



UMEÅ UNIVERSITY

MicroRNA expression profiles in prostate cancer bone metastases

Functional effects of
microRNA-23c, -375, and -4328

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Front cover: Spheres of C4-2B prostate cancer cells grown in Matrigel® with staining of PSA (red), Ki67 (green) and nuclei (blue). Cover image reproduced with the kind permission of Susanne Gidlund and Sofia Halin Bergström.

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Abstract

Non-coding microRNAs (miRNAs) function as post-transcriptional regulators of gene expression by interacting with messenger RNA. Dysregulation of miRNAs has many possible consequences, including tumor-suppressive or -promoting ones, and restoring or preventing the effects of miRNA alteration has therapeutic potential.

Metastatic prostate cancer (PC) spreads to the bone and is treated with castration therapy. Eventually, metastases relapse into castration-resistant PC (CRPC) growth. Recently, our laboratory described metastatic PC subtypes, termed MetA-C, defined based on transcriptomic differences and linked to different morphology and prognosis. Patients with MetB metastases have particularly poor prognosis.

The overall aim of the thesis was to identify novel biomarkers and therapeutic targets for metastatic PC, with a focus on miRNAs. The specific aims were to: (1) identify miRNAs associated with PC progression into bone metastasis, and their functional roles; (2) verify the MetA-C subtypes, their prognostic importance, and their relation to genetic profiles in independent validation cohorts; (3) explore miRNA expression profiles of PC bone metastases, specifically in relation to the MetA-C subtypes, and whether specific miRNAs show potential to inhibit the aggressive MetB subtype.

Study 1: Differentially expressed miRNAs ($n=79$) were identified by comparing miRNA levels in bone metastatic ($n=14$) or localized PC ($n=7$) samples to benign samples ($n=7$) using microarray analysis. Downregulation of miRNA-23c and -4328 was verified by qRT-PCR analysis, including a larger cohort of bone metastases ($n=67$). Overexpression of miRNA-23c or -4328 in PC cells resulted in attenuated cell growth in vitro. High levels of miRNA-23c were detected in extracellular vesicles shed from overexpressing cells. Overexpression of miRNA-23c did not obviously affect tumor growth or angiogenesis in vivo.

Study 2: The existence and prognostic value of the MetA-C subtypes was verified by transcriptomic analysis of bone metastasis samples ($n=103$), and by subtyping publicly available data from metastatic samples ($n=573$) from external patient cohorts. The MetB subtype was associated with high tumor-cell proliferation, low androgen receptor activity, and poor prognosis in all cohorts, and provided independent prognostic information in addition to genetic aberrations.

Study 3: The miRNA profiles of 96 bone metastasis samples from Study 2 were examined using microarray analysis. Four sample clusters not obviously related to the MetA-C subtypes were observed. Expression levels of miRNA-375, however, were inversely related to MetB. MiRNA-375 overexpression in C4-2B resulted in a cellular switch of subtype, from being dominant MetB to dominant MetA. In parallel, reduced cell growth and signs of increased cell adhesion were observed.

In conclusion, altered miRNA profiles may contribute to progression of PC into bone metastasis, and to the development of different metastasis subtypes. The MetB subtype is associated with poor prognosis and low expression of miRNA-375. Therapy stratification based on the MetA-C subtypes should be considered in the future. Restoration of miRNA-375 in MetB tumors may offer a novel treatment option.

Abbreviations

3' UTR	three prime untranslated region
ADT	androgen deprivation therapy
Ago	argonaute
AR	androgen receptor
ARSI	androgen-receptor signaling inhibitors
ATM	ataxia-telangiectasia mutated
BRCA1/2	breast cancer gene 1 and 2
CCR4-NOT	carbon catabolite repression—negative on TATA-less
CD31	cluster of differentiation 31
CNA	copy-number alteration
CNV	copy-number variant
CpG	cytidine-phosphate-guanine
CRPC	castration-resistant prostate cancer
CT	cycle threshold
CTC	circulating tumor cell
CYP17	17 α -hydroxylase/C17,20-lyase
DDX6	DEAD-box helicase 6
DGCR8	DiGeorge critical region 8
DNA	deoxyribonucleic acid
E-cadherin	epithelial cadherin
ECM	extracellular matrix
EGFR	epidermal growth-factor receptor
EMT	epithelial mesenchymal transition
ER	endoplasmic reticulum
ERG	erythroblast transformation-specific related gene
ESCRT	endosomal sorting complex required for transport
ETS	erythroblast transformation-specific
EZH2	enhancer of zeste homolog 2
FBS–HI	fetal bovine serum – heat inactivated
FOXA1	forkhead box protein A1

FOXM1	forkhead box protein M1
FOLH1	folate hydrolase 1
FSH	follicle-stimulating hormone
GalNAc	N-Acetylgalactosamine
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Gn	gonadotropin
GnRH	gonadotropin-releasing hormone
GSEA	gene set enrichment analysis
HN	hormone naïve
IDH1	isocitrate dehydrogenase 1
ISUP	International Society of Urological Pathology
KLK2	kallikrein-related peptidase 2
KLK3	kallikrein-related peptidase 3
LC-MS/MS	liquid chromatography-mass spectrometry/mass spectrometry
LH	luteinizing hormone
mCRPC	metastatic castration-resistant prostate cancer
miRNA	microRNA
mRNA	messengerRNA
NEPC	neuroendocrine prostate cancer
NES	normalized enrichment score
NKX3-1	NK3 homeobox 1
PABC	polyadenylation-binding protein complex
PAM	prediction analysis of microarray
PARP	poly (ADP-ribose) polymerase
PBS	phosphate-buffered saline
PC	prostate cancer
PECAM-1	platelet endothelial cell adhesion molecule 1
PI3K	phosphoinositide 3-kinase
poly(A)	poly adenylic acid
pri-miRNA	primary microRNA
pre-miRNA	precursor microRNA
PSA	prostate-specific antigen

PTEN	phosphatase and tensin homolog
RB1	retinoblastoma transcriptional corepressor 1
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
SEC	size exclusion chromatography
Sec23A	Sec23 homolog A
shMIMIC	short hairpin MIMIC
shRNA	short hairpin RNA
SNV	single nucleotide variant
SPOP	speckle-type pox virus and zinc finger protein
TCGA	the cancer genome atlas
TME	tumor microenvironment
TNM	tumor node metastasis
TNRC6	trinucleotide repeat-containing gene 6 protein
TMPRSS2	transmembrane protease serine 2
p53	tumor protein 53
VEGF-A	vascular endothelial growth factor-A
YAP1	yes-associated protein 1
ZEB1	zinc finger E-box binding homeobox 1

Scientific papers

Paper I

Järemo H, Semenas J, Bergström SH, Lundholm M, Thysell E, Widmark A, Crnalic S, Ylitalo EB, Bergh A, Brattsand M, Wikström P. **Investigating microRNA Profiles in Prostate Cancer Bone Metastases and Functional Effects of microRNA-23c and microRNA-4328.** *Cancers (Basel)*. 2023 Apr 24;15(9):2437.

Paper II

Thysell E, Köhn L, Semenas J, Järemo H, Freyhult E, Lundholm M, Thellenberg Karlsson C, Damber JE, Widmark A, Crnalic S, Josefsson A, Welén K, Nilsson RJA, Bergh A, Wikström P. **Clinical and biological relevance of the transcriptomic-based prostate cancer metastasis subtypes MetA-C.** *Mol Oncol*. 2022 Feb;16(4):846-859.

Paper III

Järemo H, Semenas J, Bergström SH, Thysell E, Lundholm M, Freyhult E, Josefsson A, Welén K, Widmark A, Crnalic S, Bergh A, Brattsand M, Wikström P (2023). **The microRNA landscape of prostate cancer bone metastases and the role of miRNA-375 in modulating phenotypic characteristics of metastatic cells.** Manuscript.

Additional publications

Paper IV

Voss G, Hafliðadóttir BS, Järemo H, Persson M, Catela Ivkovic T, Wikström P, Ceder Y. **Regulation of cell-cell adhesion in prostate cancer cells by microRNA-96 through upregulation of E-Cadherin and EpCAM.** Carcinogenesis. 2020 Jul 14;41(7):865-874.

Paper V

Bergström SH, Järemo H, Nilsson M, Adamo HH, Bergh A. **Prostate tumors downregulate microseminoprotein-beta (MSMB) in the surrounding benign prostate epithelium and this response is associated with tumor aggressiveness.** Prostate. 2018 Mar;78(4):257-265.

Populärvetenskaplig sammanfattning

MikroRNA (miRNA) är små molekyler som finjusterar cellens proteinproduktion genom att förhindra att information i messengerRNA (mRNA) översätts till protein. Vid prostatacancer (PC) kan miRNA-nivåer i elakartade celler minska, öka eller förbli oförändrade. Vidare kan miRNA nedreglera nivåer av såväl tumörhämmande som stimulerande proteiner. Därigenom skulle olika miRNAs potentiellt kunna användas för PC-diagnostik och prognos samt för val av behandling. Via små membrantransportörer (extracellulära vesiklar) kan miRNA utsöndras från celler och på så sätt kan en PC-cell påverka sin omgivning.

Hos män är PC vanligt och då denna cancer oftast växer långsamt lever många drabbade länge. Om canceren är lokaliserad till prostatakörteln kan patienter botas med operation och/eller strålbehandling. Diagnosen ställs med magnetkameraundersökning och genom att vävnad undersöks i mikroskop. En del drabbas av aggressiv sjukdom med spridning, framför allt till skelettet, och ibland upptäcks canceren först när den är spridd. Metastaserad PC behandlas i första hand med kastration, vilket innebär att androgennivån (testosteron) i blodet minskas med hjälp av läkemedel eller kirurgi. Trots till en början god effekt hos de flesta patienter, så fortsätter tumören efter en tid att växa och den är då kastrationsresistent. Kastrationsresistent PC behandlas med cellgift samt genom ytterligare hämning av testosteronsyntes och blockering av dess receptor med läkemedel. Inga biomarkörer används idag för att rutinmässigt förutsäga patienters svar på tillgängliga behandlingar.

Vår forskargrupp har beskrivit tre undergrupper (subtyper) av metastaserad PC samt en mRNA-panel som kan särskilja dessa. Subtyperna som benämns MetA, MetB och MetC (MetA-C) har olika molekylära och vävnadsmässiga egenskaper. MetA är vanligast förekommande, androgenkänslig och växer relativt långsamt. MetB-metastaser utmärks tvärt emot av låg androgenkänslighet, snabb tillväxt och dålig prognos efter kastrationsbehandling. MetC-metastaser är minst vanligt förekommande och kännetecknas av en stor bindvävskomponent. Vi tror att dessa subtyper är av stor prognostisk betydelse och att de skulle kunna användas för att välja samt utveckla lämpliga behandlingar för individuella patienter.

Delarbete nr. 1 syftade till att identifiera miRNA av betydelse för PC-tillväxt och metastasutveckling samt till att undersöka deras verkningsmekanismer. Studien undersökte nivåer av 2019 olika miRNA i

14 benmetastasprover och jämförelse gjordes med nivåer i lokal PC och normal vävnad (7 prover från vardera). Resultaten visades generellt låga miRNA-nivåer i skelettmetastaser. Vidare identifierades 9 miRNA (inklusive miRNA-23c och -4328) vars nivåer minskade både i lokaliserad och metastaserad PC jämfört med nivåer i normal prostatavävnad. Effekter av miRNA-23c och -4328 studerades i experimentella modellsystem för PC. Överuttryck av miRNA-23c eller -4328 i PC-celler gjorde att cellerna växte långsammare då de odlades i laboratoriet. Vidare upptäcktes att PC-celler som överuttryckte miRNA-23c utsöndrade betydande mängder av molekylen via extracellulära vesiklar. Dock hämmades inte tumörtillväxt i mus av miRNA-23c och vi kunde inte heller se någon uppenbar effekt på tumörernas kärlbildning.

I artikel nr. II var syftet att verifiera existensen av metastassubtyperna MetA-C och deras kliniska och biologiska betydelse. Detta gjordes via klassificering av metastasprover från flertalet patientkohorter bestående av 103 egeninsamlade och mRNA-profilerade prover (intern kohort) och 573 prover redan insamlade och analyserade av andra (externa kohorter). Klassificeringen av MetA-C baserades på tumörprovernas nivåer av 157 utvalda MetA-C-associerade mRNA, och resultaten verifierade tidigare resultat rörande MetA-C samt gav några nya insikter. Nästan varje metastasprov innehöll en blandning av MetA-C. Den dominerande subtypen var oftast MetA (78% av proverna). Efter kastrationsbehandling ökade andelen MetB jämfört med obehandlad vävnad, och patienter med högre andel MetB hade sämre prognos efter kastration. Även de externa patientkohorterna kunde klassificeras utifrån MetA-C med liknande resultat. Då de även innehöll metastasprover från andra organ än ben kunde det konstateras att MetB anrikades i PC levermetastaser, medan MetA anrikades i lymfkörtelmetastaser.

Syftet med delarbete nr. III var att identifiera miRNA som kunde kopplas till förekomst av metastassubtyperna MetA, B eller C. Vidare ville vi undersöka om sådana miRNA kunde påverka egenskaper kopplade till MetA (androgenreceptoraktivitet) och MetB (celltillväxt) hos PC-celler odlade i laboratoriet (cellinjer). Resultaten från analys av mRNA i metastasprover (delarbete nr. II) kombinerades med miRNA-data från samma prover (n=98). På så vis upptäcktes att miRNA-375 var tydligt associerad till MetB och att lägre miRNA-375-nivåer uttrycktes i tumörer med mer MetB-innehåll. Parallellt upptäcktes att det var möjligt att studera egenskaper kopplade till MetA och MetB i en tumörcellinje som kallas C4-2B. När dessa celler exponerades för höga halter av miR-375 växte de långsammare. Produktionen av miRNA-375 ökade i sin tur av att cellerna stimulerades med androgen. Med hjälp av en metod som kallas

in situ hybridisering var det även möjligt att visa att miRNA-375 bildas i epiteliala PC-celler. Det är därför möjligt att miRNA-375 bidrar till epitelial celldifferentiering och till den lägre celltillväxt som ses i MetA jämfört med MetB.

Sammanfattningsvis är det troligt att förändrade uttrycksmönster av miRNA bidrar till PC-progression och till utveckling av olika subtyper av metastaserad PC. MetB är associerad till dålig prognos. Därför bör information om metastassubtyp vägas in vid beslut om vilken behandling som ska ges. Behandling med miRNA-375 skulle kunna vara en möjlig terapi för att uppnå minskad celltillväxt, särskilt hos patienter med metastaser av subtyp MetB.

Introduction

Cancer

Cancer is characterized by growth beyond physiological boundaries due to uncontrolled cell division, together with the potential of the malignant cells to invade and disseminate (1). Cancer is a leading cause of death worldwide, and the Global Cancer Observatory registered approximately 1.9×10^7 diagnosed cases and 1.0×10^7 cancer deaths in 2020 (2).

The hallmarks of cancer

A number of biological hallmarks, acquired by malignant cells during carcinogenesis, have been defined (3). These include (a) sustaining proliferative signaling, (b) evading growth suppressors, (c) inducing angiogenesis, (d) enabling replicative immortality, (e) resisting cell death, and (f) activating invasion and metastasis. In 2011, (g) deregulating cellular metabolism and (h) avoiding immune destruction were added as emerging hallmarks, but are now considered to be core hallmarks (4). In addition, two enabling factors were suggested, supporting multiple hallmark capabilities, (I) tumor promoting inflammation and (II) genome instability and mutation.

