It has also been discussed that the loss of differentiation should be one major hallmark of cancer. (Floor et al., 2012; Hanahan & Weinberg, 2011)

There is a global consensus that prostate cancer is multifocal, which means that primary tumors have different focus points and are often genetically distinct. This is the case usually in latent tumors, but there is evidence that advanced prostate cancer is homozygous. Prostate cancer can also be a slow-grower. Healthy men at the age of 20 at youngest have been shown to have histologic focuses of prostate cancer, which means that cancer initiation takes place at early age. Not everyone generates clinical symptoms and that is why clinical prostate cancer is thought to arise from different pathological pathways than that of latent ones. (Shen & Abate-Shen, 2010)

Prostate intraepithelial neoplasia (PIN) is thought to be a precursor for prostate cancer although it is not fully understood since not all PIN-lesions develop into carcinomas. The basal cell layer is lost from glands in prostate cancer. PIN is characterized by reduction of basal cells but they are not totally lost. In addition, luminal epithelial hyperplasia, enlargement of nuclei and nucleoli, cytoplasmic hyperchromasia and nuclear atypia is also seen in PIN (Lee et al., 2011). Prostate cancer is unique from other epithelial cancers because it doesn't have typical subtypes that can be distinguished histopathologically with certain prognosis or treatment response even though >95% of prostate cancers are adenocarcinomas. Probably the second most significant subtype is small-cell carcinoma which is also called neuroendocrine prostate cancer with <2% of cases. There are a total of 13 different subtypes of prostate cancer of which 8 of them are variants of prostate adenocarcinoma. There are also some evidence that subtyping could be made molecularly by defining oncogenic pathways (Grignon, 2004; Shen & Abate-Shen, 2010).

Androgens are important and essential for prostate growth. The main circulatory androgen is testosterone and it is secreted primarily from testes. Approximately 90% of it is converted into dihydrotestosterone (DHT) in prostate cells by 5α-reductase. DHT has five times higher affinity to androgen receptors (AR) than testosterone. Binding of DHT to AR stimulates proliferation and inhibits apoptosis, the two key mechanisms for any kind of growth and cancer. One effective treatment for

androgen-dependent prostate cancers is androgen ablation therapy, where GnRH super-agonists and AR-antagonists are used to inhibit the effect of androgens. Unfortunately, in many cases patient develops recurrent androgen-independent prostate cancer (AIPC). There are several pathways how androgen-independency can occur and currently there is no cure for AIPC. Hormone-refractory prostate cancer and castration-resistant prostate cancer are synonyms for AIPC. (Feldman & Feldman, 2001)

2.3. Genetic aberrations in prostate cancer

Deletions, gains, amplifications and translocations of chromosomic material are frequent in prostate cancer specimens and they are causally proved to be associated with prostate carcinogenesis. Effects of these aberrations to gene copy number alterations have been studied. Gains at 8q and losses at 3p, 8p, 10q, 13q and 17p have been mapped to be of great importance (Shen & Abate-Shen, 2010). Many different studies have found also several other regions, influence of which on carcinogenesis needs to be further studied (Dong, 2006; Gurel et al., 2008; Saramaki et al., 2006). These changes are one of the reasons why certain oncogenes gain their function and certain tumor suppressor genes lose theirs. For prostate cancer, it is thought that since deletions are far more common than gains, loss of tumor suppressor genes is of greater importance for early development of prostate cancer (Leinonen, 2007).

Genes NKX3.1, PTEN and TP53 are common tumor suppressor genes in prostate cancer. NKX3.1 is down-regulated epigenetically and is thought to have critical role in carcinogenesis initiation. PTEN is also considered to be lost early in cancer initiation and it has been connected to aggressive form of prostate cancer and especially to development of castration-resistance (Shen & Abate-Shen, 2010). TP53 is the most common gene that undergoes a mutation in human cancers. It has a major role in cell cycle as a key regulator from phase G1 to phase S. It is considered to be mutated in late events of prostate cancer (Porkka & Visakorpi, 2004). MYC is an oncogene that is up-regulated in prostate cancer but also in PIN-lesions which is why it could play a role in cancer initiation. Ki-67 is a proliferation marker, expression of which rises in prostate cancer. It can be used as a biomarker that predicts high chance of recurrence (Buhmeida et al., 2006).

3. MATERIALS AND METHODS

3.1. Clinical prostate TMAs

Study material consisted of previously collected prostatectomy and hormone-refractory tumor samples that have been processed using tissue micro array (TMA) technology. The use of clinical material has been approved by the ethical committee of Tampere University Hospital and the National Authority for Medicolegal Affairs (Helsinki, Finland). Target protein, UNC13B or UBAP2 in this case, is immunohistochemically stained brown and their epitopes serve as antigens for antibodies. Staining was made with PowerVision+™ Poly-HRP IHC kit which is biotin-free and contains anti-Mouse/Rabbit primary antibodies. To exclude non-cancer samples, each sample was compared to two-color triple antibody cocktail -stained samples (Fig. 1), which has antibodies against α-methylacyl coenzyme A racemase (AMACR), high molecular weight keratin and tumor protein p63. AMACR is known to be highly sensitive luminal marker in neoplastic prostate epithelial cells, whereas p63 and high molecular weight keratin stain basal cells that are not present in malignant prostate glands (Sung et al., 2007). With this staining, also samples with prostate intraepithelial neoplasia (PIN), which isn't carcinoma, could be excluded (Fig. 2) (DeMarzo et al., 2003). Unclear TMAs were also excluded from the analysis.

