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Large-scale evaluation of *SLC18A2* in prostate cancer reveals diagnostic and prognostic biomarker potential at three molecular levels

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ABSTRACT

Limitations of current diagnostic and prognostic tools for prostate cancer (PC) have led to over-diagnosis and over-treatment. Here, we investigate the biomarker potential of the *SLC18A2* (*VMAT2*) gene for PC at three molecular levels. Thus, *SLC18A2* promoter methylation was analyzed in 767 malignant and 78 benign radical prostatectomy (RP) samples using methylation-specific qPCR and Illumina 450K methylation microarray data. SLC18A2 transcript levels were assessed in 412 malignant and 45 benign RP samples using RNAseq data. SLC18A2 protein was evaluated by immunohistochemistry in 502 malignant and 305 benign RP samples. Cancer-specificity of molecular changes was tested using Mann–Whitney U tests and/or receiver operating characteristic (ROC) analyses. Log rank, uni- and multivariate Cox regression tests were used for survival analyses. We found that *SLC18A2* promoter hypermethylation was highly cancer-specific (area under the curve (AUC): 0.923–0.976) and associated with biochemical recurrence (BCR) after RP in univariate analyses. SLC18A2 transcript levels were reduced in PC and had independent prognostic value for BCR after RP (multivariate HR 0.13, $P < 0.05$). Likewise, SLC18A2 protein was

Abbreviations: AN, Adjacent normal; BPH, Benign prostatic hyperplasia; CRPC, Castration-resistant prostate cancer; PIN, Prostate intraepithelial neoplasia; MPC, Metastatic prostate cancer; RP, Radical prostatectomy; BCR, Biochemical recurrence; HR, Hazard ratio; FF, Fresh-frozen; FFPE, Formalin-fixed paraffin-embedded; ROC analysis, Receiver operating characteristic analysis; PSA, Serum prostate-specific antigen; TCGA, The Cancer Genome Atlas; TMA, Tissue microarray; qMSP, Quantitative methylation-specific PCR; LMD, Laser capture microdissection.

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down-regulated in PC (AUC 0.898) and had independent prognostic value for BCR (multivariate HR 0.51, $P < 0.05$). Reduced SLC18A2 protein expression was also associated with poor overall survival in univariate analysis (HR 0.29, $P < 0.05$).

Our results highlight *SLC18A2* as a new promising methylation marker candidate for PC diagnosis. Furthermore, SLC18A2 expression (RNA and protein) showed promising prognostic potential beyond routine clinicopathological variables. Thus, novel *SLC18A2*-based molecular tests could have useful future applications for PC detection and identification of high-risk patients.

1. Introduction

Prostate cancer is the most frequent male cancer in the developed world (Jemal et al., 2011). Whereas most prostate cancers are relatively slow growing and tumors localized to the prostate can be cured by radical prostatectomy (RP) or radiation therapy, metastatic prostate cancer is incurable. Thus, early detection and treatment is essential. Many prostate cancers, however, may not cause clinical symptoms within the patient's lifetime and as treatment is associated with severe side-effects, only men with aggressive prostate cancer should be treated (Canfield et al., 2014; Popiolek et al., 2013). Prostate cancer is definitively diagnosed by histopathological evaluation of prostate biopsies, but the initial indicator is often increased serum prostate specific antigen (PSA). Serum PSA has relatively high sensitivity, but rather low specificity for prostate cancer. Its use has therefore led to a large increase in prostate cancer diagnoses and treatments. Due to the high prevalence of indolent prostate cancers and lack of accurate prognostic tools to guide treatment decisions, a significant number of men may be over-treated (Canfield et al., 2014). Novel molecular markers may aid in early detection and in determining the aggressiveness of prostate cancer, but are generally not used in current clinical practice.

Prostate cancer is characterized by aberrant DNA hypermethylation of promoter-associated CpG islands at a large number of genes (Baylin et al., 2001; Kim et al., 2011). A subset of these genes show reduced mRNA expression upon DNA methylation, and hence are potential drivers of cancer development (Sproul et al., 2012) and often considered candidate tumor suppressors (Yang and Park, 2012). Candidate DNA methylation markers for prostate cancer have been reported to be hypermethylated in 70–100% of prostate cancer samples (Ahmed, 2010; Haldrup et al., 2013; Kristensen et al., 2014; Park, 2015), and are thus highly attractive for diagnostic applications. In addition, a few candidate DNA methylation markers have demonstrated promising prognostic value for prediction of PSA recurrence after RP (Banez et al., 2010; Haldrup et al., 2013; Kristensen et al., 2014; Schatz et al., 2010; Strand et al., 2014; Weiss et al., 2009).

The solute carrier family 18 member 2 (*SLC18A2*) gene encodes the transmembrane protein vesicular monoamine transporter 2 (SLC18A2/VMAT2). SLC18A2 protein localization and function is well characterized in neuronal cells where it facilitates uptake of monoamines into cytoplasmic large dense core and synaptic vesicles (Lawal and Krantz, 2013). SLC18A2 is highly expressed in monoamine secreting neuroendocrine tumors (Graff et al., 2001; Jakobsen et al., 2001; Saveanu et al., 2011; Uccella et al., 2006). In contrast, we previously reported a reduction in SLC18A2 immunoreactivity levels in 506 prostate adenocarcinoma samples when compared to 70 benign prostate tissue samples (Sorensen et al., 2009). In addition, in our previous study, reduced SLC18A2 protein levels had independent prognostic value for PSA recurrence after RP in multivariate Cox regression analysis including standard clinicopathological parameters, but this was not validated in an independent cohort (Sorensen et al., 2009). Notably, our previous results indicated hypermethylation of the *SLC18A2* promoter-associated CpG island and reduced SLC18A2 mRNA expression in prostate cancer, but was based only on small patient sample sets ($n < 21$) (Sorensen et al., 2009).

We here aimed to evaluate the biomarker potential of *SLC18A2* promoter methylation and RNA expression for PC, and to independently validate our previous finding that SLC18A2 protein immunohistochemistry has prognostic potential (Sorensen et al., 2009). To this end we analyzed hundreds of patient samples for *SLC18A2* promoter methylation, SLC18A2 mRNA and protein levels. We found that the *SLC18A2* promoter was frequently hypermethylated in prostate cancer in three distinct datasets and, furthermore, was significantly associated with PSA recurrence in two independent RP cohorts in univariate, but not in multivariate analysis corrected for routine clinicopathological factors. Moreover, SLC18A2 mRNA levels were reduced in prostate cancer, and low mRNA levels had prognostic value for PSA recurrence after RP independent of standard clinicopathological parameters. This is the first report to demonstrate a significant association between *SLC18A2* methylation and transcript levels and PC prognosis. Finally, we also confirmed that SLC18A2 protein was down-regulated in prostate cancer by immunohistochemical analysis of a new tissue microarray (TMA) representing 506 RP patients. Importantly, in this large independent RP cohort, loss of SLC18A2 protein had independent prognostic value for PSA recurrence in multivariate Cox regression analysis, thus successfully validating our previous results (Sorensen et al., 2009). Notably, loss of SLC18A2 protein was also associated with poor overall survival. Hence, the present work for the first time demonstrate a significant

association between loss of SLC18A2 protein and overall survival after RP.