As research has progressed, further emerging hallmarks have been proposed, including (i) unlocking phenotypic plasticity and (j) the phenomenon of senescent cells. Two enabling characteristics were also added: (III) non-mutational epigenetic reprogramming and (IV) polymorphic microbiomes. The former is proposed to facilitate acquisition of (i) unlocking phenotypic plasticity (5).

Tumor microenvironment

The tumor microenvironment (TME) is the proximal environment surrounding a tumor cell. TME composition differs between tumor types, but in general contains immune cells, fibroblasts, pericytes, smooth muscle cells, blood vessels, nerve fibers, lymphatics, and an extracellular matrix (ECM) (6). The ECM is a network of fibrous proteins and glycoprotein macromolecules that provides structural support for cells. Collagens are the most abundant proteins in the ECM (7). Cross-talk between the tumor and cells in the TME is believed to influence tumor progression (8).

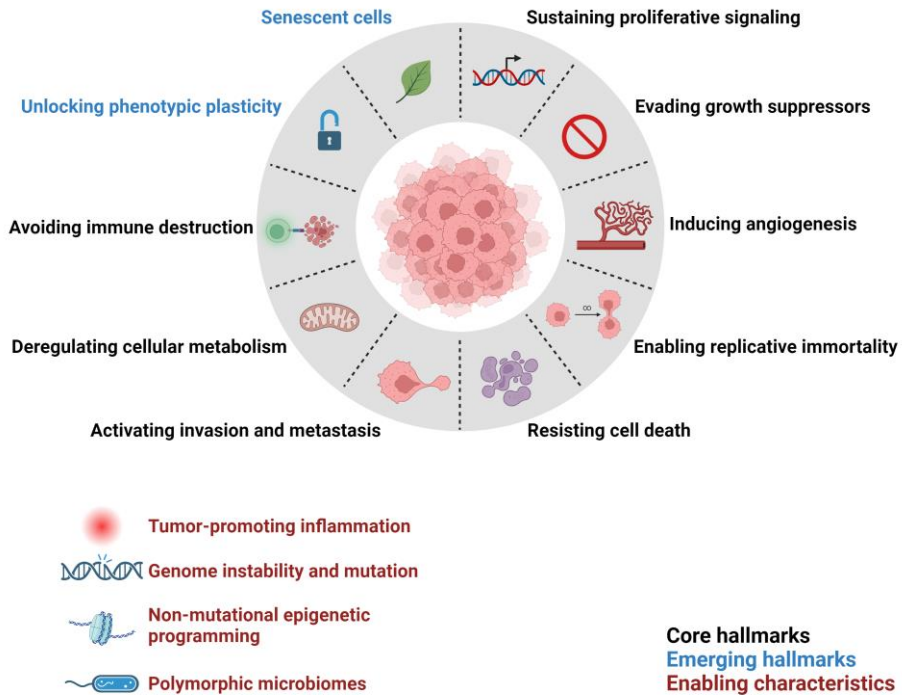


Figure 1. The hallmarks of cancer. Core hallmarks are marked in black, emerging hallmarks in blue, and enabling characteristics in red. This figure was modified from Hanahan et al. *Cancer Discov.* 2022;12(1):31–46 and created with BioRender.com (BioRender, Toronto, Canada).

Tumor vascularization

Tumors vascularize either by stimulating outgrowth from pre-existing vessels (angiogenesis) or through the formation of new vessels (vasculogenesis) (9). Angiogenesis is regulated by pro- and anti-angiogenetic factors, including vascular endothelial growth factor-A (VEGF-A) and thrombospondin 1. They bind to endothelial receptors, thus activating and impeding angiogenesis, respectively. When pro-angiogenetic signaling is dominant, vessel formation is initiated. An adequate blood supply is essential for malignant cells as the need for nutrients and oxygen increases as tumors grow, as is the necessity to remove waste. In the early stages of cancer an angiogenic switch instigates quiescent vasculature to develop new capillaries, thereby allowing solid tumors to grow beyond the size of a few mm². The switch is induced by hypoxia and oncogene signaling, causing the upregulation of VEGF-A (4).

Metastasis

Metastatic disease accounts for the majority of cancer-related mortality, but its pathophysiology remains poorly understood. A series of steps summarizing the metastatic process has been proposed: **(1)** local invasion and dissemination, **(2)** intravasation to the blood or lymphatic system and surviving in the circulation, **(3)** extravasation, and **(4)** colonization of the remote organ (10-13). Colonization is viewed as the rate-limiting and most inefficient stage of the metastatic cascade.

The epithelial cells of the prostate are polar, and reside on a basement membrane. They adhere both to one another and the adjacent tissue. Malignant cells lose polarity and tight cell-cell contact, thereby adopting a migratory and invasive phenotype. One theory explaining the changes in phenotype is epithelial-mesenchymal transition (10). This involves a down- and upregulation of the adhesion molecules epithelial-cadherin and neural-cadherin, respectively, reorganization of the cytoskeleton, and enhanced cell mobility (11).

In the blood, circulating tumor cells (CTCs) must evade the immune system and withstand mechanical and oxidative stress. CTCs express pro-survival proteins and downregulate expression of histocompatibility class-1 molecules to avoid recognition by the immune system. Furthermore, platelets coat circulating tumor cells (CTCs), providing protection (14-16).

CTCs eventually reach capillaries and may extravasate (16). Prostate cancer (PC) cells often metastasize to the bone marrow (17). The “seed and soil hypothesis” is based on the observation that different tumor types establish metastases in specific organs. This implies that a predisposed environment is necessary for the cancer cell to colonize and grow (18-20). Subsequent work has verified the existence of a “pre-metastatic niche”, an environment “primed” by the primary tumor prior to the arrival of the CTC (21-24). In contrast, the “anatomical/mechanical hypothesis” states that the site of the primary tumor and the anatomy of the blood and lymphatic vasculature determine where CTCs will spread (25). Later reviews reason that anatomical/mechanical factors likely control regional metastasis, but do not fully explain the formation of distant metastasis (26).

To colonize the distant organ disseminated tumor cells must adapt to their new microenvironment, and cells can stay dormant for years until the formation of macro-metastases (16, 27).

Prostate cancer

Epidemiology

In Sweden PC is the most common cancer among men, with roughly 1.0×10^4 new diagnosed cases in 2021. Annually, the malignancy causes approximately 2.0×10^3 deaths, making it the fourth most common cancer mortality in Sweden (28). Worldwide, PC accounts for about 7% of all diagnosed malignancies and nearly 4% of all fatalities due to cancer. PC is more common in industrial countries, even when adjusting for the age of populations (2, 29).

Anatomy, function, and morphology

The prostate is located in front of the rectum, below the urinary bladder, and surrounds the urethra. The main function of the organ is to secrete prostate fluid to the semen in order to contribute to sperm viability. The prostate can be divided into central, peripheral, and transition zones (Figure 2A). Nearly 80% of prostate tumors arise in the peripheral zone (29).

The prostate is composed of glands lined by epithelial cells that form two layers; an inner layer of columnar luminal cells, and an outer layer of cuboidal basal cells, with small quantities of neuroendocrine cells distributed in the epithelium (30, 31) (Figure 2B). The fibromuscular stroma, consisting of endothelial cells, fibroblasts, immunological cells, and smooth muscle cells, fills the compartments between the glands (32). The majority of PCs are adenocarcinomas (90–95%), but the cell of origin responsible for malignant transformation is unclear. The tumor bulk is constituted of more or less well differentiated epithelial cells with a luminal phenotype. PC is therefore hypothesized to originate from either luminal or basal epithelial cells that partly differentiate to luminal cells (33). Prostate tumors are often multifocal, and each foci can differ from others in aggressiveness (34). Neuroendocrine PC (NEPC) is a phenotype with a particularly poor prognosis that typically arises in response to treatment selection pressure (35).

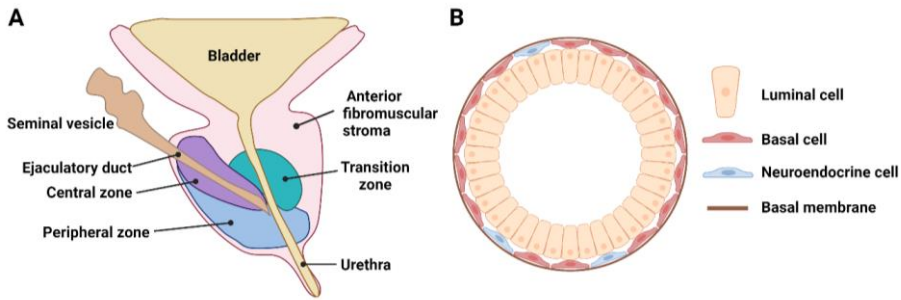


Figure 2. The anatomy and morphology of the prostate. (A) The anatomical location and regions of the prostate: central zone, peripheral zone, and transition zone. (B) Schematic representation of a prostate duct and acini; an inner layer of columnar luminal epithelium is surrounded by cuboidal basal cells. Neuroendocrine cells are interspersed in the epithelium. A layer of extracellular matrix called the ‘basal membrane’ lines the basal side of the duct. This figure was modified from Rebello et al. *Nat Rev Dis Primers*. 2021;7(1):9, and created with BioRender.com.

The aggressiveness of PC is histologically graded based on the Gleason and International Society of Urological Pathology (ISUP) grading systems (36, 37). Using microscopy, the pathologist determines the dominant Gleason pattern, ranging from 1 to 5, for the former system (38). The Gleason sum is the dominant pattern added to the highest grade of the second most frequently observed pattern (36). The sum ranges from 2 to 10, and a higher Gleason sum means a greater aggressiveness. The ISUP grading is a new grading system that divides PCs into five groups (Table 1) (37). The Gleason and ISUP grading systems are only used to grade untreated primary tumors, and thus not for metastases.

Table 1. The ISUP grade groups.

Grade group	Gleason sum
1	≤ 6
2	3 + 4 = 7
3	4 + 3 = 7
4	8
5	9–10

Molecular pathogenesis and pathophysiology of prostate cancer

Androgen-receptor signaling

Gonadotropin-releasing hormone (GnRH) is synthesized from the hypothalamus, and releases luteinizing hormone (LH) by activating GnRH receptors on gonadotroph cells in the anterior pituitary gland of the hypophysis. LH acts on the testes by stimulating testosterone production (Figure 3).

An androgen receptor (AR) is a nuclear receptor that is activated by the binding of androgens. It functions as a transcription factor, and regulates the transcription of androgen-receptive genes. When activated, its conformation changes and it traffics to the nucleus. The receptor binds to genes with androgen-response elements in their promoter or enhancer regions, thereby regulating transcription. Androgens and AR signaling control the development of normal prostate tissue, and PC growth is in most cases AR-dependent. Examples of androgen-responsive genes include kallikrein-related peptidase 2 and 3 (KLK2, KLK3), NK3 homeobox 1 (NKX3-1), transmembrane serine protease 2 (TMPRSS2), and folate hydrolase 1 (FOLH1) (39). The AR also regulates cellular processes such as apoptosis, differentiation, and proliferation (40-42). The receptor is expressed in epithelial cells and cells in the prostate stroma. Stromal AR signaling is of importance in the hormonal regulation of a normal prostate, PC, and response to castration (43-45).

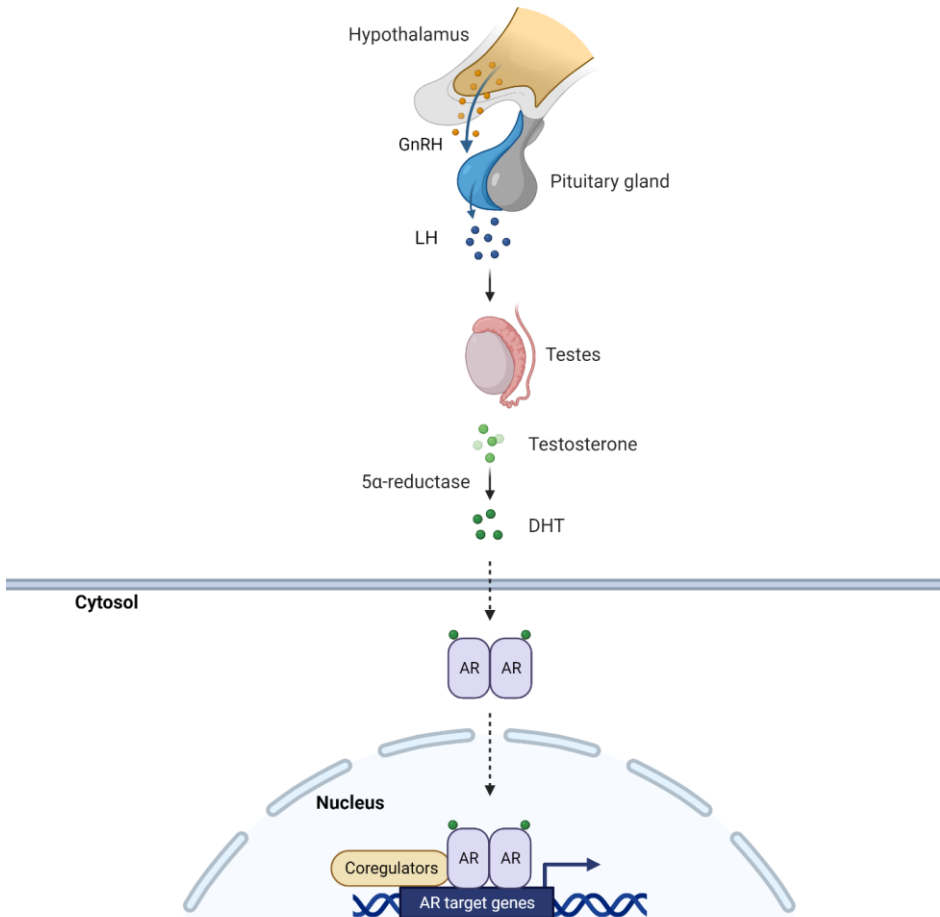


Figure 3. The hypothalamic-pituitary-testicular axis and AR signaling.

The hypothalamus releases GnRH, which stimulates the release of LH from the anterior pituitary gland. LH stimulates testosterone production of the testes. 5 α -reductase converts testosterone to DHT. An androgen receptor is activated by the binding of DHT, and translocates to the nucleus where it acts as a transcription factor. This figure was created with BioRender.com. AR, androgen receptor; DHT, dihydrotestosterone; GnRH, gonadotropin releasing hormone; LH, luteinizing hormone.

Genomic alterations

It is hypothesized that genetic alterations accumulate over time and instigate the malignant transformation of prostate epithelial cells (29). The most common genetic alterations in PC are gene fusions and DNA copy-number alterations (CNAs). In contrast, point mutations are less

common, and the overall mutational burden is lower in PC as compared to other tumor types (46).

The cancer genome atlas (TCGA) includes a comprehensive study of genomic, transcriptomic, and epigenomic profiles of primary prostate cancer (46). The erythroblast transformation-specific (ETS) related gene (ERG) encodes a DNA-binding transcription factor that regulates functions such as cell cycle, invasion, differentiation, and proliferation. When ERG is fused with TMPRSS2 its expression becomes androgen-regulated (47). The TMPRSS2-ERG fusion is the most common genomic abnormality in PC, and is found in approximately 50% of all PC cases (46). Such fusions occur early and persist in advanced PC (47).

Phosphatase and tensin homolog (PTEN) aberrations constitute the second most frequent alteration, present in 17% of all tumors (46). Loss of PTEN enhances phosphoinositide 3-kinase (PI3K) signaling (48). The PI3K signaling pathway connects with the mitogen-activated protein kinase signaling pathway. Collectively, the two signaling pathways were altered in approximately 25% of all PCs in one study (46), which also found an inactivation of DNA damage-repair genes (e.g., ataxia-telangiectasia mutated (ATM), cyclin dependent kinase 12 (CDK12), breast cancer gene 1 and 2 (BRCA1, BRCA2)) in 19% of PC samples. Other frequently mutated genes are the tumor suppressor speckle-type pox virus and zinc finger protein (SPOP) (11%) and tumor protein 53 (p53) (8%) (49).