Analysis of expression levels were made manually from digitally photographed TMAs with computer. The special camera used to image these microarray blocks was provided by Fimlab Laboratoriot Oy. IIPMooViewer 2.0 beta and JVSView softwares were used to view samples digitally. Intensity of expression levels were scored on a scale of zero to three, from no expression at all to strong expression. To maximize the consistency of scoring, samples were analyzed three times.

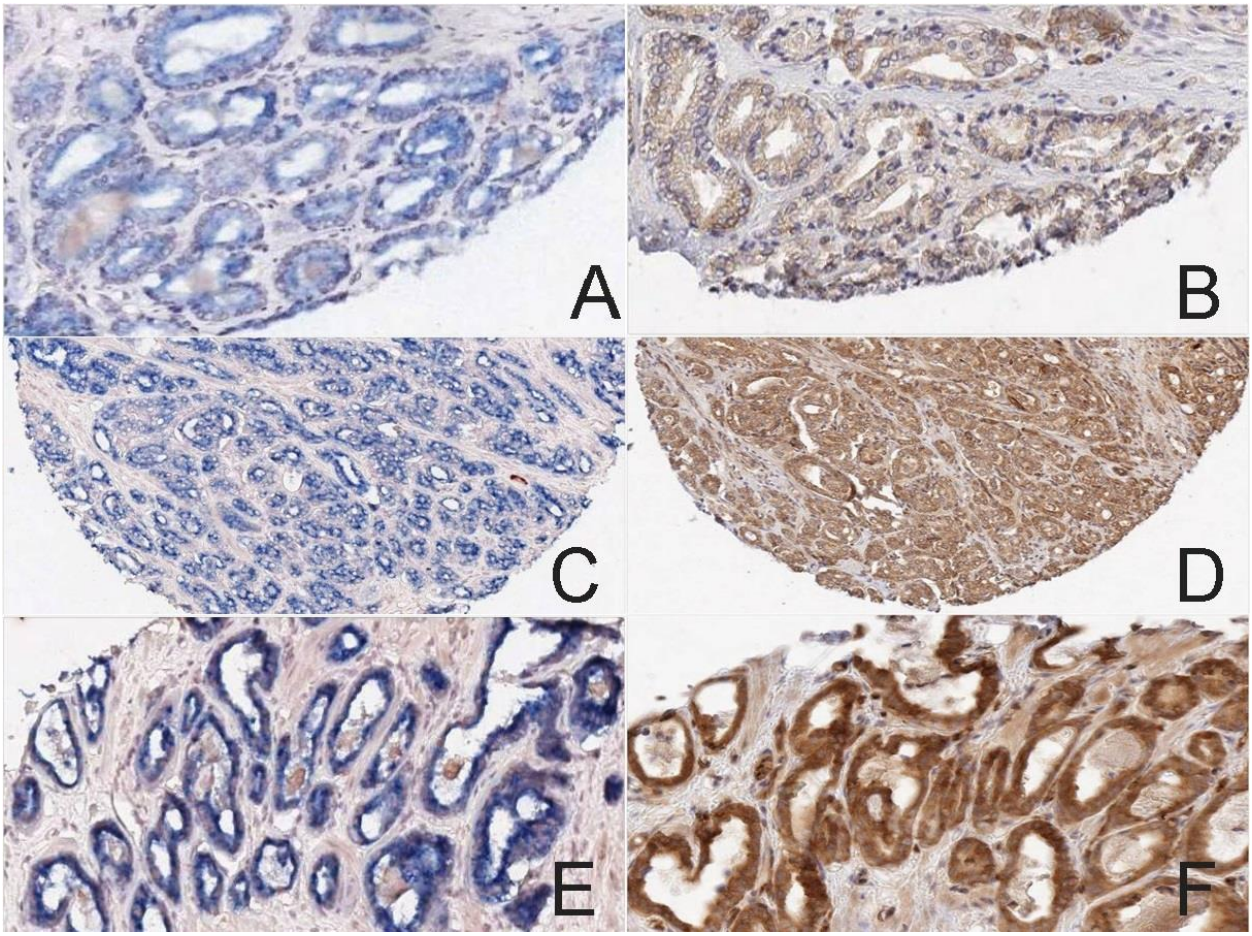


Figure 1. *Representative screen captures of TMAs from each scoring groups. Frames A, C and E are TMAs stained with two-color triple antibody cocktail. Pairs A-B, C-D and E-F are from same patient and biopsy but from different level. Frames B, D and F are TMAs stained against target protein with intensity scores 1, 2 and 3, respectively. It can clearly be seen from frames A, C and E that AMACR stains luminal cells blue. If basal cells existed, they would have been stained dark brown with p63 and high molecular weight keratin.*