2. Materials and methods

2.1. Patient samples used for quantitative methylation-specific PCR (qMSP) analyses

Two previously described RP cohorts consisting of consecutive curatively intended RPs of histologically verified clinically localized prostate cancer were used for qMSP (Florl et al., 2004; Haldrup et al., 2013; Kristensen et al., 2014).

Briefly, RP cohort 1 consisted of formalin-fixed paraffin-embedded (FFPE) RP samples collected at Departments of Urology, Aarhus University Hospital, Denmark (1997–2005) and University Hospital Zurich, Switzerland (1993–2001), previously used for TMA construction (Heeboll et al., 2009; Mortezavi et al., 2011). Hematoxylin and eosin (HE) stained slides were evaluated by a trained pathologist, and representative regions with >90% tumor content were marked. Punch biopsies were obtained from the corresponding FFPE blocks. After quality control and exclusion of patients based on pre/post-endocrine treatment or lack of follow-up, the final analysis comprised 280 RP patients (Supplementary Table S1, Supplementary Figure S1A). Similarly, RP cohort 2 samples were collected at Departments of Urology, Heinrich Heine University, Germany (1993–2002), Tampere University Hospital, Finland (1992–2003), and Karolinska University Hospital, Sweden (2003–2007). Fresh-frozen (FF) tissue specimens with >70% tumor content were selected and the final analysis comprised 171 RP patients (Supplementary Table S1, Supplementary Figure S1A).

Samples of adjacent normal (AN, n = 18), benign prostatic hyperplasia (BPH, n = 17), prostate intraepithelial neoplasia (PIN, n = 11), primary tumors from metastatic prostate cancer (MPC, n = 26), and from castration-resistant prostate cancer (CRPC, n = 13) used for qMSP (Supplementary Table S2), were previously described (Kristensen et al., 2014). Briefly, for AN and PIN, genomic DNA was extracted from punch biopsies of FFPE RP specimens. For BPH, MPC, and CRPC, genomic DNA was extracted from FFPE tissue from transurethral resections of the prostate.

2.2. qMSP

DNA from Danish FFPE samples was extracted using gDNA Eliminator columns from the miRneasy FFPE Kit (Qiagen). DNA from Swiss FFPE and German FF samples was extracted using the blood and cell culture DNA kit (Qiagen). DNA from Finnish and Swedish FF samples was extracted using AllPrep Mini DNA/RNA kit (Qiagen). DNA was bisulfite converted using the EZ-96 DNA Methylation-Gold Kit™ (Zymo), as previously described (Haldrup et al., 2013).

The qMSP primers (forward: 5′-TTTAAGGTATTCGGTTACGCGT-3′, reverse: 5′-TCGCTACGCAAAAAAAACTACCG-3′) and probe (6-FAM-5′-TTCGGGGAAGAGGCGCGGTCG-3′-BHQ-1) targeted the *SLC18A2* promoter-associated CpG island, the amplicon covered positions 119000425-119000518 on chromosome 10 (hg19 annotation). qMSP reactions (5 μL) were run in triplicate on 5 ng bisulfite converted DNA using 3 pmol of each primer, 1 pmol probe, and Taqman Universal Mastermix no UNG (Applied Biosystems). Reactions were pipetted using the Biomek 3000 robot (Beckman Coulter) and run on Applied Biosystem's 7900HT real time thermal cycler in 384-well plates (50 °C 2:00 min, 95 °C 10:00 min, 50× (95 °C 0:15 min, 58 °C 1:00 min)). As previously described (Haldrup et al., 2013), bisulfite converted and un-converted CpGenome Universal Methylated and Unmethylated DNA (Millipore), and a standard curve based on serially diluted methylated DNA were included on each plate. Results were normalized to the control assay *MYOD1* (Haldrup et al., 2013), which targets a genomic region without CpG sites, and excluded from further analysis if two out of three Ct values for *MYOD1* exceeded 36. Outliers (>2 Cts lower/higher than the other Ct values) were removed. Samples were considered negative for methylation if ≥2 methylation specific reactions did not amplify.

2.3. RP TMA

A TMA was constructed from archived FFPE tissue samples from 552 curatively intended RPs of histologically verified clinically localized prostate cancer, operated from 1998 to 2009 at Department of Urology, Aarhus University Hospital, Denmark. Based on HE stained sections, a trained pathologist (SH) identified a representative malignant area for each patient and a representative area of matched AN tissue from 305 (randomly selected) of the 552 RP patients. In all cases, Gleason scores were reassigned according to International Society of Urological Pathology criteria (Epstein et al., 2005). Three punch biopsies from malignant tissues (n = 1656) and two punch biopsies from AN tissues (n = 602) were used for TMA construction (1 mm core diameter, 16 individual blocks) on the TMA master (3DHISTECH) using Pannoramic Viewer (3DHISTECH). Six patients subsequently withdrew consent, leaving 546 patients for evaluation (Supplementary Table S3). For all patients, the most recent clinical follow-up of recurrence-free survival, overall survival, and prostate cancer-specific survival was completed in May 2015.

2.4. Immunohistochemistry

For each TMA block, a slide of 2.5 μm was used for SLC18A2 immunohistochemistry as previously described (Sorensen et al., 2009). Briefly, epitopes were demasked with TEG buffer, sections were stained with rabbit polyclonal SLC18A2 antibody (AB1767, Chemicon, Western blot demonstrating specificity for SLC18A2 previously shown in Sorensen et al. (2009)) in a 1:300 dilution in TBS with 1% bovine serum albumin. The EnVision+ System was used for secondary staining (HRP Labeled Polymer Anti-Rabbit K4003, DakoCytomation). Stained sections were scanned and scored in Pannoramic Viewer (3DHISTECH). In total 1426 malignant (86.1% of all malignant cores) and 556 benign (92.4% of all benign) cores could be evaluated and were scored by a trained pathologist (SH) and a medical doctor (ASL) with extensive experience in prostate histology. For epithelial cells in each core, the predominant cytoplasmic localization of SLC18A2 was scored as apical or diffuse, and was given a score for staining intensity (0, no or weak; 1, moderate; 2, strong).

2.5. Patients used for SLC18A2 protein analysis

Of the 552 patients included on the TMA, 6 withdrew consent, 30 had received pre/post-endocrine treatment, 22 had PSA recurrence within the first three months after RP, and 42 were previously used for SLC18A2 immunohistochemistry (Sorensen et al., 2009) and hence excluded. For 49 patients ≥2 malignant cores were lost during TMA processing, leaving 403 patients for data analysis (Supplementary Table S3, Supplementary Figure S1B). Of the 305 RP patients with two AN cores on the TMA, 72 were excluded based on pre- or post-endocrine treatment, PSA recurrence within the first three months after RP, or loss of cores during TMA processing (Supplementary Figure S1B). Thus, 233 patients with AN tissue were used for data analysis. In total, 1131 malignant and 466 AN cores were used for data analysis.