The genomic landscape of metastatic PC differs from primary PC, and the mutational burden is higher (29). Alterations in the AR-signaling pathway are frequent, and present in ~70% of all CRPC (46, 50, 51). After initial remission due to castration, the malignancy eventually relapses and becomes castration-resistant. Despite low testosterone levels, it then shows sustained AR signaling (52). Possible mechanisms include amplification and overexpression of the AR receptor, AR mutations, and the production of active AR splice variants (52, 53).

PTEN mutations are also enriched in metastatic castration-resistant prostate cancer (mCRPC), and detected in >40% of CRPC samples (50). The PI3K pathway is activated in >45% of mCRPC cases (54). As compared to localized PC, loss of TP53 (~8% vs. 50%) and RB1 (~1% vs. 21%) are more common in mCRPC (46, 50). Aberrations in DNA damage-repair genes are found in ~20% of mCRPC (50).

Epigenetic alterations

Epigenetics involves the modification of gene expression without altering the DNA sequence (55). Epigenetic and genetic alterations coordinate in regulating gene expression during cancer initiation and progression (46, 56, 57). Epigenetic mechanisms can silence tumor-suppressor genes and activate oncogenes, and are implicated in mediating therapeutic resistance (58, 59).

DNA methylation is one of the more well-understood epigenetic mechanisms of PC. Specific enzymes, termed “writers” and “erasers”, add and remove methyl groups from cytosines adjacent to guanines (CpG sites), respectively. Such sites frequently occur in specific regions of the genes (CpG islands), often close to regulatory promoter and enhancer regions. In malignancy, CpG islands are often aberrantly hyper- or hypomethylated, with an impact on gene transcription (58).

DNA methylation patterns distinguish primary PC from benign tissue (56, 60). Promoter hypermethylation occurs early in the disease and associates with lower expression of tumor suppressor genes (58, 61). The glutathione-S-transferase promoter is frequently hypermethylated and linked to PC carcinogenesis (58, 62). Loss of PTEN expression is often caused by hypermethylation of its promoter region (63). The homeobox gene family of transcription factors is recurrently hypermethylated in PC (64). TCGA’s epigenomic profiling reveals that specific methylation patterns are associated with genomic subgroups of primary PC (56).

Genome-wide hypomethylation is more frequent in metastatic PC, and associated with chromosomal instability and oncogene activation (57, 58, 65, 66). Regions with more differential hypomethylation have elevated somatic mutational rates, which suggests that global hypomethylation in cancer contributes to genomic instability (57). While the AR promoter was found to be hypomethylated in the majority of mCRPCs in one study, a subgroup of patients displayed a hypermethylation of the AR promoter region. This group of patients had a particularly poor prognosis (61).

Risk assessment and treatment stratification

In the Swedish guidelines, treatment strategies for PC are based on clinical tumor, node, and metastasis (TNM) staging (Table 2), life expectancy, and the risk of metastatic spread in the case of localized disease (37). The risk of metastatic spread is classified as ‘very low’, ‘low’,

‘intermediate’, ‘high’, or ‘very high’ based on T stage, Gleason Score, and serum prostate-specific antigen (PSA) level at diagnosis.

Treatment strategies

Watchful waiting includes regular clinical follow-ups with prostate palpation and PSA testing. The intention is not to curatively treat, but to detect cancer progression and treat to alleviate symptoms. It is an option in the case of comorbidity and a short life expectancy. **Active surveillance** includes regular clinical follow-ups with palpation, PSA assessment, and prostate biopsies, with the intention to treat curatively if the malignancy progresses. Curative treatment options include the surgical removal of the prostate and seminal vesicles (**radical prostatectomy**) and **radiation therapy**.

Androgen deprivation therapy (ADT) is achieved by castration, either through orchiectomy (removal of the gonads) or GnRH analogs/antagonists. GnRH analogs (**goserelin, leuprorelin, buserelin**) bind to GnRH receptors in the pituitary gland. Initially, they overstimulate LH and follicle-stimulating hormone (FSH) production, thereby increasing levels of circulating testosterone produced by the gonads. Eventually, testosterone declines, leading to PC regress. GnRH antagonists (**degarelix**) bind competitively to GnRH receptors, thereby reducing circulating LH, with a following decrease in testosterone production in the gonads.

Bicalutamide is an AR antagonist which binds to receptors, thereby blocking their activation by androgens (67). Second-generation AR antagonists (**apalutamide, darolutamide, enzalutamide**) are more potent than bicalutamide as they also prevent the nuclear translocation of the receptor and its interaction with DNA (37, 68).

Abiraterone acetate inhibits 17 α -hydroxylase/C17,20-lyase (CYP17), a vital enzyme in androgen biosynthesis. Similar to GnRH analogs, the drug reduces gonadal androgen production; however, unlike GnRH analogs it also inhibits synthesis in adrenal glands and cancer cells (69).

Docetaxel and cabazitaxel are taxane-based chemotherapeutics that induce mitotic arrest by affecting the stability of microtubules (70).

Radium-223 dichloride mimics Ca²⁺ and binds selectively to hydroxyapatite in the bone. It accumulates at sites of active bone

remodeling, where it emits alpha radiation that inhibits tumor growth and abnormal bone formation (71).

The poly (ADP-ribose) polymerase (PARP)-inhibitor olaparib is approved for patients with DNA-repair gene mutations and loss of BRCA1 and BRCA2 function (37, 72).

Table 2. Clinical tumor, node, and metastasis staging (TNM), adapted from the Swedish guidelines (37).

Clinical T-stage (cT)	
To	No evidence of primary tumor
Tx	Primary tumor cannot be assessed
T1	Clinically inapparent tumor that is not palpable
T2	Tumor that is palpable and confined within the prostate
T3	Palpable tumor with extracapsular extension
T4	Tumor invades adjacent structures other than seminal vesicles: bladder, external sphincter, rectum, or pelvic wall
Clinical N-stage (cN)	
No	No regional lymph node metastasis
Nx	Regional lymph nodes cannot be assessed
N1	Regional lymph node metastasis
Clinical M-stage (cM)	
Mo	No distant metastasis
M1	Distant metastasis

Localized prostate cancer

As there is considered to be an overdiagnosis of low-risk PC, the Swedish guidelines strongly recommend against any local therapy. Instead, such patients are to be managed by active surveillance, with the option to switch to treatment with curative intent if needed. Intermediate- and high-risk PC is treated with radiation therapy or radical prostatectomy, which can be combined with castration for the high-risk group (37).

Metastasized prostate cancer

Castration therapy is recommended in both the clinical N1 and M1 stages (Table 2) as first-line treatment of hormone-sensitive disease. In the past decade the treatment strategy has changed, with early addition of docetaxel, with or without further androgen-signaling inhibition (apa-

dar-, or enzalutamide or abiraterone acetate). This double or triple treatment has been shown to increase life expectancy (73, 74). Some studies have found benefit in adding local radiotherapy of the primary tumor if the sites of metastasis are few (75, 76).

Castration-resistant prostate cancer

Castration resistance is defined as a biochemical recurrence (rising PSA) of metastases or formation of new metastases during ADT, and is treated with continued castration therapy at the clinical M0 and M1 stage (Table 2). Androgen-signaling inhibition is recommended as add-on treatment at the M0 stage. The first-line treatment for distant metastasis (M1) is abiraterone, docetaxel, or enzalutamide. Second- and third-line options depend on the first-line drug, but include abiraterone, cabazitaxel, docetaxel, enzalutamide, a PARP-inhibitor, or radium-223.

Tumor heterogeneity

PC is a heterogenous disease as judged from molecular, morphological, and clinical characteristics. From a molecular perspective, **intra-tumoral** (within the same tumor), **inter-tumoral** (tumor to tumor within the same patient), and **inter-patient** (between patients) divergencies exist. Heterogeneity can be explained by genomic and epigenetic variations that ultimately affect the phenotype of cancer cells (34).

Frequently, multiple tumor foci exist in affected prostate glands (>80%). Foci usually differ in both histological grade and morphology (34). Scientific studies investigating PCs harboring multiple foci often fail to demonstrate shared genomic mutations, indicating a multiclonal origin (77, 78). **Inter-tumoral** heterogeneity poses a diagnostic challenge, making it essential to acquire tissue samples from all relevant lesions in the organ (34). In contrast, it is hypothesized that multiple distant PC metastases often originate from a single clone of the primary tumor, since they share a multitude of genomic changes (79-81). Despite originating from a single clone, metastases still exhibit subclonal heterogeneity due to parallel clonal development (34).

Intra-tumoral heterogeneity results from multiple subclones with diverging characteristics within the same tumor (34, 72, 82). Most localized PC tumors (~60%) encompass multiple subclonal populations (34, 83). Tumor evolution may explain intra-tumoral heterogeneity (84, 85). Accumulation of genomic alterations in cancer cells and

environmental pressure can lead to differences in fitness (86-88). Fitness advantage is usually facilitated by beneficial mutations conferring a growth advantage (driver mutations). Through selection pressure, clones with a fitness advantage can outcompete less well-adapted clones (34). In advanced stages, most PCs relapse due to therapy-resistance, to which intra-tumoral heterogeneity is a contributing factor. Therapies such as castration induce environmental selection pressure and may ultimately alter the clonal composition of the tumor, as clones with fitness advantages that facilitate therapy-resistance increase in number.

Clinically, at presentation PCs range from being localized to the prostate gland with an indolent and favorable prognosis, to rapidly progressing metastatic disease. In advanced disease, there are multiple treatment options available for men with CRPC, with different mechanisms of action. Thus, responses to treatment vary substantially due to **inter-patient** PC heterogeneity. Understanding PC heterogeneity is becoming increasingly important due to the prospect of tailoring treatment to the molecular biology of the tumor (54, 89). Advances in sequencing (e.g., single-cell sequencing) and in-situ techniques (e.g., digital spatial profiling) have enabled researchers to study clonal heterogeneity and tumor evolution over time and in relation to treatment response (90).

Genomic and transcriptomic prostate cancer biomarkers

Molecular subtyping of PC is a promising approach for predicting prognosis and response to therapy. Instead of relying on one or a few biomarkers only, molecular subtyping is based on broad gene expression patterns linked to biological processes. A number of PC classifications based on genomic, transcriptomic, and epigenomic alterations have been suggested (46, 57, 91-93), a few of which are summarized herein.

In Sweden, genetic testing is carried out upon suspicion of hereditary PC in order to detect potential BRCA2 mutations (37). In addition, in the ongoing Prostate-Biomarker trial, treatment decisions for mCRPC patients are based on genomic biomarkers in circulating tumor DNA (89). Unlike other malignancies (e.g., breast, bladder), no molecular classification system for PC is being routinely used to estimate prognosis and tailor treatment decisions as yet in Sweden (94).

Genomic subtyping

The cancer genome atlas classification

TCGA profiling identified seven subtypes based on genomic alterations, defined either by gene fusions of *TMPRSS2* with the ETS family of transcription factors, or mutations in *SPOP*, the forkhead box protein A1 (*FOXA1*), or isocitrate dehydrogenase 1 (*IDH1*). Of all analyzed tumors ($n=333$), 74% could be categorized as one of the genomic subtypes (46). The classification system is, however, not yet used to guide treatment decisions or for prognostic predictions.

Transcriptomic biomarkers and subtyping

Oncotype DX Genomic prostate score

A biopsy-based 17-gene genomic prostate score (Genomic Health, Redwood City, CA, US) was developed to stratify primary PC into low and high risk of aggressive disease (95). Prostatectomy specimens from patients with disease recurrence ($n=111$) and diagnostic needle biopsies of patients who later underwent radical prostatectomy ($n=167$) were analyzed. The gene panel, consisting of 12 cancer-related genes and 5 reference genes, improved prediction of adverse pathology, biochemical recurrence, distant metastasis, and PC-related death (96-100).

Decipher®

The Decipher® PC test (GenomeDx Biosciences, San Diego, CA, US) is a commercially available prognostic test. It measures the combined expression of 22 transcripts in samples obtained through radical prostatectomy, utilizing a microarray platform. The test predicts risk of early metastasis and mortality (101-105).

Prostate cancer classification system

The PC classification system of 1, 2, and 3 (PCS1–3) was established by analyzing more than 4600 benign prostate, primary PC, metastatic, and CRPC specimens (92). The classification is based on a gene panel consisting of 37 genes. In primary PC, the subgroup PCS1 accounted for 6%, while PCS2 and 3 were equally prevalent (both 47%). In contrast, PCS2 proved to be the least prevalent subtype in metastatic PC and CRPC. Luminal and basal epithelial markers revealed PCS1 and PCS2 as being luminal, whereas PCS3 displayed a basal subtype. PCS1 related to a poor clinical outcome, whereas PCS2 featured a high AR activity (92). In an independent analysis of a separate sample cohort using the PCS1–3 classifier, 77% of the metastatic samples were instead classified as PCS2 (106, 107).

Luminal and basal subtyping with prediction analysis of microarray 50 signature (PAM50)

The commercially available prediction analysis of microarray 50 (PAM50)/Prosigna signature (Veracyte Inc, San Francisco, CA, US), originally intended for molecular breast cancer subtyping, can also be used to define PC subtypes ($n=3$) with luminal- or basal-like features (91), making it possible to stratify outcomes and predict responses to treatment that targets AR signaling. An analysis of radical prostatectomy specimens ($n=3782$) found that the basal subtype was the most frequently occurring (37.1%), as compared to luminal A (34.2%) and luminal B (28.5%). Luminal B cancers displayed worse outcomes. A variation in AR signaling (high in luminal A and B) and PAM50 proliferation score (high in luminal B and basal) was detected. The luminal B subtype seemed to greatly benefit from ADT, presumably due to a high tumor-proliferation rate in combination with androgen responsiveness. Subsequently, 634 metastatic CRPC samples were stratified into basal (55%) and luminal (45%) clusters. The luminal subtype benefited from androgen-signaling inhibitors (108).

MetA-C

A classification of bone metastases was developed using a transcriptome-wide analysis of hormone naïve ($n=12$) and CRPC ($n=60$) bone metastasis (93). Unsupervised cluster analysis of the samples revealed distinct subtypes of metastases, termed MetA, MetB, and MetC. These subtypes were examined using gene-set enrichment analysis and immunohistochemistry, and in relation to patient survival (Figure 4). MetA, the most frequently occurring subtype, displayed a lower cell proliferation, a higher expression of AR-regulated genes, and a more favorable outcome after ADT compared to subtypes MetB and -C. MetB, the second most prevalent subgroup, demonstrated a poorer prognosis following ADT, a higher cell proliferation, and lower expression of AR-regulated genes compared to the others. MetC was the least common PC subgroup and showed an abundant stroma and increased expression of markers reflecting stroma-epithelial interactions.

Proteomic profiling of the overlapping samples revealed similar subtypes (109). By comparing primary PCs and metastases from the same patients, it was shown that Ki67 and PSA immunohistochemical staining of the primary tumor could be used predict an adverse outcome for the MetB subtype (110). Hypo- and hypermethylation of the AR promoter region may well explain the higher and lower expression of AR-regulated genes present in MetA and MetB metastases, respectively (61).