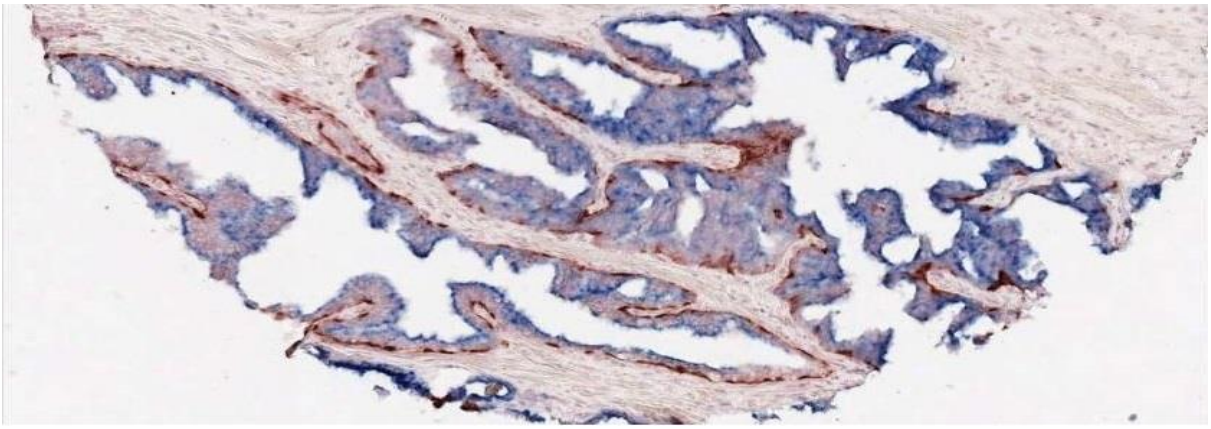


Figure 2. *Two-color triple antibody cocktail stained sample with PIN-lesion. AMACR stains luminal cells blue, but there are also basal cells left. Basal cells stain brown with p63 and high molecular weight keratin. These samples were excluded.*

3.2. Statistical analyses

Intensity level scores were compared to PSA-levels, Gleason scores, progression free survival, survival, age at diagnosis, tumors T-stage, Ki-67 expression and AR expression. These data have been collected previously. Data from primary prostatectomy samples were also compared with hormone refractory samples. Kruskal-Wallis one-way ANOVA was used to test the association between score intensity and PSA-levels, progression free survival, Gleason-score, age at diagnosis and tumor T-stage. For survival curves, log-rank Mantel-Cox test was used. To compare intensity scores between primary prostatectomy and hormone refractory groups, chi-square was used. Data were analysed with GraphPad Prism 5.0 and Microsoft Office Excel 2007 –softwares.

4. RESULTS

Staining intensity scoring was performed to 477 primary prostatectomy samples. A total of 173 samples met exclusion criteria. For hormone-refractory tumor samples, a total of 230 samples were scored and 86 samples were excluded from analysis. Eventually, no specimen was scored zero. Numbers for each scoring group are demonstrated on tables 1 and 2.

	Score intensity				
Staining	**1**	**2**	**3**	**Total**	**Excluded***
UNC13B	48 (21,4%)	125 (55,8%)	51 (22,8%)	224 (100%)	101
UBAP2	27 (10,7%)	121 (47,8%)	105 (41,5%)	253 (100%)	72

*****Samples without cancer and missing samples were excluded

Table 1. *Score intensities in primary prostatectomy samples.*

	Score intensity				
Staining	**1**	**2**	**3**	**Total**	**Excluded***
UNC13B	6 (5,4%)	76 (67,9%)	30 (26,8%)	112 (100%)	46
UBAP2	10 (8,5%)	46 (39,0%)	62 (52,5%)	118 (100%)	40

*****Samples without cancer and missing samples were excluded

Table 2. *Score intensities in hormone-refractory samples.*

Progression free survival was measured in months. Progression free survival didn't vary between scoring groups for UNC13B and UBAP2 (p=0.7985 and p=0.7917, respectively). Survival curves are shown in figure 3.