2.6. RNAseq and DNA methylation array data

In-house Illumina 450K DNA methylation array and matched RNAseq data from 19 malignant and 11 AN RP samples were used to assess *SLC18A2* promoter methylation and mRNA expression. Briefly, Veritas™ 704 (Arcturus) was used for laser capture micro-dissection (LMD) of malignant and AN cells, respectively. RNA was extracted using the RNeasy micro kit (Qiagen), and genomic DNA was extracted using the Puregene system (Qiagen). RNAseq libraries (Scriptseq™ Complete Gold Kit version II (Illumina)) were analyzed on the Illumina HiSeq2000 (15–25 million reads/sample). Fragments per kilobase of exon per million fragments mapped (FPKM) values were calculated using the Tuxedo suite (Trapnell et al., 2012). DNA was analyzed on the Illumina 450K beadchip array by AROS Applied Biotechnology A/S and data was analyzed using ChAMP (1.2.0) in R (3.0.2) (Feber et al., 2014; Morris et al., 2014). Each CpG site was assigned a β-value ranging from 0 (unmethylated) to 1 (fully methylated). Of the 16 CpG sites in *SLC18A2* interrogated by the 450K array, one site (cg15520443) was excluded because of poor detection p-values. 450K array and RNAseq data are available upon request.

2.7. Public data

RNAseq, Illumina 450K DNA methylation array, and clinical data for 297 RP patients (including 34 matched AN samples) were downloaded from The Cancer Genome Atlas (TCGA, http://cancergenome.nih.gov/). Furthermore, RNAseq FPKM values and clinical data for an American cohort of 106 RP patients were downloaded from GEO (GSE54460) (Long et al., 2014).

2.8. Statistical analysis

Unless stated otherwise, statistical analyses were conducted in R (3.0.2, http://www.r-project.org/).

Mean SLC18A2 immunohistochemistry scores were calculated separately for malignant and benign cores from each patient. Mean apical and mean diffuse scores were calculated using only cores with predominantly apical or diffuse staining patterns, respectively.

Kaplan–Meier analysis and two-sided log-rank tests, as well as uni- and multivariate Cox regression analyses were conducted in STATA v10.1 (STATA, College Station, TX) using prostate cancer-specific survival, overall survival, or PSA recurrence (Danish, Swiss, Swedish, and German samples cutoff ≥ 0.2 ng/mL, Finnish samples cutoff ≥ 0.5 ng/mL, based on local clinical practice) as endpoint. Patients that had not experienced PSA recurrence or death, respectively, were censored at their last normal PSA measurement. For multivariate Cox regression analysis, all clinicopathological parameters significant in univariate analysis were included.

2.9. Ethical approval

The study was approved by the relevant ethical committees in each country. Written informed consent was obtained from all patients.

3. Results

3.1. Correlation of SLC18A2 promoter methylation with mRNA and protein levels

We previously reported significant down-regulation of SLC18A2 protein in prostate cancer in a combined patient set from Denmark and Switzerland (Sorensen et al., 2009). In the same study, but using only a very small patient sample set, *SLC18A2* seemed to be a common target of aberrant promoter hypermethylation and to have reduced transcript levels in prostate cancer as compared to non-malignant prostate tissue.

Here, we assessed the correlation between *SLC18A2* promoter methylation and mRNA expression in matching 450K DNA methylation array and RNAseq data from in-house (11 AN/19 malignant) and TCGA (34 AN/297 malignant) patient sets. Prostate cancer samples were significantly hypermethylated compared to AN samples at four CpG sites near the *SLC18A2* transcription start site in both datasets ($P < 0.05$, Figure 1A–C, Supplementary Table S4), whereas none of the intragenic CpG sites were differentially methylated (Figure 1A). SLC18A2 mRNA was significantly downregulated in prostate cancer samples ($P < 0.05$, Figure 1D and 1E), and was inversely correlated with each of the four cancer-specifically hypermethylated CpG sites (P-values < 0.05, Figure 1F and 1G, Supplementary Table 4). Thus, promoter methylation may negatively affect *SLC18A2* transcription, consistent with our previous observation that 5-aza-2'-deoxycytidine treatments can stimulate SLC18A2 mRNA expression in prostate cancer cell lines (Sorensen et al., 2009).

We further used qMSP to evaluate promoter methylation in another set of prostate tissue samples (6 AN, 7 BPH, 8 PIN, 86 RP, 28 MPC, and 27 CRPC, qMSP assay localization in Figure 1B and 1C) from Danish patients analyzed for SLC18A2 protein levels in our previous TMA study (Sorensen et al., 2009). Promoter methylation was significantly inversely correlated to protein levels (Spearman's rho −0.396, P-value < 0.05, Supplementary Figure S2). Together these results for three independent datasets strongly indicate that aberrant