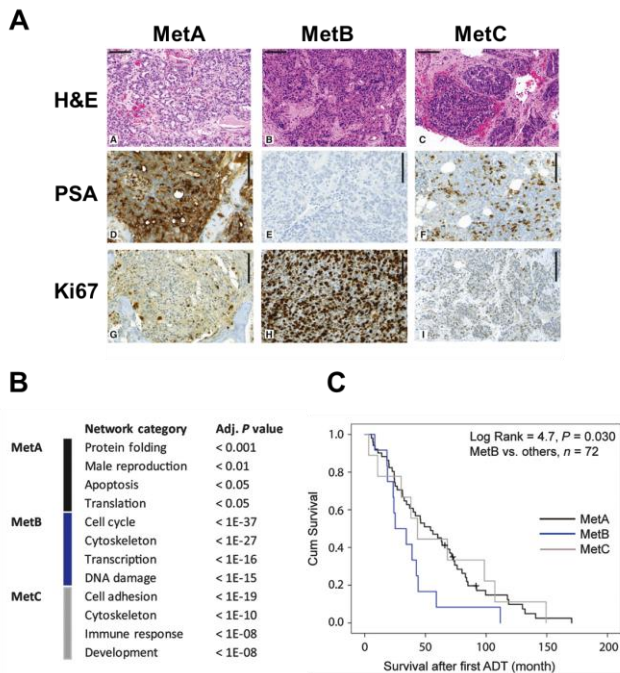


Figure 4. The MetA-C bone metastatic subtypes. (A) MetA-C morphology displayed by immunohistochemical staining with hematoxylin eosin (upper panel), PSA (middle panel), and Ki67 (lower panel). (B) Networks related to the MetA-C subtypes. (C) Kaplan-Meier analysis relating MetA-C subtype to survival after ADT. This figure is adapted from Thysell et al. *Mol Oncol.* 2019;13(8):1763–77. ADT, androgen deprivation therapy; Ki67, antigen kiel 67; MetA-C, MetA, MetB and MetC; PSA, prostate specific antigen.

Unmet clinical needs

It is important to reduce overdiagnosis and overtreatment of low-risk, indolent PC and magnetic resonance imaging before biopsies has been shown to reduce diagnosis of low-risk tumors (111). In the diagnosed cancers it is still important to identify aggressive tumors early on, and more reliable prognostic biomarkers are therefore needed.

Organ-confined PC is possible to cure, but not bone metastatic disease. Therefore, novel therapeutic strategies for metastatic PC are wanted. Such strategies could entail individualized treatment based on the molecular biology of the tumor. However, there are no validated treatment

predictive biomarkers based on molecular subtyping used in the clinical routine for patients with PC in Sweden yet.

Furthermore, patients often develop resistance to systemic treatment, and there is an unmet clinical need to identify biomarkers to monitor treatment response to detect therapeutic resistance early after the initiation of treatment, to reduce unnecessary side-effects and costs (112).

Prostate cancer cell lines

Cell lines are experimentally immortalized cells that can proliferate indefinitely. Cell lines established from patient materials are often used as model systems for different diseases. There are several commercially available cell lines that are frequently used in PC research; the ones that were used in this thesis are listed in Table 3, together with their characteristic features.

Table 3. The PC cell lines used in this thesis, and their morphology, origin, AR expression, androgen sensitivity and androgen response.

Cell line	Origin	AR expression	PSA expression	Ref.
22Rv1	relapsed mouse xenograft	Yes	Yes	(113)
C4-2B	castration resistant subline of LNCaP	Yes	Yes	(114)
DU145	brain metastasis	No	No	(115)
LNCaP	lymph node metastasis	Yes	Yes	(116)
PC-3	bone metastasis	No	No	(117)
PNT1A	normal prostate	No*	No*	(118)
RWPE-1	normal prostate	Yes	Yes	(119)
WPMY-1	stroma, normal prostate	Yes	Yes	(119)

*Brattsand et al., unpublished results.

MicroRNA

MicroRNAs (miRNAs) are non-coding RNAs with an average length of approximately 22 nucleotides, and were discovered roughly 30 years ago (120, 121). They serve as post-transcriptional regulators of gene expression, capable of fine-tuning the expression of many genes simultaneously (122, 123). Today, about 550 miRNAs genes have been confidently annotated in the human genome (124). Many miRNA families are conserved among species. Deletion of conserved miRNA genes has been demonstrated to cause severe abnormalities in phenotype, indicating their importance (125).

Functions of microRNAs

miRNAs mainly act by repressing protein translation, which is followed by mRNA decay (126, 127). Other mechanisms of action have been described, including translational activation and transcriptional regulation (126). Argonaute proteins (Ago) are catalytic components in the RNA-induced silencing complex (RISC), and mediate post-transcriptional gene silencing (128, 129). When bound to Ago, miRNAs guide RISC to target mRNAs as recognized by partial base pairing, mainly between the mRNA 3' untranslated region (3'UTR) and the miRNA seed region (130). The seed region involves nucleotides 2–8 in the 5' end of the miRNA. Repression efficacy is dependent on which nucleotides in the seed region base pair with the mRNA (125). Cleavage of the mRNA is unusual in human cells, but may occur if miRNA-mRNA base pairing is extensive (125). More frequently, Ago recruits the adaptor protein trinucleotide repeat-containing gene 6 (TNRC6), which interacts with polyadenylic acid (poly(A))-binding protein complexes (PABPC). The latter associates with the poly(A)-tail of the mRNA and recruits the deadenylate carbon catabolite repression–negative on TATA-less (CCR4-NOT) complex. CCR4-NOT shortens the poly(A)-tail, leading to a destabilization and degradation of the mRNA (125). CCR4-NOT further interacts with DEAD-box helicase 6 (DDX6), which in turn inhibits translational initiation (125).

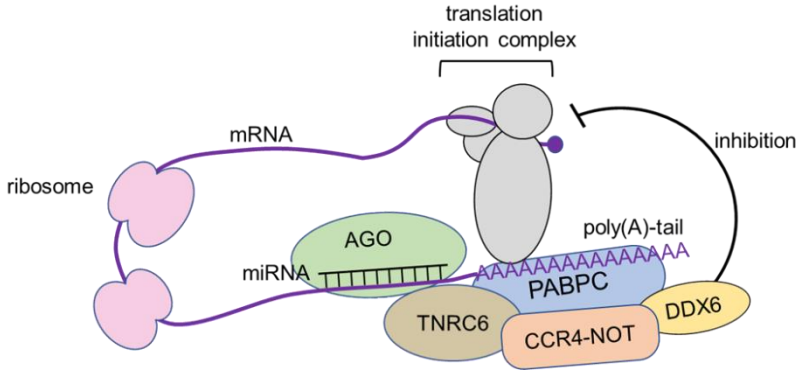


Figure 5. Assembly of the RNA-induced silencing complex. Ago recruits TNRC6, which interacts with PABPCs associated to the mRNA poly(A)-tail. This recruits the enzyme CCR4-NOT, which shortens the poly(A) tail via deadenylation and interacts with DDX6. The latter enzyme inhibits the translation initiation complex. This figure is modified from Bartel et al., *Cell*. 2018;173(1):20–51. AGO, argonaute; CCR4-NOT, carbon catabolite repression–negative on TATA-less; DDX6, DEAD-box helicase 6; PABPC, poly(A) binding protein complex; poly(A)-tail, polyadenylic acid-tail; TNRC6, trinucleotide repeat-containing gene 6.

Regulation and maturation of microRNAs

miRNA expression is tightly regulated (131). Commonly, their genes are either located within the introns of protein-coding genes (intragenic), in which case they are co-regulated with protein coding genes, or are found between genes (intergenic), in which case they are regulated by their own promoter (126, 132). miRNA genes are either transcribed as individual transcripts (monocistronic) or multiple genes are transcribed simultaneously as a single transcript (polycistronic). Initially, RNA polymerase II transcribes long primary miRNAs (pri-miRNA) containing hairpin structures (133). Subsequently, the transcripts are cut into shorter precursor miRNAs (pre-miRNAs) by the microprocessor complex, including DiGeorge critical region 8 (DGCR8) and Drosha. Exportin 5 transports the pre-miRNAs to the cytoplasm, where they are cleaved into duplexes by the Dicer enzyme. The guide strand loads into Ago, whereas the passenger strand is often degraded (126) (Figure 6). Pri-miRNA transcripts can also bypass processing by Drosha and/or Dicer through non-canonical biogenesis (134).

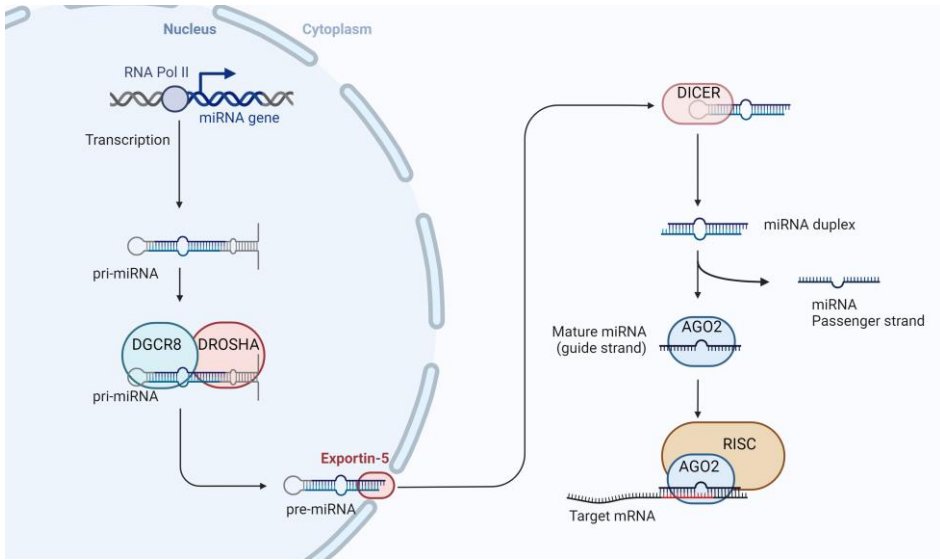


Figure 6. The canonical miRNA biogenesis pathway. After transcription of miRNA genes by RNA pol II, pri-miRNA enters the miRNA biogenesis pathway after being identified by DGCR8 and are subsequently cut by Drosha. Pre-miRNAs are transported to the cytoplasm by Exportin 5. They are then further processed by Dicer to form a miRNA duplex consisting of two strands. The guide strand of the miRNA duplex is loaded into Ago. Subsequently, Ago associates with the RISC and miRNAs to transport the guide strand to target specific mRNAs. This figure was created in BioRender.com. Ago, argonaute; DGCR8, DiGeorge critical region 8; pre-miRNA, precursor miRNA; pri-miRNA, primary microRNA; RISC, RNA-induced silencing complex; RNA Pol II, RNA polymerase II.

MicroRNA in cancer

There are numerous reports of aberrantly expressed miRNAs in cancer, and specific microRNAs are proposed to function as tumor suppressors or stimulators (133, 135). One of the first studies demonstrating the involvement of miRNA in cancer showed that loss of miRNA-15 and -16 genes was associated with B cell chronic lymphocytic leukemia (136). Many tumor types display an overall miRNA downregulation (135). A dysregulated miRNA expression can result from methylation of miRNA gene promoters or alterations (losses or amplifications) of miRNA genes (137). Lower Drosha and Dicer levels due to hypoxia (138, 139) and phosphorylation of Ago constitute other mechanisms that affect miRNA expression in cancer (140).

It is presumed that miRNAs reflect PC treatment responses, and that their dysregulation contributes to treatment resistance (141). Multiple miRNAs are reported as being important in the process of metastatic dissemination (139). A well-described example is the miRNA-200 family, which regulates EMT by interacting with zinc finger E-box binding homeobox 1/2 (ZEB1/2) directly (142-144).

In prostate cells, miRNAs can regulate AR, either directly by interacting with AR 3'UTR (145, 146), or indirectly by targeting AR co-regulators or other factors that regulate AR gene expression (147). Conversely, AR can directly or indirectly regulate miRNA transcription, or regulate components of the miRNA biogenesis pathway and impact the processing rate of miRNAs (147, 148).

Therapeutic potential

miRNA-based cancer therapeutics are intended to replenish downregulated, tumor-suppressive miRNAs or deplete levels of oncogenic miRNA in the affected tissue. miRNA mimics are synthetic miRNAs that mimic the function of the endogenous molecule. Anti-miRNAs inhibit endogenous miRNAs by binding via base pairing, preventing the miRNAs from targeting mRNA. miRNA sponges are synthetic RNA constructs with multiple binding sites for one or several miRNAs, used to sequester and thus inhibit miRNA function. Short hairpin RNAs mimic endogenous miRNA precursors and enter the miRNA biogenesis pathway. Delivery methods for miRNA therapeutics that are currently under development include encapsulation in lipid nanoparticles, polymers, viral vectors, bacterial minicells, bacteriophages and exosomes, and conjugation to antibodies, metal nanoparticles, N-Acetylgalactosamine and cell-penetrating peptides (149).

Pathological changes in miRNA expression have been linked to most cancer types, and miRNA-based treatment of cancer is thus a promising approach (135). One advantage of miRNA-based therapeutics is that one or several miRNAs can act on multiple genes in a pathway, causing a potent effect (122, 142). However, multiple miRNA targets can also lead to undesired on- and off-target effects that need to be minimized (150, 151). Although a number of RNA-based drugs are approved by the Food and Drug Administration in the USA and the European Medicines Agency, the clinical translation of miRNA-based therapeutics has faced many challenges due to poor efficacy and delivery and toxicity (150).

MicroRNA-23c

The miRNA-23c gene is located on chromosome X according to the miRBase entry, and is not clustered with any other miRNAs (152). There is not enough data for a confident annotation. Only two studies have examined miRNA-23c in PC (153, 154), they both detected miRNA-23c in exosomes from PC cell lines. In the first study, increased levels of miRNA-23c were found in exosomes from paclitaxel-resistant PC3 and DU145 cells as compared to exosomes from non-resistant cell lines (154). In the second study, high levels of exosomal miRNA-23c were found to inhibit angiogenesis in in-vitro assays and a mouse model (153). In addition, FOXM1 was found to negatively regulate miRNA-23c expression (153).

Other members of the miRNA-23 family (-23a and -23b) share the same seed sequence, and thus are likely to target similar mRNAs. Furthermore, miRNA-23b clusters with miRNA-27b and -24-1 on chromosome 9. miRNA-23b is suggested to function as a tumor suppressor (155, 156) and has been reported as downregulated in PC tissue (157, 158).

MicroRNA-375

miRNA-375 has been extensively studied as a PC biomarker (159-163), but conflicting standpoints exist with respect to its functional role in PC (164). Most publications describe an upregulation of the miRNA in PC specimens as compared to benign tissue, suggesting that miRNA-375 has a tumor-promoting role (165-170). In two studies, miRNA-375 overexpression and silencing using PC cell lines have indicated that it also acts as a tumor suppressor (165, 168). In a third study, overexpressing miRNA-375 instead had an oncogenic impact on PC-3 cells (166). It has been hypothesized that miRNA-375 functions as an oncogene during PC genesis, and as a tumor suppressor in more advanced disease (165).

A fourth study found that docetaxel treatment of DU145 and PC-3 cells increased levels of miRNA-375 (171). After overexpressing miRNA-375 cell growth of both cell lines was reduced, with a subsequent docetaxel resistance. Experimental data demonstrated a reduction in the number of apoptotic cells after combined treatment with docetaxel and miRNA-375, and offered a tenable explanation for the resistance (171).

