PERFIL DE EXPRESIÓN DE miRNA´S EN CÁNCER DE PRÓSTATA DE INICIO TEMPRANO E IDENTIFICACIÓN DE SUS POSIBLES GENES BLANCO RELACIONADOS CON LA CARCINOGÉNESIS

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DOCUMENTO DE TESIS PRESENTADO COMO REQUISITO PARA OPTAR AL TÍTULO DE DOCTOR EN CIENCIAS BIOMÉDICAS Y BIOLÓGICAS

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1. TABLA DE CONTENIDO

CONTENIDO

1. TABLA DE CONTENIDO **4**

2. LISTA DE FIGURAS **6**

3. LISTA DE PUBLICACIONES **7**

4. LISTA DE ABREVIATURAS **9**

5. RESUMEN **10**

6. INTRODUCCIÓN GENERAL **12**

7. OBJETIVOS **32**

8. INTRODUCCIÓN A LOS CAPÍTULOS **33**

8.1. CAPÍTULO I **34**

Cáncer de próstata de inicio temprano

8.2. CAPÍTULO II **57**

Perfil de expresión de microRNAs en cáncer de próstata de inicio temprano y vías moleculares relacionadas

8.3. CAPÍTULO III **81**

Comparación del perfil de coexpresión de lncrRNA-mRNAs en pacientes con cáncer de próstata de ascendencia africana

8.4. CAPÍTULO IV **94**

Expresión de CCR7 en cáncer de próstata de inicio temprano

9. CONCLUSIONES **109**

10. REFERENCIAS BIBLIOGRÁFICAS **111**

11. ANEXOS **119**

2. LISTA DE FIGURAS

Figura 1. Clasificación molecular del cáncer de próstata primario.

Figura 2. Clasificación histopatológica del adenocarcinoma convencional prostático.

Figura 3. Activación e inhibición de genes involucrados en la progresión del cáncer de próstata.

Figura 4. Vías de señalización celular alteradas en el cáncer de próstata.

Figura 5. Biogénesis de los miRNAs.

3. LISTA DE PUBLICACIONES

1. Capítulo I: Cáncer de próstata de inicio temprano

- **Parra-Medina R**, Ramírez-Clavijo S. *Cáncer de próstata de inicio temprano. ¿Una nueva entidad?* Rev Mex Urol. 2021;81(3):pp. 1-13

- **Rafael Parra-Medina**, Juan José Chaves, Julián Barahona-Correa, César Payan-Gómez, Sandra Ramírez-Clavijo, Daniel Fernández Ávila, Diego Rosselli. *Prevalence and demographic characteristics of prostate cancer in Colombia: data from the National Health Registry 2009-2019.* Urol Colomb 2021;30(3):e204–e209.

2. Capítulo II: Perfil de expresión de microRNAs en cáncer de próstata de inicio temprano y vías moleculares relacionadas

- Vladimir A. Valera*, **Rafael Parra-Medina*,** Beatriz A. Walter, Peter Pinto, Maria J Merino. *microRNA Expression Profiling in Young Prostate Cancer Patients*. Journal of Cancer. 2020; 11(14): 4106-4114.

- **Rafael Parra-Medina**, Liliana López-Kleine, Sandra Ramírez-Clavijo, César Payán-Gómez. *Identification of candidate miRNAs in early-onset and late-onset prostate cancer by network analysis.* Scientific reports. 2020. 10:12345

3. Capítulo III: Comparación del perfil de coexpresión de lncrRNA-mRNAs en pacientes con cáncer de próstata de ascendencia africana

- **Rafael Parra-Medina**, Liliana López-Kleine, Sandra Ramírez-Clavijo, César Payán-Gómez. *Coexpression network analysis identified lncRNAs-mRNAs with potential relevance in African ancestry prostate cancer* Future Sci. OA. 2021. FSO749

4. Capítulo IV: Expresión de CCR7 en cáncer de próstata de inicio temprano

Artículo sometido y revisado con cambios menores:

- **Rafael Parra-Medina**, César Payán-Gómez, Natalia L. Acosta -Vega, Sandra Ramírez-Clavijo. *Expression of Chemokine (C-C motif) receptor 7 (CCR7) in young prostate cancer patients and in metastatic cancer cells*

5. Anexo

Parra-Medina R, Ramírez-Clavijo S. *Why not to use punch biopsies in formalin-fixed paraffin-embedded samples of prostate cancer tissue for DNA and RNA extraction?* African Journal of Urology. 2021. 27:154

4. LISTA DE ABREVIATURAS

AR: Receptor de andrógenos

ADN: Ácido desoxirribonucleico

ARN: Ácido ribonucleico

CP: Cáncer de próstata

CPITa: Cáncer de próstata de inicio tardío

CPITe: Cáncer de próstata de inicio temprano

GG: Grado grupo

GS: Score de Gleason

mRNA: ARN mensajero

miRNA: Micro ARN

NCCN: National Comprehensive Cancer Network

LncRNA: ARN largos no codificantes

PSA: Antígeno prostático

TCGA: The Cancer Genome Atlas Research Network

5. RESUMEN

El diagnóstico de cáncer de próstata (CP) en pacientes jóvenes se ha definido como CP de inicio temprano (CPITe). La edad de corte no está establecida, varios autores consideran como inicio temprano si el paciente es menor de 50 años al momento del diagnóstico, sin embargo, otros estudios lo han establecido en menores de 60, 55 y 45 años de edad. En Estados Unidos en el año 2012 cerca del 10% de los hombres con CP (241.740 individuos) fueron diagnosticados antes de los 55 años. En Colombia el análisis del registro nacional de salud, mostró que la incidencia de pacientes con CPITe también ha estado incrementando desde el 2015 con una incidencia para el 2019 de 0.14 por mil habitantes (791 casos en 5 años).