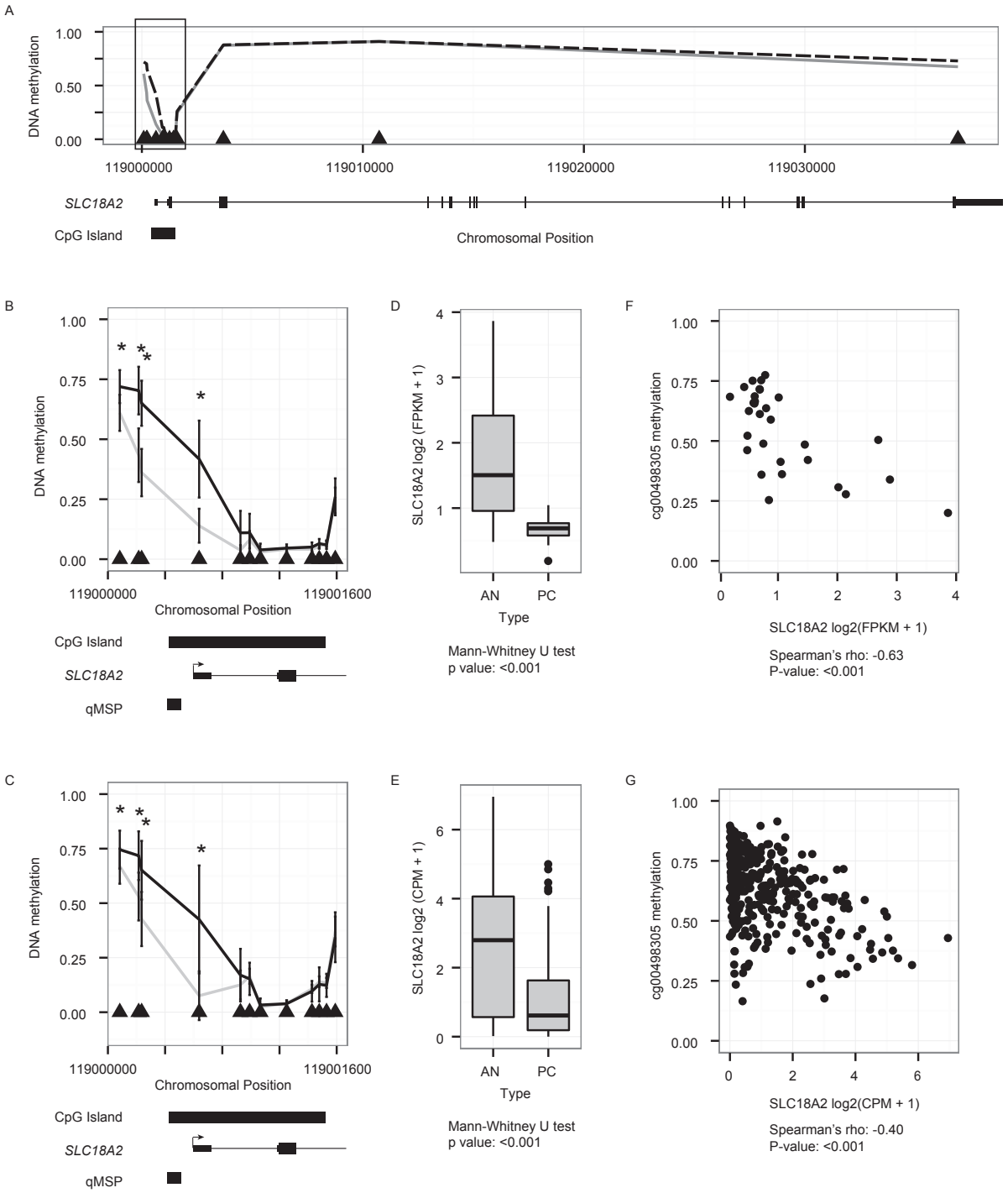

Figure 1 – Methylation status of CpG sites around the SLC18A2 transcription start site (450K array data) and SLC18A2 mRNA expression (RNAseq) in two independent sample sets. A: Mean DNA methylation levels in 11 AN (grey line) and 19 prostate cancer (black line) laser micro-dissected (LMD) samples across the SLC18A2 locus. B: Zoom in on boxed area of A, same data. C: Zoom in on boxed area of A, data from TCGA samples (34 AN (grey line) and 297 prostate cancer (black line)). D: mRNA expression in LMD samples. E: mRNA expression in TCGA samples. F: Correlation between DNA methylation at cg00498305 and mRNA expression in LMD samples. G: Correlation between DNA methylation at cg00498305 and mRNA expression in TCGA samples. Black triangles in A–C: Illumina 450K probe (CpG) site positions. A–C: The location of CpG island, SLC18A2 gene, and qMSP assay drawn to scale based on GENCODE version 19 coordinates.

promoter hypermethylation contribute to *SLC18A2* silencing in prostate cancer.

3.2. *Diagnostic potential of* SLC18A2 *promoter methylation*

To assess the diagnostic potential of *SLC18A2* promoter methylation for prostate cancer, we analyzed promoter methylation by qMSP in 280 RP (RP cohort 1), 18 AN, 15 BPH, 11 PIN, 31 MPC, and 29 CRPC samples, including samples used for correlation analyses above (Supplementary Tables S1 and S2).

SLC18A2 promoter methylation levels were significantly higher in RP than in AN, BPH, and PIN samples (P-value < 0.05, Figure 2A), whereas methylation levels in all malignant sample types (RP, MPC, and CRPC) were similar. Compared to benign prostate samples (AN and BPH), RP samples showed cancer-specific hypermethylation (ROC analysis, AUC: 0.967, Figure 2B and 2C). At a fixed specificity of 97%, the sensitivity of *SLC18A2* hypermethylation for prostate cancer was 86%. The cancer-specificity of *SLC18A2* promoter hypermethylation was confirmed using in-house (11 AN/19 prostate cancer, AUC 0.957, Supplementary Table S4) and TCGA data (34 AN/297 RP, AUC 0.923, Figure 2D, Supplementary Table S4). These findings strongly indicate that *SLC18A2* promoter hypermethylation is highly cancer-specific, and may have a diagnostic potential similar to that of other top candidate DNA methylation markers for prostate cancer (Haldrup et al., 2013; Kristensen et al., 2014).

3.3. *Prognostic potential of* SLC18A2 *promoter methylation*

Having previously reported an association between reduced SLC18A2 protein and short PSA-recurrence free survival after RP (Sorensen et al., 2009), we here investigated if cancer-associated *SLC18A2* promoter methylation might also have prognostic potential, using RP cohort 1 ($n = 280$) and an independent German/Finnish/Swedish RP cohort (RP cohort 2, $n = 171$). High *SLC18A2* methylation was significantly correlated with high pathological Gleason score, advanced pathological T-stage, positive surgical resection margin status, and high pre-operative PSA levels in at least one of the two RP cohorts (Supplementary Table S5).

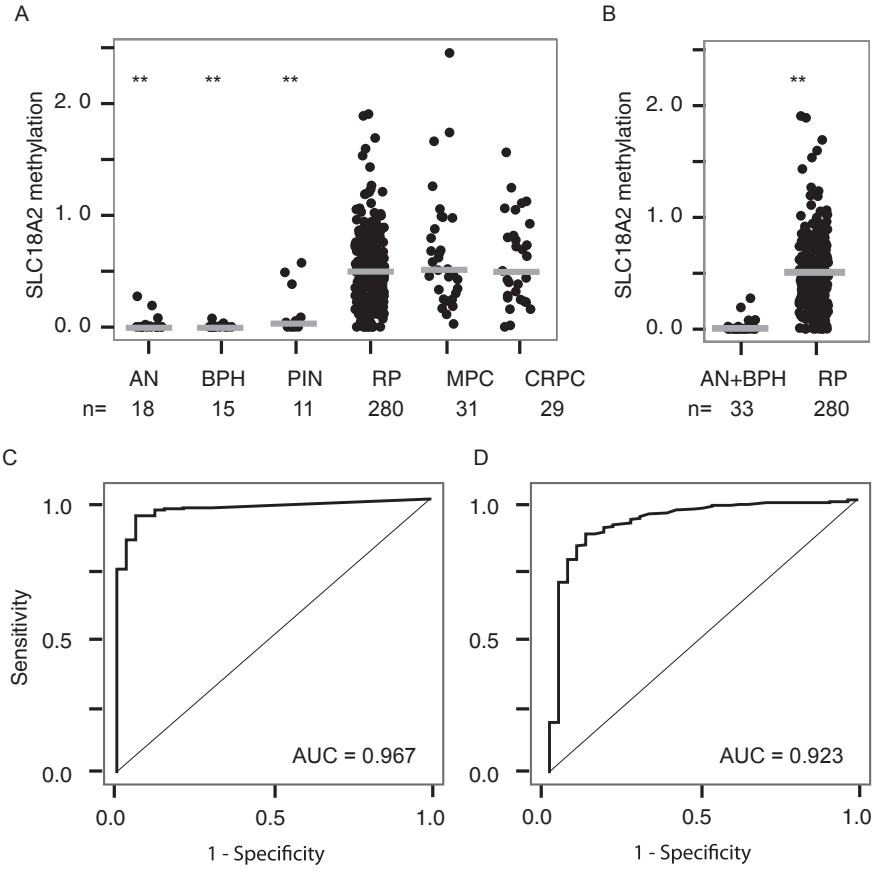

Figure 2 – DNA methylation in non-malignant and malignant prostate tissue samples according to qMSP. A: SLC18A2 promoter methylation levels determined by qMSP in RP cohort 1 samples, as well as in non-malignant (AN, BPH, PIN) and malignant (RP, MPC, CRPC) prostate samples. Methylation levels in RP samples were compared to other sample groups using Mann–Whitney U tests, grey line: median, **P < 0.0001. B: Comparison of methylation levels in benign samples (AN and BPH) versus RP (cancer) samples. C: Receiver operated characteristic analysis of cancer-specificity of SLC18A2 DNA methylation in samples from B. D: Receiver operated characteristic analysis of cancer-specificity of SLC18A2 DNA methylation in TCGA data: 34 AN versus 297 prostate cancer samples, DNA methylation of the CpG site with the highest AUC (cg15173134) is shown.

