UNIVERSIDADE FEDERAL DE UBERLÂNDIA
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PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

Desenvolvimento e caracterização de aptâmeros ligantes específicos ao gene PCA3 e implicações na modulação da expressão gênica de células tumorais transfectadas

Karina Marangoni

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Co-Orientadora

Uberlândia, 25 de Julho de 2011.

Eu aprendi...

...que ignorar os fatos não os altera;
...que quando você planeja se nivelar com alguém, apenas está permitindo que essa pessoa continue a magoar você;
...que o amor, e não o tempo, é que cura todas as feridas;
...que ninguém é perfeito até que você se apaixone por essa pessoa;
...que a vida é dura, mas eu sou mais ainda;
...que as oportunidades nunca são perdidas; alguém vai aproveitar as que você perdeu.
...que quando o ancoradouro se torna amargo a felicidade vai aportar em outro lugar;
...que não posso escolher como me sinto, mas posso escolher o que fazer a respeito;
...que todos querem viver no topo da montanha, mas toda felicidade e crescimento ocorre quando você está escalando-a;
..que quanto menos tempo tenho, mais coisas consigo fazer.

(William Shakespeare)

Dedico este trabalho...

Aos meus pais Nadir e Celso,

... meu porto seguro.

A minha filha Ana Laura,

...minha luz, minha vida.

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LISTA DE ABREVIATURAS

%	porcentagem
[ ]	concentração
A	adenina
ADARB1	*Adenosine Deaminase RNA-specific B1*
AIP	atrofia inflamatória da próstata
ANXA1	*Annexin A1*
AR	*Androgen Receptor*
B2M	*β2-microglobulina*
ºC	graus Celsius
C	citosina
CA	motivo repetitivo Citosina-Adenina
CG	motivo repetitivo Citosina-Guanina
Ca^{2+}	íons cálcio
CaP	câncer de próstata
cDNA	ácido desoxirribonucléico complementar
cGMP	guanosina cíclica monofosfato
DHT	dihidrotestosterona
DNA	ácido desoxirribonucléico
EEF1A1	*Eukaryotic Translation Elongation Factor 1 Alpha 1*
EGF	fator de crescimento epidermal
eRNA	RNA de "*efference*"
FOLH1	*Folate Hydrolase (Prostate-specific Membrane Antigen 1)*
FGF	fator de crescimento de fibroblastos
fRNA	RNA funcional
FSH	hormônio folículo-estimulante
g	grama
G	guanosina
GF	fatores de crescimento
GMP	guanosina monofosfato
GnRH	hormônio de liberação da gonadotropina

HPB	hiperplasia prostática benigna
INCA	Instituto Nacional do Câncer
Hsp90	proteína *Heat Shock* – 90 KDa
IGF	fator de crescimento tipo insulina
IgE	imunoglobulina E
Kb	kilobases
KDa	kilodaltons
KGF	fator de crescimento de queratinócito
KLK2	*Kallikrein Related Peptidase 2*
KLK3	*Kallikrein Related Peptidase 3*
KRT5	*Keratin 5*
KRT6	*Keratin 6*
KRT8	*Keratin 8*
KRT14	*Keratin 14*
KRT18	*Keratin 18*
LH	hormônio luteinizante
LSP1	*Lymphocyte-specific Protein 1*
M	molar
mL	mililitro
mg	miligrama
$MgCl_2$	cloreto de magnésio
miRNA	microRNAs
mM	milimolar
MMLV	*Murine Moloney Leukemia Virus Reverse Transcriptase*
NaCl	cloreto de sódio
ncRNA	RNA não codificante
$NADPH_2$	nicotinamida adenina dinucleotídeo fosfato hidrogênio
NIP	neoplasia intraepitelial prostática
nmRNA	RNA não mensageiro
p53	*tumor protein 53*
p21	*tumor protein 21*
pb	pares de base
PCA3	*Prostate Cancer Antigen 3*

PCR	reação em cadeia da polimerase
pKA	proteína quinase A
pKC	proteína quinase C
PKG1	proteína quinase cGMP dependente tipo 1
PKG2	proteína quinase cGMP dependente tipo 2
pmoles	picomoles
PRDM4	*PR Domain Containing 4*
pRNA	promotor RNA
PSA	antígeno prostático específico
PTPase	proteína tirosina fosfatase
RISC	complexo indutor do silenciamento de RNA
RNA	ácido ribonucléico
RNAm	ácido ribonucléico mensageiro
RNase	ribonuclease
RNAsel	*5'- Oligoadenilato-dependente Ribonuclease L*
RT	transcrição reversa
RTU	ressecção transuretral
SELEX	Systematic Evolution of Ligands by EXponential enrichment
SDS	duodecil sulfato de sódio
siRNA	pequeno RNA de interferência
SMAP1	*Small ArfGAP 1*
snmRNA	pequeno RNA não mensageiro
snoRNA	pequeno RNA nucleolar
snRNA	pequeno RNA nuclear
SRD5A2	*Steroid-5-alpha-reductase Alpha Polypeptide 2*
ssDNA	*single strand* DNA
TAC	*transit-amplifying cells*
Taq	*Thermus aquaticus*
TBE	tampão Tris/Borato/EDTA
TNF-α	fator de crescimento transformante alfa
TNM	*tumor-node-metastasis*
TLR3	*Toll-Like Receptor 3*
TLR4	*Toll-Like Receptor 4*

TLR6	*Toll-Like Receptor 6*
TLR9	*Toll-Like Receptor 9*
rRNA	RNA ribossomal
tRNA	RNA de transferência
U	unidade de enzima
μg	micrograma
μl	microlitro
μM	micromolar
UV	ultravioleta
USTR	ultrasonografia transretal
UTR	região não traduzida
VEGF	fator de crescimento endotelial vascular
γIFN	Interferon gama

APRESENTAÇÃO

Muitos estudos têm sido feitos na tentativa de compreender os mecanismos moleculares complexos envolvidos na oncogênese e progressão do câncer de próstata (CaP). As metodologias biotecnológicas atuais, especialmente estudos genômicos, estão adicionando aspectos importantes nesta área. A maioria dos trabalhos em biologia do câncer descreve extensivamente a acumulação de mutações e de outros problemas (deleções, aumento ou diminuição da expressão) em oncogenes e supressores tumorais. A identificação recente de novas classes de ncRNAs (RNAs não codificantes) envolvidos em etapas importantes da formação e da progressão do câncer, reforça o papel destes transcritos no processo de tumorigênese. Além disso, alguns dos ncRNAs recentemente identificados podem agora ser usados como novos alvos para o desenvolvimento de drogas e para o diagnóstico e prognóstico precoces, sugerindo que esta nova classe de transcritos possa ser tão importante na biologia do câncer quanto os genes codificadores de proteína. O gene *Prostate Cancer Antigen 3* (*PCA3)* é expresso especificamente na próstata e apresenta-se superexpresso no CaP em relação aos tecidos de hiperplasia prostática benigna (HPB) e tecidos normais. O fato do *PCA3* ser um ncRNA próstata-específico, o torna um marcador promissor para o diagnóstico precoce do tumor prostático, além de fornecer ferramenta poderosa para o desenvolvimento de novas estratégias de tratamentos para pacientes com CaP. A importância deste transcrito como marcador molecular no diagnóstico do CaP tem sido descrita em amostras biológicas como urina, sangue periférico, biópsias e fluido prostático. No entanto as funções deste transcrito no desenvolvimento e na progressão do CaP são desconhecidas. Nas últimas três décadas, o anticorpo foi a molécula escolhida para o desenvolvimento de ensaios diagnósticos. A descoberta dos aptâmeros cuja afinidade e especificidade assemelham-se aos anticorpos terá um impacto futuro nos diagnósticos. A metodologia SELEX pode ser usada contra uma grande variedade de moléculas alvo produzindo aptâmeros altamente seletivos, de interesse terapêutico e diagnóstico. Os ciclos iterativos de seleção e amplificação permitem a identificação de aptâmeros de RNA contra ácidos nucléicos ou proteínas. Assim como estes alvos medeiam o controle da

expressão de um gene, os aptâmeros cognatos constituem moduladores artificiais específicos de processos biológicos, imitando o comportamento das sequências antisense. Interessantemente, a regulação do gene pode igualmente ser conseguida por meio da associação entre aptâmeros de RNA e moléculas pequenas. Baseado nestes dados, o objetivo deste trabalho foi selecionar e caracterizar aptâmeros ligantes específicos ao *PCA3*, e avaliar os efeitos celulares e moleculares causados pela transfecção destas moléculas, em linhagens celulares de câncer de próstata. Embora o *PCA3* seja um alvo terapêutico excelente para o câncer, nenhum antagonista foi desenvolvido ainda. Este estudo poderá auxiliar na determinação das alterações genéticas envolvidas na transformação tumoral, fornecendo subsídios sobre os mecanismos de ação do gene *PCA3* como possível modulador da expressão de genes-chave para o surgimento e progressão do tumor de próstata.

CAPÍTULO I

FUNDAMENTAÇÃO TEÓRICA

Epidemiologia e Etiologia do Câncer de Próstata

Em termos de valores absolutos, o câncer de próstata (CaP) é o sexto tipo mais comum no mundo e o mais prevalente em homens, representando cerca de 10% do total de cânceres. As taxas de incidência do CaP são cerca de seis vezes maiores nos países desenvolvidos, comparados aos países em desenvolvimento[1].

O número de novos casos de CaP para o Brasil em 2010 foi de 52.350, correspondendo a um risco estimado de 54 novos casos a cada 100 mil homens.

Em 2010, o Instituto Nacional do Câncer (INCA) estimou que a alta incidência do câncer de próstata no Brasil pode ter sido influenciada especialmente em regiões onde o rastreamento através do teste Antígeno Prostático Específico (PSA) é comum. No Brasil e no mundo, esse aumento nas taxas de incidência ao longo dos anos pode ser decorrente do aumento da expectativa de vida da população, da evolução dos métodos diagnósticos e da melhoria da qualidade dos sistemas de informação do país [1].

A mortalidade por CaP apresenta uma magnitude mais baixa que a incidência, contudo o perfil ascendente é semelhante, sendo considerado de bom prognóstico quando diagnosticado e tratado oportunamente. Entretanto, os métodos de rastreamento disponíveis atualmente, como o PSA, não mostraram, até o momento, sucesso em reduzir a mortalidade, além de levar a muitas cirurgias desnecessárias causando prejuízos financeiros e na qualidade de vida dos pacientes[1].

Em relação à etiologia do CaP, esta permanece obscura e seus tumores variam desde a forma indolente, com baixas taxas de evolução, para forma extremamente agressiva, com rápidas taxas de crescimento (Gimba et al., 2003). Algumas causas multifatoriais, tais como a interação entre fatores genéticos, hormonais (andrógenos), alimentares e ambientais, podem estar relacionadas com o desenvolvimento do tumor. Dentre os fatores de risco bem estabelecidos que amplamente associam-se ao CaP estão a idade, etnia, a dieta e o estilo de vida (Klein et al., 2007).

[1] http://www2.inca.gov.br/wps/wcm/connect/tiposdecancer/site/home/prostata/definicao. Acesso em 02 de abril de 2011.

Além disso, estudos epidemiológicos têm demonstrado aumento de risco ao desenvolvimento do tumor associado às infecções sexualmente transmissíveis, sugerindo que a inflamação pode estar relacionada com a etiologia do CaP, já que células produzem óxido nítrico e espécies oxigênio reativas com potencial de causar dano celular ou genômico na próstata (Pagano et al., 2004).

Estudos mais recentes relatam que, as contribuições sobre o conhecimento da malignidade de agentes infecciosos, como bactérias e vírus, tem sido útil na compreensão de como os processos celulares básicos culminam no câncer (Dennis et al., 2002; Hayes et al., 2000; Pagano et al., 2004).

Caracterização da Glândula Prostática

A próstata é um órgão fibromuscular e glandular masculino, com formato esférico, situada abaixo da bexiga urinária (Figura 1). Ela envolve a parte inicial da uretra masculina e os ductos ejaculadores, desembocando no colículo seminal na parte prostática da uretra (Guyton e Hall, 1997).

Anatomicamente a próstata é dividida em 5 zonas: zona fibromuscular anterior, que contém 30% do volume da próstata, nenhum elemento glandular e praticamente composta somente de músculo liso; zona central, que contém 25% de tecido glandular; zona periférica onde origina-se 70% dos CaP; zona pré-prostática, que tem função de esfíncter durante a ejaculação para prevenir o refluxo do líquido seminal para a bexiga; e a zona de transição, que apresenta pelo menos 5% da massa glandular normal da próstata, entretanto, tem importante função por ser o local de origem da hiperplasia prostática benigna (Figura 2) (Coffey, 1992).

A próstata é uma glândula acessória do sistema genital masculino responsável pela secreção e armazenamento de uma ampla gama de produtos do líquido seminal. Sua secreção fornece aos espermatozóides condições ideais de sobrevivência e viabilidade durante e após a ejaculação (Vilamaior et al., 2000).

O epitélio secretor da próstata normal é composto por vários tipos celulares inter-relacionados: células-tronco, células basais, células de amplificação

transitória ou "*transit-amplifying cells*" (TAC), células intermediárias, células neuroendócrinas e células luminais secretoras (Corradi et al., 2009).

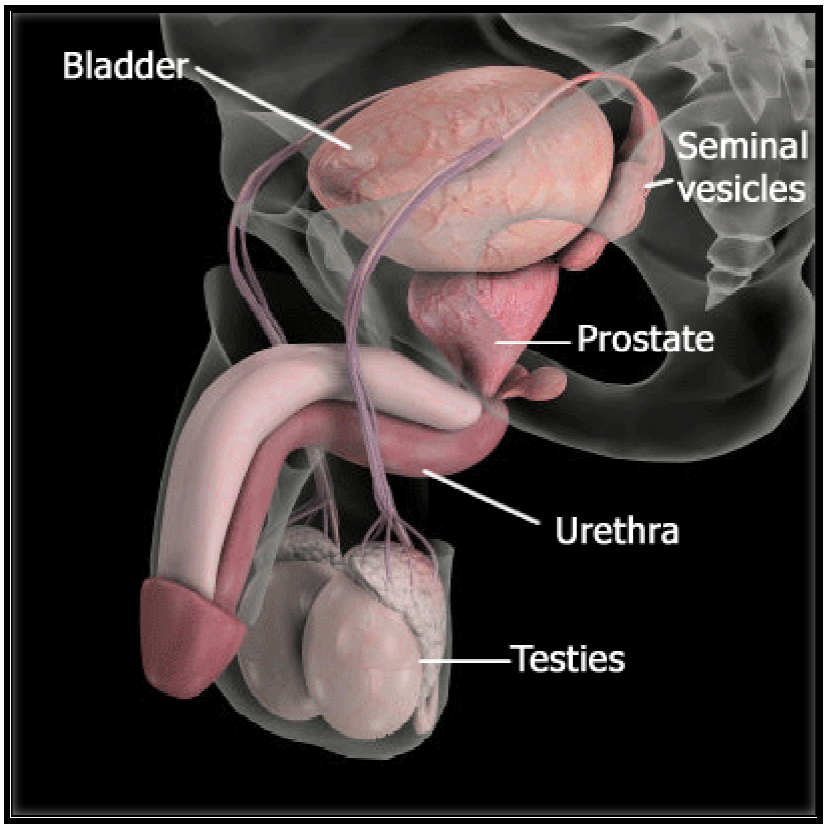

Figura 1. Localização da glândula prostática[2].

A diferenciação das células epiteliais prostáticas é um processo hierárquico que requer a divisão das células-tronco e o recrutamento de algumas das células-filhas em direção às células progenitoras intermediárias. No caso da próstata, estas correspondem sequencialmente às células basais, às TAC e às células intermediárias. Assim, à medida que as TACs se proliferam, são translocadas para a região luminal e alteram progressivamente o perfil de expressão gênica em direção às células luminais terminalmente diferenciadas (Schalken , 2005).

Esses tipos celulares diferem não apenas quanto à localização e à capacidade de originar outras células, mas também quanto à morfologia, ao padrão de expressão gênica e a resposta a estímulos fisiológicos (Corradi et al., 2009).

[2] www.mumbaiurologist.com/enlarged_prostate.html

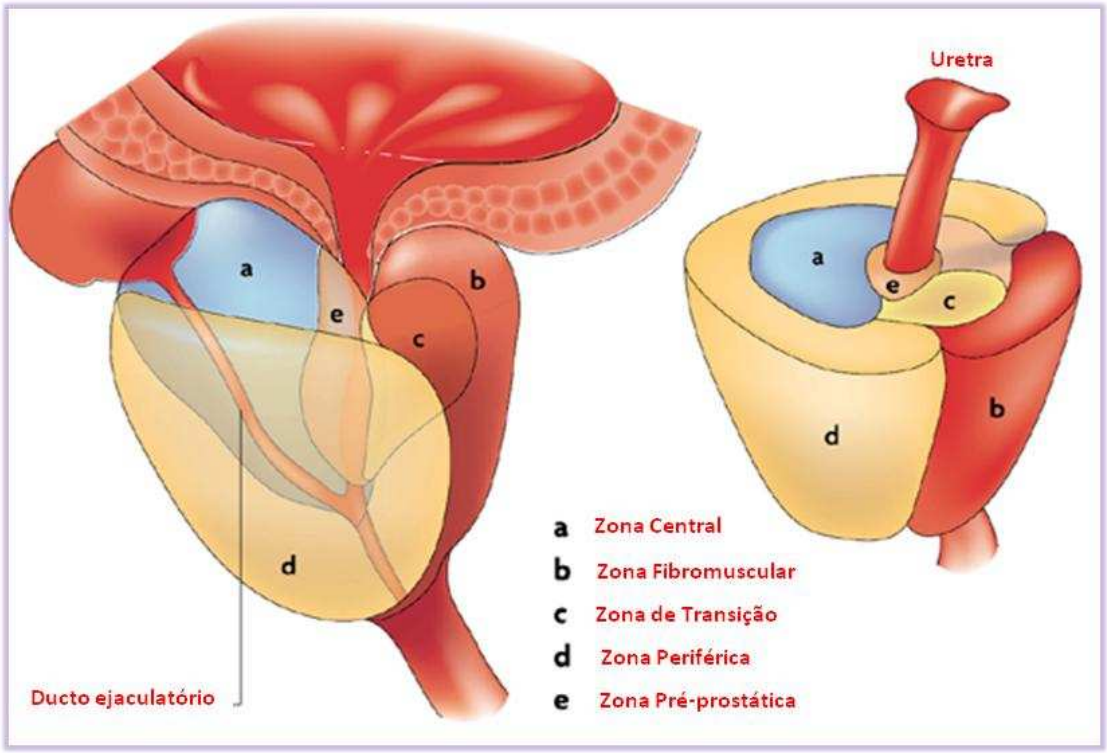

Figura 2. Divisão anatômica da glândula prostática (De Marzo et al., 2007).

Mecanismo de Regulação Geral da Próstata

O controle das funções sexuais masculinas inicia-se com a secreção do hormônio de liberação da gonadotrofina (GnRH) pelo hipotálamo. Esse hormônio estimula a hipófise anterior a secretar o hormônio luteinizante (LH) e o hormônio folículo-estimulante (FSH). O FSH estimula a espermatogênese e o LH estimula as células intersticiais de *Leydig* a secretarem o hormônio masculino, testosterona (Guyton e Hall, 1997; Griffiths e Morton, 1999).

Após ser liberada na corrente sanguínea, a testosterona entra nas células prostáticas por difusão passiva e é transformada, no citoplasma, em 5α-dihidrotestosterona (DHT) pela enzima 5α-reductase (Griffiths e Morton, 1999). A DHT se liga ao receptor de andrógeno (AR) e é transportada ao núcleo celular, onde favorece a síntese de RNA (Srougi, 1998).

Histologicamente é composta por unidades secretora túbulo-acinares, envoltas por um estroma rico em células musculares lisas. O controle da diferenciação e da atividade prostática é bastante complexo e, conforme

salientado por Schalken (2005) depende de uma soma de fatores extrínsecos (hormonais, imunológicos, alimentares e genéticos) e intrínsecos.

Os andrógenos têm sido apontados como o fator extrínseco de maior importância, atuando na homeostase do órgão e também nos eventos que levam ao seu desenvolvimento embrionário e à maturação funcional. A forma hidroxilada da testosterona, a DHT, é a de maior ação na próstata, devido à alta afinidade com o AR. Vários outros hormônios, como os estrógenos, os glicocorticóides, a prolactina e a insulina, também têm sido implicados no controle da histofisiologia prostática (Corradi et al., 2009).

A supremacia dos andrógenos na regulação da função e crescimento prostático é devida a sua ação direta, via ligação com ARs ou via mecanismos indiretos decorrentes do estímulo à secreção dos fatores de crescimento (Corradi et al., 2004).

Durante o desenvolvimento embrionário, por exemplo, as células mesenquimais do complexo prostático em desenvolvimento são as primeiras a expressar *AR* e, então, sob o estímulo androgênico liberam fatores como o FGF-10 que agem sobre as células epiteliais induzindo a formação dos ductos prostáticos e seu padrão de ramificação característico. Por sua vez, a ação direta dos andrógenos se aplica principalmente para a regulação da atividade secretora das células epiteliais (Corradi et al., 2009).

Doenças da Próstata

A próstata é um dos órgãos mais sujeitos a alterações durante o envelhecimento, apresentando frequentemente, um crescimento disfuncional, como é o caso da HPB, bem como a alta ocorrência de tumores malignos, como o CaP. As doenças da próstata levam a um prejuízo na qualidade de vida e os tumores malignos são a segunda causa de mortalidade em alguns países ocidentais. Por esse motivo, a compreensão do controle do funcionamento e a diferenciação das células prostáticas são de grande relevância (Simone et al., 2009).

A prostatite é uma doença prostática comum, sendo rara em adolescentes, mas frequente em homens adultos. Ocorre em formas distintas, as quais têm causas, características clínicas e sequelas diferentes. As formas mais comuns são: prostatites bacterianas agudas e crônicas e prostatodinia (próstata dolorida). O tratamento clínico do paciente torna-se possível a partir do diagnóstico e da estratégia terapêutica específica e adequada (Meares, 1992).

A HPB caracteriza-se por aumento notório do componente glandular prostático em relação aos componentes intersticial e capsular, acarretando por conseqüência, aumento volumétrico da próstata. Esse aumento benigno, com maior incidência na região de transição (Simone et al., 2009), ocorre após os 35 anos de idade, sendo responsável por vários sintomas do trato urinário inferior em homens (Catalona et al., 2002; Krumholtz et al., 2002; Catalona, 2004).

O CaP corresponde a uma alteração no balanço entre a proliferação e a morte celular. Durante os estágios iniciais do crescimento do câncer, as células irão responder aos mesmos fatores regulatórios (hormônio-dependente), embora as taxas de proliferação celular sejam maiores do que as de morte celular. A disfunção no processo regulatório associada a mutações genéticas reflete graus de respostas anormais dos fatores de crescimento, gerando um processo que leva a formação de clones autônomos de células malignas, com crescimento promovido por via autócrina que passa a não responder ao controle androgênico (hormônio-independente) (Schalken, 2005).

Aproximadamente 70% dos cânceres desenvolvem-se multifocalmente na zona periférica (Simone et al., 2009), menos de 5% na zona central e acima de 25% originam-se na zona de transição (cânceres denominados incidentais, quando removidos por ressecção transuretral (RTU) (Jarmulowicz, 1999).

Os cânceres da zona de transição apresentam menor potencial maligno do que aqueles que surgem da zona periférica (Bostwick, 1999). A organização anatômica da glândula inclui um tecido fibromuscular entre a zona central e a zona periférica que de certo modo limita a expansão de carcinomas da zona de transição para os feixes neurovasculares e ductos ejaculatórios, as duas maiores rotas para a expansão extra-glandular (Jarmulowicz, 1999).

Tem sido sugerido que a atrofia inflamatória da próstata (AIP) e a neoplasia intraepitelial prostática (NIP) de alto grau sejam precursores de muitos

carcinomas prostáticos, por apresentarem frequência aumentada nas próstatas com adenocarcinoma e alterações moleculares genéticas comuns. Por essas e outras características, a NIP tem sido hipotetizada como precursora do câncer de próstata (De Marzo et al., 2001).

A longa duração de inflamações crônicas, em muitos órgãos, incluindo o fígado, o estômago e grande parte do intestino, tem sido associada ao desenvolvimento de carcinoma. A carcinogênese é o resultado de danos tissulares locais recorrentes e regeneração na presença de oxigênio e nitrogênio altamente reativos liberados pelas células inflamatórias. Essas moléculas reativas podem interagir com o DNA no epitélio em proliferação produzindo permanentes alterações genômicas (Bonkhoff et al., 1994; De Marzo et al., 2001).

Diagnóstico Clínico

O rastreamento de alterações prostáticas é realizado principalmente por 4 etapas: o exame físico (toque retal), dosagem PSA, ultrasonografia transretal da próstata (USTR) e biópsia (Figura 3).

A *American Cancer Society*, juntamente com *American Urology Association* recomendam a realização anual do toque retal e da dosagem do PSA em homens a partir dos 50 anos. Nos grupos de alto risco, como em homens com história familiar de neoplasia, as avaliações devem iniciar aos 40 anos de idade (Chamberlain et al., 1996; Selly et al., 1996).

A realização de biópsias em pacientes é indicada para os casos com área de maior consistência na glândula e/ou com elevação do nível sérico do PSA (Srougi, 1998).

O estadiamento clínico e o resultado histopatológico dos pacientes com câncer de próstata são de fundamental importância para o planejamento terapêutico. No estadiamento clínico utiliza-se o estadiamento anátomo-patológico dos adenocarcinomas (TNM-*Tumor/Node/Metastasis*) (Anexo I). Estas classificações fornecem informações sobre o desenvolvimento do tumor e o grau de acometimento do mesmo (Srougi, 1998).