Por otro lado, se ha observado que algunas características clínicas del CPITe y de inicio tardío son similares, pero en pacientes jóvenes con alto riesgo de desarrollar la enfermedad el tumor presenta mayor agresividad. A nivel molecular se han encontrado algunas diferencias entre los tumores provenientes de pacientes jóvenes y viejos. En nuestros estudios observamos diferencia en los niveles de expresión de diferentes miRNAs comparando jóvenes y viejos, algunos de ellos conocidos por participar en el desarrollo de CP y otros por primera vez reportada su participación en CP. Adicionalmente en el estudio de análisis de vías y redes moleculares observamos que las vías moleculares de los dos grupos tienden a ser similares. Sin embargo, destacamos vías moleculares exclusivas de los tumores provenientes de pacientes jóvenes que son un hallazgo clave que aporta al entendimiento de la patogénesis de estos pacientes. Redes de coexpresión de miRNAs fueron obtenidas una exclusiva para los jóvenes mientras que en los viejos hubo dos. Finalmente, con el objetivo de identificar genes blancos correlacionados con miRNAs hub, mediante el uso de herramientas bioinformáticas encontramos genes involucrados en vías, tales como inflamación, MAPKs, metaloproteinasas, entre otros. El análisis estadístico permitió establecer que algunos genes coexpresados con miRNAs fueron exclusivos de los tumores provenientes de pacientes con CP de ascendencia africana.

Con el objetivo de identificar vías moleculares similares entre pacientes jóvenes con CP y pacientes con CP de ascendencia africana realizamos un estudio de expresión de redes observando genes involucrados en diferentes vías moleculares sobrerrepresentadas en pacientes con ascendencia africana, una de las vías fue la de las MAPK, la cual también mostró serlo para los pacientes con CPITe de diferentes ascendencias.

Finalmente, para lograr el último objetivo de la tesis doctoral sobre determinar la expresión de uno de los genes que se hallen asociados a lesiones tumorales de CPITe. El gen elegido fue CCR7 debido a que en el estudio de análisis de redes se encontró coexpresado con miRNAs sobre expresados exclusivos de pacientes jóvenes. CCR7 codifica para un receptor de quimiocina necesario para el tráfico y localización de diferentes células del sistema inmune. La expresión de CCR7 ha sido poco estudiado en CP, con eventual participación en la promoción de eventos de invasión como en la transición epitelio-mesénquima (EMT), angiogénesis y metástasis en varios tipos de tumores. En nuestro estudio, CCR7 estuvo presente en el 65% de los tumores provenientes de los pacientes más jóvenes (≤ 50 años) y en los de mayor grado histológico (≥ grade grupo 3 Gleason). Adicionalmente observamos que todos los pacientes con metástasis presentaron expresión de CCR7 en las células tumorales. Estos resultados son coherentes con el análisis de datos tomados de TCGA de cáncer de próstata en donde se halló que la detección de CCR7 se asoció con la presencia de metástasis (FC: 2.6, p: 0.03).

6. INTRODUCCIÓN GENERAL

6.1 Cáncer de próstata

El cáncer de Próstata (CP) es el segundo tipo de cáncer más frecuente y la quinta causa de muerte en hombres a nivel mundial. Según el informe de GLOBCAN de 2020, en el mundo la incidencia anual es de 1.4 millones y la mortalidad es de 375.304 casos anuales (1). La tasa de mortalidad secundario a CP ha disminuido desde los años 90´s en países desarrollados secundario al uso de pruebas de detección temprana de la enfermedad y los avances en los tratamientos, a diferencia de países menos desarrollados en donde el acceso de pruebas de tamizaje como PSA es limitado y los tratamientos poco efectivos (1).

La presencia de CP se sospecha en pacientes con niveles elevados de antígeno prostático (PSA) en sangre y alteración en el examen de tacto rectal. En esos casos se solicita biopsias de próstata para confirmar el diagnóstico por histopatología. El subtipo tumoral más frecuente es el adenocarcinoma acinar (aproximadamente el 99% de los casos), le sigue el carcinoma ductal (0.14%), luego el adenocarcinoma mucinoso (0.10%), después el carcinoma de célula pequeña (0.056%), y por último otros subtipos histopatológicos menos frecuentes (2).

La etiología del CP al igual que otros tumores es multicausal, en donde los componentes genéticos, medio ambiental y étnico son factores relevantes para calcular el riesgo. Entre los factores genéticos se ha destacado mutaciones puntuales (BRCA1 y BRCA2) o condiciones genéticas (Síndrome de Lynch). Entre el grupo de factores de riesgo medio ambientales se ha asociado con el consumo de cigarrillo, aumento de la masa corporal, y algunos factores nutricionales. En cuanto a los factores asociado a la raza, se ha observado que los pacientes de raza negra y del caribe tienen mayor incidencia global de la enfermedad (1).