Previous work has confirmed Sec23A and yes-associated protein 1 (YAP1) as direct targets of miRNA-375 (171, 172). Sec23A mediates protein transport from the endoplasmic reticulum (ER) to the Golgi apparatus, and miRNA-375 is proposed to reduce the growth of DU145 cells by affecting levels of Sec23A (172). Another study corroborates YAP1 as a

direct target of miRNA-375 (168). YAP1 is a transcriptional co-regulator in the Hippo pathway, and is involved in the regulation of proliferation and apoptosis (173). In addition, YAP1 interacts with AR in the nucleus of LNCaP cells, thereby positively regulating transcription of AR-responsive genes, such as KLK3 and TMPRSS2 (174).

Two studies link miRNA-375 and AR signaling (166, 175). The methylation status of the miRNA-375 promoter has been investigated in various PC cell lines (175). Hypermethylation of the promoter and low miRNA-375 expression were features of cell lines with low AR activity (PC-3, DU145), while the opposite was found for cell lines with high AR activity (LNCaP, C4-2B, 22Rv1). Furthermore, the activity of DNA methyltransferases was found to be higher in AR-negative cells, and has been suggested as a possible explanation for the differences in methylation status between cell lines (175).

miRNA-375 further associates with an epithelial phenotype, and is proposed to regulate mesenchymal epithelial transition (168). Transcription of miRNA-375 is inversely regulated by transcription factor ZEB1 (168). In-situ hybridization indeed revealed that miRNA-375 localizes in the cytoplasm of luminal epithelial cells (166, 167) and detected enhanced miRNA-375 levels in PC as compared to healthy tissue (166). Subsequent work restricted miRNA-375 expression to the nucleoli of PC cells (167, 176), but the findings may have been due to cross-hybridization of probes to ribosomal RNA, and thus an artefact (177). It is debated whether or not miRNAs are present in the nucleoli (178).

MicroRNA-4328

The miRNA-4328 gene is according to miRBase located on chromosome X, and is not clustered with any other miRNAs (152). There is one previous study of miRNA-4328 in relation to PC, in which they find a downregulation of the miRNA in PC (179). Two studies report a possible connection between miRNA-4328 and epidermal growth factor receptor (EGFR) expression, one in colorectal cancer (180) and one in lung cancer (181). However, no functional evidence was presented. In a third study, a downregulation of miRNA-4328 inhibited the migration and proliferation of an EGFR-resistant lung cancer cell line (182). miRNA-4328 was also found to be downregulated in mucinous cystadenocarcinoma of the appendix (183).

Extracellular vesicles

Extracellular vesicles (EV) are a heterogeneous group of endogenous, nano-sized particles, enclosed by a lipid bilayer. EVs are generally divided into exosomes and microvesicles based on size and subcellular origin (Figure 7) (184). Apoptotic bodies constitute a third category of vesicles not discussed in this thesis. Exosomes are smaller (~40–120nm) than microvesicles (~50–1000nm) (185). Due to overlap in terms of both size and protein markers, vesicle subgroups are difficult to distinguish between, and many isolation procedures yield a heterogeneous vesicle population (185). Thus, the term ‘EV’ here refers to both categories, i.e., exosomes and microvesicles.

Most, if not all, cells release EVs, and their content depends on the cell of origin. According to previous work EVs contain RNA, proteins (internal and membrane-bound), lipids, and possibly DNA. However, the association between EVs and double-stranded DNA is disputed, and the latter may have been a contaminant from the EV isolation procedure (186). EVs are proposed to be a way for cells to communicate, either by transferring their content between cells or interacting with cell surface receptors to elicit a response in the recipient cell (187). Recipient cells internalize EVs either through endocytosis or fusion with the cell membrane.

Evidence suggests that EVs contain mRNA and miRNA with a functional impact on recipient cells (188-191). This was initially demonstrated by transferring mouse mRNA in EVs to human mast cells, where it translated into protein (188). EVs are detected in all examined biological fluids, and are released by cell lines in culture. EV content is protected from extracellular degrading enzymes, making them attractive as both possible liquid biomarkers and drug-delivery vehicles (188, 192).

The biogenesis of extracellular vesicles

Exosomes originate from late endosomes called ‘multivesicular endosomes’, i.e., membrane-bound intracellular organelles. They sort material entering the cell via endocytosis. Inward budding of late endosomal membranes forms intraluminal vesicles. A late endosome containing intraluminal vesicles is now called a ‘multivesicular endosome’. The endosomal sorting complex required for transport (ESCRT) participates in intraluminal vesicle formation, although it is tenable that EV biogenesis also occurs independently of ESCRT. Multivesicular endosomes are either degraded by lysosomes or fused to

the cell membrane, where intraluminal vesicles are discharged as exosomes (Figure 7). In contrast, microvesicles release from the cell membrane through outward budding (Figure 7) (185).

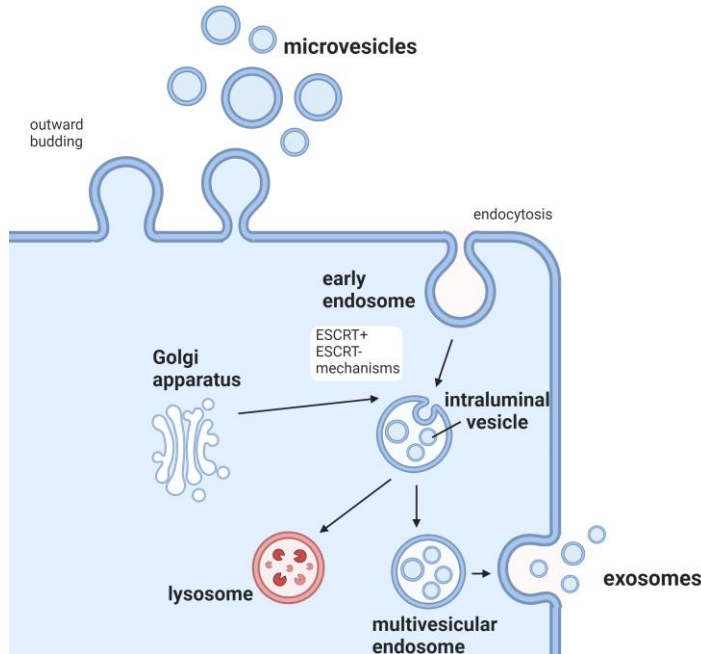


Figure 7. Schematic figure showing the subcellular origin of exosomes and microvesicles. The Golgi apparatus and the endoplasmic reticulum contribute to endosome formation and cargo. Inward budding of the endosomal membrane forms multivesicular endosomes containing intraluminal vesicles. Multivesicular endosomes fuse with the plasma membrane, releasing intraluminal vesicles as exosomes in the extracellular space or fusing with lysosomes for degradation. The ESCRT participates in intraluminal vesicle formation and sorting. Microvesicles are formed by outward budding of the cell membrane. This illustration was modified from van Niel G et al. *Nat Rev Mol Cell Biol.* 2018;19(4):213–28, and created with BioRender.com. ESCRT, Endosomal sorting complex required for transport.

MicroRNA in extracellular vesicles

Previous work differs with respect to the RNA content of EVs, likely due to the use of different EV- and RNA-isolation and RNA-quantification methods (193, 194). EVs mainly contain shorter non-coding RNA species and RNA fragments (186, 192, 195, 196). An enrichment of miRNAs in EVs

as compared to the cell source (197) has been described, although other work supports the contrary notion (198, 199). EV RNA composition partly reflects the cell source, but not entirely. Some RNA species and specific RNA sequences are more abundant in EVs (197). In addition, exosomes and microvesicles differ with respect to RNA content (197), and microvesicles have profiles that more closely resemble the cell source (186, 197).

It has been hypothesized that specific miRNAs are actively sorted into EVs. The RNA binding proteins heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNPA2B1) and synaptotagmin binding cytoplasmic RNA interacting protein (SYNCRIP) facilitate the sorting of miRNAs in exosomes by recognizing specific sequence motifs of molecules (200, 201). Recent work confirmed the finding of miRNA exosome sorting motifs in multiple cell lines (202). Passive mechanisms of miRNA sorting have also been described, and miRNA EV content increases after miRNA overexpression in the cell source (203).

Extracellular vesicles and microRNAs in cancer

Tumor-derived EVs transfer oncogenic cargo, thereby impacting both the microenvironment and distant sites of the body (185). Such cargo includes chemokine receptors, growth factors, oncogenes, oncoproteins, oncogenic mRNA, and miRNAs (204). It is further hypothesized that tumor cells use EVs to dispose of tumor-suppressing miRNAs (205, 206). Further, EVs support cancer growth by altering the function of recipient cells (190, 207). Examples include cells in the pre-metastatic niche (208-211), endothelial cells (212, 213), immune cells, and cancer-associated fibroblasts (CAFs) (214, 215). EVs also mediate therapeutic resistance (216).

Multiple studies have shown an elevated EV production in malignant cells as compared to their non-malignant counterparts (204, 207). Tumor-derived EVs also have different miRNA (159, 190, 217) and protein (218, 219) compositions. Since EV content partly reflects parental cells, it is tenable that EVs are suitable biomarkers for detecting cancer and monitoring disease progression and treatment responses (220-222).

Aims of the thesis

The overall aim was to identify biomarkers and novel therapeutic targets for aggressive PC, focusing on miRNAs.

Specific aims

Paper I

- To identify miRNAs associated with PC progression from localized to metastasized disease.
- To select a few miRNAs (miRNA-23c and -4328) that had not been investigated in relation to PC and evaluate them with respect to their functional effects on PC growth.

Paper II

- To verify the MetA-C metastasis subtypes and their prognostic importance in independent validation cohorts.
- To explore the relationships between the MetA-C subtypes and genetic profiles.

Paper III

- To explore the miRNA signatures of PC bone metastases, specifically in relation to the MetA-C metastasis subtypes.
- To functionally evaluate whether miRNA-375 can inhibit the aggressive MetB subtype.

Material and methods

Patients and samples

Paper I: Tissue samples from localized PC ($n=13$) and adjacent benign prostatic tissue ($n=13$) were collected from radical prostatectomy specimens. Tissue from PC bone metastases ($n=67$) was acquired during surgery for spinal-cord compression. Of those, 15 were hormone naïve (HN), four were short-term castrated, and the remaining 48 were CRPC. All samples were collected at the Umeå University Hospital between 2003 and 2014. Clinical patient characteristics are listed in Table 4.

Paper II and III: 103 metastatic PC samples from 67 patients were included in the analysis presented in Paper II. Of those, 77 were from the spine, 22 from crista core biopsies, 3 from lymph nodes, and 1 from dermis. For some patients ($n=14$), multiple samples were obtained from the same metastasis on a single occasion. The mean values of the replicates were calculated and used for statistical evaluations. Tissue from a few men ($n=6$) were sampled on different occasions, with treatment given in between, and handled as separate cases in the survival analysis. Thus, 73 different sampling occasions were evaluated. Of the 67 patients, 15 had not previously received ADT, 10 had received short-term ADT, and 42 had a diagnosed CRPC. Papers II and III used the same bone metastasis samples, except one crista core biopsy that was not available during the analysis presented in Paper III. The metastases derived from lymph nodes and dermis were excluded from the analysis presented in Paper III, as were two extra samples that had been wrongly classified as spine during the analysis presented in Paper II. The samples were collected at Umeå University Hospital or Sahlgrenska University Hospital. Clinical patient characteristics are listed in Table 4.

External cohorts

The Abida cohort included fresh frozen needle biopsies ($n=444$) from 429 CRPC patients (15 patients had biopsies taken twice) (223). The biopsies were collected from PC metastatic tissue in lymph nodes (37%), bone (36%), and the liver (14%). The remainder consisted of prostate, lung, and soft tissues (13%). Whole-exome sequencing and RNA sequencing were carried out on 444 and 332 samples, respectively. The “Abida polyA” library contained 266 samples and the “Abida capture” library 208 samples, with some of the samples overlapping. Matching DNA-sequencing data was available in 331 cases.

The Quigley cohort consisted of metastatic CRPC biopsies ($n=101$) originating from bone-(43%), lymph nodes (39%), the liver (11%), and soft tissue (8%) (224). RNA and whole-genome sequencing were carried out on all samples ($n=101$).

Table 4. An overview of the study populations investigated in Study I–III.

	Paper I	Paper II	Paper III
Patients			
Total number	67	67	62
Age at diagnosis (yrs)	69 (63–76)	70 (66–77)	70 (66–77)
Age at metastasis surgery (yrs)	73 (67–79)	74 (70–78)	74 (69–78)
Serum PSA diagnosis (ng/ml)	110 (47–750)	110 (20–870)	120 (23–900)
Serum PSA metastasis surgery (ng/ml)	290 (86–980)	150 (39–910)	160 (39–910)
Gleason score at diagnosis			
6		5 (8%)	5 (8%)
7	18 (27%)	13 (19%)	12 (19%)
8–10	28 (42%)	28 (42%)	24 (39%)
Not available	21 (31%)	21 (31%)	21 (34%)
Primary treatment			
Radical prostatectomy	2 (3%)	9 (13%)	9 (14%)
Radiotherapy	8 (12%)	8 (12%)	8 (13%)
Castration	57 (85%)	50 (75%)	45 (73%)
Castration therapy^a			
None (hormone naïve)	15 (22%)	15 (22%)	15 (24%)
Short-term ^b	4 (6.0%)	10 (15%)	10 (16%)
Long-term	48 (72%)	42 (63%)	37 (60%)
CRPC treatment			
Bicalutamide	36 (54%)	26 (39%)	22 (35%)
Chemotherapy	9 (13%)	13 (19%)	11 (18%)
Abiraterone acetate	1 (1.5%)	6 (9%)	6 (10%)
Enzalutamide	1 (1.5%)	5 (7%)	5 (8%)
Ra223	5 (7%)	3 (5%)	3 (5%)
Zoledronic acid/Denosumab	5 (7%)	5 (7%)	4 (6%)

Continuous variables given as median (25th–75th percentiles).

Categorical variables are given as numbers (%).

^aCastration therapies given prior to collection of metastasis tissue samples included surgical ablation, LHRH/GnRH agonist therapy, or bicalutamide treatment.

^bCastration therapy for 1 day to 3 months before metastasis tissue sampling.

RNA analyses

RNA isolation

To isolate miRNA from biopsies, the Allprep DNA/RNA/Protein Mini Kit (Qiagen, Hilden, Germany) then RNeasy MinElute Cleanup Kit (Qiagen)

were employed. The Allprep DNA/RNA/miRNA Universal Kit (Qiagen) was used to isolate total RNA, including miRNA, from cell lines (Qiagen).

Microarray analysis

Paper I: Microarray profiling of tissue samples quantified miRNA levels using the 3D-Gene® miRNA array platform (Toray Industries Inc., Tokyo, Japan) at the TATAA Biocenter (Gothenburg, Sweden).

Paper II: Clariom D™ human arrays (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) were used for mRNA profiling using a bioinformatic and expression-analysis platform (Karolinska Institute, Stockholm, Sweden).

Paper III: GeneChip™ miRNA 4.1 Arrays (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) were used for miRNA quantification at the Array and Analysis facility at Uppsala University (Uppsala, Sweden).

Reverse transcription quantitative real-time polymerase chain reactions

For miRNA quantification, the TaqMan™ MicroRNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, US) and miRNA-specific stem-loop RT primers (Applied Biosystems) were used for cDNA synthesis. TaqMan™ Small RNA Assays (Applied Biosystems) and the TaqMan™ Universal MasterMix No UNG (Applied Biosystems) were employed for real-time quantitative PCR and relative quantification of miRNA levels. Small nucleolar RNAs (C/D Box 48 (RNU48), C/D Box 47 (U47), or H/ACA Box 66 (RNU66) (Applied Biosystems)) were used as endogenous controls. A geometric mean was calculated for at least two of the small nucleolar RNAs as it controls for outlying values and abundance differences between genes better than arithmetic mean (225).