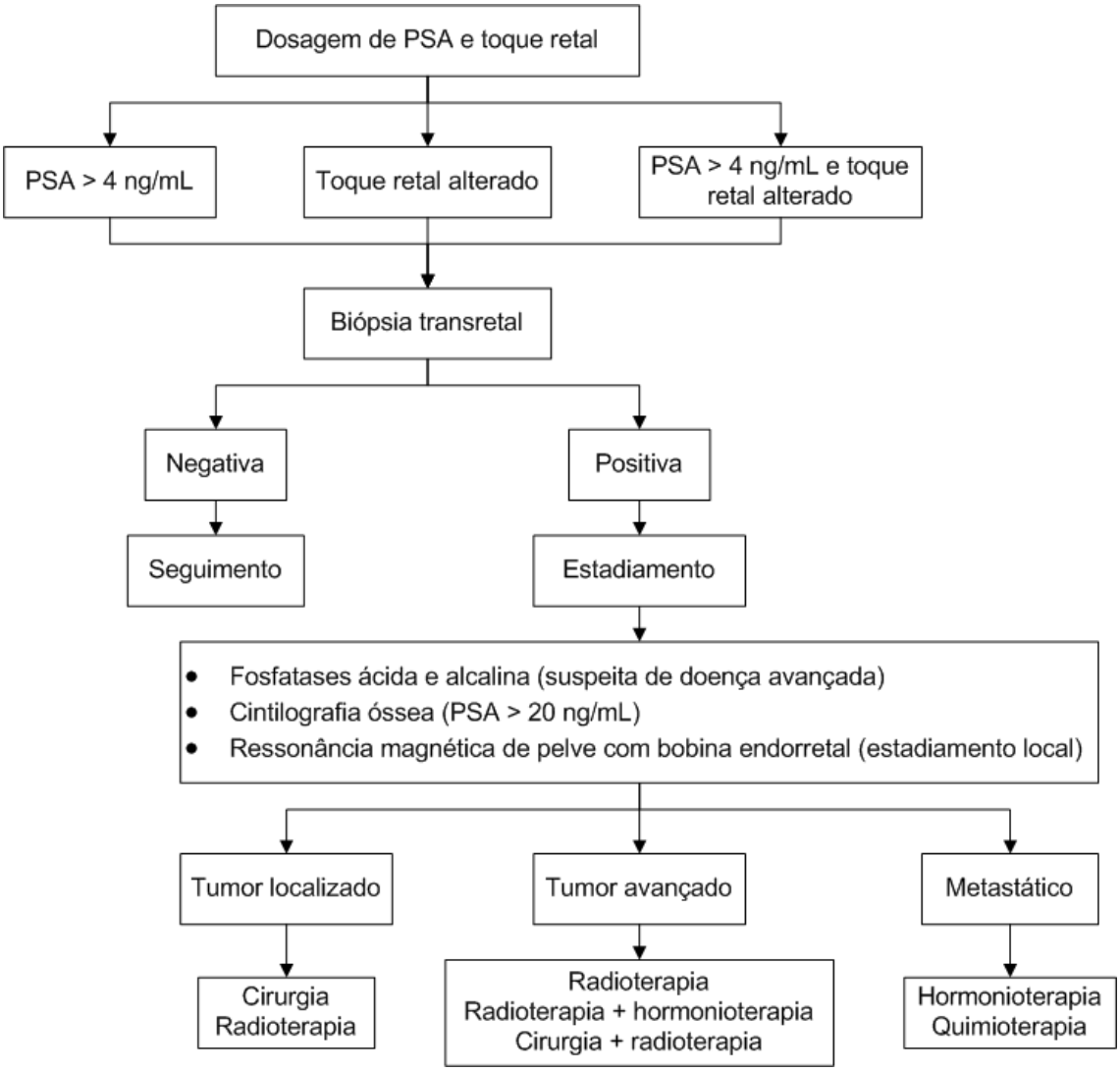

Figura 3. Abordagem diagnóstica e terapêutica do câncer de próstata (Arap e Coelho, 2010) [3].

A análise histopatológica da neoplasia é baseada na escala de Gleason (1977) (Anexo II). Este sistema valoriza o padrão glandular e a relação entre as glândulas e o estroma prostático, classificando os tumores em cinco graus, sendo grau 1 as lesões mais diferenciadas e as de grau 5 as mais indiferenciadas. Como o adenocarcinoma da próstata apresenta mais de um padrão histológico, o diagnóstico final na escala de Gleason é dado pela soma dos graus de padrão primário (área predominante) e de padrão secundário (área de menor representatividade). Assim as neoplasias mais diferenciadas são classificadas como grau 2 (1+1) e as mais indiferenciadas grau 10 (5+5) (McIntyre et al., 2004).

[3] http://www.medicinanet.com.br/conteudos/revisoes/3016/cancer_de_prostata.htm.

Aspectos Moleculares

Os estudos têm mostrado que há existência de mais de 100 tipos de cânceres e diversos subtipos, sendo que a maioria deles ou, praticamente todos, compartilham alterações essenciais na fisiologia celular ou nas múltiplas vias que em conjunto, ditam o crescimento tumoral. Dentre as alterações que ocorrem têm-se a auto-suficiência em sinais de crescimento, insensibilidade aos sinais de inibição de crescimento, evasão a apoptose, potencial ilimitado de replicação, autonomia angiogênica e capacidade para invadir tecidos e produzir metástases (Hanahan e Weinberg, 2000).

O câncer de próstata é originado por aberrações genéticas que inativam genes supressores de tumor e ativam proto-oncogenes (Porkka e Visakorpi, 2004). Estas alterações desorganizam a homeostase tissular por aumentar desregradamente à divisão celular ou por diminuir a apoptose, causando o aparecimento dos tumores (Gattas, 2003). A maioria das mutações é adquirida ao longo do desenvolvimento do câncer (mecanismo da tumorigênese), no entanto, algumas podem ser herdadas, resultando na predisposição à doença (Porkka e Visakorpi, 2004).

Por muito tempo atribuiu-se a origem do tumor prostático apenas à estimulação hormonal da testosterona, porém, atualmente sabe-se que o desenvolvimento tumoral é também regido por componentes genéticos e ambientais (Nwosu et al., 2001), sendo que em 10% dos casos ocorre por transmissão hereditária e os remanescentes por alterações genéticas esporádicas (Gattas, 2003).

Muitos esforços têm sido destinados ao melhor entendimento dos complexos mecanismos moleculares envolvidos na ontogênese e progressão do CaP (Gimba et al., 2003). Os métodos que são usados para caracterizar as aberrações genéticas encontradas nessa doença neoplásica incluem estudos familiares designados a mapear *loci* hereditários, estudos cromossomais para a identificação de anomalias que possam localizar oncogenes ou supressores de tumor e diversos estudos de expressão gênica (Li e Nelson, 2001; Karan et al., 2003).

Marcadores biológicos têm sido de grande importância para o diagnóstico e tratamento do CaP por mais de 50 anos e a identificação de novos genes e novos produtos envolvidos no processo tumoral tem crescido rapidamente, fornecendo ferramentas para o desenvolvimento de novas modalidades de tratamento (Bussemarkers et al., 1999; Bok e Small, 2002).

Biomarcadores Prostáticos

Vários genes prostáticos que codificam para ativadores transcricionais, reguladores, sinalizadores, receptores, supressores e oncogenes envolvidos na carcinogênese podem atuar de forma integrada entre si e com fatores ambientais, modulando a resposta individual, bem como os diferentes fenótipos da doença. Assim, a natureza maligna do câncer de próstata revela ser esta uma doença heterogênea, multifocal e multifatorial, além de apresentar um grande impacto sobre a qualidade de vida dos pacientes (Bok e Small, 2002).

De acordo com os dados publicados sobre os potenciais marcadores para o câncer de próstata e baseado nos bancos de dados *GeneCards* e *GeneName* [4], 23 genes associados com a ocorrência, desenvolvimento e progressão desta doença, foram sumarizados na Tabela 1.

[4] http://www.genecards.org; http://www.genenames.org

Tabela 1. Potenciais marcadores moleculares associados ao câncer de próstata.

Gene		*Locus*	Nº de exons	Localização subcelular	Processo celular e fisiológico	Função básica	Referências
Nome	**Aliase**						
Adenosine Deaminase RNA-specific B1	*ADARB1*	21q22.3	13	Intracelular	Edição de RNA	• Edição de dsRNA ; • Cataliza a desaminação hidrolítica da adenosine para inosina e muda a sequência de mRNAs específicos com estrutura em fita dupla.	• Flanagan et al., 2009 • Galeano et al., 2010
Androgen Receptor	*AR*	Xq11-q12	8	Núcleo	Transcrição, regulação, desenvolvimento (diferenciação sexual e transporte de moléculas pequenas)	• Regula a ação do andrógeno (proliferação e diferenciação celular em tecidos alvo); • Estimula a transcrição de genes regulados pelo andrógeno; • Supressão ou proliferação do câncer de próstata metastático.	• Lavery et al., 2011
Annexin A1	*ANXA1*	9q21.13	13	Membrana plasmática	Resposta antiinflamatória, exocitose (comunicação celular)	• Proteína de ligação a fosfolipídeos cálcio dependente; • Modulação da resposta antiinflamatória (atividade inibitória fosfolipase A2); • Promove fusão de membrana, regulando a via MAPK/ERKg.	• Inokuch et al., 2009
Eukaryotic Translation Elongation Factor 1 Alpha 1	*EEF1A1*	6q14.1	8	Citoplasma	Proliferação, apoptose, tradução	• Potencial atividade supressora tumoral mediada pela interação com ZBTB16; • Fator de elongação que promove a ligação dos tRNAs a sub-unidade ribossomal 80S.	• Kim et al., 2009
Folate Hydrolase (Prostate-specific Membrane Antigen 1)	*FOLH1*	11p11.2	19	Membrana plasmática	Atividade de Folato hidrolase	• Participa da conversão do neurotransmissor (NAAG) em NAA e glutamato livre.	• Ghosh et al., 2005
Kallikrein Related Peptidase	*KLK2*	19q13.41	5	Extracelular	Degradação (cascata proteolítica)	• Aumento da expressão influencia a susceptibidade ao CaP; • Medeiam o crescimento e invasão tumoral pela participação na cascata proteolítica; • Serino protease altamente específica da próstata, excretada principalmente no fluido seminal;	• Punglia et al., 2006 • Lilja et al., 2007 • Andriole et al., 2009 • Schroder et al., 2009 • Parikh et al., 2010
	KLK3	19q13.3-.4	5			• Serino protease com função na liquefação do coágulo seminal; • Expressão andrógeno-dependente; • Inibi angiogênese; • Inibe a motilidade da célula tumoral.	

Keratin	*KRT5* *KRT6* *KRT8* *KRT14* *KRT18*	12q11-q13 12q12-q21 12q13 17q12-q21 12q13.13	9 9 8 8 8	Citoplasma (citoesqueleto)	Organização do citoesqueleto e biogênese	• Mantêm a integridade da estrutura celular e tem função na transdução de sinal e diferenciação celular.	• Magin et al., 2007 • Oshima, 2007 • Uitto et al., 2007 • McLean et al., 2007 • Moll et al., 2008
Lymphocyte-specific Protein 1	*LSP1*	11p15.5	11	Citoplasma (citoesqueleto)	Motilidade celular	• Regula a motilidade neutrofílica, e a migração transendotelial;	• Howard et al., 1998 • Pulford et al., 1999 • Li et al., 2000
PR Domain Containing 4	*PRDM4*	12q23-q24.1	11	Núcleo	Ciclo celular	• Controle da diferenciação celular e tumorigênese.	• Yang et al., 1999 • Nishikawa et al., 2007
Prostate Cancer Antigen 3	*PCA3*	9q21-22	4	Núcleo / Citoplasma	RNA não codificante	• Regulação da expressão gênica	• Bussemakers et al., 1999 • Torres et al., 2007 • Marangoni et al., 2008 • Bourdoumis et al., 2010 • Salagierski et al., 2010
5′- Oligoadenilato-dependente Ribonuclease L	*RNAseL*	1q25.3	6	Citoplasma (mitocôndria)	Imunidade / defesa (apoptose)	• Medeiam a resistência a infecção viral e apoptose; • Realiza papel na atividade antiviral e antiproliferativa do IFN e contribui na imunidade inata e no metabolismo celular; • Candidato a supressor tumoral.	• Martinez-Fierro et al., 2010 • Kral et al., 2011
Small ArfGAP 1	*SMAP1*	6q12-q13	10	Membrana Plasmática	Eritropoiese	• Envolvido na atividade estimulatória eritropoiética das células estromais.	• Randazzo et al., 2004 • Bernards, 2003 • Liu et al., 2005 • Natsume et al., 2006 • Bethune et al., 2006 • Weimer et al., 2008
Steroid-5-alpha-reductase Alpha Polypeptide 2	*SRD5A2*	2p23.1	5	Citoplasma (Retículo endoplasm.)	Diferenciação sexual	• Envolvido na síntese de dihidrotestosterona no trato urogenital; • Papel central na diferenciação sexual e fisiologia do andrógeno.	• Simard et al., 2003 • Kral et al., 2011

Toll-Like Receptor	*TLR2*	4q32	3	Membrana Plasmática	Imunidade / defesa (apoptose de células infectadas, reconhecimento de patógeno)	• Papel central na resposta a uma variedade de patógenos e sinais de perigo endógeno • A estimulação realiza papel crucial na homeostase das células B humanas.	• Wang et al., 2006 • Krieg, 2007 • Apetoh et al., 2007 • Krieg, 2008 • González-Reyes et al., 2010
	TLR3	4q35	5			• Receptor de reconhecimento imune de dsRNA que reconhece perfil molecular associado com patógenos microbianos, e induz a resposta imune microbiana. • Indução de TNF e IL12; • Ativação de citocinas de acordo com o perfil molecular associado ao patógeno.	
	TLR4	9q33.1	3			• Molécula multifuncional: reconhecimento LPS e produção de citocinas	
	TLR6	4p16.1	1			• Medeiam a resposta celular a lipoproteínas bacterianas.	
	TLR9	3p21.3	2			• Reconhecimento de ácidos nucléicos e ligação da cascata de sinalização que ativa as células dendríticas plasmocíticas a produzir interferon alfa.	

Funcionalidades dos RNAs não codificantes (ncRNAs)

Por RNA não codificante (ncRNA) entende-se o RNA que não codifica proteínas, embora este ncRNA contenha na sua estrutura informações variadas podendo, pois, ter funções especiais.O genoma dos mamíferos codifica milhares de ncRNA (Pang, 2007). Como referido por Correia e Correia (2007) na maioria dos mamíferos os genomas são transcritos em ncRNA sendo alguns destes ncRNA cortados ou processados em fragmentos menores. Por vezes este ncRNA é também denominado RNA não mensageiro (nmRNA), pequeno RNA não mensageiro (snmRNA) ou RNA funcional (fRNA).

A sequência do DNA da qual é transcrito um RNA não codificante é denominada gene RNA ou gene ncRNA. Nos genes RNA não codificantes incluem-se os RNA de transferência (tRNA), os RNA ribossomais (rRNA), e os pequenos (small) RNAs tais como os snoRNA, microRNA, siRNA e piRNA (Chung, 2011).

Nos tipos ou famílias de ncRNA, deixando de lado os bem conhecidos tRNA e rRNA, os pequenos RNA nucleares envolvidos no splicing (snRNA) encontram-se no interior dos núcleos das células eucariotas e os pequenos RNA nucleolares (snoRNA) são pequenas moléculas envolvidas na modificação dos rRNA, que nos nucléolos conduzem a modificações químicas (metilação ou pseudouridilação) dos rRNA e de outros genes RNA. Os miRNA são genes RNA que são o complemento inverso de porções de transcritos de outros genes mRNA e que muitas vezes inibem a expressão do gene alvo (Costa, 2005).

Os pRNA (promotor RNA) são RNAs que correspondem a regiões promotoras atuando como um suporte para ligar uma cadeia promotora dirigida pelo siRNA, originando uma remodelação epigenética e o silenciamento do gene transcriptional dirigido pelo siRNA em células humanas (Correia e Correia, 2007)

Nos ncRNA incluem-se portanto diversas classes de RNA, inclusive os pequenos RNA reguladores, e dezenas de milhares de longos transcritos inclusive complexos que se interligam e transcritos que se sobrepõem como senso e antisenso, a maioria com funções ainda ignoradas, além de ncRNA derivados de introns. Todo este conjunto de RNA parece controlar em vários

níveis a expressão dos genes, durante o funcionamento normal dos organismos e durante o seu desenvolvimento, atuando na modelação da arquitetura da cromatina, da respectiva memória epigenética, da transcrição, do *splicing* do RNA e sua edição, tradução e *turnover* (Hüttenhofer e Vogel, 2006).

Apenas uma minoria dos genomas dos organismos multicelulares corresponde a sequências que codificam proteínas e isto é tanto mais acentuado quanto mais complexos são os organismos, aumentando nestes, consequentemente, as sequências intergenes não codificantes e sequências intrônicas transcritas na sua maioria (Chung, 2011).

Nos eucariotas mais complexos existe um grande número de proteínas que interagem com diversos ácidos nucléicos, bem como com a cromatina e estão bem identificados diversos complexos de RNA:RNA e RNA:DNA que se ligam a diversos tipos de proteínas (Correia e Correia, 2007).

Nas regiões não codificadoras dos mRNA e pré-mRNA há sequências reguladoras *cis* o que parece realizar-se através do emparelhamento com sequências complementares de outros RNA e DNA formando complexos reconhecidos por outros complexos como, por exemplo, o RISC. Estão também identificadas sequências reguladoras de RNA nas regiões UTR de mRNA que interagem com sinais adequados formando estruturas secundárias ligantes à proteínas reguladoras ou a pequenos alvos moleculares controlando desta forma a estabilidade, tradução e localização dos mRNA. É o caso dos "*riboswitches*" que interagem com vitaminas, aminoácidos ou purinas desencadeando efeitos alostéricos nos mRNA regulando dessa forma diversas vias metabólicas (Louro et al., 2007).

As UTRs nos mRNA, assim como sequências codificadoras destes mRNA podem funcionar como sensores de RNA reguladores como é o caso de Mirna, alguns dos quais são codificados em introns de outros genes. Literalmente, há dezenas de milhares de ncRNA expressos nos mamíferos e muitos destes transcritos são específicos de um determinado tipo de células, tecidos ou órgãos, com localizações subcelulares também específicas, e o desenvolvimento regulado. Atualmente, nos mamíferos existem mais de 800 ncRNA funcionais descritos, excluindo tRNA, rRNA e snRNA (Matttick e Makunin, 2006).

– SELEX –

Systematic Evolution of Ligands by EXponential enrichment

A técnica

As bibliotecas combinatórias de moléculas naturais, artificiais, de peptídeos e de oligonucleotídeos são empregadas principalmente pela indústria farmacêutica para identificação de moléculas específicas ao alvo com fins terapêuticos. As bibliotecas combinatórias de oligonucleotídeos são fáceis de manipular e de ser utilizada em ciclos reiterativos de seleção, *in vitro*, não requerendo processos de síntese química dispendiosa antes da repetição do ciclo (bibliotecas naturais ou químicas artificiais) ou passagem em bacteriófagos como em bibliotecas de peptídeos (*phage display*) Os processos podem levar a perda da diversidade durante os ciclos de seleção nos passos de síntese ou o aparecimento de um códon de terminação dentro do peptídeo que resultar na deleção da sequência no caso do *phage display* (Majumder et al., 2005).

A técnica evolução sistemática de ligantes por enriquecimento exponencial (SELEX) foi simultaneamente descrita por Tuerk e Gold (1990), Ellington e Szostak (1990) e Robertson e Joyce (1990). Ellington e Szostack (1990) estudavam a probabilidade de uma dada seqüência randômica de polinucleotídeo ou peptídeo adquirir uma conformação tridimensional estável, de se ligar ao alvo ou ainda de exercer atividade catalítica que no geral é muito baixa.

Tuerk e Gold (1990) tentavam compreender a interação do sítio de reconhecimento de um ácido nucléico e uma proteína que envolve uma população de moléculas e uma diversidade de sítios de ligação como ocorre na interação do complexo protéico na transcrição gênica.

Quando há apenas um sítio de ligação no genoma, como no alvo de nucleação da proteína de capsídeo do bacteriófago de RNA R17, a análise de quais os nucleotídeos necessários para a ligação pode ser endereçadas para criar e testar um número de mutantes. Contudo, o número de ácidos nucléicos a serem analisados seria tão grande que inviabilizaria tal estudo (Majumder et al., 2005).

Neste trabalho, foi utilizado o bacteriófago T4 e o ribossomo no sítio de ligação ao RNA mensageiro que o codifica. Este sítio sobrepõe a seqüência *Shine Dalgarno* prevenindo o início da tradução e é autoregulado. O alvo mínimo foi de 36 nucleotídeos que inclui cinco nucleotídeos em hélice com oito nucleotídeos em giros (*loops*). A fim de elucidar qual a função do *loop* de nucleotídeos na interação do operador na tradução, os pesquisadores desenvolveram um método rápido para selecionar somente seqüências as quais ligavam ao alvo, numa população de seqüências randômicas, que foi denominada SELEX (Robertson e Joyce, 1990).

Seleção de aptâmeros

O processo de seleção *in vitro* mimetiza a seleção natural. Os pesquisadores Ellington e Szostak (1990) sintetizaram por meio de síntese química em fase sólida de fosforamídeos uma larga quantidade de moléculas de DNA com 100 posições randômicas flanqueadas com adição equimolar da mistura dos quatro nucleotídeos. Estas seqüências possuíam também regiões definidas nas porções 5` e 3` para hibridização dos iniciadores (*primers*), as quais continham sítios de enzima de restrição para posterior clonagem das seqüências identificadas e o promotor T7 RNA polimerase. Verificaram prontamente que a biblioteca de DNA não era transcrita eficientemente em RNA, o que os levou a amplificar primeiramente por reação em cadeia da polimerase (PCR) 20 vezes a biblioteca inicial. Quatorze sequências foram identificadas demonstrando sua randomicidade, contudo, a PCR resultou em redução da complexidade da biblioteca pela inabilidade da Taq polimerase em amplificar regiões ricas em CG especialmente na porção 5' .

Um ensaio por extensão de *primer* foi realizado para avaliar a capacidade de síntese da fita complementar à fita simples de ssDNA demonstrando somente 4 a 5% das fitas simples tiveram uma síntese completa o que resultou numa redução em 20-25 vezes no tamanho da biblioteca. Além disso, 2/3 das seqüências amplificadas continham mutações na região constante rica em CG após a PCR o que levou a redução da diversidade de seqüências a 10^{13} seqüências. Os alvos utilizados na seleção foram diversos corantes que mimetizaram cofatores metabólitos como *Cibacron Blue* que se liga ao sítio de ligação de diversas dehidrogenases da NAD, *Reactive Red 20*,

Reactive Yellow 86, *Reactive Brown* 10, *Reactive green* 19 e *Reactive Blue 4* (Gold, 1999).

No primeiro ciclo de seleção, apenas 1% da biblioteca de RNA foi recuperada, contudo no segundo ciclo, recuperou-se 70% das moléculas de RNA. As seqüências identificadas após a seleção receberam o nome de aptâmero, do latim *aptus* que significa ligar (Gold, 1999).

Tuerk e Gold (1990) partiram de uma população randômica de 65536 seqüências e identificaram uma região de 8 nucleotídeos que interagiam com a T4 DNA polimerase e duas seqüências, uma idêntica presente no bacterófago e a outra variante desta seqüência em quatro posições.

A utilização de oligonucleotídeos como ligantes ou inibidores de proteínas baseia-se na propriedade de reconhecimento específico de epítopos protéicos pelas moléculas de RNA ou DNA, semelhante à interação RNA-proteína e DNA-proteína existente na célula. Tais ligações ocorrem pela interação de cargas entre o aptâmero e o alvo de seleção. As ligações ocorrem pela formação de pontes de hidrogênio entre os aminoácidos do alvo e a cadeia do aptâmero ou ligação de van der Waals entre as cadeias de açúcar e grupamentos fosfato (Gold, 1999).

Uma nova contribuição foi dada em 1990 por Robertson e Joyce quanto a identificação de RNAs com atividade catalítica pela seleção *in vitro* sendo denominados aptazimas (Hesselberth et al., 2003). Em 1998, Ulrich e cols. selecionaram aptâmeros capazes de inibir a atividade do receptor ionotrópico nicotínico, atuando como antagonistas. Demonstrou-se que além da alta capacidade de reconhecimento e de afinidade, o aptâmero pode modular funções protéicas de conformações do canal do receptor, isto é, atuar como antagonista ou de proteger o sítio específico de ligação, modulando, portanto o estado conformacional protéico em consequência a atividade do receptor.

Após ciclos reiterativos de enriquecimento, associados ao aumento de estringência com emprego inicial da biblioteca combinatória constituída de 10^{13} a 10^{15} moléculas de oligonucleotídeos de RNA ou DNA, há seleção do aptâmero com estrutura adequada e capacidade de interação à molécula alvo. A amplificação da biblioteca de cDNA é realizada utilizando a PCR e obtenção da biblioteca de RNA, por transcrição *in vitro* utilizando a enzima T7 RNA polimerase. Durante os ciclos de seleção *in vitro* de aptâmeros com alta

afinidade, recupera-se o RNA ligado ao receptor no sítio específico do alvo. Este é purificado e utilizado como molde nas reações de RT-PCR (Ulrich et al., 1998).