También se ha determinado que el desarrollo de CP tiene una correlación positiva con edad avanzada (3). El CP se considera una enfermedad asociada a personas mayores de edad con un pico de incidencia del 80% a los 65 años (4). Sin embargo, desde 1990 se ha reportado un 2% de aumento anual del número de hombres con diagnóstico de CP entre los 15 y 40 años (5) y en autopsias de individuos occidentales con edades entre los 40 y 50 años la prevalencia es del 20 al 30% (6,7).

La detección de CP en pacientes cada vez más jóvenes se relaciona de manera directa con el aumento del uso de la prueba para determinar los niveles de PSA en sangre como método de tamizaje y detección temprana de la enfermedad. Entre los años 2010 y 2020 la incidencia de CP en hombres menores de 50 años se incrementó cinco veces (8), lo que llevó a considerar esta enfermedad como un problema emergente de salud pública (5,9,10) con impacto en los costos del sistema salud. En los últimos años el costo del tratamiento del CP ha tenido un incremento más rápido que otros tipos de tumores (11), en donde se ha observado que los costos varían según el estadio de la enfermedad, siendo mayores para los estadios avanzados (12)

La detección temprana del CP se ha asociado con disminución de la morbilidad y mortalidad, por tal motivo las nuevas recomendaciones de la sociedad europea de urología (2021) sugieren iniciar la detección de la enfermedad con el tamizaje de PSA desde los 50 años en hombres sin factores de riesgo. Desde los 45 años en pacientes con antecedentes familiares de CP y en pacientes de descendencia africana. Y desde los 40 años en pacientes con mutaciones en el gen BRCA2 (13)

6.2. Clasificación molecular del cáncer de próstata

En el año 2015, el 'The Cancer Genome Atlas Research Network' (TCGA) público dio a conocer el sistema de clasificación molecular de CP primario basado en la presencia de alteraciones génicas como por ejemplo mutaciones puntuales, alteración en el número de copias de algunos genes, metilación del DNA, y también cambios en la expresión de algunos genes como por ejemplo los que codifican para miRNAs y proteínas. En este estudio incluyeron 333 muestras de pacientes

con CP a quienes se les realizó un estudio molecular extenso con múltiples plataformas, las cuales incluían secuenciación del genoma completo, exoma, secuenciación de ARN, secuenciación de miRNA, matrices de polimorfismos de nucleótido simple (SNPs), de metilación de ADN y de proteínas de fase inversa.

A partir de los resultados definieron dos grupos con CP (14), el primero incluye los tumores que presentaron alteraciones genéticas de tipo fusión de genes como ERG, ETV 1, ETV4, FL1, y el segundo abarca los que tenían mutaciones puntuales en genes tipo SPOP, FOXA1 e IDH1. El subtipo molecular más frecuente reportado en TCGA es ERG (46%), seguido de SPOP1 (11%), ETV1 (8%), ETV4 (4%), FOXA1 (3%), FL1 (1%), IDH1 (1%), y otras alteraciones genéticas (26%) (Figura 1) (14). Adicionalmente, se pudo conocer otros genes involucrados en la oncogénesis del CP como BRAF, CTNNB1, HRAS, ATM, NKX3-1, AKT1, y ZMYM3, y además entender el efecto de la alteración de genes previamente identificados como SPOP, TP53, FOXA1, PTEN, MED12, y CDKN1B en los diferentes subtipos tumorales (14).