For mRNA quantification, the SuperScript™ VILO™ MasterMix (Invitrogen, Waltham, MA, US) was used for first-strand cDNA synthesis. The TaqMan™ Gene Expression MasterMix (Applied Biosystems) and TaqMan™ Gene Expression Assays (Applied Biosystems) were utilized for real-time quantitative PCR. The GAPDH transcript was used as a reference point (endogenous control). Cycle threshold (CT) values were analyzed in triplicate with the 7900 HT Real-Time PCR System (Ambion, Austin, TX, US). The relative expression of the genes of interest were determined using the $\Delta\Delta CT$ method.

MetA-C sample classification

The MetA-C subtypes had been previously defined using unsupervised cluster analysis of whole-genome transcript levels in 72 PC bone metastases ($n=72$) (93). A panel of transcripts separating MetA-C were defined ($n=60$ per subtype, together $n=180$) based on the lowest p -values and fold changes ≥ 1.5 . 157 of the 180 transcripts were found to be steadily related to the subtypes in the Quigley and Abida cohorts (223, 224).

During the analysis presented in Papers II and III, the CIBERSORT tool was used to determine MetA-C abundances (0–100%) in each sample by comparing the expression levels of the 157 subtype-specific transcripts to those of reference samples representative of each subtype (226).

Estimation of epithelial tumor cell content

Estimations were based on histological examination of metastatic tissue sections by a pathologist in the analysis presented in Paper II. In the analysis presented in Paper III they were based on mRNA expression profiles and estimated using R statistical software (v4.2.2, R Core Team) and the ESTIMATE (v1.0.13) package (227).

AR activity, proliferation, and NEPC scores

Each sample was scored based on the expression levels of a panel of pre-determined transcripts associated with canonical AR activity described in (228), proliferation (cell-cycle activity) described in (229), and NEPC defined in (230).

Cell culturing

Cell lines

22Rv1, C4-2B, LNCaP, PC-3, PNT1A, RWPE-1, and WPMY-1 cells were acquired from the American Type Culture Collection (Manassas, VA, US). RWPE-1 cells were cultured in a keratinocyte serum-free medium with the recommended supplements (Gibco, Thermo Fisher Scientific, Waltham, MA, US). All other cell lines were grown in a RPMI-1640+GlutaMAX medium supplemented with 10% heat-inactivated fetal bovine serum (FBS-HI), 10 mM HEPES, 1 mM of sodium pyruvate, 100 U penicillin/mL, and 100 μ g streptomycin/mL (Gibco). Cell lines transduced with inducible lentiviral vectors (Paper III) were maintained in tetracycline depleted FBS-HI (Gibco, Thermo Fisher Scientific). All cell cultures were maintained in a humidified environment at 37°C and 5% CO₂.

Lentiviral transductions

In the analysis presented in Paper I, shMIMIC pSMART_hCMV/TurboGFP non-inducible lentiviral vectors (Horizon Discovery, Waterbeach, UK) were used to overexpress miRNAs in PC cell lines.

In the analysis presented in Paper III, shMIMIC Human Inducible microRNA mEF1a-TurboGFP (Horizon Discovery) was used. After transduction and antibiotic selection, single cells were allowed to grow and form colonies; these colonies were then picked and propagated individually. miRNA expression was induced by adding doxycycline in the cell-culturing media.

Proteomic analysis

In the analysis presented in Paper I, liquid chromatography with tandem mass spectrometry (LC-MS/MS) was employed to identify up- and downregulated proteins following miRNA overexpression in PC cell lines (22Rv1, PC-3). The analysis was performed at the Proteomics Core Facility at Sahlgrenska Academy at the University of Gothenburg. Triplicate protein samples of 22Rv1 and PC-3 cells overexpressing miRNA-23c or miRNA-4328 were analyzed in relation to protein levels in non-target control (NTC) cells. Equal amounts of all samples were pooled and used as a reference sample. A normalized abundance ratio for each protein was calculated by dividing the normalized peptide abundance per sample with the normalized peptide abundance of the reference sample and used for statistical calculations.

Proliferation and viability assays

The CellTiter-Glo® Luminescent Cell Viability Assay for 2D or 3D cultures (Promega, Madison, WI, US) determined the growth rate of cells in culture by measuring the quantity of adenosine triphosphate (ATP) as an estimate of metabolic activity. In the analysis presented in Paper I, growth rate was determined by measuring fluorescent intensity (turboGFP) over time using a Minimax 300 imaging cytometer (Molecular Devices).

Extracellular vesicle isolation and characterization

Cells for EV isolation were cultured in media; EVs of bovine origin had been depleted from the FBS-HI by ultracentrifugation. After 72 hours, the conditioned media was carefully removed and viable cells, apoptotic bodies, and microvesicles were removed through fractionated

centrifugation (231). The cleared conditioned media was stored at -80°C until EV isolation.

Prior to the EV isolation, the cleared conditioned media was concentrated through ultrafiltration using 100kDa molecular weight cut-off Amicon Ultra Centrifugal filter units (Merck Millipore, Burlington, MA, US). Size exclusion chromatography (SEC) using the qEV original column was performed (Izon Science, Christchurch, New Zealand) (232, 233). Fractions were collected ($n=24$), and protein concentrations were determined on a spectrophotometer by measuring absorbance at 280nm. Protein-free fractions were pooled and concentrated using Amicon Ultra Centrifugal filter units (Merck Millipore) prior to nucleic acid isolation using a qEV RNA extraction kit (Izon Science).

Nanoparticle tracking analysis was performed on the pooled and concentrated samples on a NanoSight NS300 (Malvern Panalytical, Salisbury, UK) to characterize the particles based on concentration and size distribution (234, 235).

Animal model

In Paper I, stably transduced PC-3 cell lines (NTC or miRNA-23c overexpressing) were injected subcutaneously in nude immunocompromised, athymic male BALB/c mice (Charles River Laboratories, Wilmington, MA, US). Tumor size was measured twice weekly and at endpoint using calipers. Each group included 10 mice, which were sacrificed after 37 days (when the first tumor measured $\geq 1000\text{mm}^3$). Fresh tumor tissue was frozen in liquid nitrogen and then stored at -80°C for later RNA isolation. In addition, tumor and lung tissue were fixed in formalin and paraffin-embedded for immunohistochemistry.

Immunohistochemistry

Immunohistochemical staining was conducted using the Discovery ULTRA system (Roche, Basel, Switzerland). In the analysis presented in Paper I, a CD31 antibody (PECAM-1) was used to stain endothelial cells in tumor sections. Quantification of vessel density was carried out using QuPath Open Software for Bioimage Analysis (v 0.4.2) (236). In the analysis presented in Paper III, Ki67 and PSA immunostaining was used to assess proliferation and androgen response, respectively, in cell lines cultured in matrigel.

In-situ hybridization

In the analysis presented in Paper III, the cellular localization of miRNA-375 was detected using in-situ hybridization in sections from clinical metastasis samples and from cells grown in Matrigel®. A miRCURY locked nucleic acid double-digoxigenin detection probe was obtained from Qiagen, and the protocol was conducted using the automatic Discovery ULTRA system (Roche).

Statistics

All univariate statistical analyses and graphs were undertaken using GraphPad Prism (GraphPad Software, Boston, Massachusetts USA). Multivariate statistical analyses were performed in SPSS® (IBM Corp, Armonk, NY, US), R statistical software (v4.2.2, R Core Team), or SIMCA® (Umetrics AB, Umeå, Sweden). GSEA was performed using the GSEA Software (v4.1.0, Broad Institute, Cambridge, Massachusetts, USA), and pathway analysis using the MetaCore software (GeneGo, Thomson Reuters, New York, NY, USA).

Kaplan-Meier survival analysis

In the work presented in Papers II and III, survival after ADT or metastasis surgery was illustrated using Kaplan-Meier plots, with the long-rank test revealing differences with respect to significance. In the analysis presented in Paper II, Cox survival analysis was performed in parallel, using both univariate and multivariate statistics.

Results and discussion

Paper I: Investigating microRNA profiles in prostate cancer bone metastases and functional effects of microRNA-23c and microRNA-4328

Overview

The miRNA profile of primary PC is well studied; in contrast, the miRNA expression of PC bone metastases is less studied. In this study we therefore aimed to investigate which specific miRNAs contribute to the progression of PC to metastatic disease. Such miRNAs could possibly serve as biomarkers of aggressive PC or as therapeutic targets.

Expression levels of miRNAs ($n=2018$) were analyzed by a microarray in benign prostate ($n=7$), localized PC ($n=7$), and CRPC ($n=14$) tissue samples. Differentially expressed miRNAs were identified; two of these, miRNAs-23c and -4328, had not been studied in PC at that time. Their expressions were further investigated in a larger cohort of samples ($n=91$) using qRT-PCR. The miRNAs were overexpressed in two cell lines (22Rv1 and PC-3). Stably miRNA-overexpressing cells were profiled by LC-MS/MS, followed by GSEA, to detect affected hallmarks. Effects on cell growth and migration and response to enzalutamide and simvastatin treatment were tested. A recent publication found high levels of miRNA-23c in EVs shed from PC cells, which in turn inhibited angiogenesis in bone metastases (153). Levels of miRNAs-23c and -4328 were therefore quantified in EVs from overexpressing cells. Effects on cell growth and angiogenesis after miRNA-23c overexpression were evaluated using a mouse model.

Results

MicroRNA profiling of prostate cancer and bone metastasis samples

The expression levels of 1510 miRNAs were above the detection threshold. Subsequently, their levels in localized PC, CRPC bone metastases, and benign prostate tissue were compared. Some miRNAs ($n=79$) were expressed differentially in localized PC or CRPC bone metastatic samples. Most of these were downregulated ($n=75$) in CRPC, although a few were upregulated ($n=4$). Further analysis showed that miRNAs -1, -23c, -143-3p, -143-5p, -145-3p, -205-5p, -221-3p, -222-3p, and -4328 are

progressively downregulated through disease progression (benign > PC > bone metastasis). Thereafter, validation in a larger sample cohort (localized PC ($n=12$), bone metastasis ($n=67$), and benign ($n=12$)) was carried out. The downregulation of miRNAs-23c and -4328 was confirmed in PC bone metastases as compared to benign using qRT-PCR.

Endogenous microRNA levels in prostate cancer cell lines

Determinations of levels of miRNAs-23c and -4328 were carried out in benign epithelial (PNT1A, RWPE-1), myoepithelial (WPMY-1), and bone metastatic (22Rv1, PC-3) cell lines, as well as one lymph-node-metastasis-derived (LNCaP) cell line. Levels of both miRNAs were low in the bone-metastasis-derived cell lines (22Rv1, PC-3), and we therefore decided to study effects of overexpression of miRNAs-23c and -4328 in those cell lines. In contrast, as compared to PC-3 cells, levels of both miRNAs were elevated in LNCaP cells; the highest levels were found in the RWPE-1 (for miRNA-23c) and WPMY-1 (for miRNA-4328) cell lines.

microRNA overexpression and effects on cell growth

A cell-viability assay revealed that overexpression of both miRNAs reduced the cell growth of both 22Rv1 and PC-3 cells as compared to NTC cells. Live cell imaging of cell fluorescent intensity confirmed these observations.

Uncovering direct targets and affected gene sets using proteomic profiling

Proteomic profiling (LC-MS/MS) of 22Rv1 and PC-3 cells overexpressing miRNAs-23c, -4328, or an NTC sequence disclosed potential miRNA targets by identifying differentially up- or downregulated proteins. Differentially expressed proteins were compared with predicted miRNA targets from the TargetScan database (237).

In the proteomic data, GSEA was used to explore differentially enriched and depleted expression levels of predefined sets of genes connected to biological pathways. Both cell lines displayed a depletion of “glycolysis” and “coagulation” following miRNA-23c overexpression. Both cell lines displayed an enrichment of “early estrogen response” and depleted “KRAS signaling”, “glycolysis”, and “complement” following miRNA-4328 overexpression.

Following miRNA-4328 overexpression, the gene sets “androgen response” and “cholesterol homeostasis” were enriched in 22Rv1 cells. Further, overexpression of miRNA-23c affected “fatty acid metabolism”. This motivated us to determine the half-maximal inhibitory concentration

(IC₅₀) of enzalutamide (an anti-androgen) and simvastatin (a cholesterol-synthesis inhibitor) in 22Rv1 cells. In this setting, neither miRNAs-23c nor -4328 overexpression affected the IC₅₀ of the drugs.

The gene set “epithelial mesenchymal transition” was depleted in 22Rv1 and PC-3 cells overexpressing miRNAs-4328 and -23c, respectively. However, as estimated based on the wound-healing assay, none of the affected migration of PC-3 cells. The 22Rv1 cell line, including the 22Rv1 NTC cells, did not migrate at all in the assay.

MicroRNAs-23c and -4328 in extracellular vesicles

EVs isolated from conditioned cell-culture media of 22Rv1 and PC-3 cells overexpressing miRNA-23c had enriched miRNA-23c content as compared to EVs originating from NTC cells (150- and 2000-fold, respectively). In contrast, miRNA-4328 was not enriched or only slightly enriched (3-fold) in EVs originating from overexpressing 22Rv1 and PC-3 cells, respectively.

Mouse model

Subcutaneous inoculation of PC-3 cells overexpressing miRNA-23c in mice did not affect tumor size nor weight in vivo as compared to tumors formed from NTC cells. miRNA-23c overexpression was confirmed by qRT-PCR at endpoint (75-fold increase). Immunohistochemistry (CD31 staining) disclosed that miRNA-23c overexpression did not affect the morphology nor vessel density of PC-3 tumors subcutaneously grown in mice.

Discussion

It was anticipated that not all miRNAs analyzed ($n=2018$) on the microarray would be detectable in prostate tissue. As compared to benign tissue, 79 of the 1510 detectable miRNAs were differentially expressed in PC or CPRC, indicating their possible impact on the disease. Of those, the majority ($n=75$) were downregulated in PC bone metastases. It is known that hypoxia affects miRNA biogenesis by influencing miRNA-processing enzymes such as Drosha, Dicer, and Ago (133). The hypoxic environment in the bone metastatic environment (238) offers a tenable explanation as to why most of the differentially expressed miRNAs in bone metastases were downregulated. A limited set of miRNAs ($n=9$) were sequentially downregulated in both PC and CRPC, suggesting that they could be of importance for disease progression. Of those miRNAs, miRNAs-1-3p, -143/-145, -205-5p, -221/-222 have already been demonstrated by others to function as tumor suppressors, and to be downregulated in PC (145,

239-241). In addition, a downregulation of miRNAs-1-3p and -221/222 was previously confirmed in metastatic PC (239, 241), which aligns with the results presented herein.

The clinical tissue samples from prostate tumors and bone metastases were heterogenous in cellular composition, and contained varying degrees of both epithelial and stromal cells. The determination of endogenous levels of miRNAs-23c and -4328 in different cell lines was carried out to estimate the cellular origin of the miRNAs, and to select the appropriate cell lines for overexpression. Ideally the baseline miRNA level should not be too high in such cell lines to minimize interference of the endogenous transcript. The highest endogenous levels of miRNA-23c were detected in non-malignant epithelial RWPE-1 cells, suggesting that epithelial prostate cells express this miRNA. These levels declined progressively in malignant LNCaP and the more aggressive cell lines, 22Rv1 and PC-3 cells, leading to the selection of 22Rv1 and PC-3 cell lines for reconstitution of miRNA-23c and -4328 levels. We were, however, not able to detect miRNA-23c in prostate tissue using in-situ hybridization to confirm the epithelial expression (results not shown). The lowest miRNA-4328 levels were also found in the 22Rv1 and PC-3 cells. High miRNA-4328 baseline levels were detected in WPMY-1, LNCaP, and PNT1A cell lines, suggesting that this miRNA may be expressed in both stromal and epithelial cells.