Para utilização do aptâmero como produto intracelular com expressão no interior da célula, utiliza-se bibliotecas de RNA não modificados que resulta na seleção de intrâmeros. Caso o alvo da seleção seja a extracelular e o aptâmero possa ser um candidato à droga em potencial, utiliza-se a biblioteca com RNA-resistente a nucleases que consiste na utilização de ribonucleotídeos de pirimidinas modificadas que aumentam a estabilidade do RNA em meios fisiológicos. Se for necessário identificar aptâmeros que serão acoplados a coluna de cromatografia de afinidade para purificar a proteína, por exemplo, utiliza-se a biblioteca de aptâmeros de DNA que pode ser reutilizado após lavagens alcalinas (Gold, 1999).

A fim de aumentar a estabilidade das moléculas selecionadas têm se realizado seleções utilizando enantiômeros, conhecidos como *spiegelmers*, que não são reconhecidos pelas nucleases (Klussmann et al., 1996). Entretanto, a utilização de tais ribonucleotídeos tem encarecido o processo de seleção, pois se requer modificações nos quatro ribonucleotídeos para síntese de aptâmeros.

O emprego de ribonucleotídeos de pirimidina modificados no anel da ribose na posição 2'OH por 2'F ou 2'NH2 resulta em moléculas com maior estabilidade e menor custo. A meia-vida da molécula de RNA passa de oito segundos a 86 horas com modificação 2'F e a 174 horas com modificação 2'NH2. Contudo, as moléculas de RNA selecionadas modificadas com 2'F têm maior rigidez em sua estrutura e afinidade ao alvo comparado a aptâmeros com 2'NH2. O intuito é adicionar nucleotídeos modificados tanto no processo de seleção de DNA ou de RNA que possam ser incorporadas pelas enzimas Taq polimerase, T7 RNA polimerase e enzimas transcriptase reversas, que resultam em aptâmeros mais estáveis a fluídos biológicos além de não interferirem no pareamento de *Watson-Crick*. Todas estas modificações são necessárias a fim de sintetizar-se o aptâmero em escala industrial para aplicação terapêutica (Pagratis et al., 1997).

Outras modificações têm sido introduzidas para aumentar o tempo de meia-vida e de distribuição dos aptâmeros em sistemas fisiológicos como a

adição de um grupamento lipídico inserido no aptâmero para ancoragem em lipossomos (Willis et al., 1998).

Propriedade dos aptâmeros

Os aptâmeros são pouco imunogênicos. Ao contrário da produção de anticorpos monoclonais, a produção em larga escala do aptâmero é menos dispendiosa e sua área de atuação é versátil, isto é, o aptâmero pode discernir domínios extracelulares ou intracelulares, alcançar epítopos pequenos e protegidos de vírus, os quais não podem ser distingüidos por anticorpos como pode ser utilizado em terapias celulares de DNA através da inserção em plasmídeos (Gopinath et al., 2006).

Os aptâmeros identificados por SELEX possuem afinidade e especificidade de ligação aos seus alvos de forma semelhante ao anticorpo monoclonal com constantes de dissociação da ordem de nanomolar a picomolar (Jayasena, 1999); de discernir aminoácidos e oligonucleotídeos como ATP e dATP (Sassanfar e Szostak, 1993), inclusive estereoisômeros e proteínas com conformações diferentes resultantes de variantes de *splicing* alternativo como de proteína quinase C (pKC) (Conrad et al., 1994), estados conformacionais de uma mesma proteína (Hess et al., 2000), diferenciar entre vasculatura tumoral e normal (Blank et al., 2001) ou de diferenciar células em dois estágios distintos de diferenciação no caso de células PC12 (Wang et al., 2003).

O aptâmero selecionado pode ser empregado como ferramenta diagnóstica em técnicas de citometria de fluxo, arranjos (*arrays*), imunofluorescência, testes fluorescentes e testes em placas de Elisa (Majumder et al., 2005; Ulrich et al., 2006). O aptâmero pode ser expresso intracelularmente sendo denominado intrâmero e interagir em porteínas envolvidas com regulação de vias intracelulares de transdução de sinal (Famulok et al., 2001) ou ainda atuar como sensores, aptasensores (Minunni et al., 2004).

Aplicação clínica dos aptâmeros

Tal a especificidade e afinidade de ligação dos aptâmeros selecionados, este pode ser utilizado como agente terapêutico contra moléculas de

imunoglobulina E (IgE), interferon gama (γIFN), α-trombina, PTPase (proteína tirosina fosfatase) demonstrando grande eficácia em testes em cultura de células e em animais (Lee et al., 2006). Em 2004, a empresa *Pfizer-Eyetech Pharmaceuticals* iniciou a comercialização do primeiro aptâmero para o tratamento de coroidal neovascularização (AMD) e melhora da acuidade visual (Ng et al., 2006) conhecido como Macugen. O aptâmero desenvolvido reconhece a isoforma específica VEGF165 (*vascular endothelial growth factor*). Outros aptâmeros que estão em fase I selecionados pela companhia *Gilead* são promissores em terapias de coagulação, como o aptâmeros análogo de heparina (Bock et al., 1992). Contudo, já há aptâmeros de segunda geração com o mesmo efeito do análogo e que demandam menor concentração para obtenção do mesmo efeito. O grupo do pesquisador *Sullenger* desenvolveu aptâmeros contra o fator de coagulação IXa que conjugados com a cadeia de colesterol aumentam o tempo de vida do aptâmero de 5-10 minutos a 1-1,5 horas (Rusconi et al., 2002) e para este aptâmero há inclusive um “antídoto” para inativar o efeito (Rusconi et al., 2004).

Os intrâmeros são utilizados como mecanismos de inativação de proteínas relacionadas ao desenvolvimento, de sinalizações intracelulares ou mesmo com fins terapêuticos para inibir a replicação viral. Por exemplo, no desenvolvimento a expressão intracelular de aptâmeros pode ser regulada através do promotor utilizado que controla sua expressão em quantidade e temporal. O promotor pode ser do tipo indutível neste caso. Intrâmeros contra kanamicina e tobramicina foram posicionados na porção 5’ não transcrita de genes. Na adição de drogas, inibiu-se a expressão gênica (Werstuck e Green, 1998).

O intrâmero permite compreender o papel de proteínas envolvidas em vias intracelulares de transdução de sinal. A proteína B52 é expressa em drosófila durante o desenvolvimento envolvido em processos de *splicing* gênico, entretanto, sua importância não estava definida. O intrâmero desenvolvido foi expresso na forma pentamérica durante o desenvolvimento e a inibição de B52 resultou na drástica redução de sobrevivência (Shi et al., 1997). A utilização do intrâmero a proteína rev envolvida com ciclo de replicação do HIV resultou na inibição da replicação do vírus em cultura de células (Good et al., 1997). Em células humanas de linfócito, houve drástica

atividade anti-viral na presença dos intrâmeros (Chaloin et al., 2002). Há intrâmeros capazes de inibir ligações na porção intracelular do domínio humano na beta 2 integrina linfócito função-antígeno associada-1 (*αLβ2 integrin adhesion molecule, LFA*) que bloqueia a ativação de vias intracelulares. Tais aptâmeros foram expressos em leucócitos para sistema de desenvolvimento de vacinas (Blind et al., 1999).

Os aptâmeros podem ser utilizados para investigar vias de transdução de sinal. Theis (2006) identificaram intrâmeros que ligam e inibem o trocador nucleotídeo guanina fator citoesina-2, mas não seu homólogo que funcionalmente é uma proteína diferente, a citoesina-1. Após seis horas de transfecção em células HeLa ainda identificou-se aptâmeros transfectados presentes nas células (Lee et al., 2006).

Para estes estudos, os alvos também podem ser o ligante ou o receptor de membrana. O aptâmero contra o VEGF inibiu a interação deste com o seu receptor na superfície celular (Jellinek et al.,1994). Aptâmeros contra o receptor de tirosina quinase RET foram selecionados após ciclos de enriquecimento e retirados de ligantes não específicos pela utilização de células que expressavam o receptor mutado; identificaram-se dois aptâmeros com representatividade de mais de 50% nas 67 sequências identificadas. Através de testes de atividade biológica em células normais as quais ocorria a ativação de RET e iniciava a sinalização para a fosforilação de ERK (*extracellular signal regulated kinase*), os aptâmeros identificados não ativaram tal sinalização intracelular. Contudo, o aptâmero identificado reconheceu apenas o receptor à membrana e não RET purificado e é um potencial candidato a ser utilizado em testes pré-clínicos (Cerchia et al., 2005).

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CAPÍTULO II

Highly specific RNA aptamer ligand to the tertiary structures of the non-coding Prostate Cancer Antigen 3 mRNA

ABSTRACT

The prostate cancer antigen 3 (*PCA3)* mRNA expression has been used as a prostate cancer (PCa) biomarker in tissue and urine clinical samples, but its function is unknown. Analysis of the *PCA3* sequence has predicted a significant tertiary configuration based on a complex double-stranded fold-back structure formed through the complementary base pairing of highly repetitive sequences. RNA aptamers were selected against the partial structure of the *PCA3* transcript encompassing exons 1, 3 and 4. Six *PCA3*-ligand aptamers with high affinity were selected and one of the most repetitive aptamer motifs was reduced and synthesized for validation. The novel biotinylated RNA aptamer (CG3) was used to detect the transcript on prostate histopathological sections in tissue microarrays (TMA) through immunohistochemical analysis. Biotin-labeled CG3 aptamer stained the cytoplasm and nucleus of PCa and BPH tissue and is absent in stromal cells, corroborating previous findings of the *PCA3* RNA cellular localization. Additional immunoassays provided final evidences that the CG3 aptamer has specific binding to the *PCA3* transcript, and may be used as an auxiliary biomarker in PCa diagnosis. Its detection in the peripheral blood and interference in the transcript function still remain to be demonstrated.

INTRODUCTION

The prostate cancer antigen 3 (*PCA3*) mRNA was found to be overexpressed in more than 95% of primary prostate cancer (PCa) tissue specimens (1), and is considered one of the most strong candidate biomarkers for PCa diagnosis, and may be used as an auxiliary tool to the measurement of serum prostate specific antigen (PSA) levels. The *PCA3* transcript is a non-coding gene, and due to the absence of a coded protein product, nucleic acid-based tests have been developed to detect its presence in PCa clinical samples, although its specific function is unknown.

Most of the newly identified ncRNAs' functions are unknown, but these transcripts are increasingly recognized as regulators of gene expression (2). The understanding of the function of non-coding RNAs (ncRNAs) has as well generated a great enthusiasm because these molecules may provide an obvious potential use as powerful new tools in cancer medicine (3). ncRNAs falls in a broad range of regulatory RNA molecules, such as ribozymes, antisense, interfering small RNAs or aptamers that are either naturally found in several cell types or are artificially designed to target gene and controlling expression (4).

Systematic Evolution of Ligands by EXponential enrichment (SELEX) has been widely used to study properties of RNA or DNA molecules that bind to proteins, small molecules, oligonucleotides and peptides among others (5). In RNA aptamer selections, a DNA pool containing random sequences is typically synthesized artificially, which are subsequently transcribed to give the starting RNA population. The major difference between SELEX and Genomic SELEX is the starting pool used for the selection (6). SELEX begins with a library of

synthetically derived random DNA molecules. Genomic SELEX starts from libraries derived from genomic DNA as a consequence, the enriched sequences will not represent a full size functional RNA, but the ligand-binding domain, or “aptamer”, within the putative transcript.

High-affinity binding RNAs are enriched from the initial pool through multiple rounds of binding of the RNAs to a given ligand. The winning molecules can be cloned and sequenced individually if only a limited number of sequences are expected. However, depending on the bait used for the selection, a large number of sequences can be expected, making high-throughput sequencing the method of choice (6).

Genomic SELEX is both alternative and complementary to *in vivo* and *in silico* approaches. Additionally, SELEX was applied to select RNA aptamers targeting RNA molecules to foster the understanding of RNA–RNA interactions (7). Interestingly, the most competitive aptamers can participate in complex regulatory networks not merely through Watson–Crick interactions with other RNAs (as in antisense RNA), but also by forming discrete secondary structures that bind DNA, RNA or protein targets (6).

The advantage of aptamers over other biochemical or chemical substances employed up to now include high potency and specificity for the target, use of *in vitro* techniques for their production, that considerably reduce production costs as well as the need for animal testing and that markedly increase specificities and quality assurance in diagnostic and therapeutic applications (4).

In this study, we have generated by *in silico* analysis a remarkable tertiary structure of the *PCA3* transcript with a significant free energy, showing

many hairpins and loops, due to the extensive base pairing within the molecule. Our hypothesis is that the conformational structure may be functional and may be required for editing or processing, which will eventually control other genes. However, looking at the perspective of PCa diagnosis, the direct use of high affinity aptamers to the *PCA3* transcript may substitute the required reverse-transcriptase PCR amplification, and detection of the *PCA3* molecule can be achieved in physiological conditions without controlling hybridization parameters, and may even be used for tissue analysis. Here, we are describing the selection of an RNA aptamer that was enriched by SELEX that specifically binds to *PCA3* mRNA, and its application was validated by tissue microarrays. This novel nucleic acid antibody-like cancer biomarker may also be useful in peripheral blood analysis; however, biosensors are still under development.

MATERIAL AND METHODS

Target Molecule

For the selection of RNA aptamers with high affinity to the target molecule, we have used a partial amplified *PCA3* transcript that consists of 277 bases, encompassing parts of exons 1, 3 and 4 (alternative splicing of exon 2), which is the most common transcript detected in 95% of the prostate cancer tissues (Figure 1) (1).

For a successful selection of aptamers with high affinity and specificity, the single target molecule should be present in sufficient amount and with high purity as demonstrated elsewhere (8). The 277-bp PCR fragment was cloned and purified, and biotin was introduced into sense strand of the *PCA3* during asymmetric PCR amplification using 5pmols of biotinylated forward-primer (5’

biotin - AGATGTTCTTTGATGCGGAGC – 3') in the reaction. PCR assay was performed as previously reported (9) with minor modifications.

DNA Source for the Genomic SELEX

In general, any source or desired protocol can be used for genomic DNA isolation, as long as the resulting DNA is of high quality and reliably represents the genome of interest. For the library construction we used 1 µg of genomic DNAs pool from human prostate cancer tissue specimens (*PCA3* positive). All PCa patients were submitted to radical prostatectomy. The library was generated by random priming of sheared DNAs, by sonication step , followed by sizing to obtain overlapping fragments of any desired size.

Primers

As described elsewhere for the Genomic SELEX, two pairs of primers called "hyb"- and "fix"-primers were used (10). The first part of the library construction consists of first- and second-strand synthesis, in which hyb-REV and hyb-FOR primers are used, respectively. Both hyb-primers consist of a unique constant sequence region, followed by ~ 9 randomized nucleotides at the 3′ terminus. These randomized regions serve to pick random genomic regions for inclusion in the library.

In order to amplify the library, fix-primers are used. The fix-primers correspond perfectly to the 5′ constant regions of respective hyb-primers (16nt FOR, 16nt REV), with addition of the T7 promoter at the 5′ end of the fix-FOR primer.

Preparation of the aptamers' genomic library

In Genomic SELEX, the primary aim of constructing a genomic library is to give every potential genomic aptamer region equal candidacy for selection, and therefore have complete coverage of the genome and amplify it evenly (Figure 2) (11).

The first strand PCR reactions consisted of: genomic DNA (1 µg), primer hyb-REV (5 pmols), *Platinum* Taq DNA Polymerase (1 U), 200 mM dNTPs (final concentration), 1.5 mmol/L MgCl2, 50 mmol/L KCl and 10 mmol/L Tris-HCl at pH 8.3. The reaction was incubated for 26 cycles at 93°C - 20s, 50°C - 40s, 72°C - 40s, preceded by initial denaturation at 93°C - 3 min and a final extension cycle at 72°C - 10 min. The 50°C step allows *Platinum* extension through hairpins in single-stranded DNA.

In order to minimize the number of fragments flanked with the same sequence on both sides, the excess of hyb-REV should be thoroughly removed prior to second strand synthesis with hyb-FOR (7). The purification was performed by DNA precipitation with ammonium acetate (7.5 M) and the pellet was ressuspended in 10 µL of water. Preparation of the genomic library finishes with the introduction of the T7 promoter through PCR amplification using the fix-primers. The second strand PCR reaction was the same as first strand synthesis. The reaction products were separated on a 2.5% agarose gel electrophoresis and fractions of various sizes (with genomic inserts ranging from about 40–700 nucleotides) that were purified according to protocol described above.

Next we amplified the library by PCR using primers of completely fixed sequences, one of which adds a T7 promoter (primer fix-FOR); thus the library can be expressed as RNA.

In vitro Selection of RNA aptamers

The first step of each round of Genomic SELEX is the construction of the RNA pool by *in vitro* transcription of the DNA library. The *in vitro* transcription reactions were performed by T7 RiboMAX™ express large scale RNA, according to manufacturer's instruction (Promega).

Next, the selection, or binding step, follows. The exact condition in which binding is performed is to be optimized. In this work, SELEX was performed using 400 pmols of biotinylated *PCA3* (277 bases) with bait and 10 nmols of random pool of 10^{14} RNA oligonucleotides (aptamers).

PCR products that contained the genome-specific biotinylated primer were batch-purified on streptavidin beads. A fresh aliquot of $1x10^{8}$ streptavidin-coated magnetic beads was washed three times with binding buffer (100 mmol L^{-1} NaCl, 20 mmol L^{-1} Tris-HCl pH 7.6, 2 mmol L^{-1} MgCl2, 5 mmol L^{-1} KCl, 1 mmol L^{-1} CaCl2, 0.02% Tween 20) before each selection round. The pool of biotinylated *PCA3* was heated to 55°C for 10 min, immediately cooled, and kept at 4°C for 15 min, followed by a short incubation (5-8 min) at room temperature before its application in the binding reaction. Binding was carried out at 37°C for 1h with mild shaking.

After immobilization, the bead/*PCA3* complex was extensively washed - 5 steps with 500 µL binding buffer - to remove unwanted reaction components. Oligonucleotides complementary to the biotinylated strand was eluted by

denaturation at 37°C for 15 min with 400 µl of 0.15 M NaOH and then for 5 more min with another 500 µl of 0.15 M NaOH. This helps to minimize the enrichment of unspecifically binding oligonucleotides and to increase the specificity of the selection (12).

In the initial selection round, the bead/*PCA3* complex were resuspended in 500 µL binding buffer containing 10 nmol of the random pool RNA library. After an incubation of this mixture at 37°C for 1h with mild shaking, the unbound oligonucleotides were removed according to washing protocol described above.

Subsequently, the bound oligonucleotides were eluted by incubating the binding complex twice with 200 µL elution buffer (40 mmol L^{-1} Tris-HCl pH 8, 10 mmol L^{-1} EDTA, 3.5 mol L^{-1} urea, 0.02% Tween 20) at 37°C for 10 min with mild shaking. The amount of RNA recovered is an indicator of enrichment of binding species in the pool. The pooled oligonucleotides were precipitated with ethanol. The RNA was then resuspended in a smaller volume of binding buffer.

During this SELEX process the selected RNA of each round has to be reverse transcribed and subsequently amplified by RT-PCR using the fix-REV and fix-FOR primers. The new RNA pool for the next SELEX round is then generated again by *in vitro* transcription (Figure 3).

Cloning and sequencing

The selected aptamers from SELEX rounds 1, 3 and 8 were amplified and cloned into pCR2.1-TOPO vector systems (TOPO TA Cloning Kit; Invitrogen). The purified sequences were submitted to a capillary sequencer (MegaBace 1000), using the DYEnamic ET Dye Terminator Cycle Sequencing

kit (GE Healthcare). Three sequencing reactions were carried out for each fragment and injected twice to minimize sequencing artifacts.

Screening and structure analysis of selected aptamers

In this article we developed a set of criteria to create a list of RNA aptamers (from 8th round of SELEX) with potential binding affinity to *PCA3*.

Our approach consisted of three steps:

(1) Step 1: Selection of sequences based on motifs.

(2) Step 2: Sequence sizes up to 21 nucleotides.

(3) Step 3: Hybridization energy characteristics between the folded-structure of the *PCA3* transcript and selected aptamers ($\Delta G_{hybrid} \leq -18.0$ kcal/mol).

Only the sequences that passed the selection criteria were forwarded to the next step. *Sfold* web server was used for hybridization (application module: *StarMir*) and for nucleic acid folding predictions of several aptamers. The secondary structure analysis was performed by means of free-energy minimization.

Reverse Binding Assay

400 pmols of synthetic biotinylated aptamers (CG3 and BC4) was incubated with washed 1x10^8 streptavidin-coated magnetic beads at 37°C for 30 min in a total volume of 200 µL of binding buffer. After the binding reaction, the unbound aptamers were removed by several washing steps with binding buffer.

The reverse binding assay was performed in three different conditions: (I) the bead/specific-aptamer complex was resuspended in a 500 µL binding buffer containing 10 nmol of the *PCA3*, (II) 10 nmols of *PCA3* pre-incubated with the specific-aptamer without biotin-labeled at 37°C for 1h with mild shaking and (III) 10 nmol of the scramble non-specific RNA. For all the three experiments were used *PCA3* RNA without biotin-labeled. Incubation, washing and elution steps were performed as reported above.

The amount of *PCA3* recovered in each approach is an indicator of affinity and specificity of the selected RNA aptamer binds *PCA3*.

Tissues and Tissue Microarray Assembly

Tissue samples were provided by the AC Camargo Hospital Tumor Bank (São Paulo / Brazil). For *In Situ Hybridization* (ISH), we evaluated a total of 161 cases, including 129 prostatic adenocarcinoma (PCa), 23 benign prostatic hyperplasia (BPH), 3 lymphomas, 3 gastric cancers and 3 pancreatic cancers. The median patient age was 63 (range, 46–86) years.

One cylinder of 1 mm was obtained from each PCa and BPH to build the tissue microarray (TMA; Beecher Instruments, Sun Prairie, WI) as previously described (13). The biotinylated RNA aptamer, named CG3, was used to detect the *PCA3* transcript through ISH analysis.

In Situ Hybridization (ISH)

Procedure

For ISH reactions tissue slides were incubated with 1µg of biotin-labeled CG3 aptamer at 37°C for 1h. Slides were washed three times with PBS and

incubated with horseradish peroxidase (HRP)-conjugated streptavidin for 5 min, washed three times with PBS, incubated with the peroxidase substrate, washed twice with PBS. The slides were counter-stained in Mayer's hematoxylin for 60 sec, blued with tap water, dehydrated, cleared, and mounted for light microscopy. Endogenous biotin was blocked with Avidin-Biotin-Complex kit (DAKO - Carpinteria, CA).

All histochemistry reactions were performed simultaneously to avoid any bias in the results due to the order of testing or differences in environmental conditions.

Staining evaluation

The CG3 aptamer staining observed in the prostate cancer and BPH TMA spots were evaluated visually. In addition, for all spot the percentage of positive cells and the staining intensity (score, 1–3) was recorded for each sample. Staining was then categorized as described in Table 1.

RESULTS AND DISCUSSION

In vitro selection of high affinity RNA aptamers specific binding to PCA3

Genomic libraries have been generated previously using a variety of methods, including restriction digestion and ligation (14) mechanical fragmentation and blunt-end ligation (15), but our approach was through PCR amplification using a single primer with a fixed 5′ end and random bases at the 3′ end, as reported elsewhere (16). The protocol (17) uses hyb-primers containing the restriction sites for switching the flanking sequence described elsewhere (18); however these are not included in the fix-primers, because may

lead to pronounced degeneracy of the recognition sequences and complications of the sequencing analysis. Therefore, we have used fixed-primers that include the entire fixed region of the hyb-primers, to ensure the fidelity of the final primers and proper alignment of the whole insert, as suggested elsewhere (7).

Human genomic DNA (from prostate cancer tissue) was denatured and annealed to an oligo with nine random nucleotides at the 3′ end and a fixed sequence at the 5′ end. Another randomized oligo with a different 5′ fixed sequence was added to the products of the first reaction, annealed and extended in the same way. We ran the extended reaction products on a gel to fractionate by size. Each fraction became the basis of a library with a different length of genomic insert. The library was completed by PCR amplification that added a T7 promoter to one of the primer annealing sites.

The SELEX method has usually been performed with 10^{14} unique oligonucleotides in the initial pool, the library used to find the winning ligand, but the number 10^{10} is an interesting number: it represents the approximate sequence complexity, the genome size, of a human genome (10).

Overall, 8 rounds of *in vitro* selection against *PCA3* were carried out. The selection step was continuously performed at 37°C. The PCR product resulting from selection round 8 was cloned and sequenced. One hundred and twenty three unique RNA sequences were identified (7 sequences were found two times) after 8 rounds of SELEX.

Specific RNA-binding is dependent on both sequence and structure of the folded molecule. Interactions can often take place as a result of direct hydrogen bonding with Watson–Crick groups of the aptamer with the ligand. Motif finding can provide insight into whether the SELEX pool has been

enriched as the result of such sequence recognition. The problem of motif finding has been given quite a bit of attention for decades; however, motif finding remains a complicated task (19). In this study, the computational approach consists of three steps that were used to pre-select sequences from the initial pool of RNA molecules and effectively led to the reduction in motifs' search, what is called of convergent evolution of RNA molecules.

In step 1, the sequences were classified according to the presence of five different motifs identified (CCAU, CCCA, UCCA, UGCC and UGUC) during the cloned rounds (1^{st}, 3^{th} and 8^{th}). It is important to emphasize that the some sequences can present more than one motif, thus they will appear in more than one family classification. Sequence alignment demonstrated that the four motifs (CCAU, CCCA, UCCA and UGCC) present one common "CC" dinucleotide reapeat (yellow) (Figure 4).

Figure 5A shows the heterogeneity behavior of the motifs among SELEX rounds, although within the round it was not happen. The CCCA, UGCC and UGUC motifs were the most frequent in the 3^{th} and 8^{th} round. The first step screening was able to selected 88 sequences out of 123.