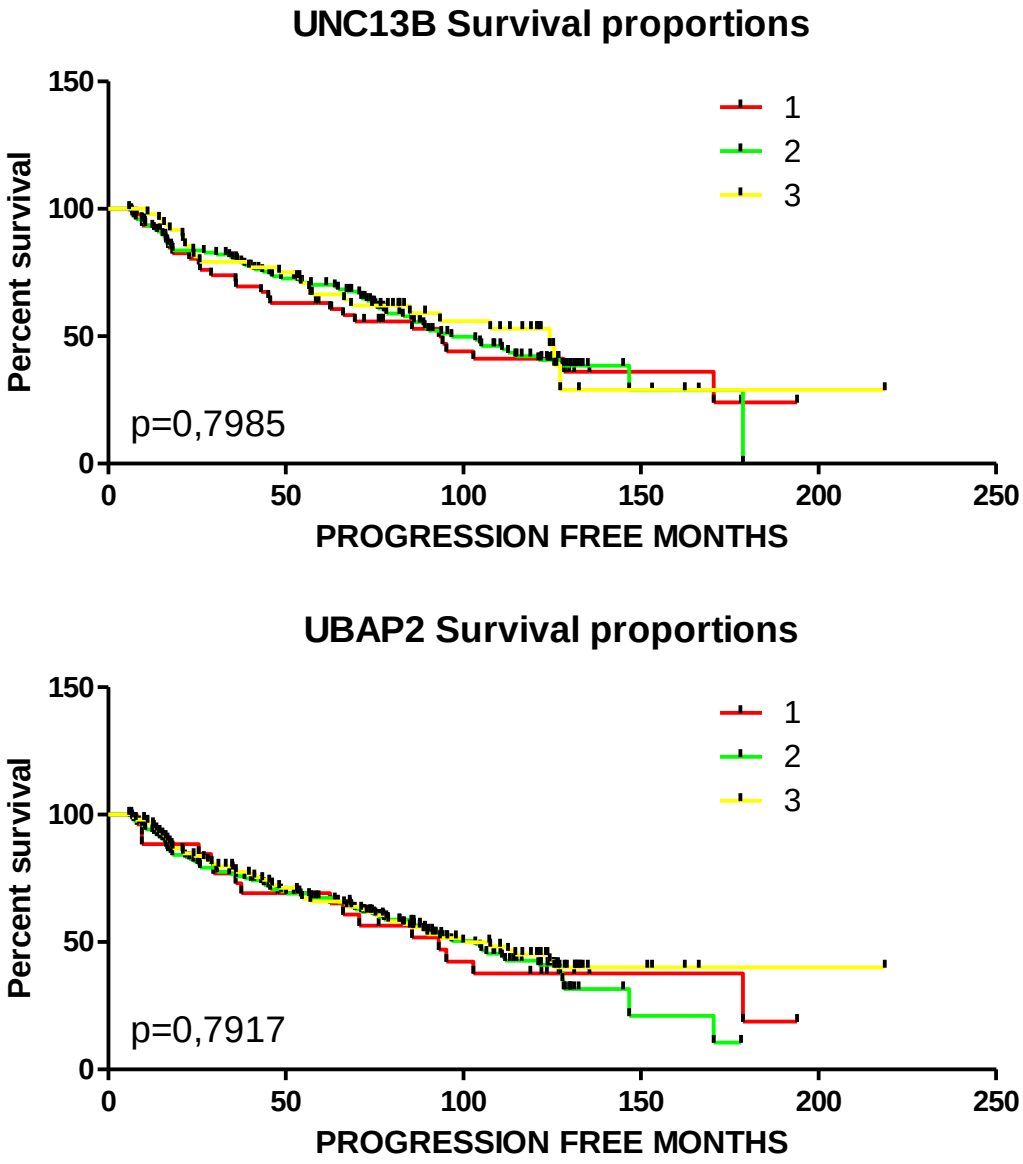


Figure 3. *Survival curves for progression free survival made with Mantel-Cox test. Intensity score groups are color coded. Difference between scoring groups was statistically insignificant.*

When comparing PSA-levels of primary prostatectomy samples to their intensity scores, no significant difference were found between groups for UBAP2 (p=0.6317). For UNC13B the difference were almost statistically significant (p=0.0632) but the trend for PSA-level was decreasing when intensity scores increased. In Gleason scoring and intensity scoring comparison, no statistically significant were found between scoring groups. Also, comparison of scoring groups to age at diagnosis and T-stage did not result in significant results. Table 3 summarizes the results of analyzed primary prostatectomy samples and the associations of clinicopathological variables with scoring and table 4 shows distribution for Gleason scores. T-stage distribution is shown in table 5.

		Score intensity			
Variables		**1**	**2**	**3**	**p-value**
PSA-level (µg/l) (mean±SD):					
	UNC13B	16.87 ± 13.99	14.64 ± 13.98	10.05 ± 6.507	0.0632
	UBAP2	16.03 ± 14.99	14.39 ± 11.59	13.53 ± 12.90	0.6317
Age at diagnosis:					
	UNC13B	63.50 ± 5.073	62.87 ± 5.183	61.54 ± 5.812	0.1684
	UBAP2	63.35 ± 5.346	62.84 ± 4.858	62.55 ± 5.704	0.8419

Table 3. *Summary of analysed data in prostatectomy specimens and associations of clinicopathological variables with scoring.*