It is tenable that differentially expressed proteins in the LC-MS/MS analysis, overlapping with predicted targets, are direct targets of miRNAs-23c and -4328. Other differentially expressed mRNAs could be indirect targets. As indicated by GSEA, both miRNAs altered hallmarks reflecting cell growth (“E2F targets”, “mitotic spindle”) and metabolism (“glycolysis”, “oxidative phosphorylation”). Accordingly, effects on cell growth were confirmed by the CellTiter-Glo™ assay. Interestingly, lamin B1 (LMNB1), one of the anticipated miRNA-23c targets in PC-3 cells, is a previously confirmed target of miRNAs-23a and -23b (242).

The hallmarks “androgen response” and “cholesterol homeostasis” were enriched in 22Rv1 cells overexpressing miRNA-4328. Thus, the IC₅₀ of enzalutamide (an AR-inhibitor) and simvastatin (a statin) treatments of such cells was determined. However, the 22Rv1 cell line partly demonstrates enzalutamide resistance due to constitutively active AR variants (243). Therefore, in this setting, cell growth in response to enzalutamide might be a less suitable endpoint. Quantifying the effects on androgen and cholesterol production in miRNA-4328 overexpressing cells as an endpoint might yield more information.

To load EVs with high levels of a specific miRNA, it is common to construct cell lines overexpressing the miRNA of interest (244). In our work, miRNA overexpression in 22Rv1 and PC-3 cells yielded higher intracellular levels of miRNA-23c than miRNA-4328, which may explain the higher miRNA-23c content found in EVs. A selective incorporation of the miRNA-23c in EVs constitutes another explanation. As miRNA content in EVs can impact tumor angiogenesis (245), this led to the hypothesis that cancer cells may impact their surroundings through accumulation of miRNA-23c in EVs.

In the mouse model used, malignant cells were injected subcutaneously and tumor take, growth, and angiogenesis were evaluated. This model did not, however, reflect the metastatic process (153). Tumor cells affect their surroundings by releasing EVs for uptake by recipient cells (188). According to a recent report, miRNA-23c in exosomes restrains angiogenesis of PC bone metastasis in mice *in vivo*, as judged from CD31 staining (153). In contrast, we were not able to reproduce this finding of EVs originating from PC-3 cells containing high levels of miRNA-23c inhibiting tumor angiogenesis *in vivo*.

The miRBase archive for miRNA sequences and annotation contains many entries of poor quality, which are frequently removed after post-hoc manual review. In order to annotate miRNAs with certainty, a set of criteria was defined to assess the likelihood that they have been processed in the miRNA biogenesis pathway and subsequently bound to Ago (152). miRNAs-23c and -4328 lack readings from both the 3' and 5' arms of the miRNA hairpin precursor, a prerequisite for canonical miRNA processing. This could mean that these two transcripts are non-canonically processed, or that one of the mature miRNAs is expressed in levels that are too low to detect. Another possibility is that miRNA-23c is an isoform of miRNAs-23a or -23b.

The high similarity in sequence between miRNA family members makes it difficult to assess the specific levels of each individual member. Microarray probes in particular, as well as some qRT-PCR reagents, are not sensitive enough to accurately discriminate between miRNA family members (246, 247).

miRNAs-23a-3p, -23b-3p, and -23c share the same seed sequence. miRNA-23b-3p has been shown to affect PC migration and invasion *in vitro* (155, 248). Our proteomic profiling also suggests an impact on migration/invasion for miRNA-23c in showing a significantly lower normalized enrichment score (NES) for the “epithelial-mesenchymal

transition” gene set. Optimal in-vivo mouse models for studying PC bone metastasis are lacking. In the case of subcutaneous inoculation of tumor cells, spontaneous spread to distant sites such as the lungs and bones rarely occurs (10). Consequently, the model does not reflect migration and invasion. However, the advantage of the model is that open surgery is not required, and it is easy to monitor tumor growth. Intravenous, intraosseous, and intracardial injections are commonly used to study metastasis, but they omit the early steps of the metastatic cascade (249). Orthotopic implantation is a further option for examining metastasis to distant organs (249).

Paper II: Clinical and biological relevance of the transcriptomic-based prostate cancer metastasis subtypes MetA-C

Overview

To verify the existence of the MetA-C subtypes and their prognostic and biological differences and relation to genetic aberrations, a validation cohort of bone metastasis samples ($n=103$), collected at the Umeå University and Sahlgrenska University hospitals, was analyzed using whole-genome microarray analysis. In addition, two external publicly available data cohorts covering metastasis samples (Abida ($n=444$) and Quigley ($n=101$)) were used (223, 224). The MetA-C contents of each metastasis sample were estimated using a panel of predefined MetA-C differentiating transcripts ($n=157$). Furthermore, all samples were assigned proliferation, AR, and NEPC scores. In our own samples the epithelial tumor content was determined by histopathological examination. In the external cohorts, liver and lymph node metastases were included, and genomic information for the samples was available. The above-mentioned information as well as clinical characteristics (age at sampling, Gleason score, serum PSA, survival time, previous treatment) were analyzed in relation to sample MetA-C content. A GSEA of the transcriptomic data was carried out to verify the previously described biological characteristics of the MetA-C subtypes (93).

Results

Intra-tumoral MetA-C subtype heterogeneity

Of all the metastatic samples profiled by microarray analysis ($n=103$), the majority (~90%) contained varying content of at least two subtypes. MetA correlated inversely with MetB and MetC content, while MetB and MetC

sample content did not correlate. Multiple samples obtained from a single metastasis displayed a high concordance with respect to MetA-C content.

The handling of replicate samples (described in the 'Material and Methods' section of this thesis) left 73 cases for survival analysis. These were classified according to their dominant subtype; the majority were classified as MetA (78%, $n=57$). The corresponding figures for MetB and MetC were 11% ($n=8$) for both subtypes.

MetA-C content in relation to CRPC and survival

As compared to HN metastases, the CRPC metastases in our own cohort displayed elevated MetB content. The MetB subtype was also associated with shorter median survival after ADT (1.6 years) as compared to the other subtypes. Accordingly, the Cox survival analysis showed that higher MetB content was associated with higher mortality after ADT. MetB was the strongest prognostic variable when analyzed in relation to other prognostic variables.

The comparison of CRPC and HN metastases showed that the former contained less MetA than the latter. When MetA was the dominant subtype, the median survival after ADT was 5.3 years. Consequently, higher MetA content was associated with reduced mortality rate after ADT in the Cox survival analysis.

MetC content did not differ in CRPC metastases as compared to ST and HN metastases. After ADT, patients with MetC as the dominant subtype had a median survival of 8.2 years. When analyzed as a continuous variable in the Cox survival analysis, MetC content was not significantly associated with cancer-related mortality rate.

MetA-C subtypes in relation to other clinical characteristics

The MetA-C subtypes did not correlate to age at diagnosis or age at metastasis surgery, Gleason score at diagnosis, or previous CRPC therapy (bicalutamide, abiraterone, enzalutamide, radium-223 isotope, or chemotherapy). Osteoclast-inhibiting remedies (zoledronic acid/denosumab) seemed to augment (+28%) and decrease (-20%) MetA and MetB content, respectively. Circulating PSA was positively correlated with MetA and negatively correlated with MetB content.

MetA-C in external-metastasis data sets (Abida and Quigley)

The MetA-C subtypes were detectable in the Quigley and Abida metastasis cohorts; MetA and MetC proved to be the most and least frequent subtypes, respectively. MetA and MetB content (%) were inversely and

strongly correlated in all cohorts. All external cohorts displayed enhanced MetA content in lymph nodes and MetB content in liver metastases.

MetA-C phenotypes

The hallmark gene sets androgen response, protein secretion, and adipogenesis were consistently enriched in MetA samples of all examined cohorts. Furthermore, MetA content was correlated positively with AR activity score, and inversely with proliferation score and NEPC-like score in both our own sample and the external Abida and Quigley cohorts.

The MetB subtype was enriched for multiple hallmarks associated with cell-cycle activity (e.g. E2F targets and G2M checkpoint) and DNA repair. The MetB subtype was negatively correlated with AR activity score and positively with proliferation score, but showed no association with NEPC-like score. In the external sample cohorts, MetB showed a positive correlation with proliferation score and NEPC-like score, and negative correlation with AR-activity score.

In MetC metastases, the epithelial-mesenchymal transition, myogenesis, and angiogenesis gene sets were positively enriched. The MetC subtype was correlated inversely with tissue epithelial content, AR activity score, and proliferation score. In contrast, a positive correlation was detected between MetC and NEPC-like score. Similar associations were found between MetC and AR activity score, proliferation, and NEPC-like score in the external cohorts.

Gene alterations in relation to MetA-C

In the Abida and Quigley samples, ETS gene fusions, single-nucleotide variants (SNVs), and copy-number variants (CNVs) were compared with the transcriptomic profiles of MetA-C. Only gains and losses that had significant implications as regards their respective RNA transcript levels were considered. ETS gene fusions were associated with higher MetA content in metastatic tissue in the Abida cohort. No such relationships were detected in the Quigley cohort. The MetB subtype systematically showed CNV losses and corresponding reductions in transcript levels for 21 genes on chromosome 13, including RB1. RB1 aberrations (deleterious SNVs and/or CNVs) were found in 18–31% (Abida polyA and capture) and 63% (Quigley) of metastases classified as MetB based on dominant MetA-C content. RB1 aberrations were correlated with a poor outcome after androgen-receptor signaling inhibitor (ARSI) (Abida capture) and a poor outcome after mCRPC diagnosis (Quigley). When information regarding RB1 aberrations and MetA-C classification was combined in the

multivariate survival analysis (Cox regression), the MetB subtype consistently provided independent prognostic information.

Discussion

In this study, MetA-C subtype heterogeneity was further explored in validation cohorts of bone metastases and in relation to clinical characteristics. As in the study in which MetA-C were defined (93), MetA constituted the most common subtype. MetA and MetB content correlated inversely, underscoring their opposing transcriptomic profiles and phenotypes. Nearly all biopsies were heterogenous in their MetA-C content. The observed MetA-C heterogeneity could result from a homogenous metastasis on the cellular level, with a subtype that did not 100% correspond to any of MetA, -B, or -C. Another scenario could be a heterogenous metastasis with a mix of different populations of cells representing MetA, -B, or -C. Analysis on the single-cell level could provide a better understanding of the intercellular heterogeneity of metastases. Replicate samples from the same metastases displayed high concordance with respect to MetA-C content, justifying the experimental design and methods chosen.

The external datasets validated the poor prognosis associated with the MetB subtype, which underscores the importance of its early diagnosis/classification to enable improvement of prognostication and therapy stratification. The enrichment of the MetB subtype in CRPC suggests either clonal selection or some level of subtype plasticity. The enrichment of MetB in CRPC could result from a selection of cell clones proliferating independently of AR signaling during ADT, already present before ADT, or evolving during therapy due to cellular plasticity, which could be examined through analysis of PC samples from the same patient before, during, and after ADT. The enrichment of MetB in CRPC could also be a result of the differences between the microenvironments of different metastatic sites. Subtype plasticity would make it possible to modulate MetB into a more MetA-like subtype that would respond better to therapy.

MetB profiles were consistently enriched for cell-cycle hallmarks and had high proliferation scores, implying that MetB patients may respond well to chemotherapy and novel cell-cycle inhibitors. Similarities between MetB and NEPC (androgen independence, high proliferation, and RB1 deletions) suggest that MetB cases might further develop into NEPC, and that MetB metastases could be sensitive to treatments suggested for NEPC, such as epigenetic therapies (72). MetB content also decreased

after zoledronic-acid treatment, suggesting that osteoclast-targeting drugs may be beneficial for MetB patients.

Serum PSA levels were positively correlated with MetA content and displayed an inverse association with MetB content, implying that the MetA subtype is more AR-driven than MetB. This result is in keeping with the original finding of an enhanced expression of AR-regulated transcripts in MetA (93). In addition, close relationships were found between MetA and AR activity score and hallmarks such as androgen response, protein secretion, and adipogenesis, indicating substantial AR activity. As compared to MetB, the MetA subtype was associated with a more favorable outcome after ADT. A tenable explanation is that MetA metastases respond well to ADT and other AR-signaling inhibitors.

The MetC subtype was not clearly associated with previous treatment, nor prognosis. MetC demonstrated close relationships with epithelial-mesenchymal transition, myogenesis, and angiogenesis, hallmarks connected to the tumor microenvironment. Further, the MetC subtype was negatively correlated with tumor epithelial content, indicating that those samples were rich in stroma. The characteristics of MetC suggest that the subtype is likely to respond to immunosuppression, inhibitors of angiogenesis, and other therapies that target processes in the tumor microenvironment.

The enrichment of MetB in PC liver metastasis and MetA in metastatic lymph-node metastasis indicates that MetA-C, like breast cancer subtypes, favor different organs for metastatic spread (250). Liver metastases are more commonly observed in NEPC, and typically arise after treatment with second-generation ARSI (abiraterone acetate, enzalutamide, apalutamide, and darolutamide). Furthermore, deletions of RB1 and p53 are associated with NEPC (35). Surprisingly, based on our observations, MetB content was not associated with previous AR-targeting therapy (abiraterone and enzalutamide).

It is well agreed that loss of RB1, especially in combination with p53 inactivation, promotes treatment resistance and impairs CRPC prognosis (223, 224, 251). As judged from the external cohorts, Abida and Quigley, RB1 was frequently lost in MetB metastases, but not in all. Therefore, the current study clearly shows that transcriptomic MetA-C classification provides more independent prognostic information in relation to outcome after AR-targeting therapies than genetic alterations alone.

The MetA-C subtypes could either derive from different cells or originate from clonal expansions of a single subclone. As all subtypes have been detected not only in CRPC metastases but in HN metastases and primary PC (93), it is unlikely that MetA-C develop solely in response to treatment. Instead, they might be inherently developed or clonally derived at an early stage, and later enriched by therapy and/or diversities between different microenvironments, such as bone marrow, lymph nodes, or the liver (3).

Paper III: The microRNA landscape of prostate cancer bone metastases and the role of miRNA-375 in modulating phenotypic characteristics of metastatic cells

Overview

The pathogenesis of MetA-C is not yet fully understood, but genetic and/or epigenetic factors could be of importance. We hypothesize that miRNAs directly or indirectly regulate the expression of MetA-C associated genes. The main purpose of the work presented in this study was therefore to investigate whether miRNAs contribute to the differences seen in the transcriptomics, biology, and phenotype of MetA-C. If so, we aimed to further determine whether such miRNAs could be used as therapeutic targets by affecting the MetA, -B, or -C characteristics of PC cells. Specifically, we hypothesized that it might be possible to modulate MetB to a less aggressive phenotype with a better response to AR-targeting therapies, thus achieving a more favorable prognosis.

First, miRNA profiles were investigated in clinical PC bone metastasis samples ($n=96$). An integrated analysis was conducted of the mRNA and miRNA expression levels in the samples, and in relation to the MetA-C content for the identified candidate miRNAs (Figure 8). In addition, an unsupervised cluster analysis of the samples was carried out based on their miRNA expression. The identified clusters were further explored using differential expression analysis of corresponding mRNA levels, immunohistochemical analysis, and in relation to patient survival and other clinical variables.

A suitable model system (the C4-2B PC cell line) was identified to study the effects of specific miRNAs on the MetA and B phenotypes. miRNA-375 was found to be downregulated in MetB metastases, and was therefore selected as a candidate for further investigation. After transduction with

an inducible vector for miRNA-375 expression, the C4-2B cells were profiled both prior to and after miRNA induction by microarray. Differentially expressed transcripts were explored using unsupervised cluster analysis and further related to affected processes in the cells.