In step 2, sequences' sizes smaller than 21 nucleotides were excluded from analyses. The identification of truncated aptamers restricted to the minimal target-binding domain requires some effort, but it has been successfully carried out to obtain functional aptamers less than 40-nucleotides long (20–27). In the majority of cases, the fixed sequence regions used for primer binding are unimportant for aptamer function and can be eliminated. Technological advances have already been made to eliminate the requirement for the fixed regions in random sequence libraries used for the SELEX process, thereby

producing short aptamer sequences (28). In our results, the average sequence length dropped from 66 bases to about 47 upon initial transcription and reverse transcription (library to first round), but only steadily declines for the remainder of the rounds, with the 8^{th} round at approximately 21 bases (Figure 5B). More than half of the sequences were eliminated in step 2 (40 out of 88).

In step 3, hybridization energy calculations between the tertiary structure target (*PCA3*) and an aptamer were predicted by *sfold* web server. At this point, only six sequences (out of 40) were specific to the *PCA3* with $\Delta G_{hybrid} \leq -18.0$. It is interesting to note that predicted hybridization analyses between aptamers and *PCA3* present 2 conserved binding-points in *PCA3,* located at the position 2 to 26 and 186 to 218 bases, which correspond to exon 1 and 3, respectively (Table 2).

There are important evidences in structure predictions, which exhibit two types of conservation: first, there are bases that covary and provide conserved secondary structure domains that form structural scaffolds; second, there are bases that are absolutely conserved. The latter nucleotides often are single-stranded or in noncanonical base pairs that directly interact with the target molecule (8).

Results presented in Table 3 show that the CG3 and BC4 aptamers have more paired-bases with *PCA3* I loop (Figure 6). It can be clearly seen that RNA bases involved in molecular recognition do not form Watson–Crick pairs with other bases. A similar conclusion can be demonstrated by Carothers et al. (28) that shows secondary structures for 11 classes of GTP aptamers. The bases with high informational content, which is important for the high-affinity binding, are always unpaired and located in loops or bulges. There are two possible

reasons for this: firstly, unpaired RNA bases are more flexible, so they can easily change their conformation to form a binding pocket and accommodate a ligand, and secondly; unpaired bases have available donor or acceptor atoms for potential formation of hydrogen bonds with the ligand (8).Therefore, we set a constraint that the secondary structure of CG3 (30 bases) and BC4 (24 bases) aptamers should have at least 26 and 14 unpaired bases, respectively.

Some authors indicated that the optimum stability is obtained with seven-base complementarily (8), thus this number of unpaired bases (26 and 14) seems optimal for us, since the higher number of unpaired bases will significantly reduce the presence of sequences with high free energy of the secondary structure while the lower number will increase the presence of sequences with low binding affinity. It is important to emphasize that the two most frequent motifs (UGCC and UGUC) were present within this unpaired bases, for both aptamers.

Our results demonstrated that the amount of *PCA3* recovered during reverse binding assay with CG3 was 2-fold higher than with BC4 (Table 4), which is an indicator of affinity and specificity of the CG3 aptamer binds *PCA3*. Thus, the CG3 aptamer turned out to be the best binding molecule and was therefore subjected to ISH assay.

Another interesting statistic of genomic aptamers is their homology with other genomic structures (6). To identify a putative homologue of the CG3 aptamer sequence, we searched by BLAST the *GenBank*/NCBI database. We found that the *SMAP1* gene (Small ArfGAP 1) showing a high degree of homology to CG3 aptamer over the entire length (data not shown). The expression of *SMAP1* in erythropoietic organs as well as the correlation with the

erythropoietic activity of the hematopoietic organs suggests that *SMAP1* is induced in stromal cells by the contact with erythroid cells, defining *SMAP1* as a key molecule that induced an erythropoietic microenvironment in hematopoietic organs (29). However, expression levels of SMAP1 associated with prostate cancer were not reported in the literature yet.

Aptamers as Histological Markers

Aptamers are versatile tools that rival antibodies in diagnostic applications. Unlike antibodies, synthetic aptamers can easily be produced with a high degree of accuracy, reproducibility and purity. Therefore, little or no batch to batch variation is expected in aptamer production. They are not sensitive to temperature and undergo reversible denaturation, thus having a much longer shelf-life. Further, aptamers can easily be conjugated to a variety of reporter molecules, chemotherapeutics, or photosensitizers at precise locations (12).

Representative images of prostate adenocarcinoma and BPH with high and low CG3 aptamer stained are shown in Figure 7. Biotin-labeled CG3 aptamer stained the cytoplasm and nucleus of prostate cancer and BPH tissue and is absent in stromal cells. Overall, 100% of the lymphoma and gastric cancer tissue specimens stained negatively in nuclei and cytoplasm. The pancreatic tumor cells were nuclei-negative, with some weakly cytoplasm staining (33%) (Table 3). This data demonstrated the specificity of the CG3 aptamers for the prostate tissues. Considering that the CG3 aptamer has specific binding to *PCA3*, our results are corroborated with findings on the cellular location of *PCA3* (30, 31) and with it's specificity in prostate cells.

Although the authors acknowledge that the exact location of the transcripts has not yet been clearly established, they mention that they found indications that most *PCA3* transcripts are located in the nucleus. The method used to reach this conclusion is, however, not mentioned.

The positivity staining for CG3 was generally somewhat stronger in nuclei of BPH cells (78%) than in cytoplasm, suggesting that the *PCA3* RNA generated into the nucleus was degraded before to be exported to the cytoplasm. On the other hand, the high *PCA3* expression in nuclei of BPH cells can be explain by *PCA3* gene detection at DNA level, binding in different DNA regions (as CpGs island) and regulating genes.

The low *PCA3* expression in cytoplasm of BPH cells can be due to the loss of these transcripts, and in this case, BPH patients with weak positive cells stained, can already develop microfocal cancer without morphologic alterations. However, due to the high *PCA3* transcripts production in the tumor, the signal staining was lost by competition, diluting across the nuclei and cytoplasm.

In prostatic adenocarcinoma specimens, we observed a homogeneous nuclei-cytoplasm staining, which probably happen due to constant *PCA3* RNA production and edition. Tumor staging for prostate adenocarcinoma was similarly moderate positivity staining. Marangoni et al. (32) found *PCA3* positive blood samples among BPH patients as well as prostatic intra-epithelial neoplasia and cancer patients but at largely similar frequencies and claimed there was no correlation to histopathological findings.

Briefly, *in silico* analysis of the *PCA3* transcript demonstrated that the molecule undergoes to significant folding with many hairpins and loops. This conformational structure may be functional, but considering that the *PCA3*

overexpression in PCa samples has become an important biomarker, we believe that the direct use of high affinity aptamers to the *PCA3* transcript may substitute the required reverse-transcriptase PCR amplification, and detection of the *PCA3* molecule can be achieved in physiological conditions without controlling hybridization parameters, and it may even be used in ISH analysis. Therefore, we have selected and characterized a significant RNA aptamer that was enriched by SELEX that specifically binds to *PCA3* mRNA, and its application was confirmed by tissue microarrays. New therapeutic and diagnostic uses of the aptamer are under investigation.

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Table 1. Scoring criteria for CG3 aptamer stained.

ISH result	Staining intensity	Fraction of stained tumor cells (%)
Negative		No staining
Weak positive	1+	≥ 10 < 50
	2+	≥ 10 < 30
Moderate positive	1+	≥ 50
	2+	≥ 30 < 60
	3+	≥ 10 < 50
Strong positive	2+	≥ 60
	3+	≥ 30

Scored positive if staining in ≥10% of the cells.

Table 2. Description of the six best aptamers sequences against *PCA3*.

Aptamer Identification	Sequence (□)	Aptamer size (ntd)	N° of aptamer ntd bindind to *PCA3* RNA (in bold)	$_{Hybrid}$ ΔG	Aptamer-*PCA3* loop binding (ntd)	Localization of Aptamer-*PCA3* binding (exon / ntd position)
CD5	N**AGGUCU**NNNUCCANN**CCCUU**	**21**	**14**	**- 20.0**	**9**	**1 (3 to 22)**
DF7	NN**AUGUGG**N**UCUCC**ANN**CCCUUGC**C	**25**	**18**	**- 24.0**	**7**	**1 (2 to 26)**
BE9	NN**UGGUUCCUUC**NNNNNNUGCCNNNNNNNN	**30**	**10**	**- 18.1**	**5**	**1 (3 to 13)**
BC4 *	N**GUUU**NN**GUGUGGAUG**CCNNN**GCU**	**24**	**16**	**- 20.7**	**10 ***	**3 (186 to 217)**
CG3 *	N**GUUUUU**NN**GUGUGUG**CCNNN**UUUG**UCNNN	**30**	**17**	**- 19.8**	**12 ***	**3 (194 to 217)**
DB11	**UGC**N**UUCC**ANN**UGUGUC**CANN**GGA**	**25**	**15**	**- 18.5**	**9**	**3 (200 to 218)**

(*) Aptamers chosen for ISH assay

(□) Motifs were underlined in the sequences.

Table 3. Summary of tissue stained with CG3 aptamer

Tissue type	Sample size	Nucleus stained (%)				Cytoplasm stained (%)			
		Negative	*Weak positive*	*Moderate positive*	*Strong positive*	*Negative*	*Weak positive*	*Moderate positive*	*Strong positive*
BPH	23	13	0	9	78	61	30	9	0
Prostatic adenocarcinoma T2	68	21	15	41	24	28	25	35	12
Prostatic adenocarcinoma T3 - T4	61	11	26	44	18	25	28	34	13
Lymphoma	3	100	0	0	0	100	0	0	0
Gastric cancer	3	100	0	0	0	100	0	0	0
Pancreatic cancer	3	100	0	0	0	67	33	0	0

Table 4. Reverse binding assay performance

Aptamers (400pmols)	***PCA3* recovered (RQ)** *Conditions*		
	I	**II**	**III**
CG3	2.512,27	235,12	*Insignificant**
BC4	1.111,35	108,02	*Insignificant**

(*) < 10

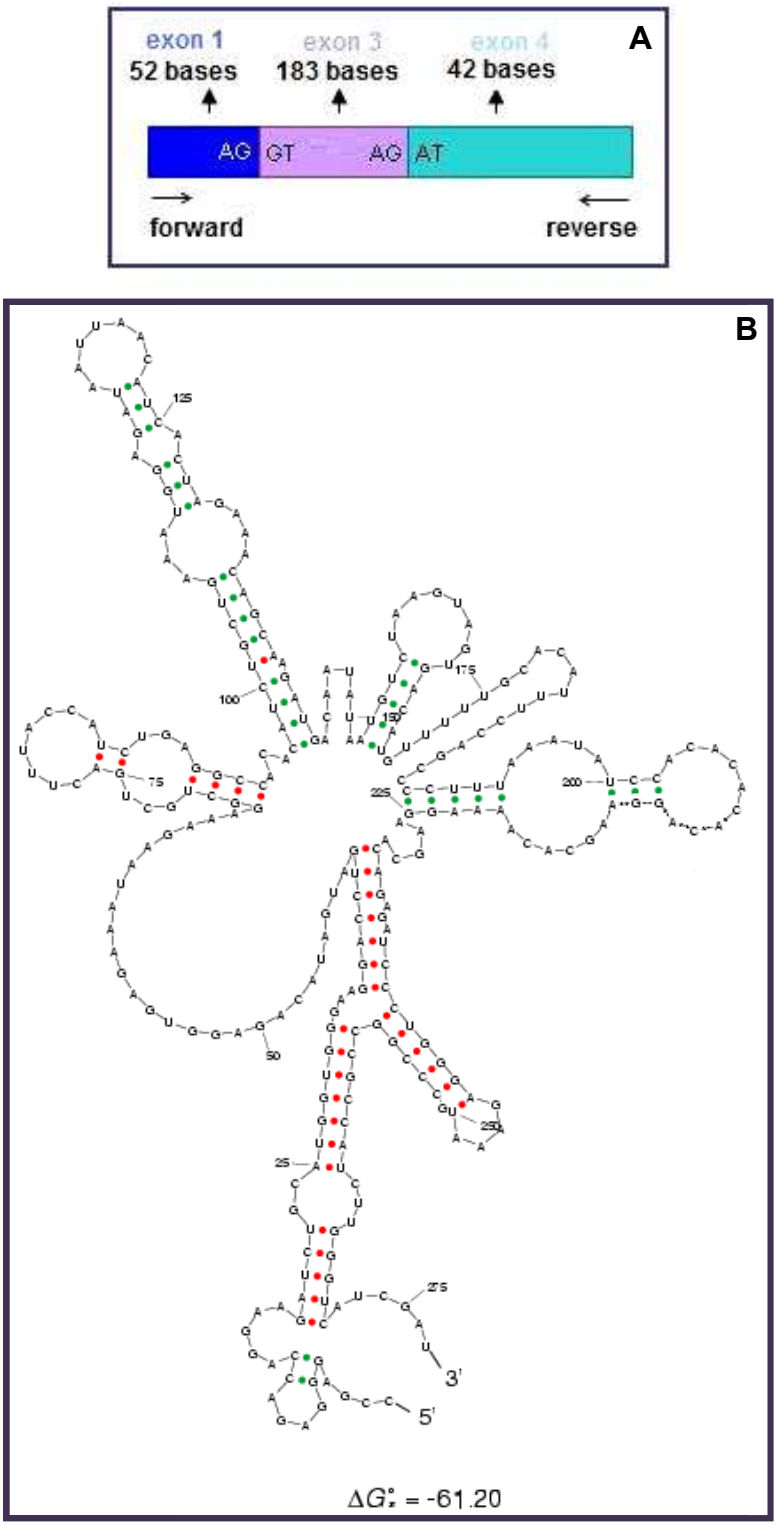
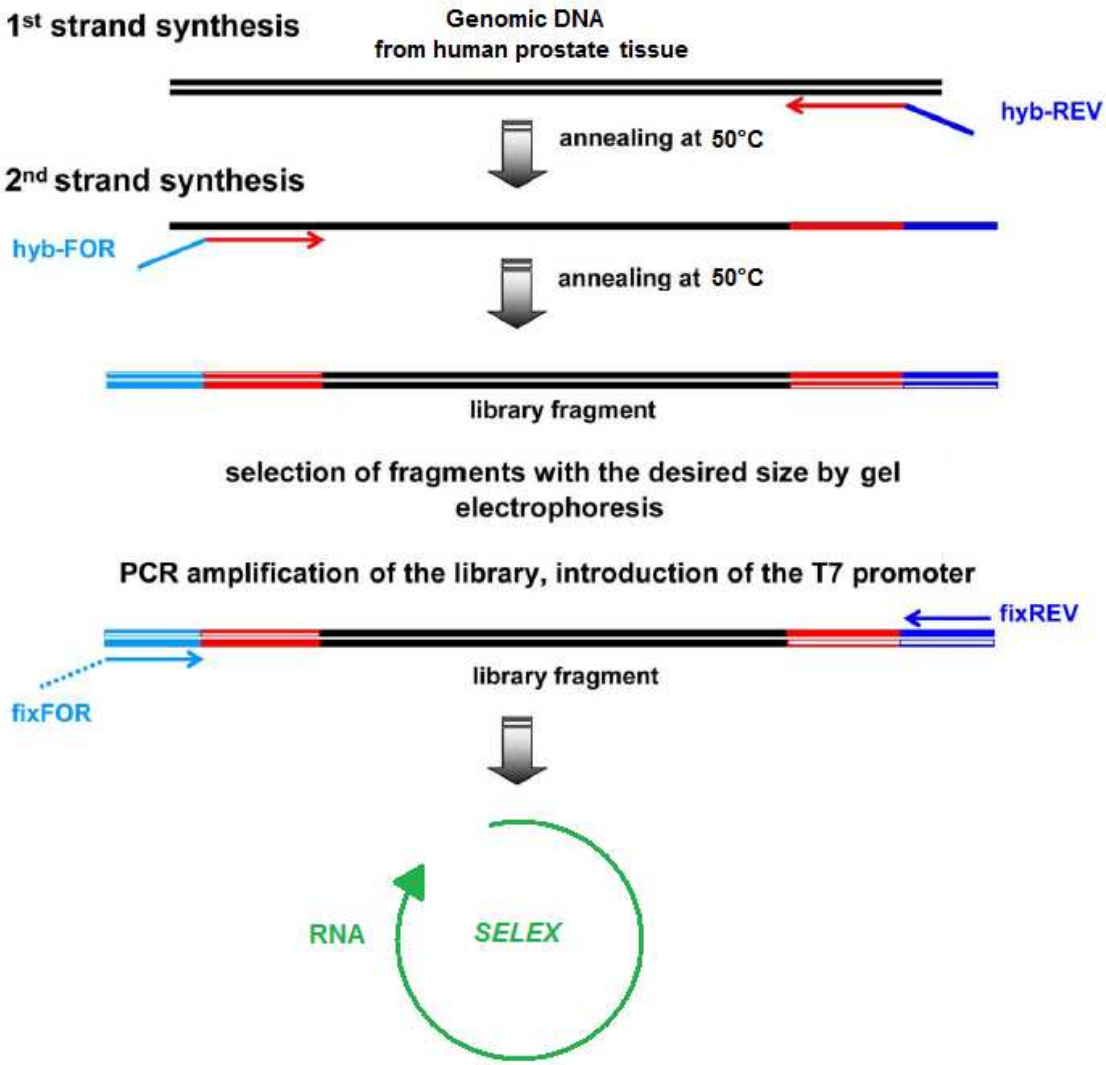


Figure 1. *PCA3* strucuture. **A.** Molecular structure of the exon 2 alternative splicing (277 bases) of non-coding *PCA3* gene. **B.** Conformacional structure prediction (by sfold program).



nine random nucleotides (red) followed by a specific sequence (light and dark blue), are used to synthesize the strands of the library. After synthesis of both strands, reaction products are size-selected by gel electrophoresis. The fraction that contains library fragments of the desired size range is eluted and amplified by PCR with the fix-primer pair. The T7 promoter (dashed) for transcription of the library into RNA is introduced through the fix-FOR primer. Zimmermann et al (7) with minor modifications.

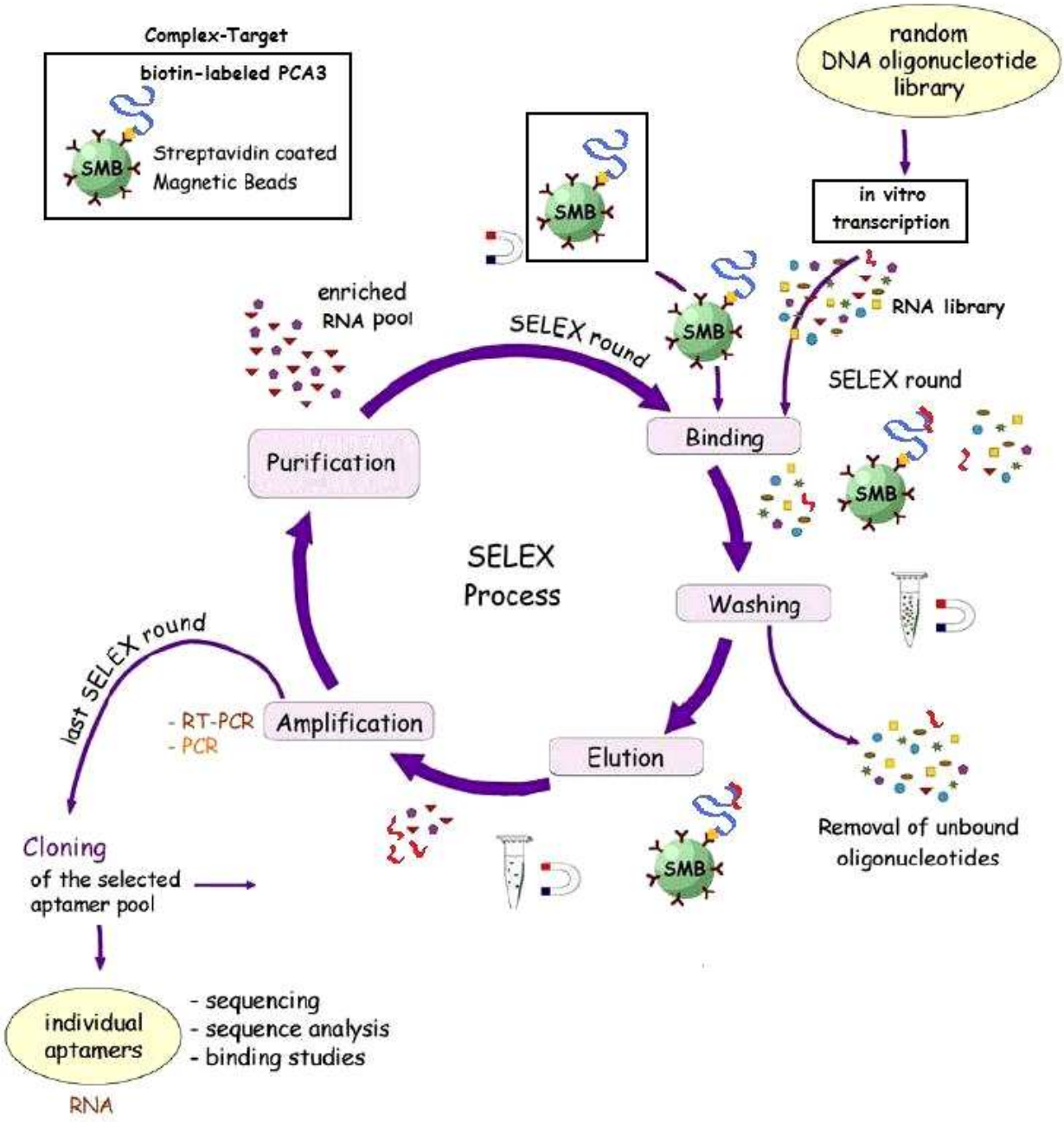


Figure 3. Schematic representation of the Genomic SELEX. Streptavidin-coated magnetic beads were used to generating RNA aptamers for specific target to the biotin-labeled *PCA3* RNA. The starting RNA combinatorial library consisting of oligonucleotides with a centrally randomized region of 60 nucleotides flanked by two specific regions that was incubated with the complex-target (streptavidin-coated magnetic beads + biotin-labeled *PCA3*) for

binding. The SELEX procedure is characterized by the repetition of successive steps consisting of selection (binding, washing and elution), amplification and purification. In the first SELEX round the library and the target molecules were incubated for binding. Unbound oligonucleotides were removed by several stringent washing steps of the binding complexes. The target-bound oligonucleotides are eluted and subsequently amplified by PCR or RT-PCR. A new enriched pool of selected oligonucleotides was generated by preparation of the relevant ssDNA for the *in vitro* transcription. This selected oligonucleotide pool is then used for the next selection round. The last SELEX round is finished after the amplification step. The enriched aptamer pool is cloned and several individual aptamers have to be characterized. Stoltenburg et al (12), with modifications.

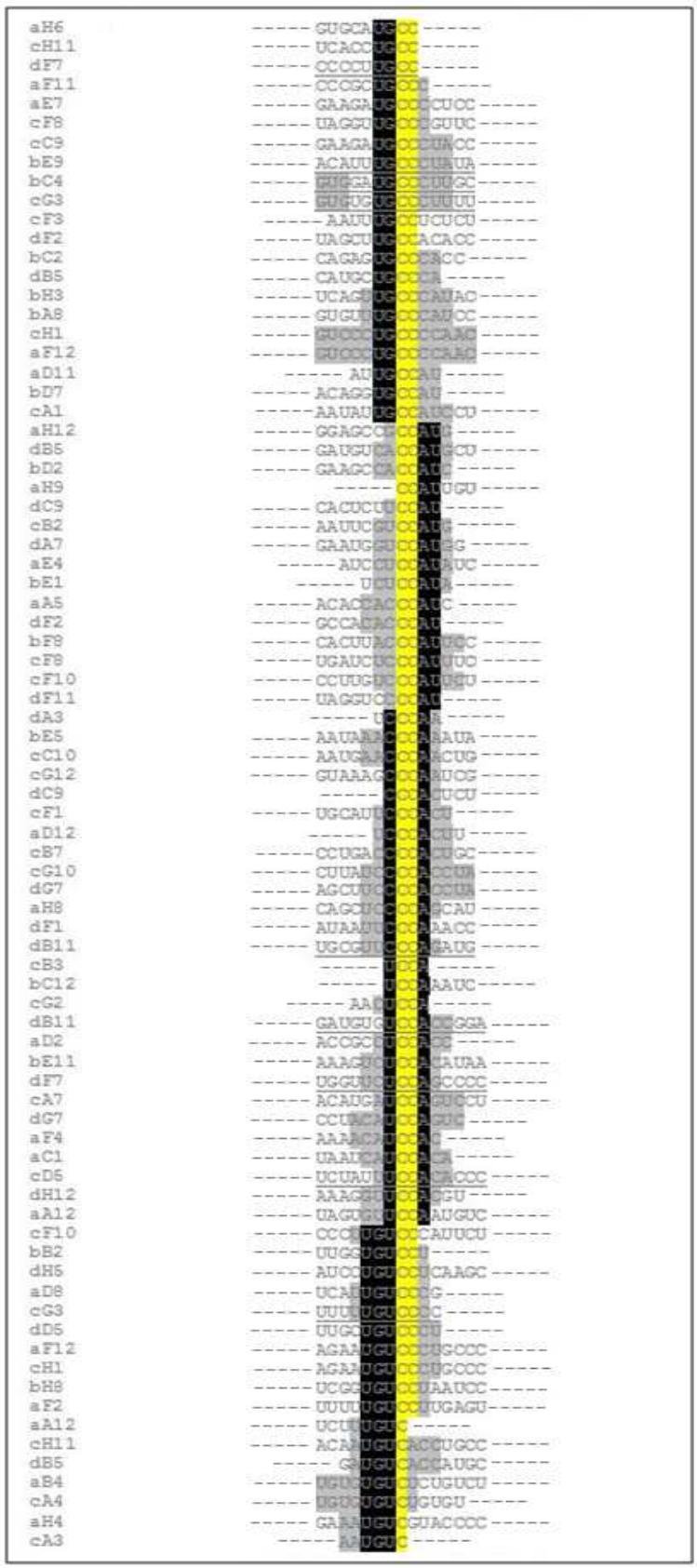


Figure 4. 8th round sequences alignment. Five motifs were identified (CCAU, CCCA, UCCA, UGCC and UGUC). The best six aptamers were underlined.