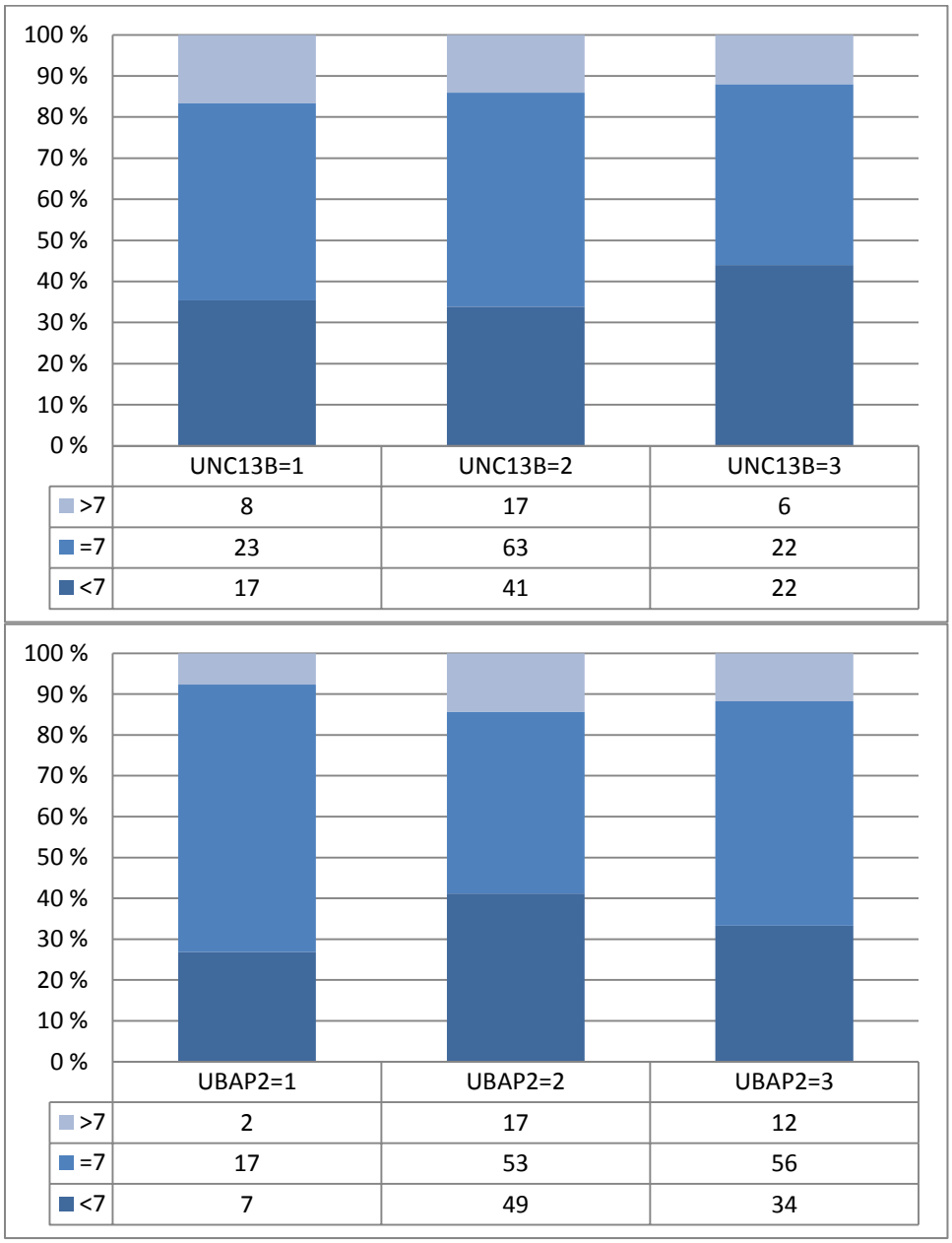


Table 4. *Gleason scores grouped to <7, 7 and >7 values and their associations to UNC13B and UBAP2 staining in prostatectomy samples. Their associations between scoring intensity and Gleason score were insignificant.*