Furthermore, in both cohorts, *SLC18A2* promoter hypermethylation was significantly associated with increased risk of PSA recurrence in univariate Cox regression analysis (HRs 1.77 and 2.34, P-value < 0.05, Table 1A and 1B), but did not retain statistical significance in multivariate analysis including established clinicopathological prognostic variables (Table 1A and 1B). Thus, *SLC18A2* promoter hypermethylation was associated with increased risk of PSA recurrence, but did not provide independent prognostic information in these cohorts.

3.4. *Diagnostic and prognostic value of SLC18A2 mRNA levels*

SLC18A2 mRNA levels were significantly down-regulated in prostate cancer samples (Figure 1D and 1E). Furthermore in ROC analysis (data not shown), mRNA levels were able to separate AN and prostate cancer samples (AUCs 0.876 (11 AN/19 malignant in-house samples from Figure 1D) and 0.714 (34 AN/297 malignant TCGA samples from Figure 1E)). Thus, mRNA levels were cancer-specifically reduced, but with lower AUCs than promoter methylation (Figure 2C and 2D).

The potential prognostic value of SLC18A2 mRNA levels was tested using publicly available RNAseq data from 96 RP patients with long-term clinical follow-up (Long et al., 2014). SLC18A2 mRNA levels were not significantly correlated with any of the clinicopathological parameters in this sample set (Supplementary Table S5). However, low SLC18A2 mRNA expression was significantly associated with PSA recurrence after RP in both uni- and multivariate Cox regression analysis (Table 1C). Thus, SLC18A2 mRNA levels had independent prognostic value for prediction of PSA recurrence in this cohort.

3.5. *Validation of diagnostic potential of SLC18A2 protein levels, as assessed by immunohistochemistry*

To independently validate our previous finding that cytoplasmic SLC18A2 protein levels were reduced in prostate cancer and associated with PSA recurrence (Sorensen et al., 2009), a new large RP TMA (n = 502) was constructed and used for SLC18A2 immunohistochemistry, using the same antibody as previously (Sorensen et al., 2009). On the new TMA, we distinguished between diffuse and apical cytoplasmic staining patterns since these could potentially reflect different cellular activities, e.g. vesicular storage versus active secretion of monoamines (Lawal and Krantz, 2013). Thus, epithelial cells in each core were given a score (0: no or weak staining, 1: moderate staining, 2: strong staining) for either predominantly apical or predominantly diffuse cytoplasmic staining (Figure 3A–L). For malignant cores, 144 (35.7%) patients had both staining patterns, whereas 175 (43.4%) and 84 (20.8%) patients had only apically or diffusely stained cores, respectively. For AN cores, 70 (30.0%) patients had both staining patterns, 121 (51.9%) had only apically, and 42 (18.0%) had only diffusely stained cores.

For each patient, mean SLC18A2 immunohistochemistry scores for malignant and AN cores were calculated, and for patients with apical and/or diffuse scores, mean apical and mean diffuse scores were also calculated. The mean SLC18A2 score for each patient, shifted from predominantly strong (median = 2) in AN cores to predominantly moderate (median = 1) in malignant samples (AUC 0.898, Figure 3M). Thus, we here successfully validated our previously published observation that cytoplasmic SLC18A2 protein levels are reduced in prostate cancer (Sorensen et al., 2009). Similarly, both mean diffuse and mean apical staining scores shifted from strong (median = 2, diffuse and apical means) to

Table 1 – Uni- and multivariate Cox regression analysis using PSA recurrence after radical prostatectomy as endpoint.

	Univariate HR (P-value)	95% CI	Multivariate HR (P-value)	95% CI
A: DNA methylation RP cohort 1				
SLC18A2 DNA meth cont.	1.77 (0.028)	1.06–2.96	0.81 (0.486)	0.45–1.46
Gleason score ≤6 vs ≥ 7	3.90 (<0.001)	2.36–6.43	2.95 (<0.001)	1.71–5.08
Pathological T-stage ≤ pT2c vs ≥ pT3a	3.26 (<0.001)	2.28–4.66	1.78 (0.005)	1.19–2.68
Margin, positive vs negative	3.16 (<0.001)	2.20–4.53	2.34 (<0.001)	1.56–3.53
Pre-operative PSA cont.	1.04 (<0.001)	1.03–1.05	1.04 (<0.001)	1.02–1.05
B: DNA methylation RP cohort 2				
SLC18A2 DNA meth cont.	2.34 (0.041)	(1.03–5.29)	1.72 (0.237)	0.70–4.20
Gleason score ≤6 vs ≥ 7	4.05 (<0.001)	(2.32–7.07)	3.72 (<0.001)	2.10–6.60
Pathological T-stage ≤ pT2c vs ≥ pT3a	2.93 (<0.001)	(1.86–4.61)	2.42 (<0.001)	1.49–3.93
Margin, positive vs negative	1.82 (0.053)	(0.99–3.32)	–	–
Pre-operative PSA cont.	1.02 (0.033)	(1.00–1.04)	1.03 (0.009)	1.01–1.05
C: mRNA, Long et al. data set				
SLC18A2 mRNA (log2(FPKM + 1))	0.19 (0.002)	0.07–0.54	0.16 (0.002)	0.05–0.50
Gleason score ≤7 vs ≥ 8	2.42 (0.019)	1.15–5.07	1.90 (0.180)	0.74–4.88
Pathological T-stage ≤ pT2c vs ≥ pT3a	1.55 (0.245)	0.74–3.22	–	–
Margin, positive vs negative	3.25 (<0.001)	1.75–6.02	2.73 (0.002)	1.45–5.14
Pre-operative PSA cont.	1.08 (<0.001)	1.05–1.11	1.07 (<0.001)	1.04–1.10

A and B: Association of SLC18A2 promoter DNA methylation in RP cohorts 1 and 2 with PSA recurrence. DNA methylation levels were determined by qMSP. C: Association of SLC18A2 mRNA expression levels with PSA recurrence, RNAseq and clinical data from Long et al. (2014). HR: Hazard ratio. CI: Confidence interval.