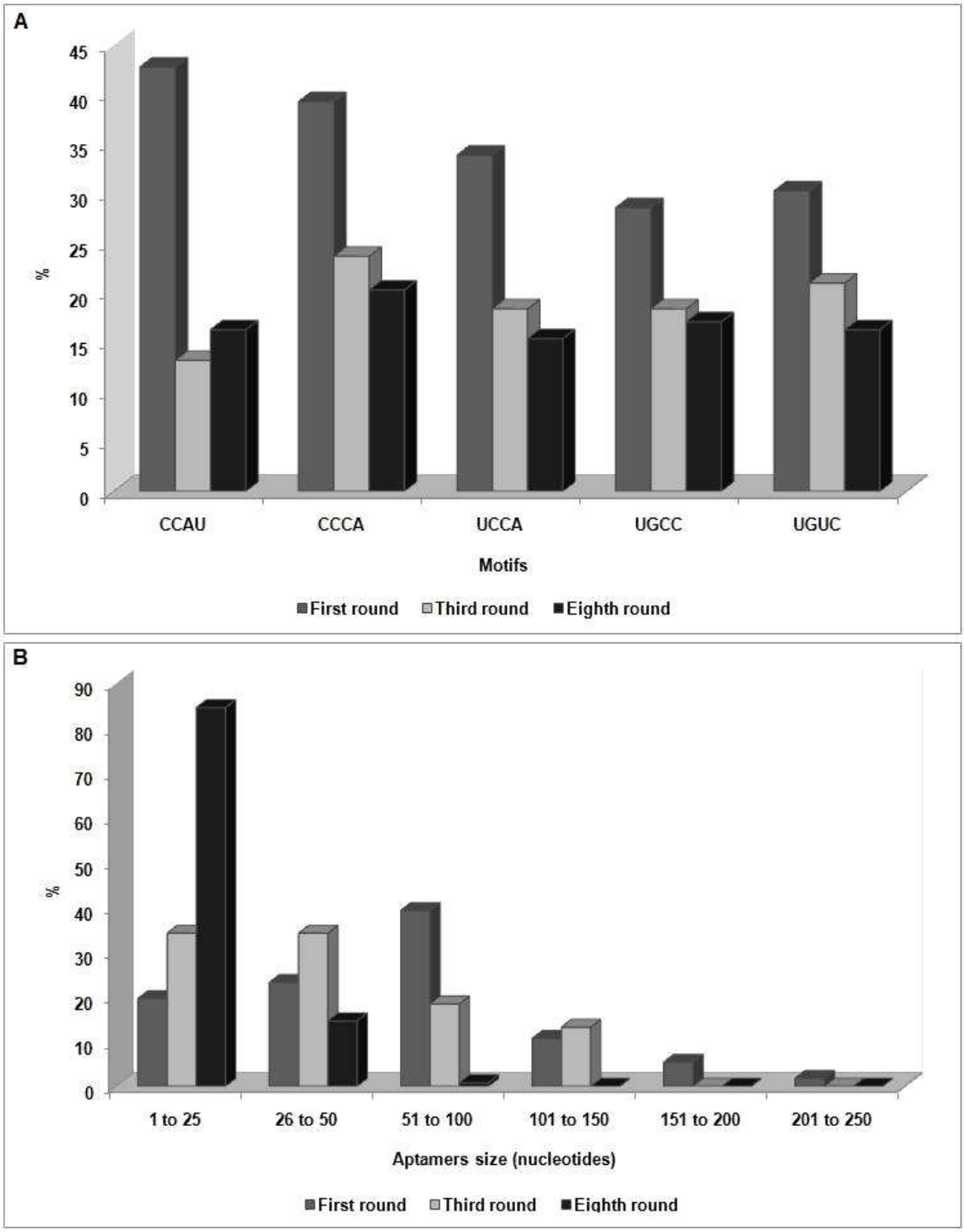
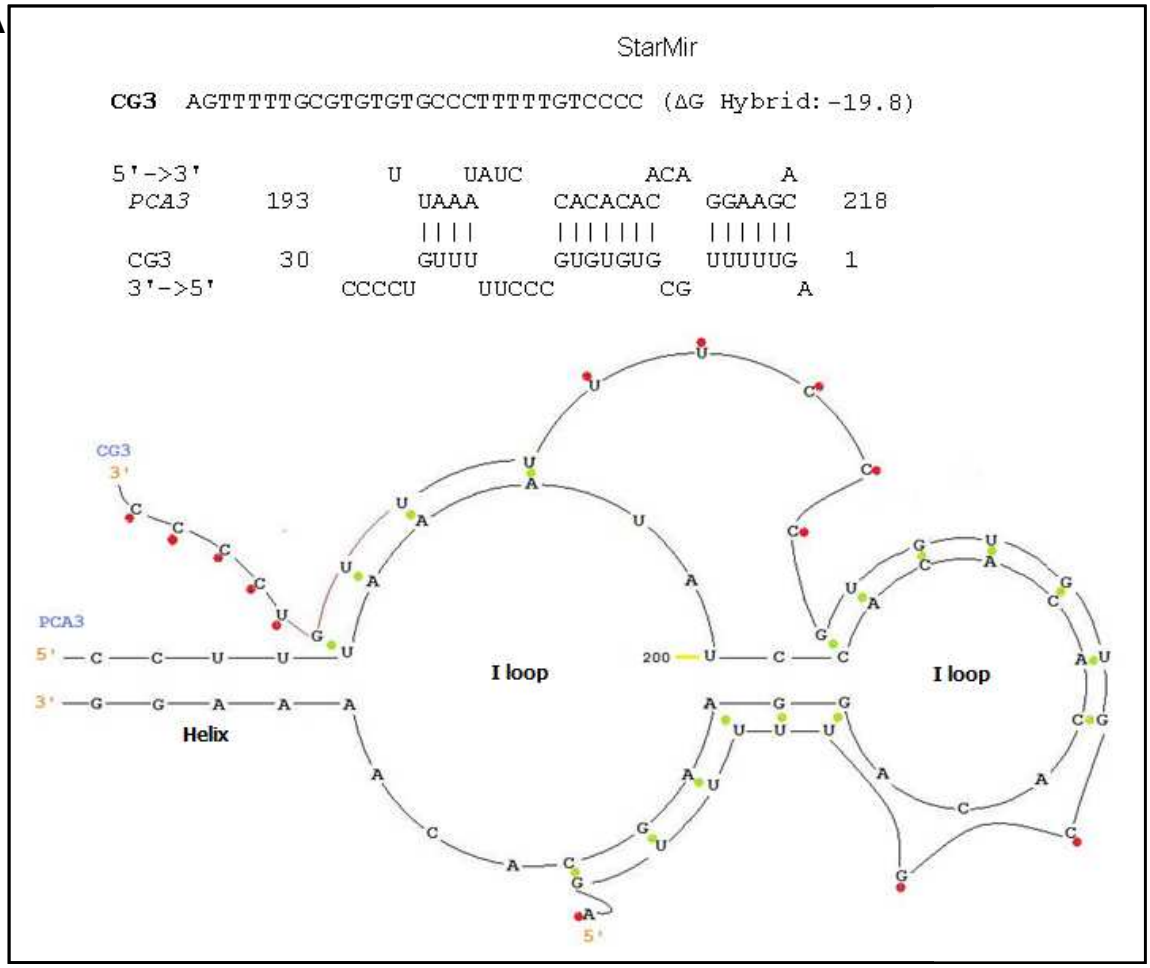


Figure 5. Frequencies of selected motifs **(A)** and sequence sizes **(B)** for 10^{15} random-sequence pools, across the sequenced rounds.

A


StarMir
CG3 AGTTTTTGCGTGTGTGCCCTTTTTGTCCCC (ΔG Hybrid: -19.8)
5'->3' U UAUC ACA A
PCA3 193 UAAA CACACAC GGAAGC 218
CG3 30 GUUU GUGUGUG UUUUUG 1
3'->5' CCCCU UUCCC CG A
CG3
3'
PCA3
5'
3'
Helix
I loop
I loop
200

B

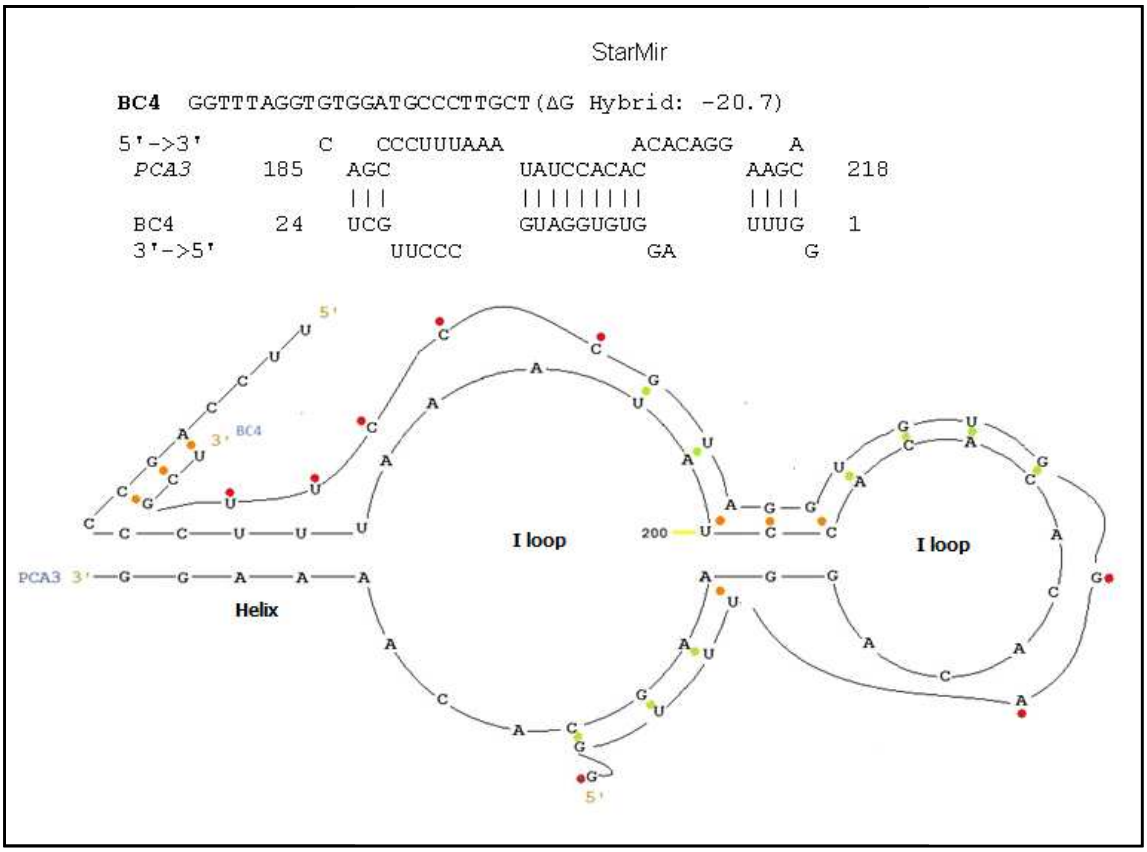
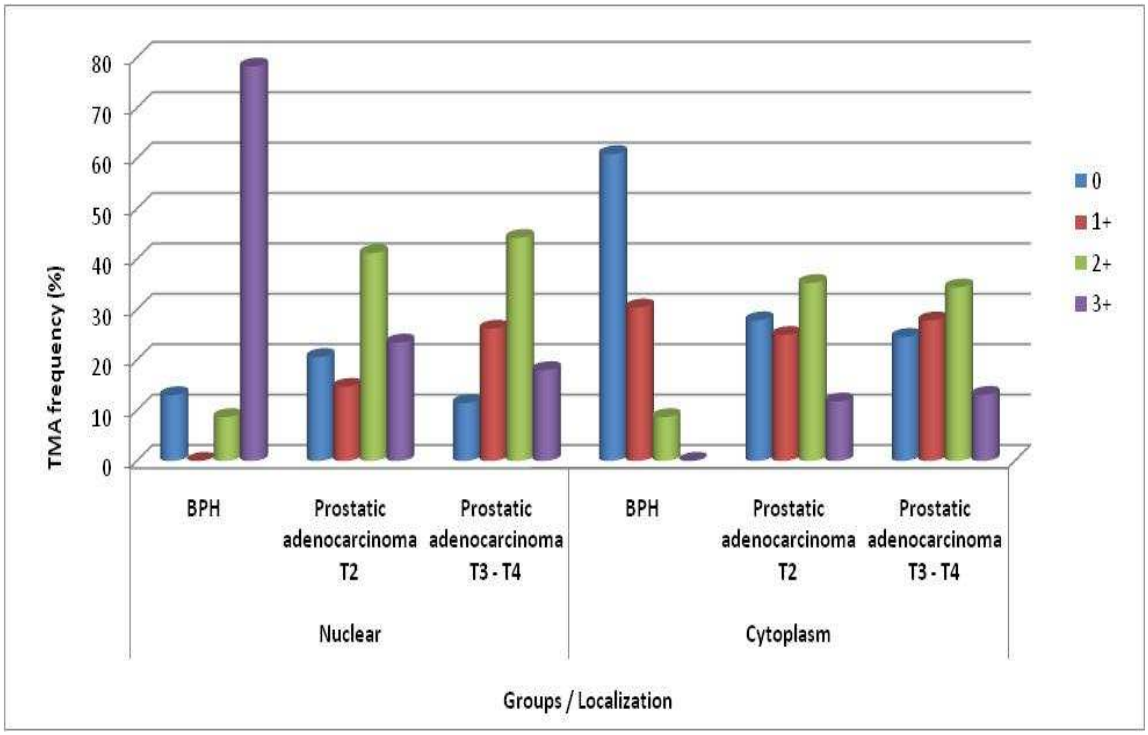
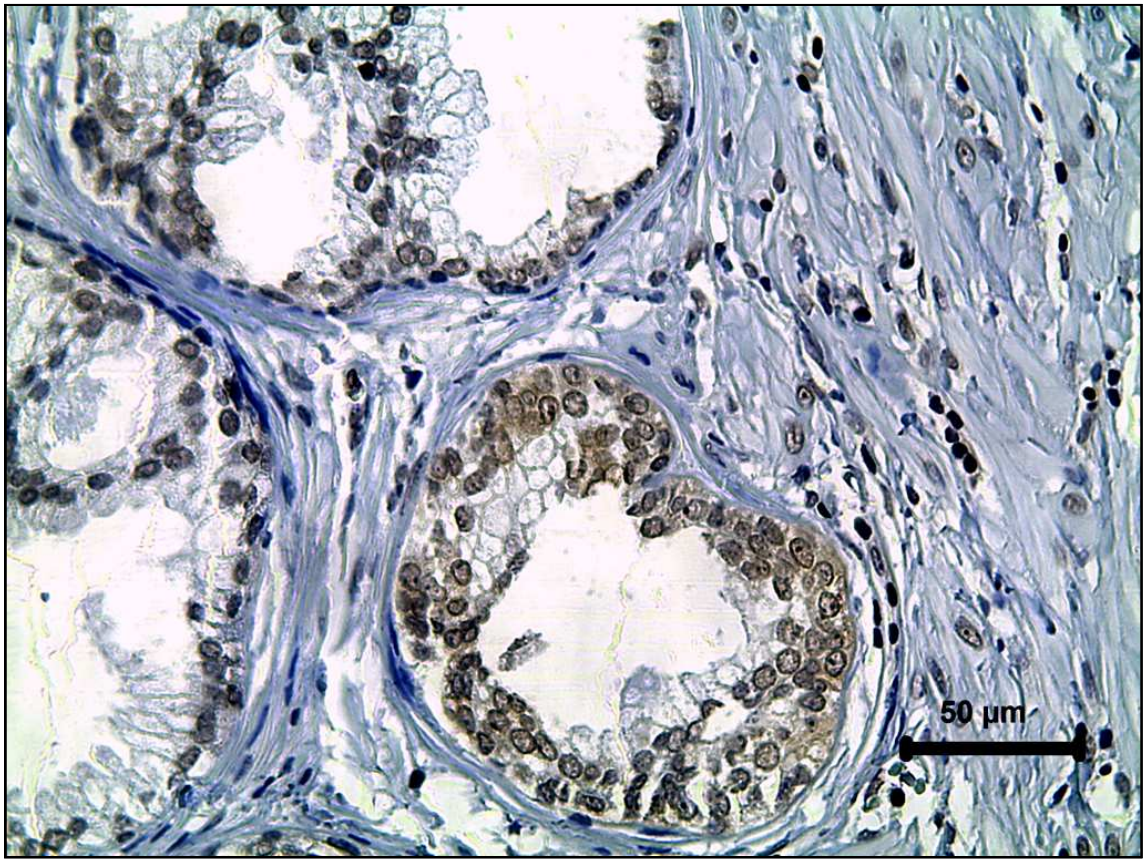


Figure 6. Structure prediction analyses. Folding predictions for best-binding CG3 **(A)** and BC4 **(B)** RNA aptamer. Red point: unpaired bases; green point: Watson-Crick based-pairing; orange point: StarMir prediction based-pairing.

A


80
70
60
50
40
30
20
10
0
TMA frequency (%)
0
1+
2+
3+
BPH
Prostatic adenocarcinoma T2
Prostatic adenocarcinoma T3 - T4
BPH
Prostatic adenocarcinoma T2
Prostatic adenocarcinoma T3 - T4
Nuclear
Cytoplasm
Groups / Localization

B1


50 µm

B2

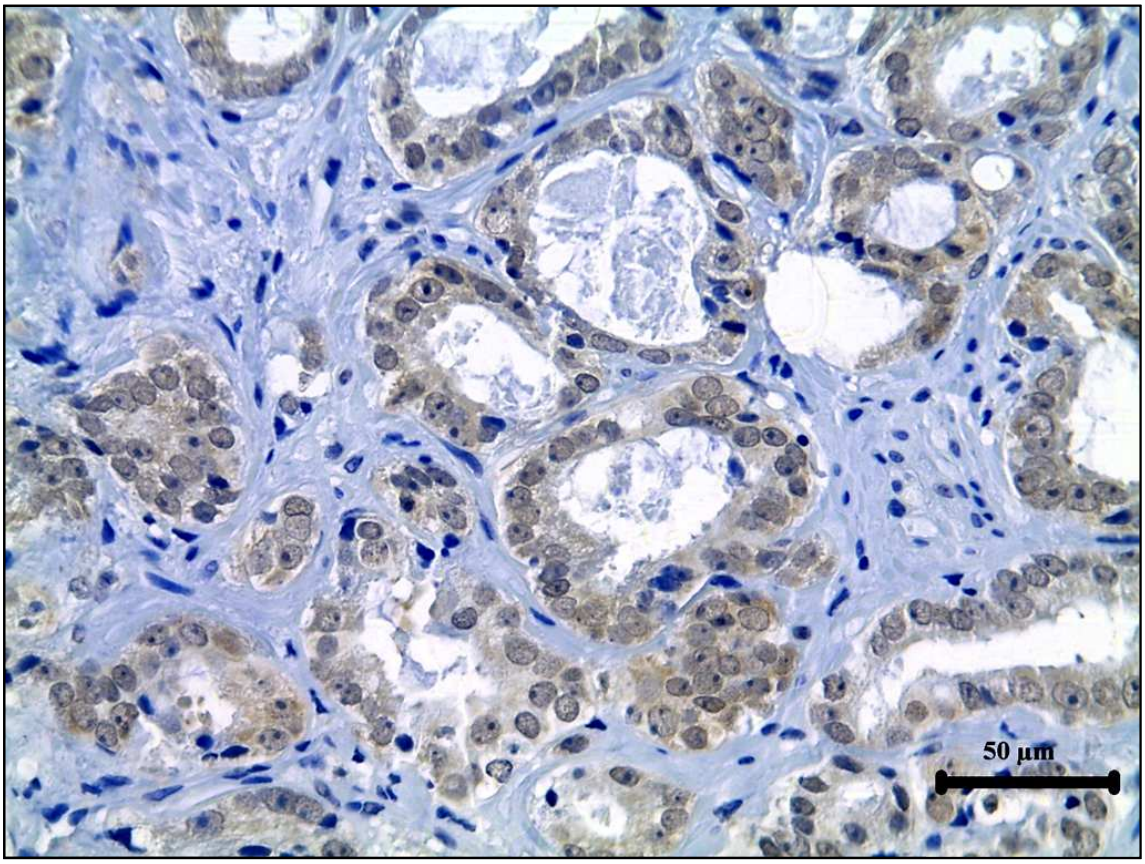


Figure 7. A. Immunoreactivity score (0 to 3) for the biotinilated CG3 aptamer probe in tissue microarray analysis (TMA) of benign prostatic hyperplasia (BPH) and prostate carcinoma (PCa) samples. **B.** Representative ISH detection of TMA samples (400x). **(1)** Represent BPH case with strong nuclear and weak cytoplasm staining; **(2)** represent PCa case with nuclear and cytoplasm staining, but with lower intensity than BPH case.

CAPÍTULO III

Prostate cancer antigen *3*-specific ligand aptamer modulates the expression of multiple genes with a potential therapeutic role

ABSTRACT

Currently, the non-coding prostate cancer antigen 3 *(PCA3)* is one of the most specific and overexperessed prostate cancer (PCa) genes, and considered a potential biomarker with unknown function. It forms hairpin-loop structures that may be required either for editing or processing. We hypothesized that disruption of its secundary strucuture by an RNA aptamer ligand probably modulates the expression of genes that may be associated with *PCA3* and consequently with PCa development. An RNA aptamer with high affinity to *PCA3* (CG3) was transfected into LNCaP and PC3 cell lines. A reverse binding assay and immunhistochemistry with tissue microarrays confirmed its specificity to the *PCA3* transcript and to PCa cells, respectively. Differential gene expression profiles of both transfected cells suggest a multigenic modulation by the *PCA3*. PCa and benign prostatic hyperplasia patients were further evaluated for the *PCA3* expression in the prostatic tissue, and besides confirming its diagnostic potential (AUC=0.80), it showed a similar pattern to the LNCaP cell. Finally, the opposite behavior of prostate cancer cell lines indicates that *PCA3*, *AR* and *TLR* signaling pathways genes are connected and their combination may be responsible for the tumor development and the transition from the hormonal to the refractory response profile.

INTRODUCTION

Most studies in cancer biology have extensively described accumulation of mutations and other alterations (deletion, over-expression or down-regulation) in protein-coding oncogenes and tumor suppressor genes. The recent identification of new classes of ncRNAs implicated in important steps of cancer occurence and progression reinforces the role of these transcripts in the process of tumorigenesis. Moreover, some of the newly identified ncRNAs can be used as new targets for drug development and for early diagnosis and prognosis suggesting that this new class of transcripts is important in cancer biology (1).

Non-coding Prostate Cancer Antigen 3 (*PCA3*) RNA was identified in 1999 and was found to be strongly over-expressed in up to 95% of tumor tissues (2). Furthermore, no *PCA3* transcripts have been detected in benign and cancerous extraprostatic tissues, demonstrating that it is the most specific prostate cancer (PCa) biomarker identified to date (3) and even be considered a target for further cancer therapies.

Over the past three decades, antibodies have been the reagent of choice for the development of diagnostic assays. Consequently, diagnostic platforms that are commonly used today were evolved to better suit antibodies. The discovery of aptamers whose affinity and specificity parallel those of antibodies is expected to have a future impact on diagnostics. The Systematic Evolution of Ligands by EXponential enrichment (SELEX) can be used against a wide variety of target molecules leading to highly selective aptamers of interest for both therapeutic and diagnostic purposes. Iterative cycles of selection and amplification allowed the identification of RNA aptamers against nucleic acids or proteins. As far as these targets mediate the control of the expression of a gene, the cognate aptamers constitute specific artificial modulators of biological processes, mimicking the behaviour of decoy or antisense sequences. In contrast to rational strategies there is no pre-requisite to the design of aptamers. Interestingly, gene regulation can also be achieved through the association between RNA aptamers and small molecules (4).

In fact, it was previously suggested that *PCA3* transcript tertiary conformation may also perturb regulatory events in transcriptional or post transcriptional levels , although nothing is known about the possible modulatory

role of this non-coding gene. *PCA3* transcripts is at the moment an excellent target for cancer therapeutic, however no antagonists have been developed yet (3).

To elucidate the mechanisms of *PCA3* may modulate the PC3 and LNCaP expression profile, we generated an RNA aptamer against this gene using the SELEX method; and transfected it in a both prostate cancer cell lines. Gene expression patterns also were measured in patients with PCa and benign prostatic hyperplasia (BPH) to obtain a comparison to the cell lines. In this study, we demonstrated a specific control of prostate cancer cell lines gene expression by *PCA3*-specific ligand aptamer, providing a potential diagnostic and therapeutic role. Additionally, qRT-PCR analyses provide a very sensitive and specific tool to detect prostate tumor cells in tissue biopsies, mainly looking for new more sensitive and discriminatory biomarkers.