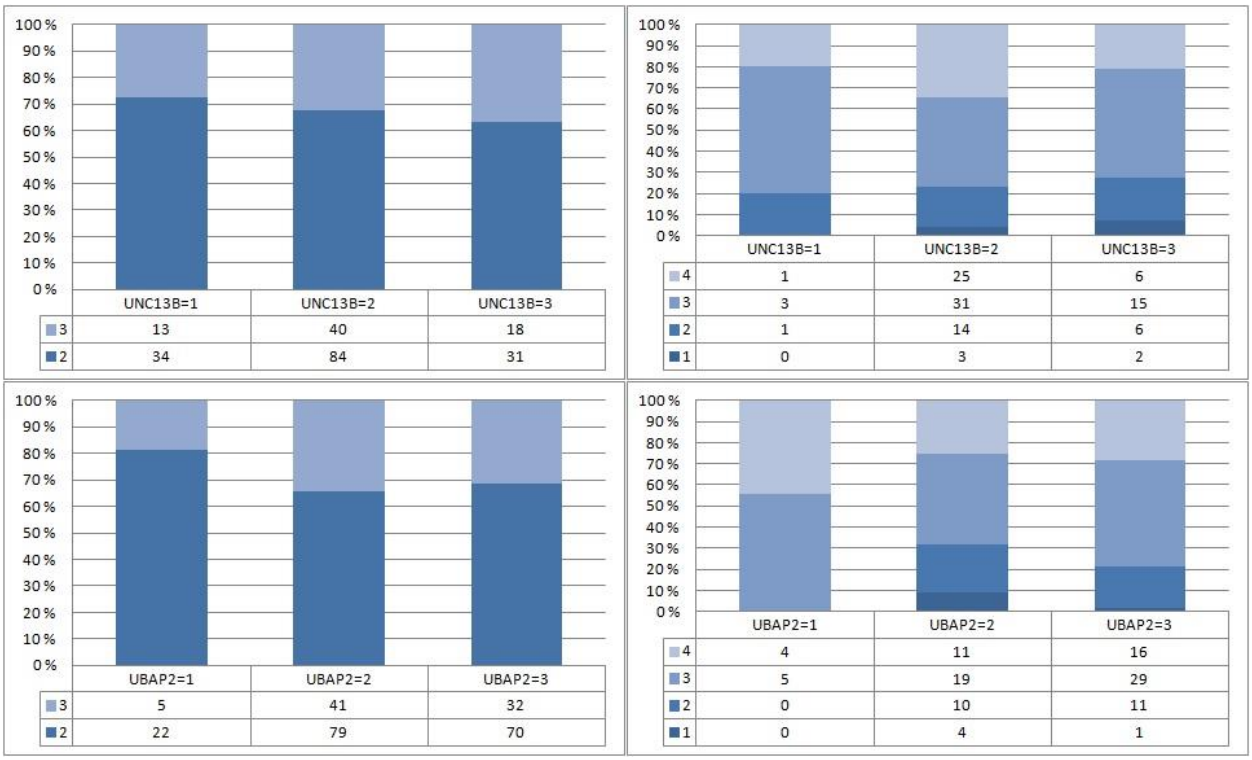
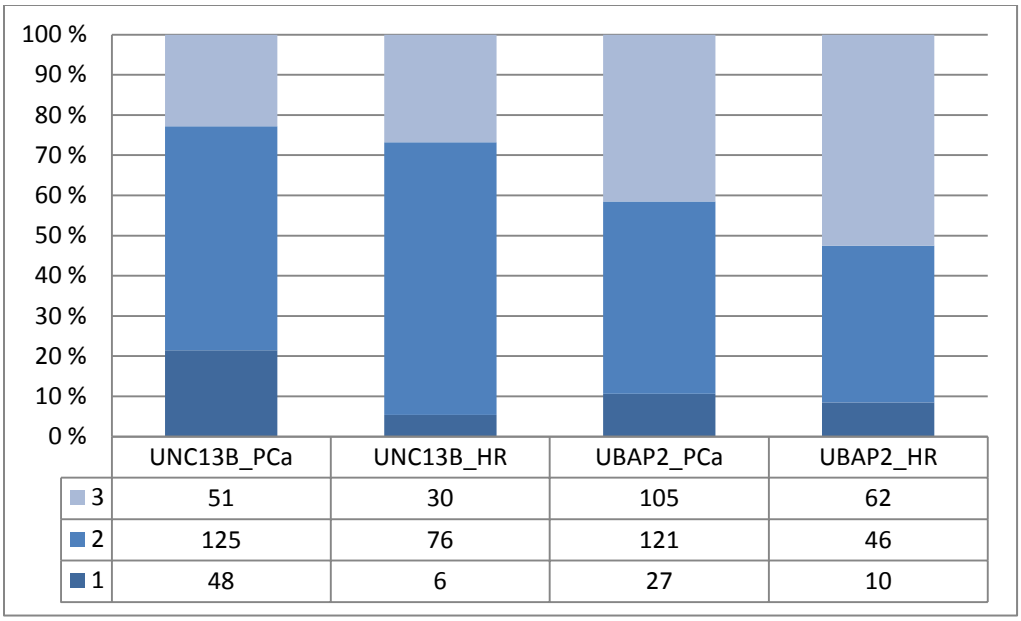


Table 5. *T-stage distribution in intensity scoring groups for UNC13B and UBAP2. Prostatectomy tables are on left and hormone-refractory on right. Differences between groups were statistically insignificant.*

In comparison between prostatectomy and hormone-refractory samples for intensity scoring, a difference was noted for UNC13B and UBAP2. The proteins stained more intense in hormone-refractory group than in prostatectomy group. For UNC13B the difference was statistically significant (p=0.0008) but for UBAP2 it was insignificant (p=0.1376). Distribution is presented in table 6.

Association of UNC13B and UBAP2 staining intensity to Ki-67 expression was also studied (figure 4). For these analysis, scores 2 and 3 were pooled to same group. Ki-67 expression increases with higher scores for UNC13B and UBAP2 ($p<0.0001$ and $p=0.0006$, respectively) in prostatectomy samples but not in hormone-refractory tumor specimens ($p=0.7326$ and $p=0.3324$, respectively).

In prostatectomy samples, androgen receptors expression also increases with higher score intensities for UNC13B and UBAP2 ($p<0.0001$ both). This is shown in table 7. Hormone-refractory tumor specimens were not included in this analysis.



	UNC13B_PCa	UNC13B_HR	UBAP2_PCa	UBAP2_HR
3	51	30	105	62
2	125	76	121	46
1	48	6	27	10

Table 6. *Score intensity distribution for both proteins stained. Score intensities are color coded. For UNC13B and UBAP2 p-values were 0.0008 and 0.1386, respectively. PCa=prostatectomy HR=Hormone-refractory.*