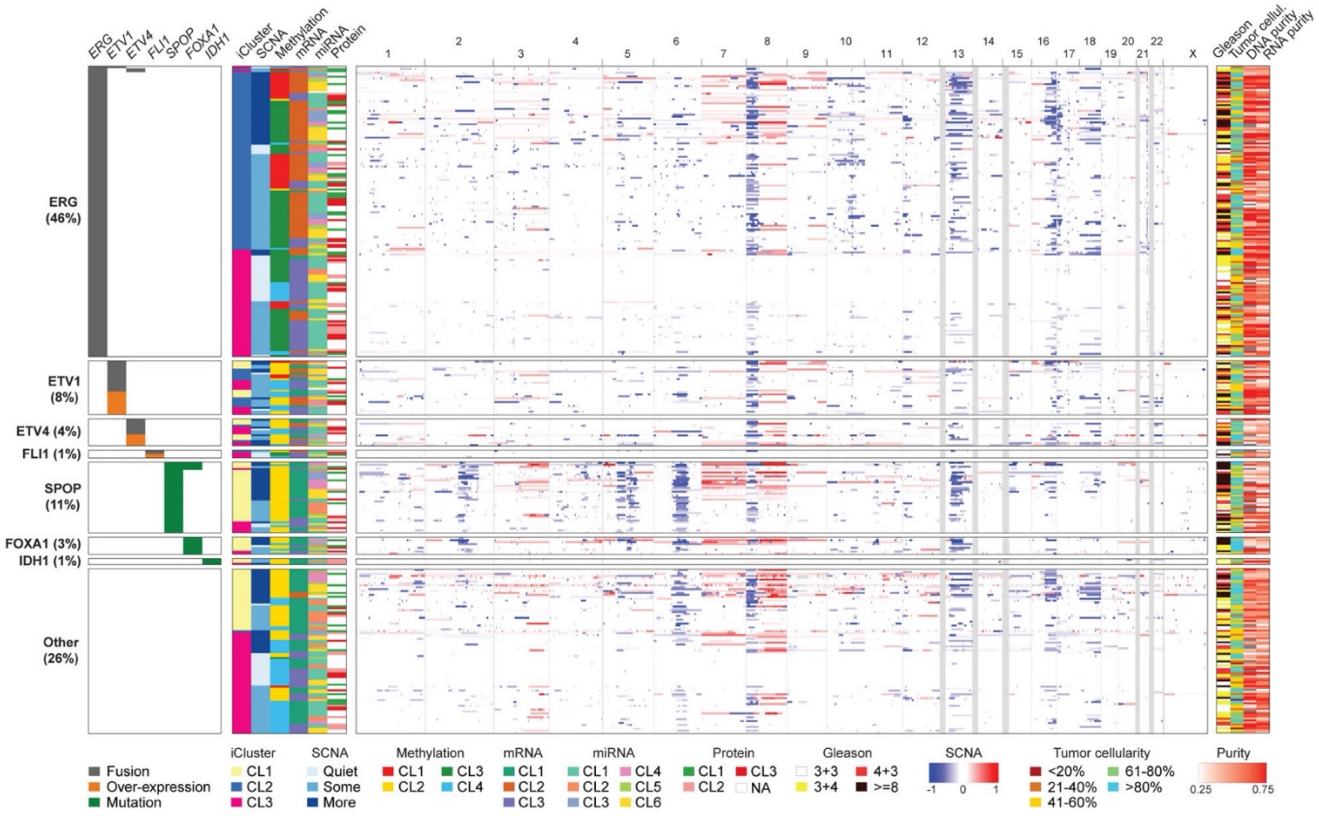

Figura 1. Clasificación molecular del cáncer de próstata primario. Figura tomada de referencia (14).

En la figura 1 se observa el perfil molecular de CP dividido en siete subtipos genómicamente distintos, definidos por la presencia de genes fusionados entre los que se encuentran ERG (46%), ETV1 / ETV4 / FLI1 (8%, 4%, 1%, respectivamente), o por mutaciones puntuales en genes tales como SPOP (11%), FOXA1 (3%) e IDH1 (1%). Algunos subtipos se correlacionaron con agrupaciones (clusters) computacionales obtenidas de los datos de las plataformas evaluadas (alteraciones del número de copias génicas somáticas, metilación del DNA, presencia de mRNA, presencia de microRNA y niveles de expresión de proteínas de las matrices de proteínas de fase inversa). El mapa de calor muestra el número de copias de DNA para todos los casos, en los cromosomas indicados por los números de izquierda a derecha. Las regiones de pérdida se indican con tonos de azul y las ganancias con tonos de rojo.

6.3. Clasificación histopatológica

La clasificación de Gleason score (GS) se usa a nivel mundial para el diagnóstico del adenocarcinoma acinar prostático, la cual evalúa las características arquitecturales de las glándulas conformadas por células malignas. El GS se divide en cinco patrones, los patrones 1, 2 y 3 se caracterizan por presentar glándulas discretamente bien formadas, el patrón 4 tiene glándulas fusadas, pobremente formadas o formando cribas, y el patrón 5 se caracteriza por la ausencia de glándulas y presencia de células malignas las cuales se disponen de manera suelta en forma de cordones o nidos sólidos (Figura 4) (15). El patólogo da una puntuación de GS basado en el primer y segundo patrón más frecuentes, es decir si el patrón más frecuente es 4 y el segundo más frecuente es 5, el diagnóstico definitivo es 4+5.

En el año 2016 se implementó una nueva clasificación basada en 5 grupos teniendo en cuenta el patrón de GS (15). En el grado grupo (GG) 1 se ubican aquellos casos con GS de 6 (3+3), en el grupo 2 los GS de 7, pero siendo más frecuente el patrón 3, es decir 3+4. En el grupo 3, se incluyen

aquellos tumores con GS de 7 con patrón más frecuente 4 (4+3), en el grupo 4 están los pacientes con GS 8 (4+4), y en el grupo 5 se clasifican los pacientes con GS 9 y 10 (4+5,5+4, 5+5).

Esta nueva clasificación en grupos se basó en el comportamiento clínico de la enfermedad, es así como los pacientes que tienen tumores clasificados en el grupo 5 tienen peor pronóstico que los del grupo 1. Teniendo en cuenta que los tumores del grupo 1 tienden a tener un crecimiento lento mientras que los del grupo 5 el crecimiento es rápido.