MATERIAL AND METHODS

Cell lines and patient samples

Two prostate cancer cell lines (LNCaP and PC3) were obtained from Dr. Etel R. P. Gimba from National Institute of Cancer (Brazil). All cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich) and incubated under a 5% CO2 atmosphere at 37°C.

Tissue samples panel consisted of 13 benign prostatic hyperplasia (BPH) patients (submitted to transurethral resection of prostate –TURP) and 50 patients with PCa (submitted to radical prostatectomy). Median age of BPH patients was 64 (range, 41 – 77 years) and median age of PCa patients was 67 (range, 59 – 82 years). Tissue samples were collected at surgery and stored at -80ºC. In accordance with institutional guidelines, all patients gave informed consent before collection of the specimens. Total RNA was extracted from cell lines and tissue samples using Trizol (Life Technologies, Inc.) according to the manufacturer's instructions. RNA quantity and purity were determined on a NanoDrop spectrophotometer (GE Heathcare).

Genomic SELEX and RNA aptamer

The SELEX procedure was described previously (Marangoni *et al*., manuscript in preparation). Briefly, random priming on denatured genomic DNA

that had been sheared by sonication only enough to reduce viscosity. Genomic DNA (1µg), Primer hyb-REV (5pmols) (5), *Platinum* Taq DNA Polymerase (1U) and 200mM dNTPs (final concentration) were mixed and the reaction was incubated for 26 cycles at 93°C - 20s, 50°C - 40s, 72°C - 40s, preceded by initial denaturation at 93°C - 3 min and a final extension cycle at 72°C - 10 min. The 50°C step allows Platinum extension through hairpins in single-stranded DNA. To remove primers from the first step, the purification was performed by DNA precipitation with ammonium acetate (7.5M) and the pellet was ressuspended in 10µL of water.

Second strand synthesis with primer hyb-FOR (5) was the same as first strand synthesis. The reaction products were separated on a 2.5% agarose gel electrophoresis and fractions of various sizes (with genomic inserts ranging from about 40–700 nucleotides) were purified according protocol described above. Since the human genome has about 6×10^9 nucleotides, each position potentially served as the starting nucleotide of an insert of every given size in ≥100 molecules of the human library.

Next we amplified the library by PCR using primers of completely fixed sequences, one of which adds a T7 promoter (primer fix-FOR) generating a library expressing RNA. Primers hyb-REV was removed to reduce the fraction of the molecules that have primer hyb-REV at both ends, and the extension protocol should not give rise to primer hyb-FOR at both ends. The unwanted molecules were eliminated from the nucleic acid library by a single cycle of transcription, which required the T7 promoter from primer fix-FOR, reverse-transcription using primer fix-REV and PCR. This library has been generated using the primers described elsewhere (5).

SELEX was performed using non-coding *PCA3* gene (277 base) with bait and a random pool of 10^{14} RNA oligonucleotides aptamers. Selection after 8 round was followed by ligation of 0.5µg of the dsDNA pool into pCR2.1-TOPO vector systems (TOPO TA cloning - Invitrogen) for sequencing.

The RNA aptamer sequence, named CG3 according sequenced reaction in plate position, was selected to the presence of enriched motifs, binding affinities to *PCA3* RNA and predicted secondary structure (Marangoni *et al*., manuscript in preparation) performed by means of the free-energy minimization algorithm according to Zuker (6) using *sfold* software.

Transient transfections

LNCaP and PC3 cells, at about 70% confluency, were transfected with CG3 aptamer using Lipofectamine 2000 according to manufacturer's instruction (Invitrogen, Frederick, MD). Briefly, cells were seeded with antibiotic-free RPMI-1640 medium on each well of 24-well plates the day before transfection. 600 ng of CG3 aptamer and 1.5µl of Lipofectamine 2000, diluted with Opti-MEM medium (Invitrogen), were mixed gently and incubated with cells. Culture medium was changed after 6 hours transfection and incubated further at 37 °C for 24 h. After, cells were monitored for gene expression profile by real-time PCR. The one control cells received Lipofectamine 2000 alone, and the other with a scramble non-specific RNAs sequences.

Cell viability

Cells were seeded on 96-well plates, 6000 cells/ well, in RPMI-1640 supplemented with 10% FCS. A standard MTT (MethylThiazolyldiphenyl-Tetrazolium bromide, Sigma-Aldrich) assay was performed using triplicates up to 24 hours after aptamer transfection.

Gene expression screening using qRT-PCR after CG3 transfection in PC3 and LNCaP cells

After CG3 aptamer cell-internalizing, the expression profile of 23 genes (Table 1) was monitored by real-time qRT-PCR. The reverse transcriptase reaction was carried out with 1µg of total RNA using a *Murine Moloney Leukemia Virus* Reverse Transcriptase (MMLV-RT) (Amersham Biosciences), with random primers. Real-time PCR was carried out using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems); information on the PCR reaction condition is available on request. Relative quantification was performed using *B2M* transcripts as an endogenous control. Cells received Lipofectamine 2000 alone was used as calibrator, the ΔΔCt as method and SYBR Green I as dye for fluorescent signal. The threshold cycle (Cts) values were set as the cycle at which the measured fluorescence intersected the cycle threshold line. Negative controls containing no template DNA were included in each batch of PCR tests and all data was calculated using the average of three

replicates. In Table 2 and 3 were demonstrated the 24 primer pairs sequences (include the *B2M* primer pair) used for each gene in this analysis and these function in cell, respectively. The differential *PCA3* expression was tested in prostate tissues from patient with PCa and BPH.

Statistical Analyses

The non parametric Mann Whitney test was used to mean comparisons between gene expression in LNCaP and PC3 cells before and after transfections with CG3 aptamer or scramble RNAs sequence. For *PCA3* expression in patient groups a cut off value was stablished and the OR *(odds ratio)* were estimated to verify the chances of PCa occurrence in the presence of high levels of transcripts. To visualize the efficacy of the markers to discriminate tumor tissue from normal tissue we summarized the data in a ROC curve. This curve plots the sensitivity (true positives) on the Y axis against - 1 the specificity (false positives) on the X axis, considering each observed value as a possible cutoff value. The AUC was calculated as a single measure for the discriminative efficacy of a marker. When a marker has no discriminative value, the ROC curve will lie close to the diagonal and the AUC is close to 0.5. When a test has strong discriminative value, the ROC curve will move up to the upper left-hand corner and the AUC will be close to 1.0. The Statistical Package for Social Sciences (SPSS) was used for these analyses. Teoretical structure binding models were predicted by the RNA folding and hybridization software *sfold* (http://sfold.wadsworth.org/cgi-bin/index.pl). Hierarchical clustering of PC3 and LNCaP cell lines transformation gene expression data was done using *GenePattern* web server (http://www.broadinstitute.org/cancer/software/genepattern/).

RESULTS

Prostate cancer cell lines: expression profile after aptamer-internalizing

The expression of all 23 genes compared between cells lines prior to the CG3 aptamer transfection was presented in the Figure 1. Differential expression was detected for 11 genes that were up-regulated in LNCaP (*KLK2, KRT5, KRT14, LSP1, PCA3, TLR2* and *TLR9*) or in PC3 (*ANXA1, FOLH1, KRT8* and *TLR4*). Although not significant, the *AR* expression was almost two-fold higher in PC3 cells comparing with LNCaP. Furthermore, teoretical structure binding

models between CG3 aptamer and each gene transcript were predicted by the hybridization software *sfold* was in Table 4. More interaction stability available (using $_{total}\Delta G$) occurred between CG3 and the sequences from variant 2 of *ADARB1* gene (-18.28), *KRT6A* (-16.83), variant 3 of *LSP1* (-16.10), *PCA3* (-13.71), *SRD5A2* (-29.20), *TLR9* (-14.89) and *TLR4* (-13.73).

The CG3 aptamer effect on the expression of 23 genes was analyzed separately for each cell line (Figure 2). Interestingly, the transfected LNCaP cell line demonstrated significantly down-regulation of *PCA3, LSP1,* and *TLR9* that presented higher total ΔG. On the other hand, up-regulation occurred for *KRT6A* and *ADARB1* genes that also presented *in silico* stable interaction with CG3 aptamer. The transcripts from *EEF1A1* and *ANXA1* were down- and up-regulated, respectively (Figure 2A). *AR* down-regulation was also observed, although not significant.

The PC3-transfected and non-transfected cells presented significant differences in 14 genes (*ADARB1, FOLH1/PSMA, KLK2, KLK3/PSA, KRT14, KRT5, KRT6A, KRT8, LSP1, PCA3, PRDM4 SRD5A2, TLR4* and *TLR9*), and three genes were down-regulated (*KRT8, PRDM4* and *TLR4*).

A cluster analysis was also generated when both non-transfected and transfected cells were analyzed (Figure 3). Both LNCaP-transfected and not-transfected cells were kept in the same group with major differences, but among 23 genes only *ADARB1, ANXA1, EEF1A1, KRT6A, LSP1, PCA3* and *TLR9* present significantly differences. There is concordance in the differential expression in only five genes (*ADARB1, PCA3, LSP1, KRT6A* and *TLR9*) between transfected cell lines. The PC3 cells were classified into two different groups/clades.

The cluster analysis for the CG3-transfected cell lines (Figure 3) indicated that they present even a more distant profile when compared to the non-transfected lines. Eight genes had a similar expression patterns with down-regulation in both transfected cell lines; *KRT8, TLR4, TLR3, PRMD4, SMAP1, KLK2, TLR6,* and *TLR2*, although grouped into different clades. Twelve genes were up-regulated in the PC3 cell and down-regulated in the LNCaP cell (*EEF1A1, KRT18, AR, PSM, PSA, PCA3, KRT6A, FOLH1/PSMA, ADARB1, LSP1, SRD5A1, KRT14, TLR9*). On the other hand, two genes were down-regulated in PC3 and up-regulated in LNCaP (*ANXA1* and *KRT5*). There were

no genes that were commonly up-regulated in both cell lines and *RNASEL* (*HPC1*) was not affected.

Significant differences among patient's groups (PCa and BPH) for *PCA3* (p= 0.019) gene expression were observed (Figure 4). *Cut-off* value for the relative level of transcript was established based on the maximum average level observed for the BPH group. Considering this, it was observed a 3.0-fold higher chance (CI 95% = 0.74 – 8.82) of having cancer when the *PCA3* expression levels were equal or higher than 3.1. For PCa patients, the Pearson's correlation coefficients obtained among the genes expression and the pre-surgical serum PSA, the patients' age on the diagnosis data were not significant. Only for Gleason score (p = 0.03) and TNM staging (p = 0.02) the Pearson correlation was significant. Similarly for BPH patients, the genes expression, serum PSA, patients' age on diagnosis were not correlated among each other. The AUC-ROC represents the diagnostic efficacy of the continuous test result, which was 0.8 (95% confidence interval, 0.96–1.00) for *PCA3*, indicating good discrimination power for this tests (Figure 5).

DISCUSSION

Aptamers can be used for therapeutic purposes as monoclonal antibodies, and can be developed for intracellular, extracellular or cell-surface targets (7). These molecules have been converted into switches for turning on and off the expression of a downstream gene, and these switches are mediated by an RNA structure which constitutes a highly selective binding site for a molecule. Upon association between the target molecule and the mRNA, the structure element undergoes a conformational change that leads to the alteration in the expression of a gene. As the regulated gene is directly related to the production of a protein, these sensors offer a direct link between the biochemical surrounding and the genetic information (4). Binding of aptamers with strong affinity and a high specificity for RNA hairpins has been previously shown (8), which were able to block translation in both cell free assays and cultured cells. Our hypothesis is that the disturbance of the *PCA3* transcript conformation may also perturb genes that are modulated by the non-coding gene, although nothing is known about the possible modulatory role of the *PCA3* expression in prostate cells.

Evidences suggest that the *AR* pre-mRNA is a target of RNA editing would be the presence of repetitive elements that could promote the formation of complex double-stranded foldback structures through the complementary base pairing of inversely spaced repeat elements (9), and this also happens to the *PCA3* mRNA (data not shown). Our bioinformatics also generated homology targets to the structure, which matched to specific hairpin sites where most of the predicted structure was not paired. The selected aptamer also demonstrated great binding specificity to prostate cancer cells, as detected by reverse binding assay (using magnetics beads) and immunohistochemistry analyses (Marangoni et al., manuscript in preparation). It was important to emphasize that the dot-blots solid phase assay was done, but was not verified if really exist binding between CG3 aptamer and another genes. For this, *in vitro* binding conformational assay would be done for each gene, individually, in liquid phase, however it was impossible to verify using linear linkage of the sequence as in dot-blot membrane (data not shown).

Prior to the transfection analysis, gene expression patterns were analyzed in both cell lineages, which determined important opposing profiles. Interestingly, PC3 cells presented high AR expression, although not significant. The expression of *AR* in PC3 cells and *KRT5* in LNCaP cells were not expected, and these are probably due mutations as have been shown elsewhere (10-12).

The interference analysis of the CG3 aptamer on gene expression demonstrated that significant differences were observed for 14 and 7 genes in the PC3 and LNCaP cell lines, respectively.

The LNCaP are luminal-like tumor cells, which were isolated from a lymph node prostate metastatic tumor (10), express a mutated *AR* (T877A) (13), and are *KRT5*-negative and *KRT8/18*-positive (14). These cells may respond to androgen signals differentially depending on different environments (11). Down-regulation of androgen receptor with anti-sense oligonucleotides (15) or siRNA (16) resulted in the suppression of cell proliferation or promotion of apoptosis in both androgen-dependent and independent sub-lines of LNCaP cells. In our study with the LNCaP cell line, the CG3 aptamer induced down-regulation of *PCA3, LSP1, EEF1A1* and *TLR9*, and up-regulation of *KRT6A, ANXA1* and *ADARB1* genes. Our data corroborate results demonstrating that

the *PCA3* expression is regulated by androgen in LNCaP cells (17), which may indicate that the aptamer may act as an inhibitor of tumor growth in androgen-dependent tumors.

Supporting data was also found with the *ANXA1* up-regulation transcripts, which codify the annexin A1, a calcium- and phospholipid-binding protein considered to play an important role in tumorigenesis. In prostate carcinoma, the annexin A1may be an essential component for maintenance of the normal epithelial phenotype, because tumors showed either complete loss or a great reduction in the level of this protein compared with patient-matched normal epithelium (18). Therefore, our data lead to the conception that the *ANXA1* up-regulation in prostate tumor tissues is a necessary event to recover and/or maintain the normal epithelial phenotype, especially in hormonal dependent tumors. This is also corroborated by other findings, which have shown *ANXA1* suppression in various cancers, and part of this down-regulation is possibly mediated by increasing levels of microRNA-196a (19).

The PC3 cell line was originally isolated from a human bone marrow prostate metastastic tumor (20). PC3 cells express *KRT5* and *KRT8/18* transcripts, but little or no endogenous androgen receptor. Thus, PC3 cells are basal intermediate-like tumor cells (14) that are highly tumorigenic. Early studies have reported that ectopic expression of *AR* driven by a strong viral promoter in PC3 cells resulted in androgen-dependent suppression of cell proliferation (21, 22). This androgen-suppressed cell growth in PC3 cells transfected with *AR* was confirmed later (23, 24). Androgen-deprivation therapy (ADT) for the suppression of androgens binding to the *AR* has been the standard prostate cancer treatment. Despite its success to suppress tumor growth, ADT eventually fails leading to recurrent tumor, even though androgen receptor remains functional in hormone-refractory prostate cancer. Interestingly, androgen receptor acts as a stimulator for prostate cancer proliferation and metastasis in stromal cells, as a survival factor of prostatic cancer epithelial luminal cells, and as a suppressor for prostate cancer basal intermediate cell growth and metastasis (25). Surprisingly and in accordance with the putative androgen receptor role in PC3 cells, our results suggest that the CG3 aptamer may lead to the induction of *AR* expression, and consequently to the inhibition

of cell proliferation or to cell growth arrest, which was confirmed by the MTT assay, with a significant inhibition (73.4%) of cell proliferation (data not shown).

Some genes were expected to have a close relationship with *AR* expression levels, such as *KLK2* and *KLK3/PSA*, because both of them have an androgen responsive element (ARE) in the promoter gene sequence. So, in LNCaP, *AR* and *KLK2* transcripts presented a similar behavior with down-regulation by CG3 aptamer, and in PC3 cells, *AR*, *KLK2* and *KLK3* were up-regulated. The opposite pattern of gene expression in both cells were expected, and this has been demonstrated elsewhere (26), in which there is a dissociation between androgen responsive regulation of malignant growth *vs*. regulation of expression of prostate specific markers. Differently, the up-regulation of *ADARB1* (adenosine deaminase RNA-specific enzyme B1) transcripts in both transfected cell lines is probably related to this RNA editing function (27). Our results are corroborated by findings in other cells lines (28), in which siRNA knockdown of *ADARB1* has significantly altered the cell viability of U2OS, an osteosarcoma cell line; however, it did not alter the proliferation of other cell lines as MSC (- transformed human fibroblasts), MCF7 (breast cancer), SK-LMS1 (leiomyosarcoma), and MG63 (osteosarcoma). Further studies are needed to clarify the mechanisms for androgen ligand-independent and *AR*-dependent regulation of genes.

Considering that the two transfected and non-transfected cell lines presented remarkable and opposite gene expression patterns, that activation or inhibition of genes under transfection differentiated them even further, and finally that due to the important intrinsic characteristics of each line previously discussed, we will now focus on individual genes that present major effects on cell biology during tumor development.

The *PRDM4* transcripts that codify PR domain protein 4 were down-expressed in both cells. This gene is located at a tumor suppressor locus on 12q23-q24 (29), and its overexpression inhibits DNA synthesis (30). Therefore, its activation by the CG3 aptamer suggests that proliferation of tumor cells may be inhibited.

Down-regulation of Toll-like receptors (TLRs) may indicate inhibition of the pro-inflammatory response and its associated cytokines, and consequently interferon-regulated pathways may be affected, which can be explained in part

by the common down-regulation of most *TLRs* except *TLR9*. Interestingly the *RNAseI* (*HPC1*, hereditary prostate cancer 1) is a gene encoding the protein RNase L that regulates cell proliferation and apoptosis through the interferon-regulated 2-5A pathway. Upon interferon-beta activation by dsRNA, the 2'-5' oligoadenylate synthetase 1 (AOS1A) is induced by binding to dsRNA and converting ATP into 2'-5' oligomers of adenosine (2-5A), which activates the RNase L that degrades viral RNA and all classes of mRNAs (31), thereby shutting off translation. The androgen receptor and the interferon-activated RNase L interact with each other in a ligand-dependent manner (32), which suggests that there is a relationship between innate immunity and tumor suppression (33). There are important evidences that human interferons are able to increase androgen receptor levels and improve adhesion potential of PC-3 androgen-insensitive cells (34). Therefore, although *RNAseI* was barely down-regulated in both cell lines, it is possible that its inhibition may be an interesting approach to control tumor growth of hormone-refractory prostate cancer; however, it is an independent event of the aptamer-induced *AR* expression, probably because of direct modulation of *AR* or by interferon-independent stimulation.

The *TLR3* expression is associated with high probability of metastasis, which is in agreement with previous studies indicating that *TLR3* expression is related to tumor aggressiveness (35-37). Our data in PC3 cells support the notion that inhibition of *TLR3* in metastatic cancers is a desirable molecular event.

The *TLR4* also seem to be involved in tumor development as well as in tumor cell escape from immune surveillance, but the endogenous ligand for *TLR4* in tumors is not known. We have shown that *TLR4* is highly expressed in both cell types, and under transfection with the CG3 aptamer both were down-regulated. Although this has not been shown in patients samples, there is a strong supporting evidence in mouse model, in which blockade of the *TLR4* pathway by either *TLR4* short interfering RNA or a cell-permeable *TLR4* inhibitory peptide reversed tumor-mediated suppression of T cell proliferation and natural killer cell activity *in vitro* and *in vivo*, and delayed tumor growth, extending the survival of tumor-bearing mice (38).

However, a different expression profile has been observed for the *TLR9*, which is highly expressed in the transfected PC3, but not in the transfected LNCaP. It is known that synthetic agonists of *TLR9*, a class of agents that induce specific immune response, exhibit antitumor activity and are currently being investigated in cancer patients (39); however, the mechanisms by which they affect signaling proteins involved in tumor growth and angiogenesis, thus leading to tumor growth inhibition, have yet to be elucidated. The *TLR9* immunomodulatory role acts by impairing epidermal growth factor receptor (EGFR) signaling and potently synergizes with anti-EGFR antibody cetuximab in GEO human colon cancer xenografts, whereas it is ineffective in VEGF-overexpressing cetuximab-resistant GEO cetuximab-resistant (GEO-CR) tumors (39).

It is interesting to note that aggressive and metastatic tumor-derived cell lines usually presents overexpression of VEGF, and the VEGF-C/VEGFR3 pathway is primarily required for lymphangiogenesis and lymphatic metastasis, an increased level of VEGF-C can also stimulate angiogenesis (40). It is possible that AR may be inhibited in LNCaP through controlling *TLR9* expression, consequently down-regulating *PCA3* (tumor-specific gene) and *LSP1* (inflammation) genes. Therefore, *TLR9* signaling may be responsive only in a hormone-dependent tumor growth, mimicking the early response in prostate tumor development. Other supporting evidences corroborate our hypothesis about the link between *TLR9* and *AR* signaling pathways (40).

It has been shown that primary and immortalized prostate epithelial cells (RWPE) exhibited increased proliferation in response to exposure to lipopolysaccharide (LPS) that triggers *TLR4*, and to CpG DNA that triggers *TLR9* (41). Furthermore, it has been shown that the androgen-independent prostate cancer cell lines, DU145 and PC3, presented *TLR9*-induced NF-kB activity, but not the androgen-sensitive LNCaP cell (42). Interestingly, blockade of NF-kB in LNCaP cells inhibited *AR* activation (43), an event also observed in our CG3-transfected LNCaP cell line. On the other hand, the reactivation of the *AR* pathway in PC3 cells was followed by increased *PCA3* and *TLR9* expression, probably leading to the suppressor of proliferation of cells as observed elsewhere after *AR* stimulation (25).

Interestingly, the dihydrotestosterone (DHT) pathway, represented by *AR* and *SRD5A1* genes, is highly activated in PC3-transfected cells, in which are usually down-regulated, as observed in the profile of non-treated PC3 cells. Additionally, the *PCA3* gene that was expected to be blocked or inhibited was surprisingly expressed in very high levels, and this expression may be related to the activation of the *AR* signaling pathway as shown elsewhere (36), or it is also possible that the *PCA3* and *AR* are co-modulated by each other. Furthermore, it is also important to emphasize that Lenti-AR transduced PC3 and DU145 lines (*AR* is lost in androgen depletion independent prostate cancers) expressed transcriptionally functional AR protein at appropriate physiological levels, and this expression and engagement of AR protein in PC3 cells resulted in transactivation of p21 and subsequent growth inhibition of these cells in culture and in mouse xenografts (24), which may explain why the *AR* is reactivated under the CG3 internalization in PC3 cells, resulting in high growth inhibition detected through the MTT assay.

The opposite behavior expect for the *PCA3* expression levels in prostate cancer cell lines was observed in patient groups. *PCA3* is over expressed by more than 95% of all prostate cancers tested (45). It can determine benign from cancerous prostate cells with an accuracy approaching 100% (46). Our data corroborate results demonstrated a 3.0-fold higher chance of having cancer when the *PCA3* expression levels were higher than 3.1. Factors regulating *PCA3* gene expression are not yet clearly defined, but the great confounds of serum PSA levels (prostate volume, age, trauma and other prostatic diseases) appear to affect *PCA3* to a much lesser degree than PSA (47). The reported and discussed data suggest that *PCA3* may benefit from combination with other predictive factors. The limited literature on this issue is developing (48) used a base model of clinical predictors, and *PCA3* scores were used to construct a nomogram recommended for use in conjunction with *PCA3* testing, leading to significant improvements of the AUC up to a maximum of 0.730.For diagnosis of tumor, *PCA3* expression was compared between patient groups (PCa and BPH). Sensitivity and specificity estimates for this gene were calculated as the AUC-ROC. *PCA3* showed diagnostic characteristics with AUC- ROC values of 0.80. Futhermore, the *PCA3* transcriptional activity were also highly correlated with disease staging (Figure 6), once its highest level is reached at the pT3

stage, when the stimulus is required for tumor cell dissemination and metastasis.

Finally, we have provided evidences that the CG3 aptamer has specific binding to *PCA3* through many assays, although it presents many predicted microRNA-like homologies to most of the genes. Additionally, immunohistochemical analysis confirmed its specificity (Marangoni et al., manuscript in preparation) and suggests that the differential gene expression profiles after transfection are possibly modulated by *PCA3*. The opposite behavior of prostate cancer cell lines indicates that *PCA3*, *AR* and *TLR9* signaling pathways genes are connected and their combination may be responsible for the tumor development and the transition from the hormonal to the refractory response profile.