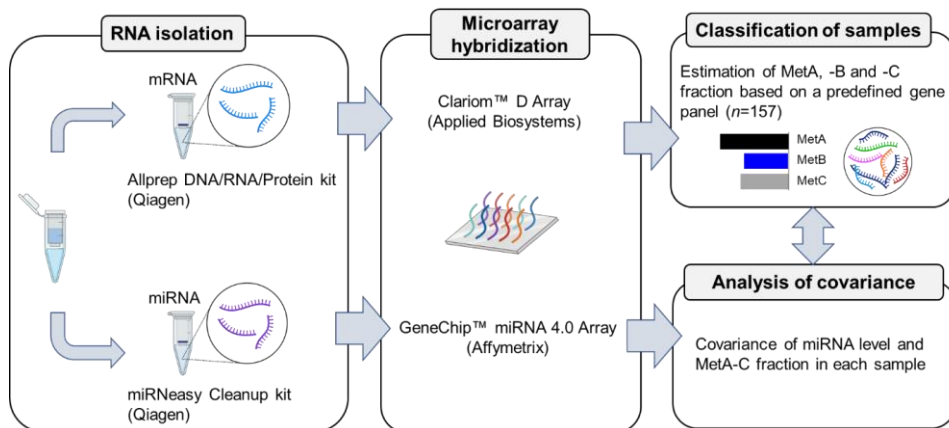


Figure 8. An integrated mRNA and miRNA classification of bone metastases. Following microarray profiling, the MetaA-C fraction of the samples was determined based on the mRNA levels. An analysis of covariance between miRNA level and MetaA-C content identified the associated miRNAs.

Results

Unsupervised cluster analysis of metastasis samples

Based on the expression levels of 459 miRNAs in 96 bone metastasis samples, an unsupervised cluster analysis separated the samples into four clusters. Cluster-unique miRNAs responsible for separating the clusters in the model were identified. The clusters were further analyzed in relation to survival and clinical variables (data not shown); no significant associations were found, although a trend towards shorter survival was seen for patients with CRPC in Clusters 2 and 4. Based on the mRNA levels of the samples included in each cluster, a differential expression analysis was carried out, where no transcripts reached significance after adjusting for multiple comparisons. When Ki67 and PSA staining of metastases was compared between clusters, an increase in Ki67 was seen in Cluster 2 compared to Clusters 1 and 3, indicating higher proliferation in Cluster 2. There were no differences in tumor-cell PSA expression.

miRNA expression in relation to MetA-C and tumor epithelial cell estimate

No clear separation between samples in relation to MetA-C sample content was observed in the principal component analysis. However, samples classified as MetB displayed high scores on the first component in the score plot. Samples with higher epithelial content displayed the same trend. Analysis of covariance after adjusting for epithelial cell purity identified six miRNAs that were negatively associated with MetA (-130b-3p, -210-3p, -433-3p, -1180-3p, -1290, and -1269b) and one miRNA that was negatively associated with MetB (-375-3p). No miRNAs were significantly associated with MetC. A number of miRNAs were associated with the tumor epithelial estimate ($n=72$), and the top positively associated candidate was miRNA-375. Through in-situ hybridization, miRNA-375 expression was detected in the cytoplasm of the tumor epithelial cells in MetA metastatic tissue.

A model for studying MetA-B phenotypic characteristics

A MetA-C classification of four PC cell lines determined that all cell lines showed most similarity with MetB at baseline. Upon DHT stimulation, the C4-2B cells shifted to resemble MetA to a greater degree. None of the cell lines were classified as MetC. Levels of miRNA-375 were lower in the C4-2B cell line than in the 22Rv1 cell line, but greater than in DU145 and PC-3 cells. Levels of miRNA-375 increased when the C4-2B cells were stimulated with DHT, and the same treatment caused a decrease in cell growth.

Effects of miRNA-375 induction

When miRNA-375 was overexpressed in the C4-2B cell line, a shift from a MetB-like profile to a MetA-like profile was observed. When the mRNA expression was analyzed following miRNA-375 induction four clusters were identified, based on differentially expressed mRNAs. These were related to increase in cell adhesion and cytoskeleton (Cluster 1), inhibition of the cell cycle (Cluster 2), increase in translation (Cluster 3), and decrease in processes involved in development, signal transduction, and cell cycle (Cluster 4). Inhibitory effects on cell growth, as indicated in Cluster 2, were confirmed by a cell-growth assay.

Androgen response and miRNA-375

As miRNA-375 was negatively associated with MetB and positively associated with MetA, the effects on MetA characteristic androgen response were tested. Seven AR-regulated transcripts were quantified in C4-2B cell lines following miRNA-375 induction by qRT-PCR. In addition, levels of YAP1, a previously confirmed transcript regulated by miRNA-

375, were analyzed. The steroidogenic enzymes CYP17A1 and AKR1C3 were upregulated by miRNA-375, while YAP1 and KLK3 levels were downregulated.

Discussion

In this study the miRNA landscape of PC bone metastases and miRNA expression in relation to the bone metastatic subtypes MetA-C were explored. We discovered four sample clusters of bone metastases based on miRNA expression patterns; this finding needs to be independently validated before further interpretation, however. Further analysis showed that miRNA-375 is negatively associated with the MetB subtype, while highly expressed in MetA. Expression levels of miRNA-375 were correlated with the estimated tumor epithelial content of the samples, indicating that miRNA-375 is expressed by the tumor epithelial cells and not by stromal cells. The tumor epithelial expression of miRNA-375 was also confirmed by in-situ hybridization.

After DHT treatment, the C4-2B cell line displayed some degree of MetA-B subtype plasticity, and was therefore used as the model system in this study. None of the profiled cell lines were classified as MetC, we believe due to the epithelial origin of the cell lines, in contrast to the high stromal content of MetC.

In wild type C4-2B cells the expression of miRNA-375 was induced by DHT stimulation, suggesting an androgen-regulated expression. Perhaps such androgen-related effects would be even more evident in the LNCaP cell line, which is more androgen responsive (10). Furthermore, the C4-2B cells displayed an increase in MetA content following miRNA-375 induction. Some of the processes affected by miRNA-375 were related to cell adhesion (upregulation) and cell-cycle progression (downregulation), which could explain the increased similarity to MetA. An in-vitro assay confirmed an inhibitory effect on cell growth following miRNA-375 induction, while the suggested effect on cell adhesion has yet to be shown. This is in line with other reports that describes miRNA-375 as a tumor-suppressor maintaining an epithelial phenotype (168). At the same time, studies have reported growth-promoting effects of miRNA-375 (166), and so its effects may be context-dependent.

We confirmed a prominent downregulation of the previously identified targets of miRNA-375 (YAP1 and Sec23a) following miRNA-375-2p induction. YAP1 is an oncogene in PC and a transcriptional co-regulator in the Hippo-signaling pathway (173). A number of transcripts were

clearly down- and upregulated following miRNA-375 induction, and may well be direct or indirect targets of the miRNA that were not previously described by others. These potential new targets need further confirmation.

YAP1 positively regulates the expression of the AR-responsive genes KLK3, PSMA, and TMPRSS2 (174), and we confirmed a downregulation of YAP1, KLK3 and PSA following miRNA-375 induction. However, miRNA-375 also induced an upregulation of steroidogenic enzymes in C4-2B cells when cultured under steroid-free conditions, suggesting that steroidogenesis and androgen production might be upregulated. This could be confirmed by measuring the cellular capacity of androgen production and/or response to steroidogenesis inhibitor abiraterone.

Conclusions

Paper I

- Most miRNAs were downregulated in PC bone metastases as compared to localized PC and benign tissue. Of the miRNAs consistently downregulated with disease progression ($n=9$), many have been previously reported as being downregulated in PC, and functionally investigated in relation to this.
- miRNAs -23c and -4328 were found to significantly inhibit the growth of PC cell lines (22Rv1, PC-3) in vitro.
- miRNA-23c was secreted in high levels in EVs, suggesting paracrine or more distant effects.
- No effect of miRNA-23c overexpression on PC-3 tumor growth was observed when inoculated subcutaneously in mice. Effects on bone metastatic growth remain to be explored.

Paper II

- The molecular subtypes MetA-C and their prognostic value in relation to AR-targeting therapy were verified in both our own validation cohort and external cohorts of metastatic PC tissue samples.
- Diverse phenotypic characteristics of MetA-C indicate that patients will require different treatments depending on metastasis subtype.
- MetA-C subtyping could, in addition to determination of RB1 alterations, improve patient prognostication and therapy stratification.

Paper III

- miRNA-375 is associated with an epithelial phenotype in clinical bone metastasis samples, and expressed in significantly lower levels with increasing MetB metastasis content.
- The growth-inhibiting effects of miRNA-375 on C4-2B cells indicate its potential as a suppressor of the aggressive MetB subtype.

- In addition to inhibiting cell proliferation, miRNA-375 may also stimulate cellular adhesion and the maintenance of a luminal, epithelial phenotype.

Concluding remarks and future perspective

The overall aim of the work presented in this thesis was to identify biomarkers and novel therapeutic targets for aggressive PC, with a specific focus on miRNAs.

The biomarker perspective

The clinical translation of prognostic miRNA biomarkers in PC tissue has been hampered by inconsistent results between studies (252). Differences in methodology and study design are possible explanations for these discrepancies (252). miRNA expression patterns are likely both cell-type- and differentiation-specific (252, 253). Therefore, differences in the stromal and epithelial composition of profiled tissue samples could impact results. Single-cell techniques and standardization of protocols for reducing biological and technical variation may result in more reproducible results, and hopefully the clinical translation of miRNAs as biomarkers will soon be implemented. In Study I we contributed with the miRNA expression profile of metastatic PC in relation to primary PC and benign tissue. The identified miRNA candidates need further validation in external cohorts and in relation to clinical parameters before their usefulness as biomarkers can be fully assessed. Tissue-based miRNA biomarkers could be detected using in-situ hybridization, qRT-PCR, microarrays, and sequencing of PC biopsies in a clinical setting. miRNAs with increasing levels in PC and bone metastases may have the potential to be further detected in liquid biopsies as diagnostic or prognostic biomarkers. Candidate miRNAs with decreasing levels in disease might be less suitable to measure in liquid biopsies, unless they can be monitored in circulating tumor cells.

Molecular classification systems for PC that have been previously proposed were initially found in localized PC, and later applied to analysis of metastatic samples. The evidence suggests that primary tumors and metastases differ with respect to phenotype, microenvironment, and response to ADT (61, 110, 238). To take such differences into account, the MetA-C classification was based directly on analyses of bone metastases. By investigating metastases instead of primary tumors, the drivers of aggressive disease and metastasis-specific therapeutic targets can be identified. Although few of the specific genes included in the panels overlap, the PAM50 and PCS1-3 classification systems have, through examination of primary tumors, been shown to be similar with respect to

their molecular profiles and prognostic ability (254). Both display similarities to and differences from the MetA-C classification. These three methods of classification segregate tumors based on the degree of proliferation and AR activity/androgen response. MetB is distinguished by a low AR activity, a high proliferation, and a particularly poor prognosis. Luminal B has the worst prognosis in the PAM50 classification system, and is characterized by high AR activity and proliferation, which differs from the MetB subtype (91). One perspective here is that the three systems of classification should be compared in clinical studies to determine the most informative panel and cut-offs for patient-stratification into AR-inhibiting therapies, chemotherapy, and other possible therapies, such as bone-targeting therapies, immunotherapy, PARP inhibitors, and others in pipeline. Easy-to-use and more cost-effective surrogate subtype markers, such as assessing immunoreactivity for Ki67 and PSA, may be even more suitable for clinical applications.

Clinical implementation of the MetA-C classification system (or other subtype classifications) as a therapy-predictive biomarker will require large prospective studies to be performed. These should involve patient stratification into different treatment protocols depending on the metastasis subtype, and subsequent evaluation of treatment responses. To enable this, the development of methods for MetA-C classification and monitoring based on tumor tissue and/or liquid biopsy analysis within clinical routine is essential. In our laboratory, a subtype-specific panel that uses qRT-PCR is under development. It has been applied to primary PC and metastasis tissue as well as circulating tumor cells (unpublished results). This panel will hopefully enable subtype prediction at an early PC stage, already at presentation, and monitoring of subtype alterations during cancer progression and changes in response to treatment. In addition, surrogate MetA and -B markers (Ki67/PSA immunostaining) have been retrospectively applied for subtype classification of primary tumor biopsies, and to predict metastasis subtype and aggressiveness (110). However, the specificity of predicting metastasis subtypes MetA-C based on analysis of primary tumor biopsies needs to be validated in further studies by transcriptomic and IHC analysis of paired tissue specimens.

To validate the findings presented in Study III, an external cohort of PC bone metastases should be analyzed in relation to the four miRNA-based sample clusters found in our analysis, and to validate the downregulation of miR-375-3p in metastasis of subtype MetB. However, the publicly available datasets of bone metastatic cohorts of adequate size do not contain miRNA expression profiles.

Identifying biomarkers may also lead to new insights regarding molecular mechanisms and novel therapeutic targets, which connects to the second part of the overall aim of this thesis.

Therapeutic target perspective

Future development of subtype-specific therapeutic strategies and targets requires improved understanding of the molecular basis of the MetA-C subtypes. Furthermore, preclinical model systems such as organoids and in-vivo models that reflect phenotypic MetA-C characteristics are needed to enable therapeutic evaluations. Based on the results presented in Paper III, pushing tumor cells from being more MetB to more MetA seems achievable at least in vitro, but remains to be tested in an in-vivo setting. If proved also in vivo, it would be possible to treat patients with miRNA-375 mimics to increase the level of this miRNA in MetB metastases. The goal would be to shift metastatic cells from a MetB-like to a MetA-like phenotype, and thereby improve prognosis. Ago immunoprecipitation assays could be used to verify direct targets of miRNA-375 involved in regulating MetA-B plasticity and verify the effects on protein expression and in functional assays. Although there are no approved miRNA-based therapeutics yet (as far as I know), there are candidates currently being tested in clinical trials (150, 255-257). In addition, several small interfering RNA-based drugs were recently approved (149).

The MetA-C subtypes have recently been analyzed in relation to methylation profiles (61). A high degree of promoter methylation of AR and AR-associated genes was seen in MetB samples, which could explain the low AR activity in those metastases. There was no association between degree of methylation and genes involved in proliferation (results not shown). Instead, the aberrations in the RB1 gene seen in MetB may be mechanistically involved. However, RB1 alterations were not present in all MetB metastases, indicating that other factors also contribute to the observed phenotype. It is likely that the MetA-C phenotypes result from a combination of genetic and epigenetic alterations, such as changes in DNA methylation, miRNA expression, and influences from the microenvironment.

The overall hypothesis of the thesis, that miRNAs are important for the progression of PC to aggressive disease and the differences seen in MetA-C phenotype, is strengthened by the results presented. The overall reduction of miRNA levels in metastatic disease may be of great relevance to metastasis development. Although we were not able to confirm the functional importance of the downregulation of miRNAs-23c and -4328, other model systems that better reflect bone metastasis may be able to

confirm their role. In addition, other miRNAs identified in our screening may be of high importance, and these warrant further studies. By combining clinical observations with results from functional experiments, we conclude that expression of miRNA-375 in metastases is likely important for keeping a more luminal, epithelial (MetA) phenotype and inhibiting the more aggressive MetB phenotype. We have identified a number of miRNAs with high expression in MetB and concurrent low expression in MetA (miRNAs-130b-3p, -210-3p, and -1180-3p, among others) in the clinical samples; these could be important regulators of the MetB phenotype and their functional role could be further investigated.

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