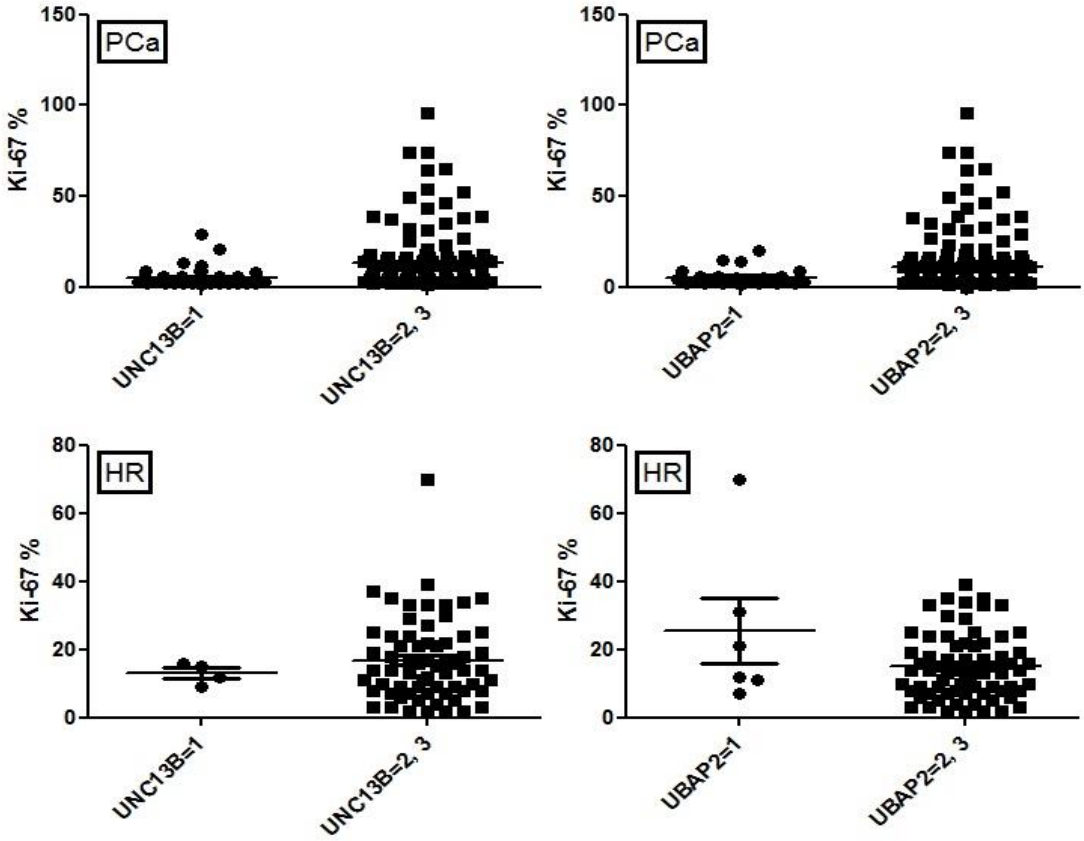
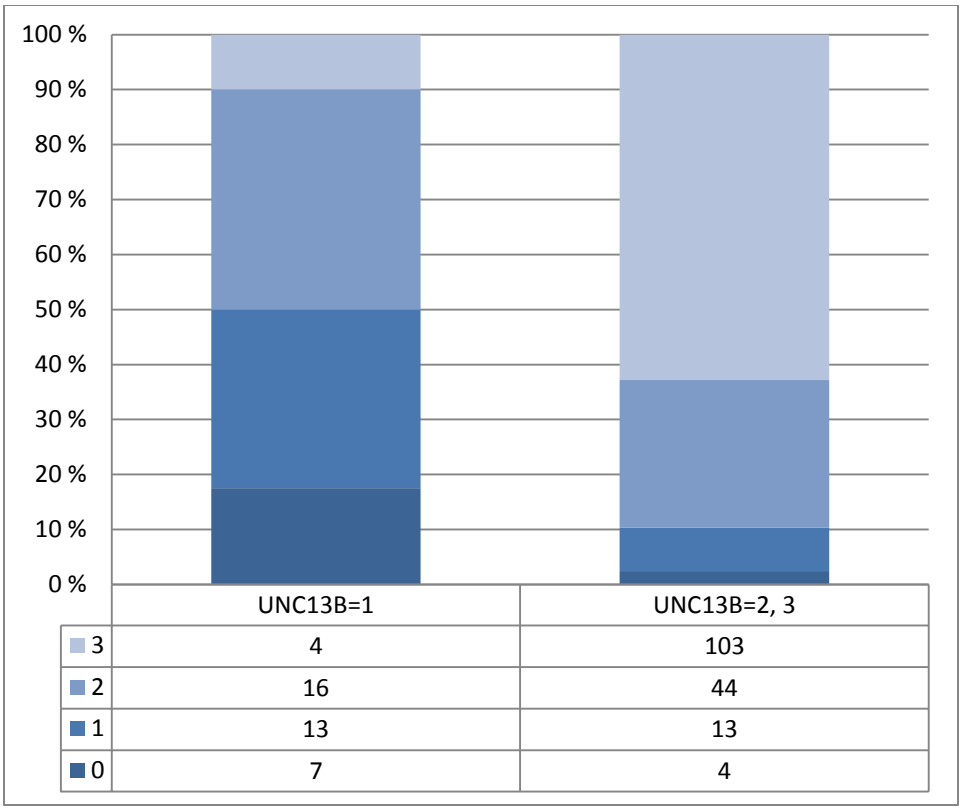
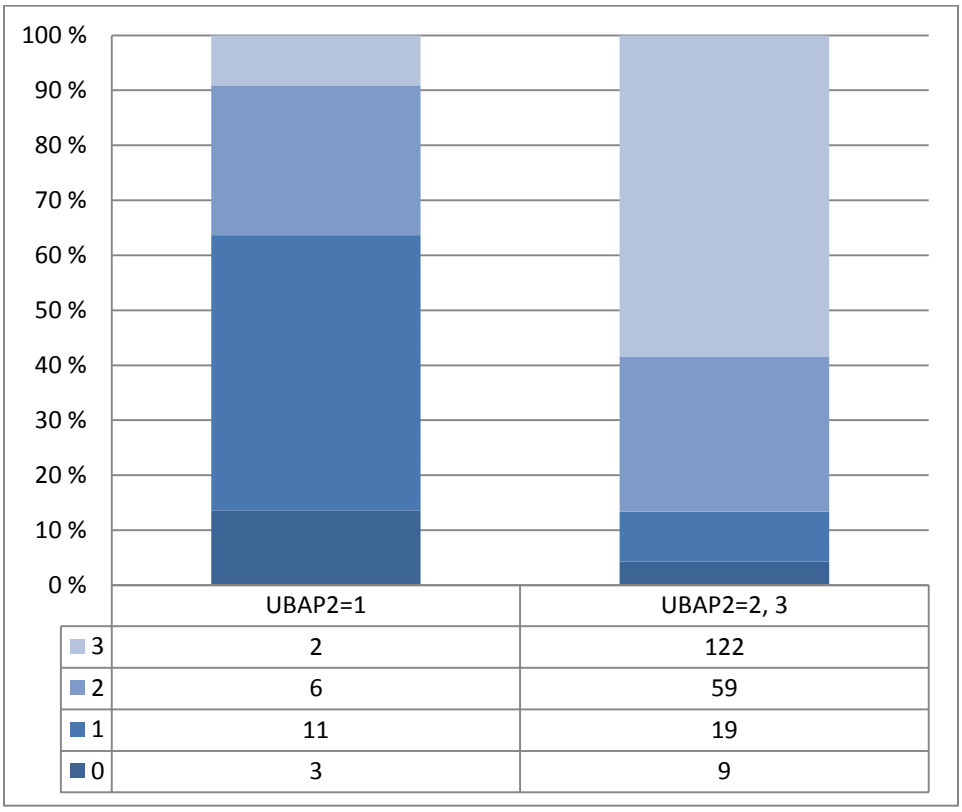