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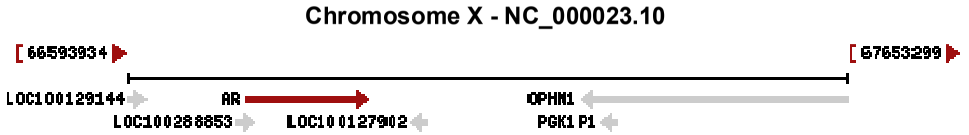
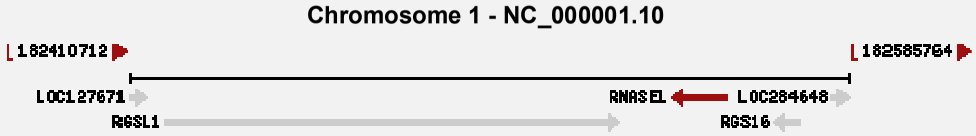
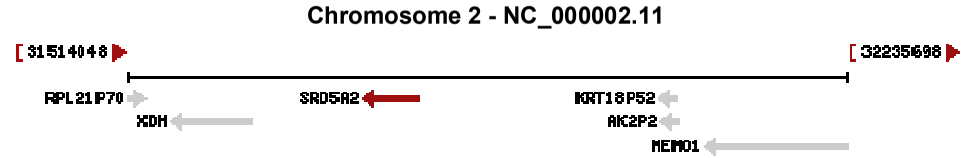
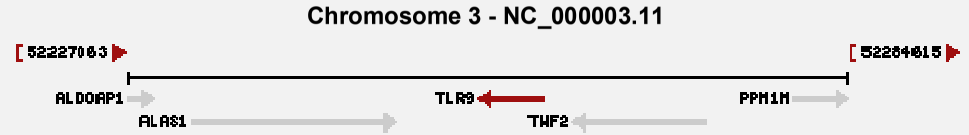
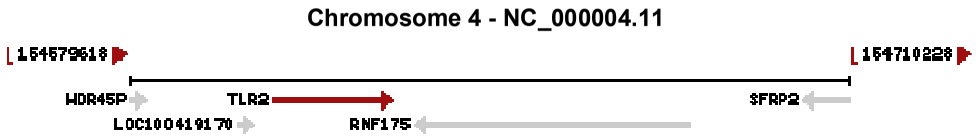
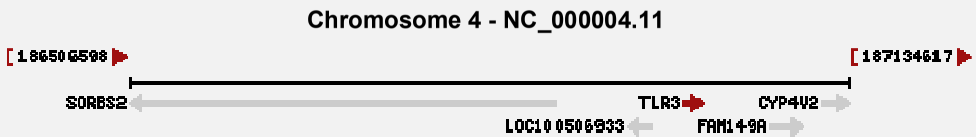
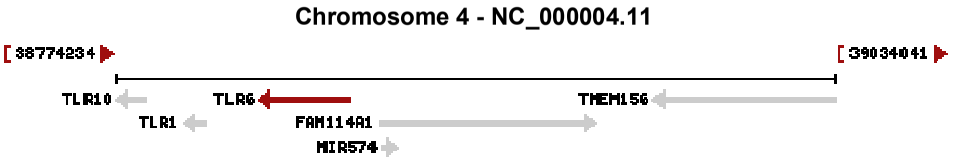
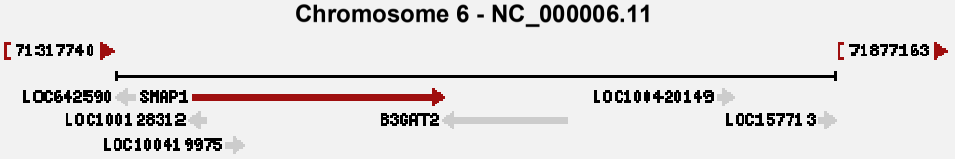
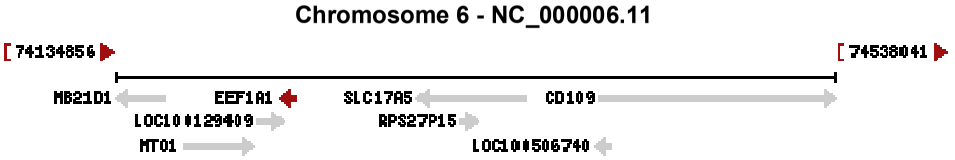
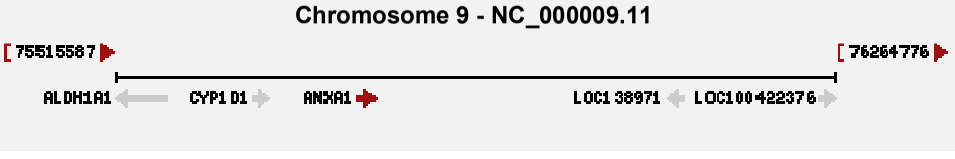
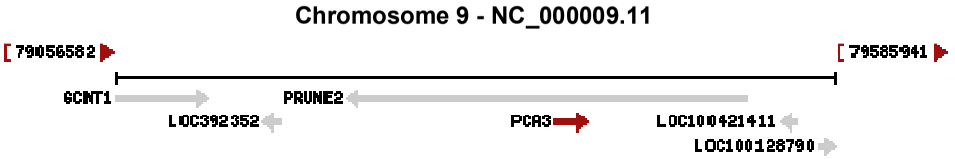
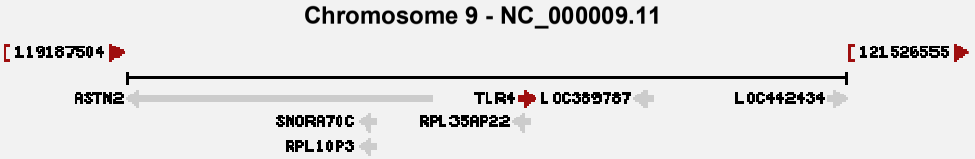
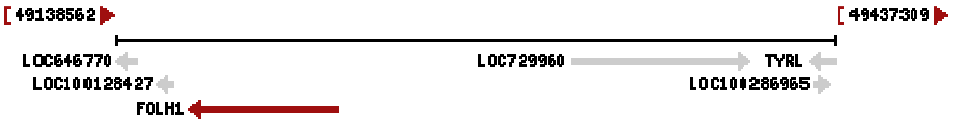
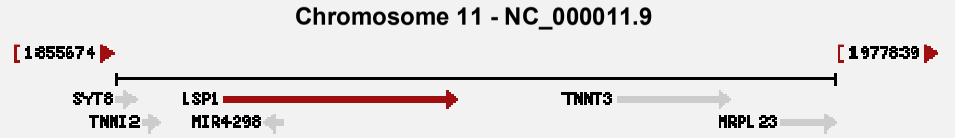
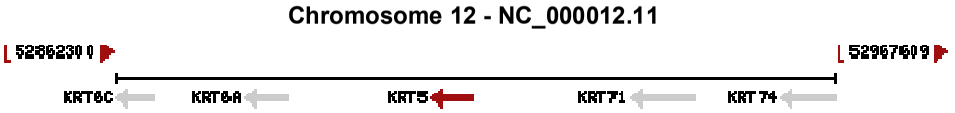
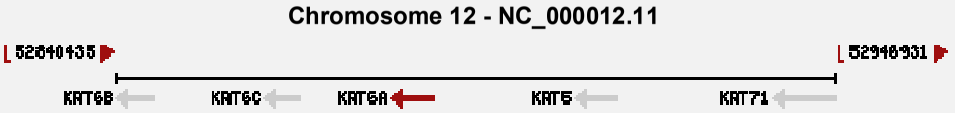
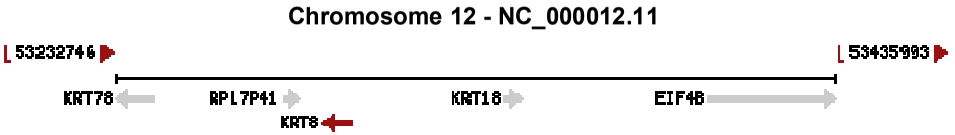
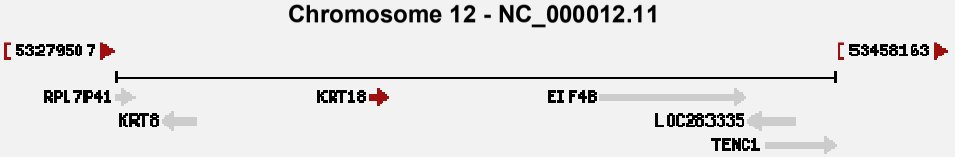
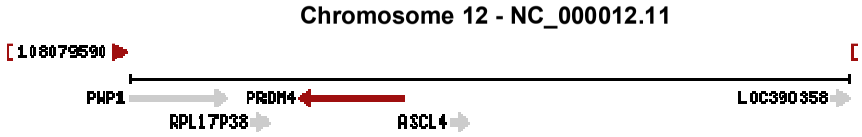
Table 1. Genes data according to HUGO Gene Nomenclature Committee (HGNC).

Approved Symbol	Approved Name	HGNC ID	Chromosome	Genomic Context
AR	androgen receptor	644	Xq11-q12	
RNASEL	ribonuclease L	10050	1q25	
SRD5A2	steroid-5-alpha-reductase, alpha polypeptide 2	11285	2p23.1	
TLR9	toll-like receptor 9	15633	3p21.3	
TLR2	toll-like receptor 2	11848	4q32	
TLR3	toll-like receptor 3	11849	4q35	

TLR6	toll-like receptor 6	16711	4p16.1	
SMAP1	small ArfGAP 1	19651	6q12-q13	
EEF1A1	eukaryotic translation elongation factor 1 alpha 1	3189	6q14.1	
ANXA1	annexin A1	533	9q21.13	
PCA3	prostate cancer antigen 3 (non-protein coding)	8637	9q21-q22	
TLR4	toll-like receptor 4	11850	9q33.1	
FOLH1	folate hydrolase (prostate-specific	3788	11p11.2	Chromosome 11 - NC_000011.9

	membrane antigen) 1			
LSP1	lymphocyte-specific protein 1	6707	11p15.5	
KRT5	keratin 5	6442	12q11-q13	
KRT6A	keratin 6A	6443	12q12-q21	
KRT8	keratin 8	6446	12q13	
KRT18	keratin 18	6430	12q13	
PRDM4	PR domain containing 4	9348	12q23-q24.1	

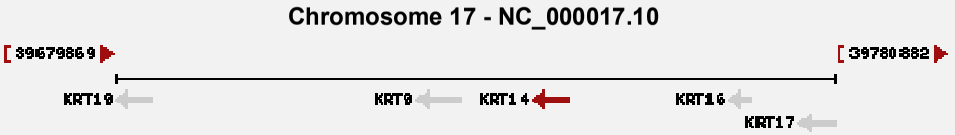
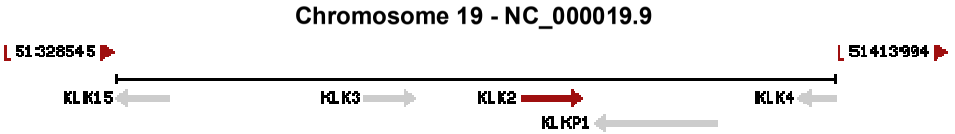
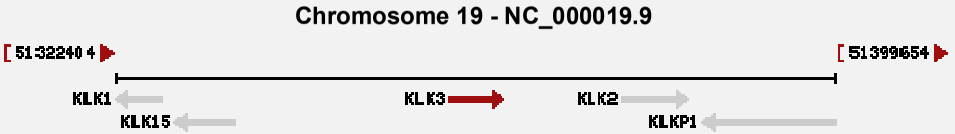
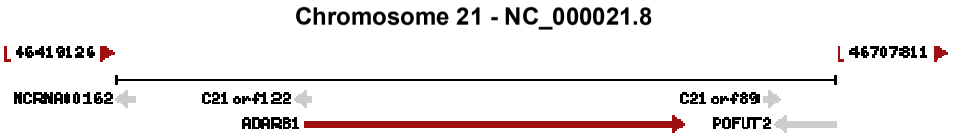
KRT14	keratin 14	6416	17q12-q21	
KLK2	kallikrein-related peptidase 2	6363	19q13.33	
KLK3	kallikrein-related peptidase 3	6364	19q13.41	
ADARB1	adenosine deaminase, RNA-specific, B1	226	21q22.3	

Table 2. Oligonucleotide sequences used for the multiple mRNA markers study.

Gene	GenBank Reference Sequence	Primer sequence 5'-3' (foward / reverse)	Nucleotide position	Amplicon (bp)
PCA3	NM_000424	AGATGTTCTTTGATGCGGAGC	1033 - 1052	277
		CTGCGGTTGGCAATCTCCT	1203 - 1310	
KRT5	NM_000424	AGATGTTCTTTGATGCGGAGC	1074 - 1094	148
		CTGCGGTTGGCAATCTCCT	1203 - 1222	
KRT6A	NM_005554	AGGGTGAGGAGTGCAGGCT	1618 - 1636	157
		CCAAGACCACTGCCATAGGAG	1754 -1774	
KRT8	NM_002273	AGCTGGAGTCTCGCCTGGA	729 - 747	80
		CAGCTCCCGGATCTCCTCT	790 - 808	
KRT14	NM_000526	ATTGAGGACCTGAGGAACAAGATT	566 - 589	128
		CGCAGGTTCAACTCTGTCTCAT	672 - 693	
KRT18	NM_000224.2	GCTCTGGGTTGACCGTGG	804 - 821	151
		GTGGTGCTCTCCTCAATCTGC	934 - 954	
ANXA1	NM_000700.1	GATTCAGATGCCAGGGCCT	672 - 690	110
		CACTCTGCGAAGTTGTGGAT	763 - 782	
LSP1	NM_002339	ATACACCCAGGCCATCGAGA	874 - 893	75
		ATGCTGGGCAGCTCTATGGA	948 - 929	
RNAsel	NM_021133	TGCCCGAAATGTCCTGTCA	1547 - 1565	124
		CAGGTGAGCAGCTTTCTTAGAATCTA	1670 - 1645	
EEF1A1	NM_001402.5	GGCTACAACCCCGACACAGT	607 - 636	92
		TTGAACCAAGGCATGTTAGCAC	677 - 698	
SMAP1	NC_000006.11	CCATGCCCAATGGGTTTATG	1362 – 1381	175
		GCCATCTGCTGATTCATCTGTG	1515 - 1536	
ADARB1	NC_000021.8	CGTGCTCATCCACACTCAAGA	1910 – 1930	126

		GCGGGTGAGATGGTATTTGGT	2017 - 2035	
PRDM4	NM_012406.**3**	TGAGCCAATCCTGGAAGAACC	2512 – 2532	166
		CACGTTTGGATGCTCGCAT	2657 - 2677	
AR	NM_001011645.1	CCACTGAGGAGACAACCAGA	2135 - 2173	236
		GTGTAAGTTGCGGAAGCCAGG	2310 - 2371	
SRD5A2	NM_000348.3	GGTACTTCTGGGCCTCTTCTG	314 - 334	211
		GAAGACACCCAAGCTAAACCG	504 - 524	
KLK2	NM_001002231.1	AGTTCTTGCGCCCCAGGAGT	532 - 551	361
		ACTAGAGGTAGGGGTGGGAC	873 - 892	
KLK3	NM_001648.2	AACCAGAGGAGTTCTTGACC	523 - 542	199
		GTGATACCTTGAAGCACACC	702 - 721	
FOLH1	NM_001014986.1	AGGGGCCAAAGGAGTCATTC	921 - 940	196
		CTCTGCAATTCCACGCCTAT	1097 - 1116	
TLR2	NM_003264.3	CGCAATGGTATCTGCAAGGG	313 - 332	162
		GGATGTCAGCACCAGAGCCT	455 - 474	
TLR3	NC_000004.11	TTCGAGAGTGCCGTCTATTTGCCA	5 - 28	92
		TGTTGGATGACTGCTAGCCTTTCC	73 - 96	
TLR4	NM_138554.3	ACCAAGAACCTGGACCTGAGCTTT	457 - 480	150
		AGAGAGGTGGCTTAGGCTCTGATA	583 - 606	
TLR6	NM_006068.3	CGAACTCTATTTTGCCCATCACA	2262 - 2284	147
		CACTGCAAATAAGTCCGCTGC	2388 - 2408	
TLR9	NM_017442.2	CCACGGCCTGGTGAACTG	751 - 768	67
		GGGTGCTGCCATGGAGAA	800 - 817	
B2M	NM_004048	CCTGCCGTGTGAACCATGT	356 - 374	94
		GCGGCATCTTCAAACCTCC	449 - 431	

Table 3. Gene's basic function

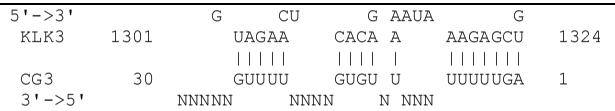
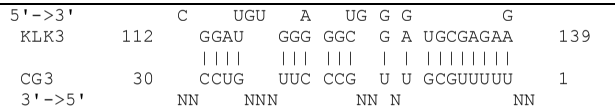
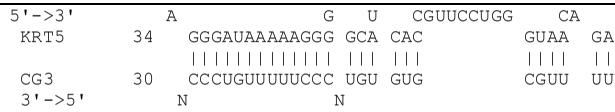
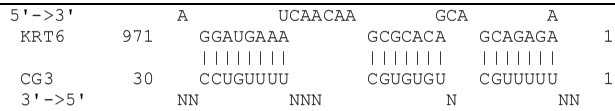
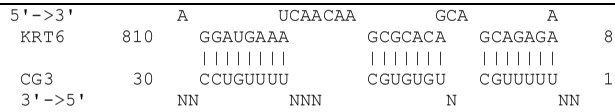
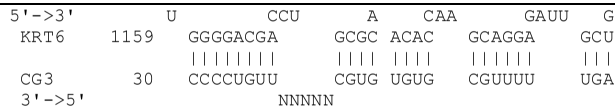
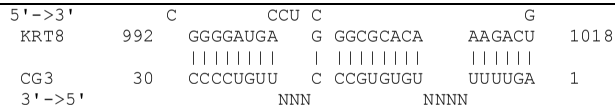
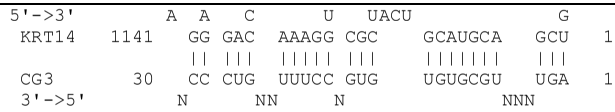
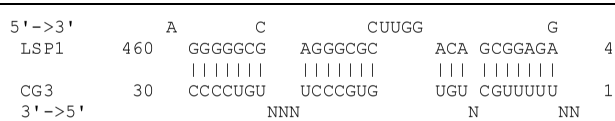
Genes	Expression / Subcellular location	Cellular and physiological process	Basic function
ADARB1	Intracellular	RNA editing	• Double stranded RNA editase 1; • Catalyze the hydrolytic deamination of adenosine to inosine and change the sequence of specific mRNAs with highly double-stranded structures.
AR	Translocation into the nucleus upon binding the hormone ligand	Transcription, regulation, development (sex differentiation, small molecule transport)	• Regulation of androgen action (cellular proliferation and differentiation in target tissues); • Steroid hormone activated transcription factor; • Stimulating transcription of androgen responsive genes; • Suppressor and a proliferator to suppress or promote prostate cancer metastasis.
ANXA1	Located on the cytosolic face of the plasma membrane	Anti-inflammatory response, exocytosis transport (cell communication)	• Calcium dependent phospholipid binding protein; • Modulating antiinflammatory response (phospholipase A2 inhibitory activity); • Promoting membrane fusion, regulating the MAPK/ERK pathway.
EEF1A1	Exported from the nucleus with tRNA	Proliferation, apoptosis, translation/synthesis.	• Potential tumor suppressor activity mediated by its interaction with ZBTB16; • Eukaryotic translation elongation factor 1, alpha 1, binding aminoacyl tRNAs to 80S ribosomes.
FOLH1	Plasma membrane	Folato hidrolase activity	• Involved in conversion of the neurotransmitter (NAAG) to NAA and free glutamate.
KLK2	Extracellular	Degradation (proteolytic cascades)	• Playing a role in prostate cancer susceptibility (increased expression); • Mediate tumor growth and invasion by participating in proteolytic cascades; • Highly prostate-specific serine protease, which is mainly excreted into the seminal fluid; • Direct transcriptional target.
KLK3			• Serine protease functioning in the liquefaction of seminal coagulum; • Androgen-mediated expression by binding of the GAGATA binding factor and AR to GAGATA and AREs, respectively; • Direct transcriptional target; • Inhibit angiogenesis; • Inhibition of prostate cell carcinoma motility.

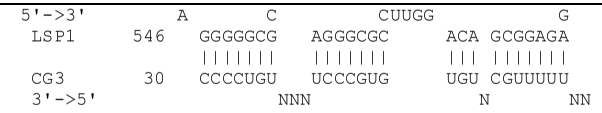
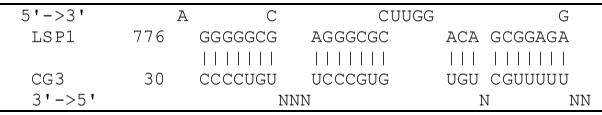
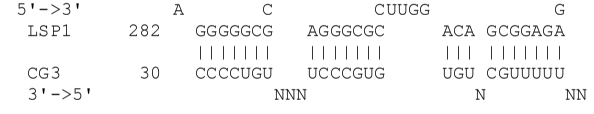
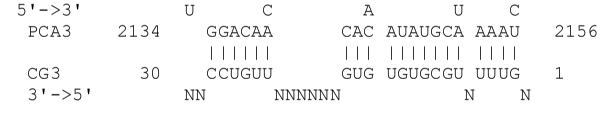
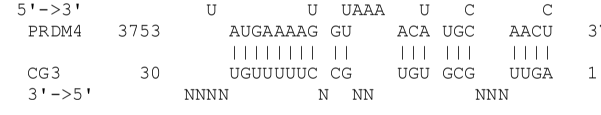
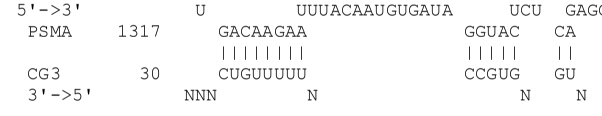
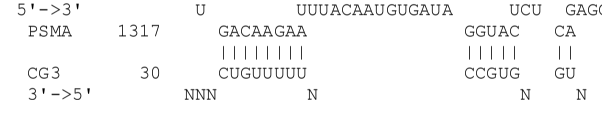
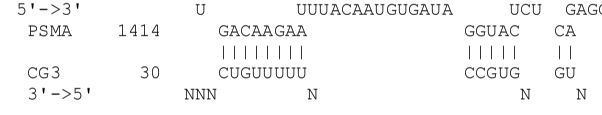
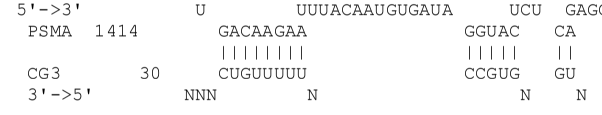
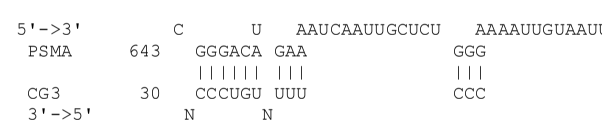
KRT5A *KRT6A* *KRT8* *KRT14* *KRT18*	Cytoplasm (cytoskeleton)	Cytoskeleton organization and biogenesis	• Plays a role in maintaining cellular structural integrity and also functions in signal transduction and cellular differentiation; • KRT8/KRT18-coexpression plays a causal role in basement membrane invasion by transformed keratinocytes.
LSP1	Cytoplasm (cytoskeleton)	Cell migration and motility	• Regulate neutrophil motility, adhesion to fibrinogen matrix proteins, and transendothelial migration; • Transmembrane signal transduction through postulated calcium-binding function.
PRDM4	Nucleus	Cell cycle	• Cell differentiation control and tumorigenesis
PCA3	Nucleus / Cytoplasm	Non-coding mRNA	• **Unknown**
RNAseL	Cytoplasm (mitochondria)	Immunity / defense (apoptosis)	• Mediating resistance to virus infection and apoptosis; • Playing a role in the antiviral and antiproliferative activities of IFN and contributes to innate immunity and cell metabolism; • Intracellular enzyme that is vital in innate immunity; • Tumor suppressor candidate.
SMAP1	Plasma membrane	Erythropoiesis	• Involved in the erythropoietic stimulatory activity of stromal cells.
SRD5A2	Cytoplasm (Endoplasmic reticulum)	Sexual differentiation	• Involved in synthesis of dihydrotestosterone in the urogenital tract; • Playing a central role in sexual differentiation and androgen physiology.
TLR2	Plasma membrane	Immunity / defense (apoptosis of infected cells, pathogen recognition)	• Plays a central role for the response to a wide variety of microbial and endogenous danger signals; • Stimulation plays a crucial role in the homeostasis of human B cells.
TLR3			• Involved in the recognition of ds-RNA; • Immune-recognition receptor that recognize molecular patterns associated with microbial pathogens, and induce antimicrobial immune responses; • Inducing TNF and IL12; • Activating cytokines according to pathogen-associated molecular patterns.

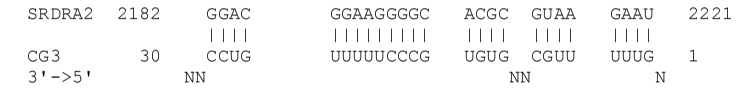
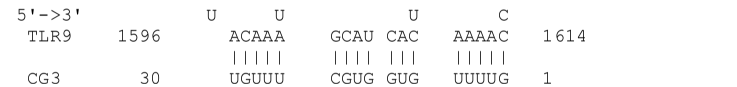
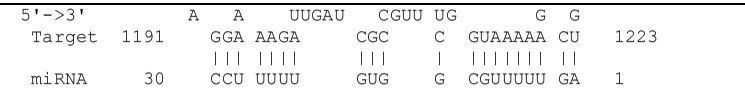
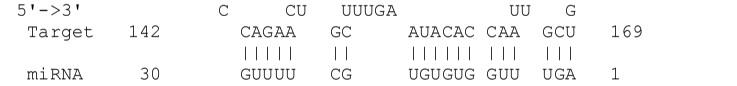
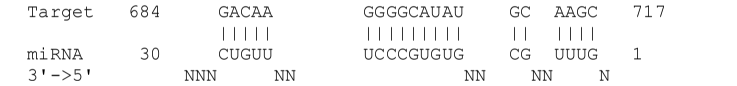
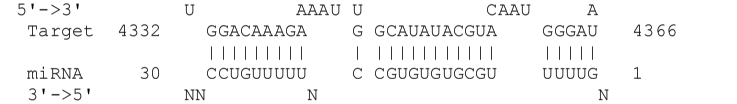
TLR4	• Multifunctional molecule participating in photoreceptor outer segment membrane recognition, oxidant production, LPS recognition, and cytokine production
TLR6	• Key factor in determining whether the first trimester trophoblast response to peptidoglycan would be apoptosis or inflammation.
TLR9	• Recognize nucleic acids and trigger signaling cascades that activate plasmacytoid dendritic cells to produce interferon-alpha; • Mediating cellular response to unmethylated CpG dinucleotides in bacterial DNA to mount an innate immune response.