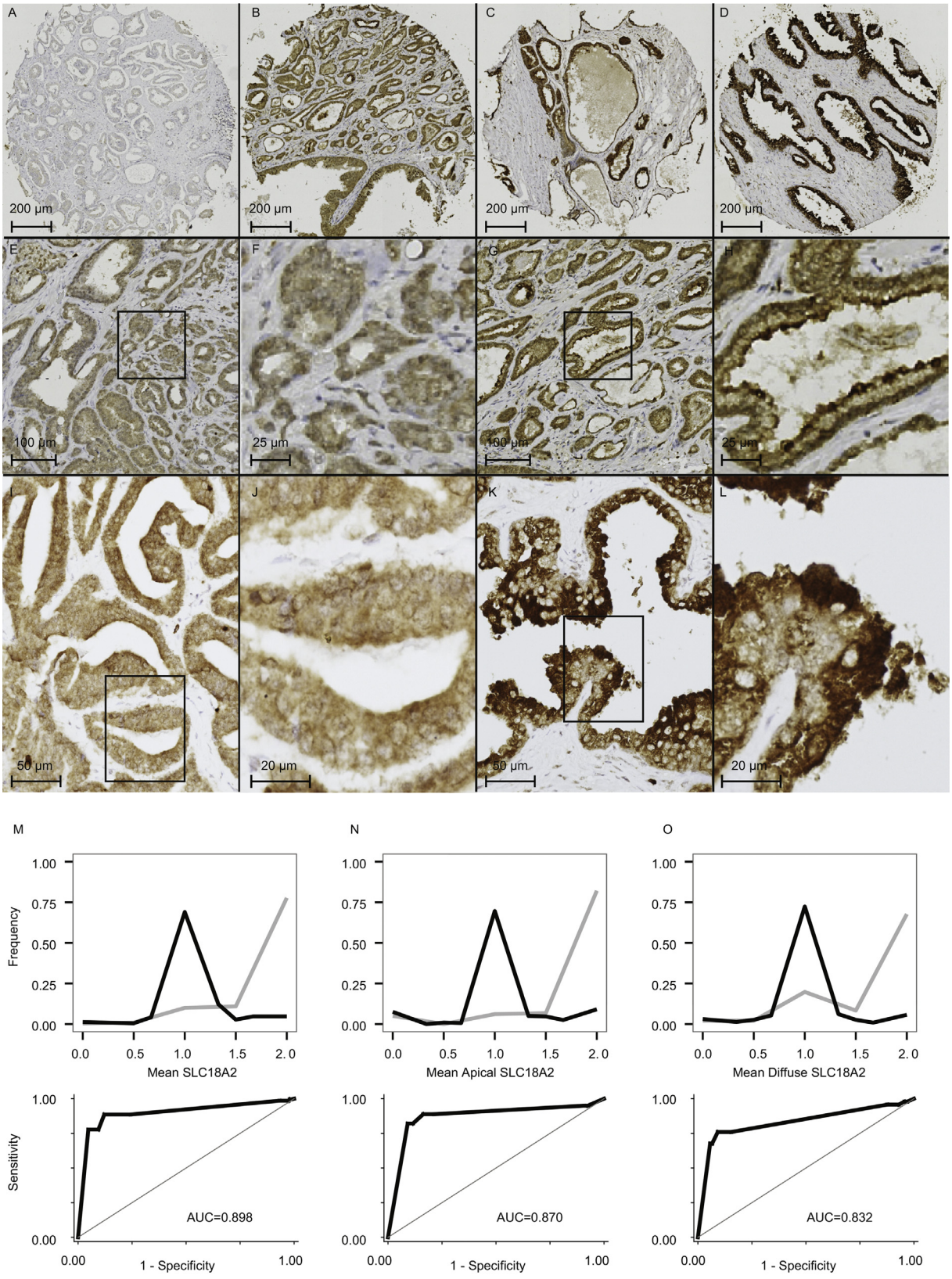

Figure 3 – Representative examples of SLC18A2 immunohistochemical stainings. A–L: SLC18A2 immunohistochemistry. A: Malignant, unstained, score = 0. B: Malignant, moderate diffuse, score = 1. C: Benign, strong diffuse, score = 2. D: Benign, strong apical, score = 2. E, F: Zoom of B. G: Malignant, moderate apical, score = 1. H: Zoom of G. I: Representative high resolution image of diffuse staining pattern. J: Zoom of I. K: Representative high resolution image of apical staining pattern. L: Zoom of K. M–O: Top: frequencies of mean SLC18A2 protein scores. Bottom: Receiver operated characteristic analyses using the same data as in top panels. M: Mean SLC18A2 in 403 malignant (black line) and 233 benign (grey line) patient samples. N: Mean apical SLC18A2 in 319 malignant (black line) and 191 benign (grey line) patient samples. O: Mean diffuse SLC18A2 in 228 malignant (black line) and 112 benign (grey line) patient samples.

Table 2 – Uni- and multivariate Cox regression analysis of SLC18A2 staining levels and time to PSA recurrence or overall death after radical prostatectomy.

	Univariate HR (P-value)	95% CI	C-index	Multivariate HR (P-value)	95% CI	C-index
A: SLC18A2 IHC score, n = 403, PSA recurrence						
SLC18A2 score ≤1 vs > 1	0.41 (<0.001)	0.26–0.66	0.566	0.51 (0.008)	0.31–0.84	0.756[a]
Gleason score ≤6 vs ≥ 7	2.90 (<0.001)	1.92–4.38	0.602	2.10 (0.001)	1.38–3.21	0.754[b]
Pathological T-stage ≤ pT2c vs ≥ pT3a	3.25 (<0.001)	2.35–4.50	0.638	2.11 (<0.001)	1.48–3.01	
Surgical margin status pos vs neg	2.80 (<0.001)	2.02–3.88	0.627	1.98 (<0.001)	1.39–2.81	
Pre-operative PSA cont.	1.05 (<0.001)	1.03–1.06	0.670	1.04 (<0.001)	1.02–1.05	
B: Apical SLC18A2 IHC score, n = 319, PSA recurrence						
Apical SLC18A2 score ≤1 vs > 1	0.28 (<0.001)	0.15–0.53	0.582	0.42 (0.015)	0.21–0.84	0.774[a]
Gleason score ≤6 vs ≥ 7	2.88 (<0.001)	1.84–4.51	0.607	1.92 (0.006)	1.21–3.06	0.766[b]
Pathological T-stage ≤ pT2c vs ≥ pT3a	3.55 (<0.001)	2.47–5.09	0.649	1.95 (0.001)	1.30–2.91	
Surgical margin status pos vs neg	3.25 (<0.001)	2.26–4.66	0.640	2.28 (<0.001)	1.54–3.38	
Pre-operative PSA cont.	1.06 (<0.001)	1.04–1.08	0.677	1.05 (<0.001)	1.03–1.06	
C: Diffuse SLC18A2 IHC score, n = 228, PSA recurrence						
Diffuse SLC18A2 score ≤1 vs > 1	0.91 (0.752)	0.49–1.68	0.501	0.70 (0.292)	0.37–1.35	0.717[a]
Gleason score ≤6 vs ≥ 7	3.34 (<0.001)	1.80–6.21	0.593	2.84 (0.001)	1.51–5.34	0.719[b]
Pathological T-stage ≤ pT2c vs ≥ pT3a	2.10 (0.002)	1.33–3.33	0.584	1.57 (0.070)	0.96–2.56	
Surgical margin status pos vs neg	2.65 (<0.001)	1.69–4.16	0.622	2.13 (0.002)	1.32–3.44	
Pre-operative PSA cont.	1.04 (<0.001)	1.03–1.06	0.679	1.03 (<0.001)	1.02–1.05	
D: SLC18A2 IHC score, n = 403, overall death						
Mean SLC18A2 score ≤1 vs > 1	0.29 (0.043)	0.09–0.96	0.585	0.39 (0.129)	0.12–1.31	0.720[a]
Gleason score ≤6 vs ≥ 7	4.08 (0.008)	1.43–11.62	0.635	3.31 (0.027)	1.15–9.53	0.705[b]
Pathological T-stage ≤ pT2c vs ≥ pT3a	2.15 (0.029)	1.08–4.27	0.611	1.66 (0.152)	0.83–3.34	
Surgical margin status pos vs neg	1.83 (0.088)	0.91–3.64	0.565	–	–	
Pre-operative PSA cont.	1.00 (0.983)	0.97–1.04	0.476	–	–	