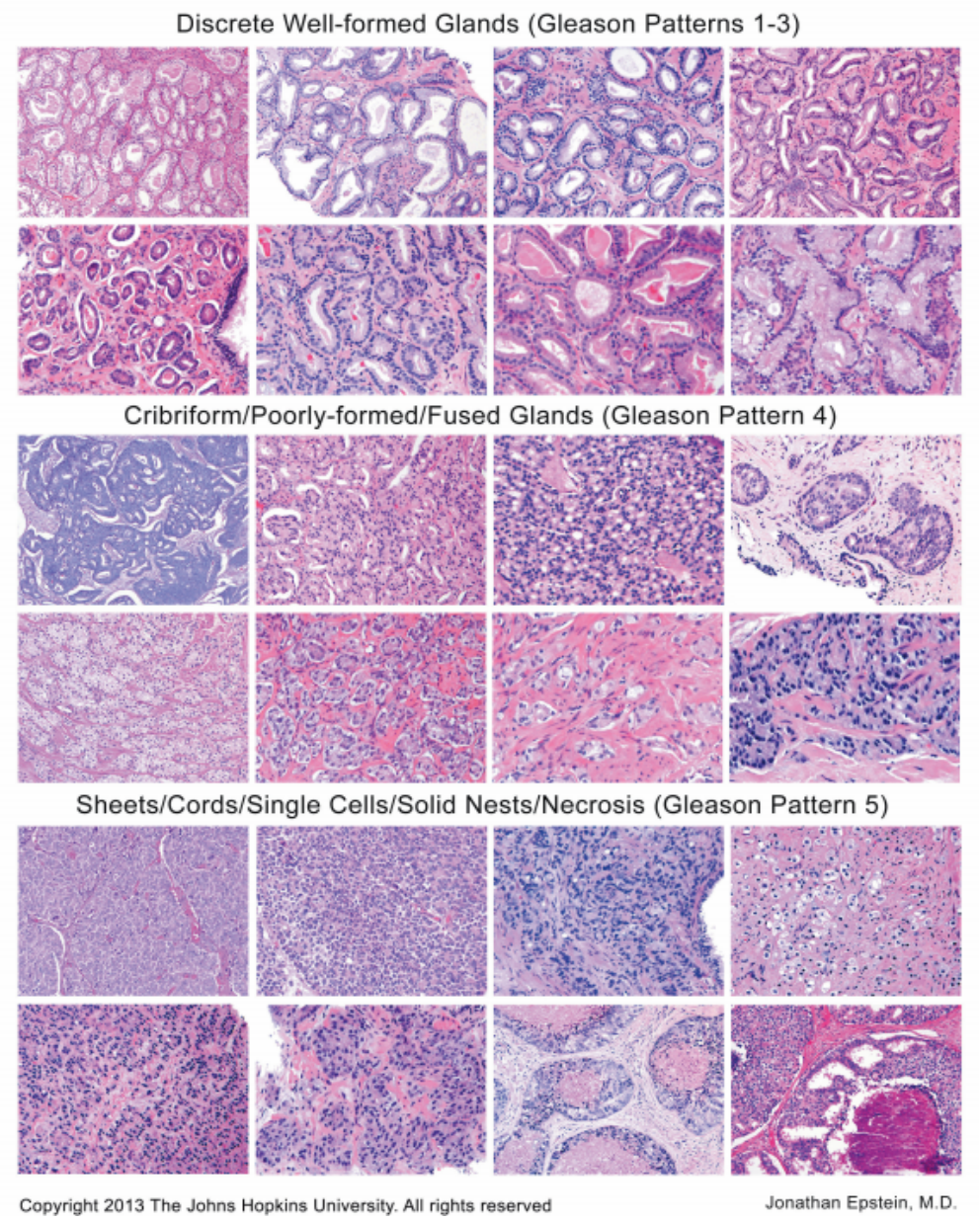

Figura 2. Clasificación histopatológica del adenocarcinoma acinar prostático basado en los patrones establecidos por Gleason. Figura tomada de la referencia (15). Coloración de Hematoxilina y eosina.

6.4. Estadio de la enfermedad

La clasificación del National Comprehensive Cancer Network (NCCN) divide el CP en nueve grupos (Tabla 1) teniendo en cuenta el sistema de clasificación del TNM, el GG del GS, los valores del PSA y los resultados de la biopsia (16).

Tabla 1. Estratificación de riesgo del cáncer de próstata

Grupos de riesgo	Criterios
Muy bajo	Todos los siguientes: - T1c - GS ≤ 6 / GG 1 - PSA <10 ng/mL - < 3 fragmentos de biopsia positiva, ≤ 50% en cada fragmento - Densidad de PSA <0.15 ng/ mL / g
Bajo	Todos los siguientes - T1 - T2a - GS ≤ 6 / GG 1 - PSA <10 ng/mL
Intermedio - Favorable	Cualquiera de los siguientes: - T2b – T2c - GS 3+4 / GG 2 - PSA 10 - 20 ng/mL

	- < 50% de positividad en los fragmentos de la biopsia
Intermedio - Desfavorable	Cualquiera de los siguientes: - T2b – T2c - GS 3+4 / GG 2 o GS 4+3 / GG 3 - PSA 10 - 20 ng/mL
Alto	Cualquiera de los siguientes: - T3a - GS 8 / GG 4 o GS 4+5 / GG 5 - PSA >20 ng/mL
Muy alto	Cualquiera de los siguientes: - T3b-T4 - Patrón primario 5 del GS - > 4 fragmentos de la biopsia con GS 8-10 / GG 4 o 5
Regional	Cualquier T, N1, M0
Metastásico	Cualquier T, cualquier N, M1

Esta estratificación del riesgo permite conocer el comportamiento de la enfermedad y de esta manera elegir el tratamiento y determinar la estrategia para el seguimiento de los pacientes con CP. Por ejemplo, los de riesgo muy bajo, bajo y algunos intermedios con respuesta favorable se les recomienda vigilancia activa, la cual consiste en hacer un control periódico con prueba de PSA cada seis meses y tacto rectal al menos una vez al año, asociado a estudios imagenológicos y biopsias de próstata cada 1 a 3 años. Mientras que el tratamiento para grupos de alto riesgo el tratamiento puede ir desde procedimiento quirúrgico acompañado de quimioterapia, radioterapia, o únicamente tratamiento paliativo (17).