Table 4. Gene transcripts binding analyses

Gene Symbol	Map *locus*	Transcript variant (mRNA) - since (ntd)	NCBI Reference Sequence	Predicted hybrid conformation (CG3 aptamer / mRNA)	ΔG_{total}	$\Delta G_{disruption}$	ΔG_{hybrid}
ADARB1	21q22.3	Variant 1 – 6924	NM_001112.3	5'->3' A ACA U G U ADARB1 1861 GCAAGAGG GC ACG AC CAAAA 1885 |||||||| || ||| || |||| CG3 30 UGUUUUUC CG UGU UG GUUUU 1 3'->5' NNNN N N N NNN	-16.277	-4.923	-21.2
		Variant 2 – 7044	NM_015833.3	5'->3' A AUUCG A U ADARB1 5441 GCAGAAA ACACA GCAGGAACU 5467 ||||||| |||| ||||||||| CG3 30 UGUUUUU UGUGU CGUUUUUGA 1 3'->5' NNNN NNNN N	-18.296	-7.004	-25.3
		Variant 3 – 3605	NM_015834.3	5'->3' C U CA A A ADARB1 2299 AG GGCAU GC AUGCAGAAGC 2321 || ||||| || |||||||||| CG3 30 UC CCGUG UG UGCGUUUUUG 1 3'->5' NNNNNNNNNN N	-12.460	-13.840	-26.3
		Variant 4 (non-coding) - 5073	NR_027673.1	5'->3' A ACA U G U ADARB1 1979 GCAAGAGG GC ACG AC CAAAA 2003 |||||||| || ||| || |||| CG3 30 UGUUUUUC CG UGU UG GUUUU 1 3'->5' NNNN N N N NNN	-14.200	-7.000	-21.2
		Variant 5 (non-coding) - 4020	NR_027672.1	5'->3' A ACA U G U ADARB1 926 GCAAGAGG GC ACG AC CAAAA 950 |||||||| || ||| || |||| CG3 30 UGUUUUUC CG UGU UG GUUUU 1 3'->5' NNNN N N N NNN	-14.831	-6.369	-21.2
		Variant 6 – 4955	NR_027674.1	5'->3' A ACA U G U ADARB1 1861 GCAAGAGG GC ACG AC CAAAA 1885 |||||||| || ||| || |||| CG3 30 UGUUUUUC CG UGU UG GUUUU 1 3'->5' NNNN N N N NNN	-14.806	-6.394	-21.2
		Variant 7 – 3485	NM_001160230.1	5'->3' C U CA A A ADARB1 2179 AG GGCAU GC AUGCAGAAGC 2201 || ||||| || |||||||||| CG3 30 UC CCGUG UG UGCGUUUUUG 1 3'->5' NNNNNNNNNN N	-12.951	-13.349	-26.3
ANXA1	9q11-q22	1399	NM_000700.1	5'->3' U AACC A AU A C ANX1 1058 GGAUGA AAAGG G UAUG GAAAAU 1087 |||||| |||| | ||| ||||| CG3 30 CCUGUU UUUCC C GUGC UUUUUG 1 3'->5' NN NNNN N N	-11.220	-7.780	-19.0
KLK3	19q13.41	Variant 1 – 1464	NM_001648.2	5'->3' G CU G AAUA G KLK3 1430 UAGAA CACA A AAGAGCU 1453 ||||| |||| | ||||||| CG3 30 GUUUU GUGU U UUUUUGA 1 3'->5' NNNNN NNNN N NNN	-9.586	-5.514	-15.1

		Variant 3 – 1906	NM_001030047.1		-10.720	-19.580	-30.3
		Variant 4 – 1335	NM_001030048.1		-9.738	-5.362	-15.1
		Variant 6 – 415	NM_001030050.1		7.214	-34.614	-27.4
KRT5	12q11-q13	2320	NM_000424.3		-11.463	-23.737	-35.2
KRT6	12q12-q14	Variant A – 2450	NM_005554.3		-16.833	-12.467	-29.3
		Variant B – 2331	NM_005555.3		-10.379	-18.921	-29.3
		Variant C – 2345	NM_173086.4		-10.148	-21.952	-32.1
KRT8	12q13	1802	NM_002273.3		-13.328	-20.372	-33.7
KRT14	17q12-q21	1653	NM_000526.4		-13.015	-14.385	-27.4
LSP1	11p15.5	Variant 1 -1756	NM_002339.2		-15.992	-19.908	-35.9

		Variant 2 -1842	NM_001013253.1		-15.945	-19.955	-35.9
		Variant 3 -2072	NM_001013254.1		-16.101	-19.799	-35.9
		Variant 4 -1578	NM_001013255.1		-15.922	-19.978	-35.9
PCA3	9q21-q22	Non-coding – 3735	NR_015342.1		-13.707	-9.293	-23.0
PRDM4	12q23-q24.1	4225	NM_012406.3		-11.938	-4.462	-16.4
FOLH1	11p11.2	Variant 1 – 2653	NM_004476.1		-5.036	-15.664	-20.7
		Variant 2 – 2560	NM_001014986.1		-4.900	-15.800	-20.7
		Variant 3 – 2737	NM_001193471.1		-3.533	-17.167	-20.7
		Variant 4 – 2644	NM_001193472.1		-3.609	-17.091	-20.7
		Variant 5 – 2266	NM_001193473.1		-3.609	-17.091	-20.7
SRD5A2	2p23.1	2446	NM_000348.3		-29.200	-0.000	

						-29.2
TLR9	3p21.3	3868	NM_017442.2		-14.892	-1.308	-16.2
EEF1A1	6q14.1	3528	NM_001402.5		-10.954	-8.746	-19.7
TLR4	9q33.1	Variant 1 – 5667	NM_138554.3		-12.757	-5.743	-18.5
		Variant 3 (non-coding) - 5787	NR_024168.1		-12.803	-14.697	-27.5
		Variant 4 (non-coding) - 5500	NR_024169.1		-13.737	-16.363	-30.1

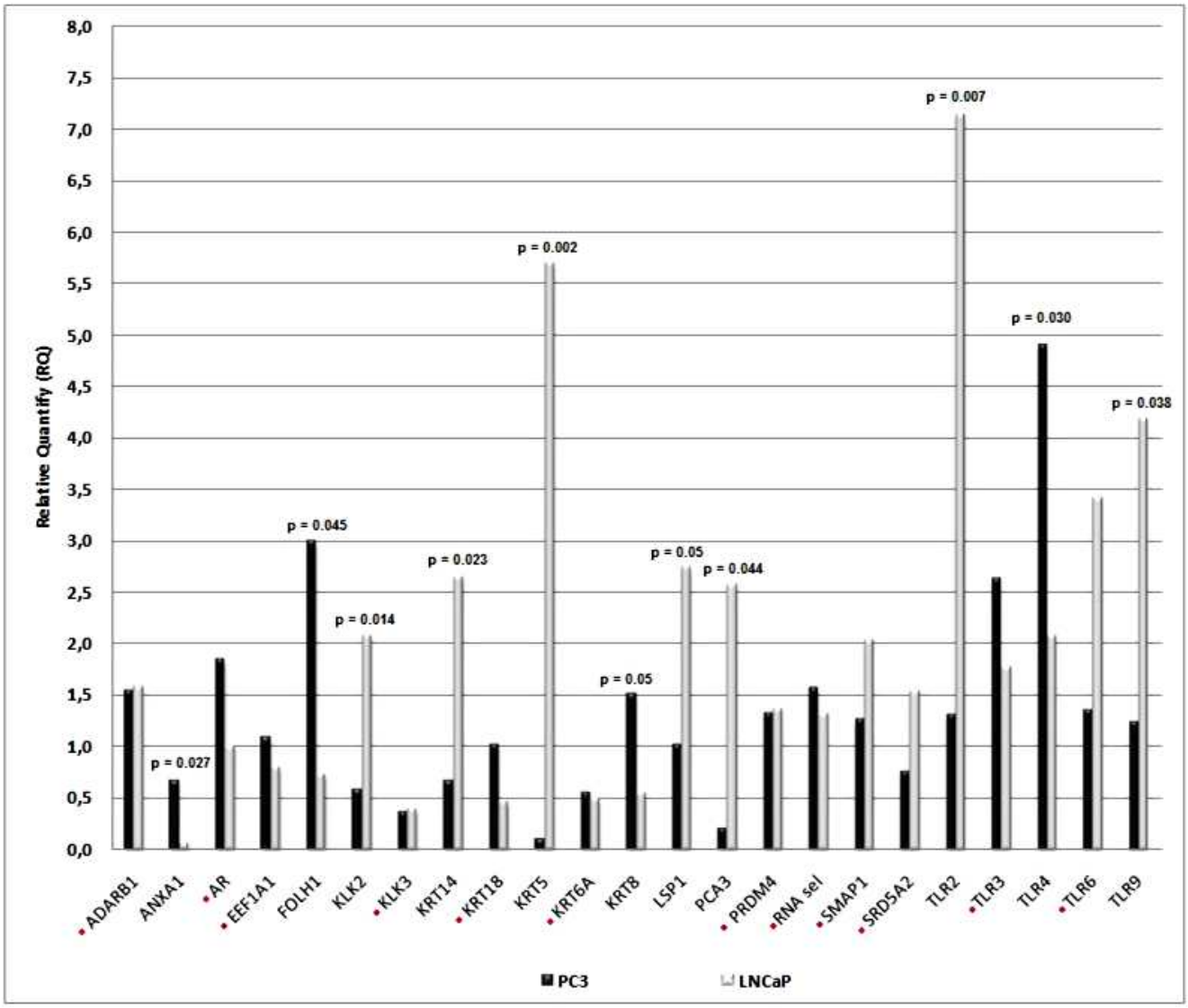


Figure 1. Analysis of differential gene expression of 23 genes between prostate cancer cell lines PC3 and LNCaP. Significant differences between lines (P<0.05) are indicated in the appropriated genes. Genes with no significant difference were identified with red point.

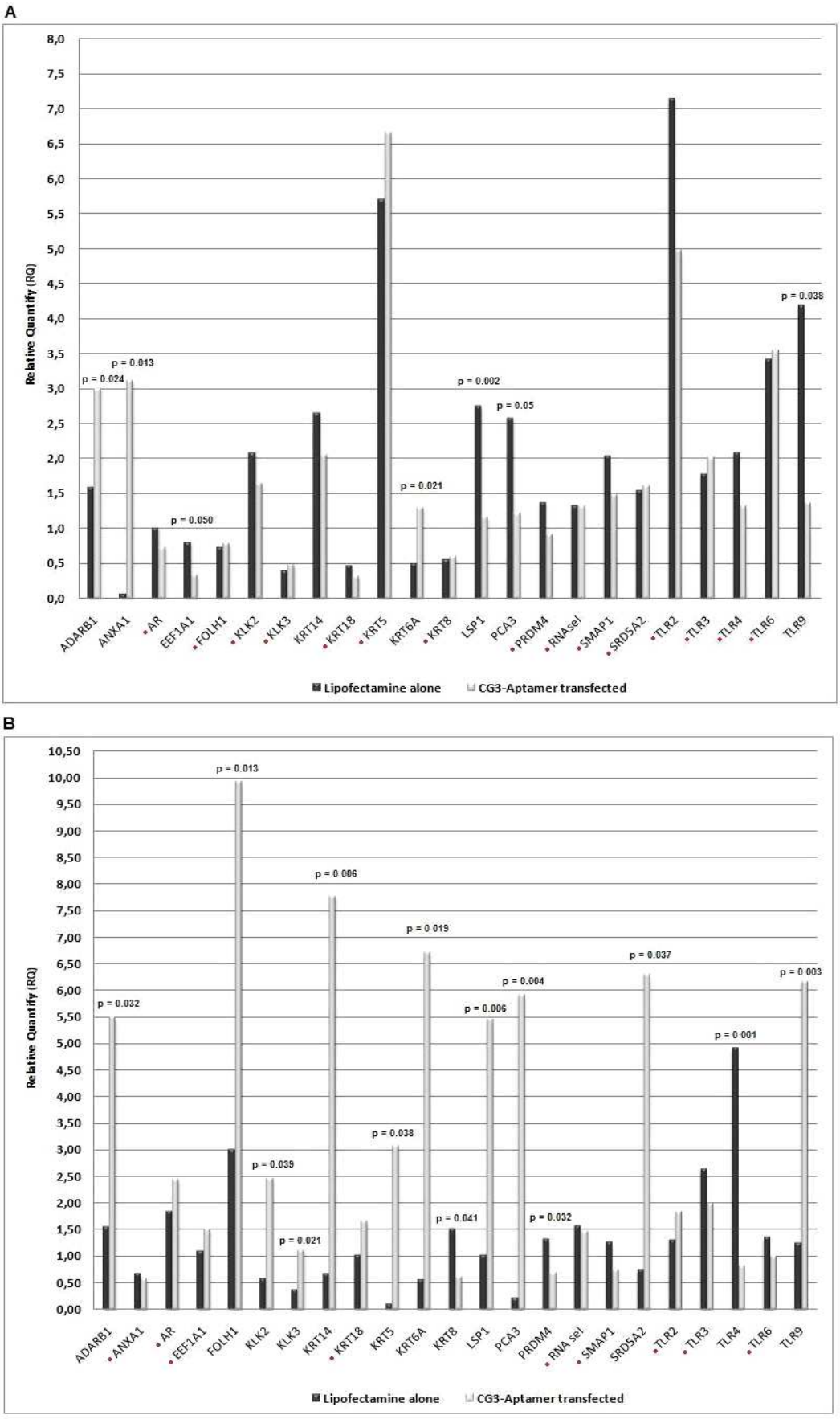


Figure 2. Differential gene expression of 23 genes for prostate cancer cell lines LNCaP **(A)** and PC3 **(B)** submitted to transfection with the CG3 aptamer designed to bind specifically to the *PCA3* gene. Genes with no significant difference were identified with red point.

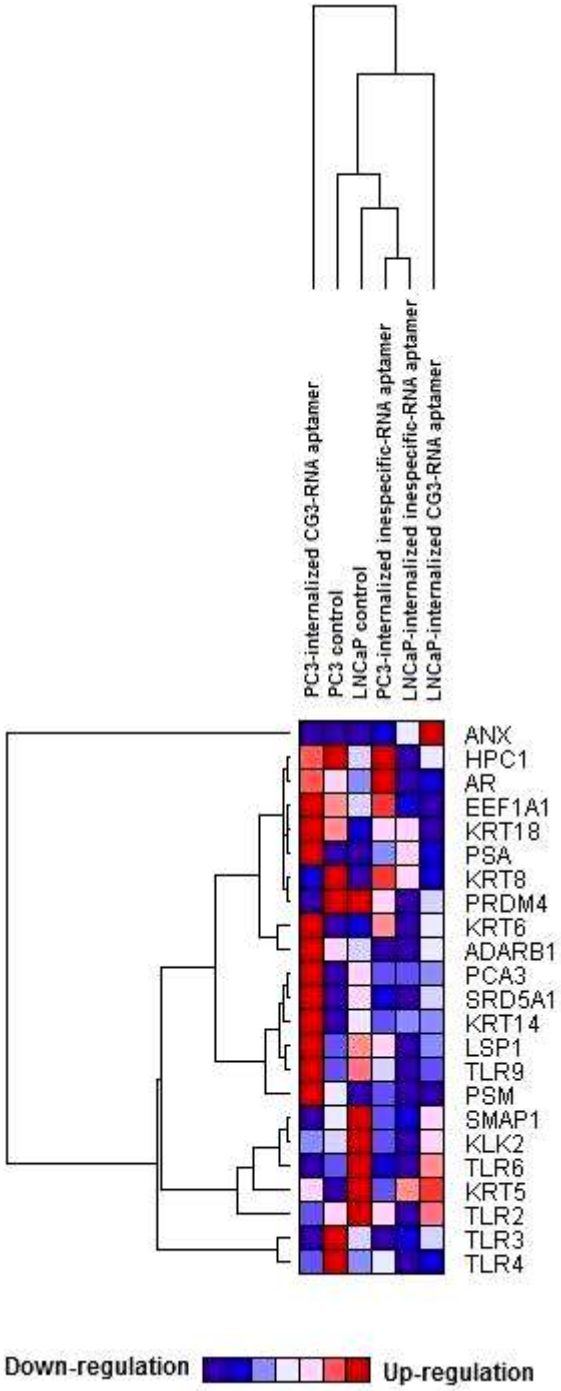


Figure 3. Cluster analysis for the expression of 23 genes considering prostate cancer cell lines and their respective CG3-aptamer transfected cell lines. Highly expressed genes are represented in red, and highly inhibited ones are in blue. Intermediate colors indicate moderate expression (pink) or moderate inhibition (light blue).

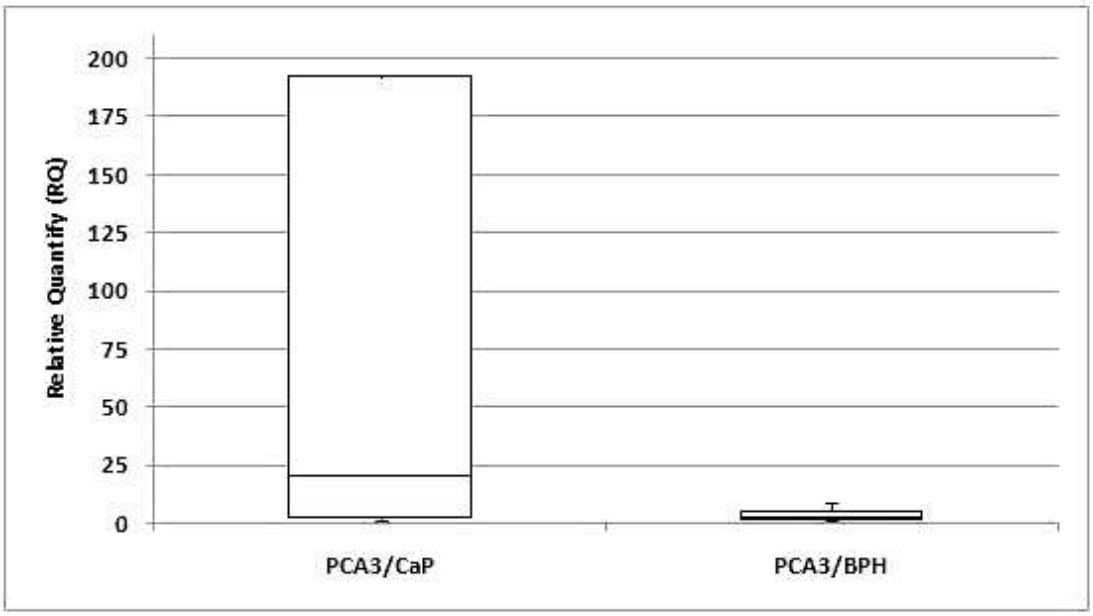
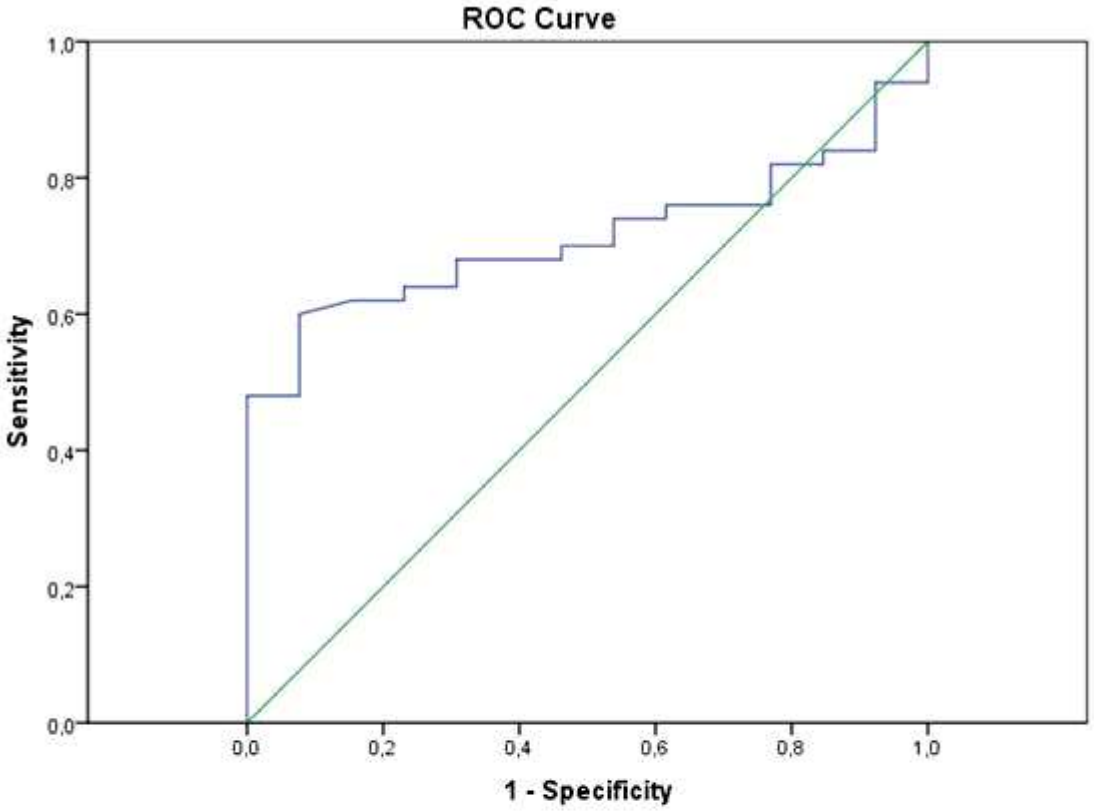


Figure 4. Expression of genes with significant difference. Expression was normalized against the *B2M* housekeeping gene. Data are displayed in a *boxplot*.



Area Under the Curve

Test Result Variable(s)	Area
PCA3	0,8

Figure 5. ROC curve to visualize the diagnostic efficacy of *PCA3* gene.

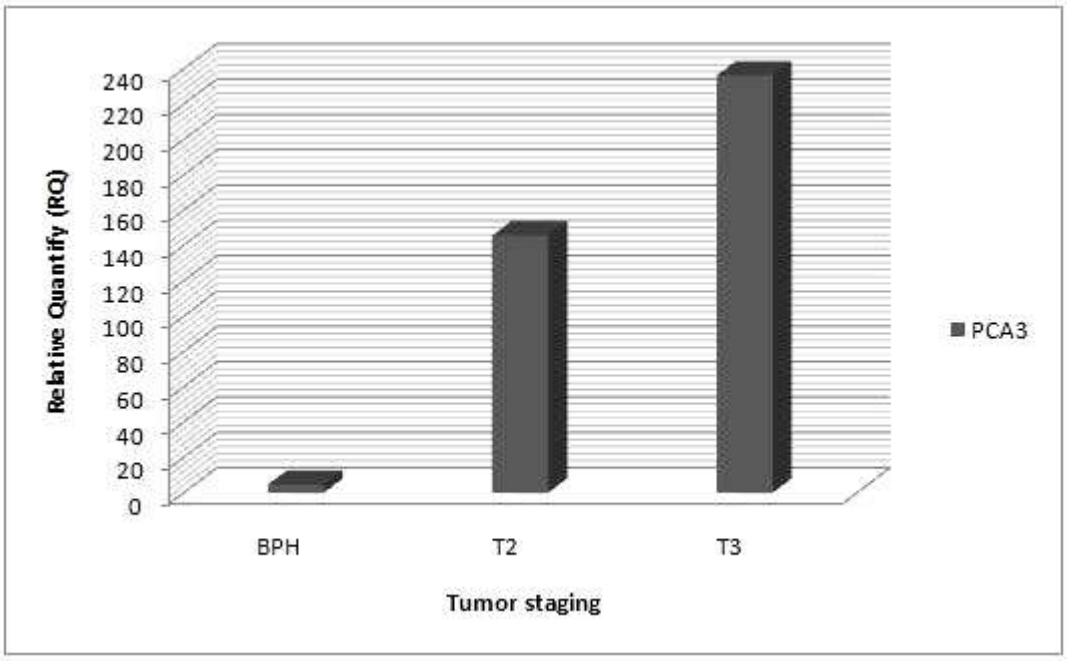


Figure 6. The average relative levels of PCa patients classified according to tumor stages.

ANEXOS

Anexo I: Estadiamento do câncer de próstata

TNM	Definição
T0	Sem evidência de tumor prostático
T1a	Tumor não palpável, < 5% do tecido ressecado na ressecção transuretral
T1b	Tumor não palpável, > 5% do tecido ressecado na ressecção transuretral
T1c	Tumor não palpável, PSA alterado
T2a	Nódulo < meio lobo
T2b	Nódulo > meio lobo
T2c	Nódulo bilateral
T3a	Extensão extraprostática mínima
T3b	Invasão do colo vesical
T3c	Invasão de vesículas seminais
T4	Invasão de parede pélvica e/ou bexiga
N0	Sem metástases em linfonodos
N1	Metástases em linfonodos regionais
M0	Sem metástases sistêmicas
M1	Metástases sistêmicas
M1a	Linfonodo(s) não regional(ais)
M1b	Osso(s)
M1c	Outra(s) localização(ões)

Anexo II: Escala de Gleason