HR: Hazard ratio. CI: Confidence interval. C-index: Harrell's C.
a Harrell's C for multivariate model including SLC18A2.
b Harrell's C for multivariate model not including SLC18A2.

moderate (median = 1, diffuse and apical means) between AN and prostate cancer (AUCs 0.870 and 0.832, Figure 3N and 3O). Thus, SLC18A2 protein levels accurately separated AN and prostate cancer tissues independently of predominant cellular localization.

3.6. *Validation of prognostic potential of SLC18A2 protein levels*

Low SLC18A2 staining levels (mean SLC18A2 score ≤ 1) were significantly associated with high pathological Gleason score, advanced pathological T-stage, and high pre-operative serum PSA (Supplementary Table S6). Furthermore, in recurrence-free survival analyses, patients in the low SLC18A2 staining group had significantly increased risk of PSA recurrence after RP (HR 0.41, P < 0.001, Table 2A, Figure 4A). Notably, low SLC18A2 staining also had significant independent prognostic value in multivariate Cox regression analysis including all routine clinicopathological parameters (HR 0.51, P = 0.008, Table 2A). Harrells's C was modestly increased upon addition of SLC18A2 to the clinicopathological parameters (Table 2A). Thus, using a large independent RP cohort, we here validate our previously published observation that loss of SLC18A2 immunoreactivity is a significant independent prognostic factor for PSA recurrence after RP (Sorensen et al., 2009).

Notably, when testing mean apical and mean diffuse SLC18A2 scores independently, only loss of apical staining was significantly associated with adverse clinicopathological parameters (Supplementary Table S6). Similarly, only loss of apical staining was associated with increased risk of PSA recurrence in uni- and multivariate survival analyses (Table 2B and 2C, Figure 4B and 4C). In accordance, Harrell's C increased by addition of the mean apical SLC18A2, but not the mean diffuse SLC18A2 immunohistochemistry score, to the full multivariate model (Table 2B and 2C). Thus, the apical levels of SLC18A2 likely underlie the association of SLC18A2 protein levels with PSA recurrence.

Of the 403 RP patients analyzed for SLC18A2 immunohistochemistry, 33 had died and 10 had died from prostate cancer (Supplementary Table S3). In Kaplan–Meier analysis, loss of SLC18A2 was borderline significantly associated with prostate cancer-specific death (p = 0.062, Figure 4D). As all prostate-cancer specific deaths occurred in patients with low SLC18A2 immunohistochemistry scores, HRs could not be estimated. However, low mean SLC18A2 score was significantly associated with overall survival after RP in both univariate Cox regression and Kaplan–Meier analyses (Table 2D, Figure 4E). Although SLC18A2 immunohistochemistry score did not have significant independent prognostic value in multivariate Cox regression analysis, it did improve Harrell's C (Table 2D). In conclusion, we here for the first time report an association between SLC18A2 protein levels and survival of prostate cancer patients.

4. Discussion

We here report that the *SLC18A2* promoter-associated CpG island was hypermethylated in prostate cancer, that SLC18A2 mRNA levels were reduced, and that both were associated

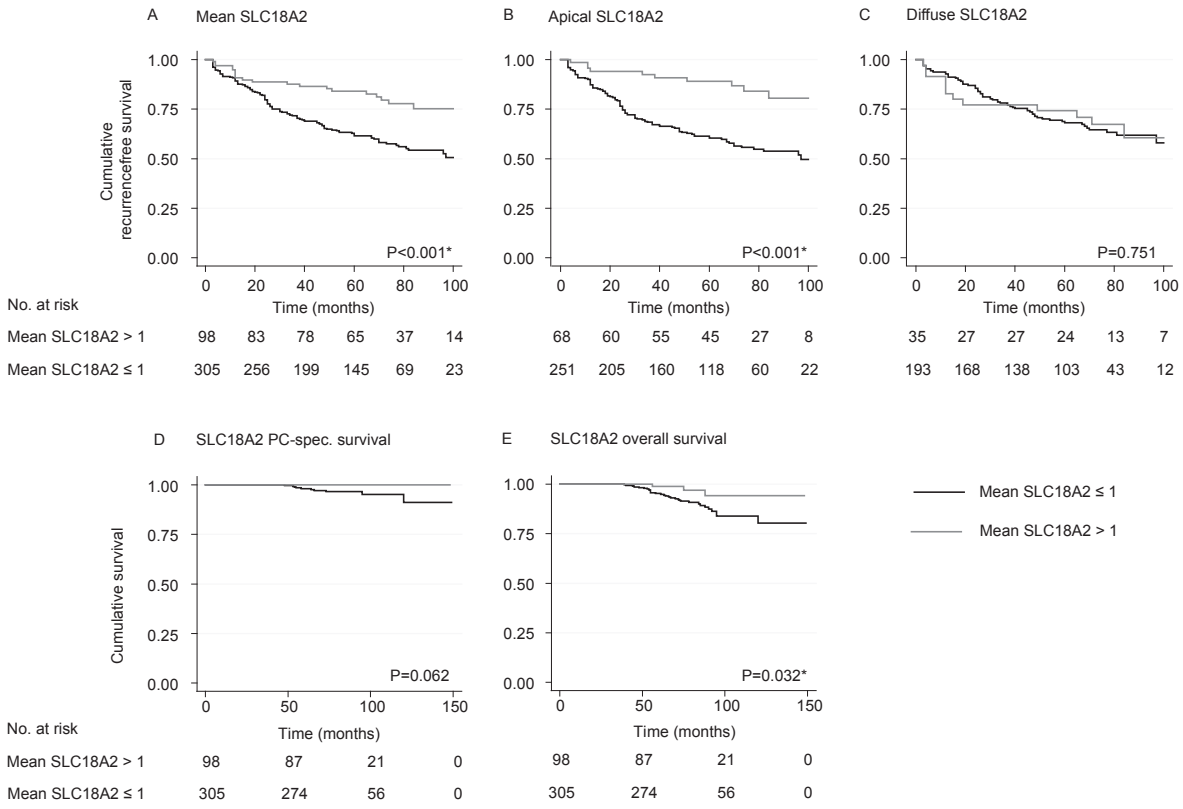

Figure 4 – SLC18A2 protein staining and survival after RP. Kaplan–Meier plots of PSA recurrence-free survival (A–C), prostate cancer-specific survival (D), and overall survival (E) for RP patients with SLC18A2 protein staining data for two or more prostate cancer cores, comparing. A: mean SLC18A2 score >1 versus ≤1. B: mean apical SLC18A2 score >1 versus ≤1. C: mean diffuse SLC18A2 score >1 versus ≤1. D: Kaplan–Meier plot of prostate cancer-specific survival of RP patients comparing mean SLC18A2 score >1 versus ≤1. E: Kaplan–Meier plot of overall survival of RP patients comparing mean SLC18A2 score >1 versus ≤1. P-values from log rank tests. *P < 0.05.