6.5. Oncogénesis de la próstata

El CP al igual que otros tumores malignos tiene una fase de inicio en donde las células van adquiriendo alteraciones moleculares sin cambios estructurales observables, pero que van transformando la actividad celular. A medida que avanza la promoción de la lesión cancerosa hacia la transformación, algunos cambios morfológicos considerados premalignos pueden observarse tales como la hiperplasia típica o atípica, que en la próstata recibe el nombre de neoplasia intraprostática (PIN). Una vez la lesión es instaurada, se le denomina adenocarcinoma localizado que puede extenderse hacia tejidos vecinos extra prostáticos. Si la lesión avanza e invade otros tejidos más alejados de la lesión inicial, genera una metástasis o adquiere un fenotipos más agresivo como el que presentan los tumores resistentes a la castración (18). Durante este proceso las células, consecuencia de la acumulación de mutaciones, van presentando la activación de proto-oncogenes o la inhibición de genes supresores de tumores involucrados en diferentes vías moleculares asociado con el desarrollo de CP (Figura 2).

En el CP se han identificado tres vías de transducción de señales (PI3K/AKT, RAS/MAPK, y STAT3) asociado a la carcinogénesis (Figura 3). En una célula normal la activación de dichas vías de señalización inicia con la unión de los ligandos sobre los receptores de factores de crecimiento, incrustados en la membrana plasmática por ejemplo EGFR, ERBB (HER2) e IGFR. Enseguida estos receptores se autofosforilan o fosforilan complejos proteicos ligados al dominio intracitoplasmático, lo cual desencadena la activación de proto-oncogens (Receptor de andrógenos (AR), TMPRSS2-ERG, TMPRSS2-ETV5) o la inhibición de genes supresores de tumores (CDKN1B, NKX3.1, PTEN), los cuales están asociados con la activación de la proliferación y sobrevida de células tumorales, al igual que el desarrollo de angiogénesis, migración e invasión.

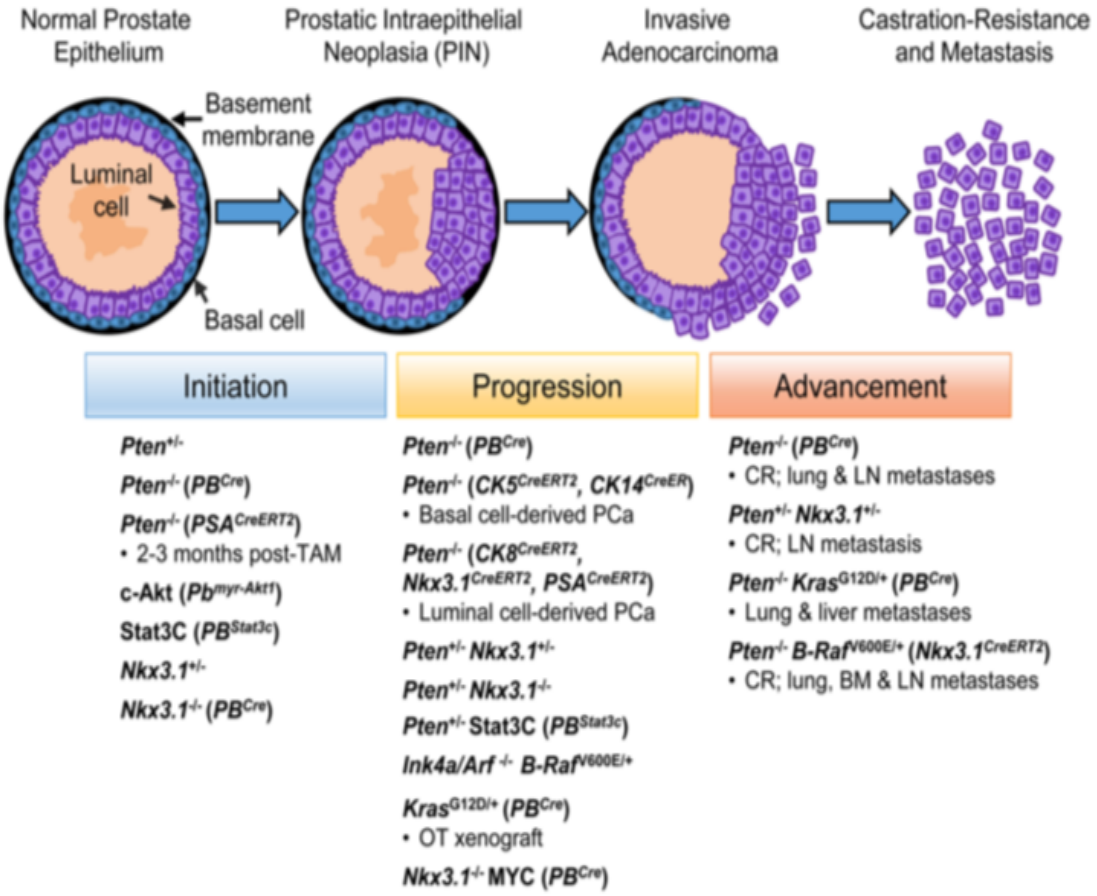

Figura 3. Cambios en la expresión de algunos genes involucrados en la fase de iniciación, progresión y avance del cáncer de próstata. Las primeras alteraciones ocurren en la fase de iniciación en donde se observa a nivel morfológico hiperplasia de células epiteliales (PIN) con la membrana basal intacta. En la fase de progresión se observa pérdida de la membrana basal y transformación en adenocarcinoma invasivo hasta progresar en metástasis o en el fenotipo resistente a la castración. Figura tomada de la referencia (18).