Figure 4. *Ki-67 percentage expressions association to UNC13B and UBAP2 score intensities. PCa=prostatectomy, HR=hormone-refractory.*



	UNC13B=1	UNC13B=2, 3
3	4	103
2	16	44
1	13	13
0	7	4



	UBAP2=1	UBAP2=2, 3
3	2	122
2	6	59
1	11	19
0	3	9

Table 7. *AR score expressions association to UNC13B and UBAP2 scoring intensities in prostatectomy samples.*

5. DISCUSSION

This research is based on a previous study which confirmed amplification in 9p13.3 in prostate cancer xenograft LuCaP35, clinical prostatectomy samples and hormone-refractory specimens. Leinonen (2007) found possible target genes, including UNC13B and UBAP2. It was observed that amplification of 9p13.3 was more common in hormone-refractory samples than in untreated prostatectomy specimens. This study's aim was to further investigate whether UNC13B and UBAP2 genes could possibly be oncogenes. A total of 477 clinical prostatectomy samples and 230 hormone-refractory specimens were included in the analysis in this study.

When prostatectomy samples were compared to hormone-refractory specimens, intensity score for UNC13B was significantly higher in hormone-refractory samples ($p=0.0008$) but insignificant for UBAP2 ($p=0.1376$). This supports the finding that 9p13.3 amplification was more common in HR specimens than in prostatectomy samples which Leinonen (2007) observed, and that UNC13B is a more potent oncogene than UBAP2. However, no association between UNC13B and UBAP2 expression to progression free survival was found. Similar results were observed when comparing Gleason scoring, age at diagnosis and T-stage to intensity scoring. PSA-levels of prostatectomy samples had almost statistically significant association to UNC13B intensity scores ($p=0.0632$) but not to UBAP2 ($p=0.6317$). However, the trend for PSA-level was decreasing when intensity scores got higher when they should have been increasing.

Expression of protein Ki-67 increased when scoring intensities of UNC13B and UBAP2 increased ($p<0.0001$ and $p=0.0006$, respectively) in prostatectomy samples but strangely not in hormone-refractory tumor specimens ($p=0.7326$ and $p=0.3324$, respectively). This might be caused by insufficient amount of data in HR groups. In prostatectomy samples, expression of androgen receptors also was higher in samples with higher score intensities for UNC13B and UBAP2 ($p<0.0001$ both).

These findings confirm previous observations that expression of UNC13B and UBAP2 increase during prostate cancer development, and show that the increased expression occurs also at the protein level. However, associations between expression levels of these proteins and clinicopathological variables were not found. Strong correlation between Ki-67 and these two proteins in prostatectomy samples could mean that UNC13B and UBAP2 would predict cancer recurrence but this needs further research. UBAP2 had only fifth best p-value ($p=0.1513$) in Leinonen (2007) study and UNC13B had the second best

(p=0.0826) when gene expression correlation with copy number status was examined. PIGO was the most promising gene and its association to protein expression and clinicopathological variables would be interesting to analyse.

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