with increased risk of PSA recurrence after RP. Furthermore, we observed that SLC18A2 protein was reduced in prostate cancer and had significant independent prognostic value for PSA recurrence after RP, validating previous data (Sorensen et al., 2009), and indicating high prognostic biomarker potential for SLC18A2 protein. In addition, our results suggested an association of reduced SLC18A2 protein with both prostate cancer-specific and overall survival after RP.

SLC18A2 promoter methylation was highly cancer-specific with AUCs, ranging from 0.923 to 0.976, similar to those of other well-described candidate promoter hypermethylation markers for prostate cancer (Goering et al., 2012; Haldrup et al., 2013; Kristensen et al., 2014). Given the significant inverse correlation between *SLC18A2* promoter hypermethylation and both mRNA and protein levels, promoter hypermethylation likely contributes to the down-regulation of *SLC18A2* in prostate cancer. The potential clinical applicability of *SLC18A2* promoter hypermethylation for early detection of prostate cancer should be further tested in clinically relevant patient samples, including prostate biopsies negative for cancer by morphological criteria. Due to field-effects, cancer-specific hypermethylation of certain gene promoters (e.g. *GSTP1*) has been shown to be detectable in morphologically benign prostate tissues adjacent to cancer, and to thus potentially indicate the need for repeat biopsy (Brikun et al., 2014; Trock et al., 2012; Troyer et al., 2009). Of note, in a separate study, we have recently observed that *SLC18A2* DNA methylation can be detected in a subset of cancer-negative diagnostic biopsies from patients with prostate cancer (Møller et al., unpublished data, manuscript in preparation). Moreover, future studies should investigate whether *SLC18A2* methylation could potentially be employed for development of minimally invasive tests for prostate cancer in urine, plasma, and serum.

Current procedures for pre-operative determination of Gleason score and T-stage are suboptimal leading to frequent under-staging and under-grading (Heidegger et al., 2015; Rapiti et al., 2013). Therefore, in clinical practice, molecular markers yielding independent prognostic information would be most valuable before RP (Arsov et al., 2012), and thus could also be used to guide treatment decisions at the time of diagnosis. Therefore, although *SLC18A2* promoter methylation levels in RP specimens did not have independent prognostic value for PSA recurrence in multivariate Cox regression analysis after correction for post-operative clinicopathological variables, our results warrant further studies of the possible prognostic value of *SLC18A2* promoter methylation if analyzed in diagnostic biopsy samples.

Reduced SLC18A2 mRNA and protein levels both had significant prognostic value for PSA recurrence after RP independently of standard clinicopathological parameters ($P < 0.05$, multivariate HRs 0.19 and 0.41, respectively). Notably, we previously demonstrated independent prognostic value of SLC18A2 protein for PSA recurrence (Sorensen et al., 2009), thus, with the present data, we independently validate the prognostic value of SLC18A2 protein. In addition, we here document an association of SLC18A2 protein levels with both prostate cancer-specific and overall survival, strongly suggesting that SLC18A2 is associated with prostate cancer aggressiveness. Adding SLC18A2 to clinicopathological parameters only modestly increased Harrell's C, as often seen for molecular biomarkers (Banez et al., 2010; Cottrell et al., 2007). However, Harrell's C may not reflect the true clinical potential of biomarkers (Janes et al., 2008; Ware, 2006). Given the current evidence, patients with low levels of SLC18A2 protein are at higher risk of PSA recurrence after RP, thus these patients could be candidates for e.g. adjuvant radiation therapy. Of note, it has recently been suggested that a genomic classifier designed for prediction of metastases after RP, may be used to stratify patients for adjuvant or salvage radiation therapy (Den et al., 2015).

A limitation to this study is the use of PSA recurrence as endpoint. More relevant clinical endpoints include PC-specific death and time to clinical progression. Thus the survival analysis should be repeated in a cohort with longer follow-up (>10 years) and more fatal events to solidly establish the association of reduced SLC18A2 protein with reduced survival. In addition, to evaluate the potential use of SLC18A2 protein levels to stratify patients prior to RP, additional study populations are needed. Thus, as for the promoter methylation, the prognostic value of SLC18A2 protein levels should be further tested in diagnostic biopsies to assess its prognostic potential prior to RP. In addition, biopsy material from active surveillance cohorts could provide information about the association between natural development of PC and SLC18A2 protein levels. Thus, further studies, also including large prospective cohorts, are needed to assess the actual clinical utility of SLC18A2 as a biomarker for aggressive prostate cancer.

The association of reduced SLC18A2 protein levels with PSA recurrence suggests that SLC18A2 may have tumor-suppressive properties. Indeed, another protein involved in monoamine regulation, monoamine oxidase A (MAOA), which degrades monoamines, is frequently up-regulated in PC and associated with poor prognosis (Wu et al., 2014). Some of the monoamines known to be transported by SLC18A2 are present in human seminal fluid (e.g. serotonin (Gonzales and Garcia-Hjarles, 1990), epinephrine, and norepinephrine (Fait et al., 2001)), and serotonin released from neuroendocrine cells may play a role in regulation of volume homeostasis inside the prostatic glandular structure (Pai and Marshall, 2011). Thus, the presence of SLC18A2 at the apical cell surface of epithelial prostate cells could suggest a role of SLC18A2 in release and/or uptake of these substrates. How loss of monoamine transport could impact cancer-development/progression is unclear, and the role of SLC18A2 in both benign and malignant prostate cells should be investigated in functional studies. Interestingly, whereas the level of apical SLC18A2 protein had a strong association with PSA recurrence (HR 0.28, $P < 0.05$), the level of diffuse staining had no association. This could reflect underlying biology, and the role of SLC18A2 in different cellular contexts should also be investigated, but is beyond the scope of the present study.

In conclusion, we established that *SLC18A2* promoter hypermethylation is highly cancer-specific, and that SLC18A2 mRNA and protein levels are significantly decreased in prostate cancer. Notably, decreased levels of SLC18A2 mRNA and protein were both significantly associated with increased risk of PSA recurrence and had significant independent prognostic value for PSA recurrence in multivariate Cox regression analysis after correction for confirmed clinicopathological factors.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2016.02.001.

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