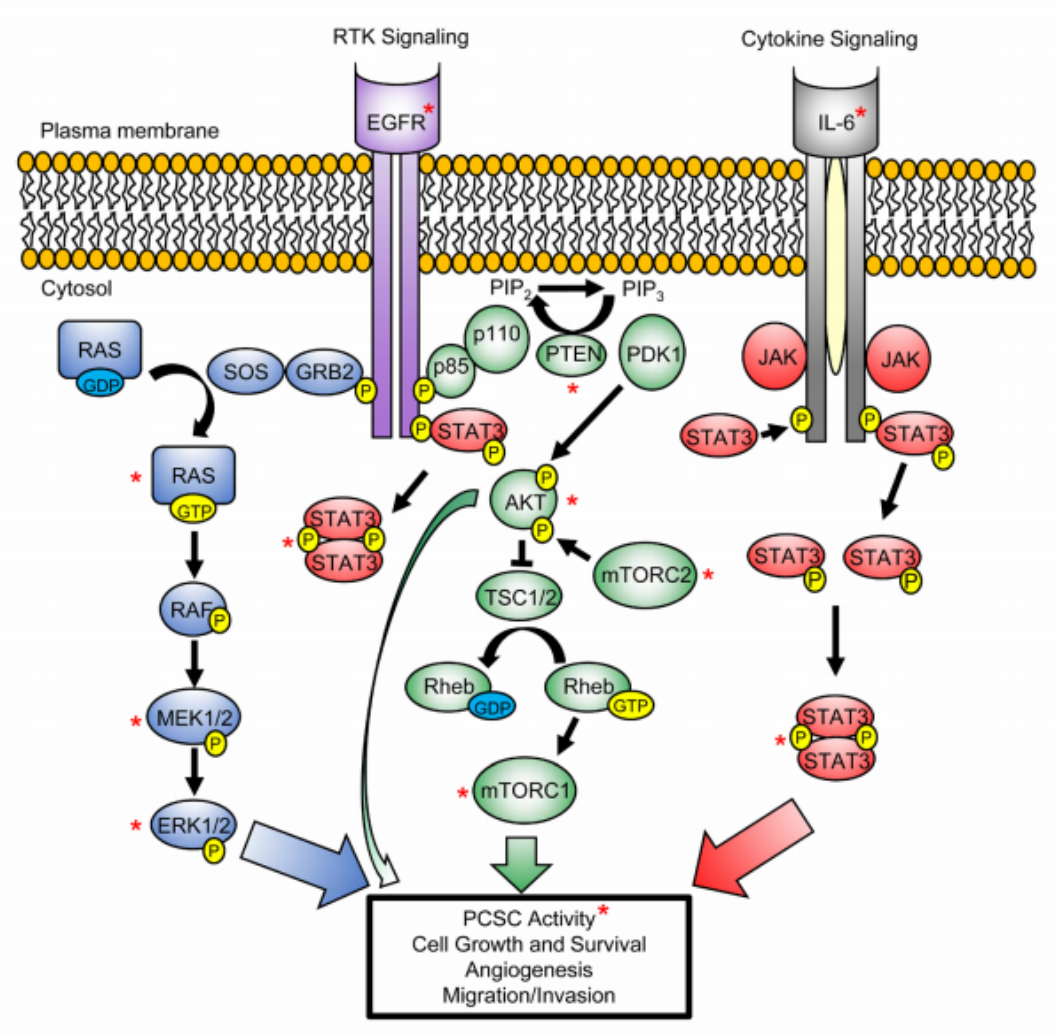

Figura 4. Vías de señalización celular que se encuentran alteradas en el cáncer de próstata (PI3K/ AKT (verde), RAS / MAPK (azul) y STAT3 (rojo)). Figura tomada de la referencia (18).

La vía de señalización más estudiada en CP es la del fosfatidilinositol3-kinasa (PI3K/AKT). La activación normal de la vía se da como consecuencia de la unión de los ligandos respectivos a los receptores de membrana tipo tirosina kinasa (RTK), como los receptores EGFR, ERBB (HER2) e IGFR, los cuales autofosforilan y fosforilan el sustrato del receptor de insulina (IRS), éste último, a la vez, fosforilara la subunidad p85 de la PI3K, el cual proviene de la conversión de PIP2 en PIP3. La quinasa dependiente de fosfoinositol (PDK1) se une a PIP3 y fosforila AKT (19). En el CP, la pérdida de la fosfatasa lipídica responsable de convertir PIP2 de nuevo en PIP3 (PTEN) favorece la activación constitutiva de AKT, que luego fosforila y activa un amplio rango de factores de

transcripción (18,20) involucrados en la apoptosis (BAD, CASP9, FOXO1) y en el ciclo celular (CDKN1B, GSK3B, mTOR) (21). En un modelo murino se describió que el complejo mTORC2 es relevante para el desarrollo de CP en los casos con pérdida de PTEN (22). La pérdida de función de PTEN (gen supresor tumoral) se ha descrito entre el 20-45% de los pacientes con CP, la cual se observa en las fases iniciales de la oncogénesis. En los últimos años se ha observado que alteraciones en este gen tiene una gran relevancia clínica, debido a que los tumores con pérdida de la función por ausencia de la proteína evaluada por inmunohistoquímica de PTEN tienen peor pronóstico, y también se ha reconocido que los casos con GS 1 y 2 con pérdida de la expresión de PTEN son considerados no aptos para un tratamiento clínico conservador como la vigilancia activa (23).

La otra vía molecular importante en CP, es la de RAS/MAPK, se ha observado que niveles elevados de las formas fosforiladas de ERK1/ERK2 se detectan a medida que aumenta el estadio tumoral (24). Se demostró que ERKI1/ERK2 está relacionado con la activación del receptor de andrógenos (AR) (25). También se reportó mutaciones en las tres isoformas de RAS (KRAS, HRAS y NRAS) asociadas con el desarrollo de CP (18,26). Finalmente, se ha descrito que la activación de STAT3 se ha asociado a mayores puntajes GS y estadios tumoral avanzados y también con disminución de la sobrevida, principalmente en los pacientes cuyo tumor presenta un fenotipo resistente a la castración (27,28). IL-6 es un activador de STAT3, en CP se ha reconocido que los pacientes con fenotipos más agresivos tienen mayor aumento de esta interleucina (29).

A nivel nuclear diferentes factores de transcripción y receptores nucleares son activados o inhibidos los cuales se unen a moléculas correguladoras con acción directa o indirecta sobre la transcripción de varios genes para configurar el complejo de inicio de la transcripción en el promotor del gen diana. El receptor nuclear más relevante en CP es AR, el cual se encuentra en el citoplasma unido a las proteínas del choque térmico (HSP), que actúan como inhibidores. Tras la unión de la hormona el receptor se disocia del complejo, se dimeriza y se transloca al núcleo (20). La activación de AKT por pérdida de PTEN genera la activación de AR, el cual se traslada al

núcleo y recluta factores de transcripción y coactivadores para promover el crecimiento, supervivencia, invasión y migración celular (20).

Uno de los factores de transcripción que tienen mayor relevancia en la carcinogenesis del CP es la proteína ERG/TMPRSS2 la cual proviene de una deleción intersticial del cromosoma 21 entre los genes ERG (21q22.2) y TMPRSS2 (21q22.3) (30). Estos dos genes tienen la misma orientación transcripcional, y la fusión se genera por la deleción de una región de aproximadamente 3 megabases en el locus 21q22 (31) que genera dos posibles productos uno es la fusión del exón 1 del gen TMPRSS2 con el exón 4 del gen ERG (T1E4) y otro es la unión del exón 1 de TMPRSS2 con el exón 2 de ERG (T1E2). El primer producto es el más frecuente y está presente entre el 40% y el 70% de los casos de CP. Estas dos gens de fusión representan aproximadamente el 80% de los reordenamientos identificados en CP (32). La expresión de ERG/TMPRSS2 se ha reconocido en las etapas iniciales como neoplasias intraepiteliales del CP y se mantiene hasta la enfermedad avanzada (33,34).

6.6. Biogénesis de los microRNAs (miRNAs)

Los microRNAs (miRNAs) son moléculas de ARN pequeñas (~ 20-22 ribonucleótidos) no codificantes con función regulatoria de la expresión de genes que hacen parte de programas genéticos de diferentes procesos biológicos. Desde su descubrimiento en 1993 en *Caenorabditis elegans* (35) se han descrito aproximadamente 1872 genes que codifican para miRNAs humanos que se procesan en ~ 2578 secuencias de miR's maduros (http://www.mirbase.org) (36). Los miRNAs regulan la expresión del 1 al 5% de todos los genes humanos (37), impidiendo la traducción de los mRNAs blanco (38) o activando su transcripción (39). En las células eucariotas, la regulación de la traducción mediada por los miRNAs ocurre en el citoplasma, en dónde éstos ejercen una acción regulatoria negativa al unirse por complementariedad a las regiones 3' no traducidas (3'UTRs) de los mRNAs sobre los que actúan. La complementariedad total conduce a la destrucción del mRNA, mientras que si es parcial se bloquea el acceso de los ribosomas sobre el mRNA(40). También se ha descrito la presencia de sitios diana para los miRNAs en regiones

codificantes y en la región 5'UTR (41). Recientemente, se ha observado que los miRNAs activan la transcripción mediante la participación en eventos epigenéticos o interactuando con promotores directamente (39,42). Por ejemplo, el miR-205 en CP puede inducir la activación de genes supresores de tumores como IL-24 e IL-32 por la unión a sitios específicos en el promotor (42).

La mayoría de los genes que codifican para miRNAs están localizados en regiones intergénicas, y muy pocos en regiones intrónicas (43). Pueden forman parte de clusters, en cuyo caso son transcritos en unidades policistrónicas, otros genes son transcritos en unidades monocistrónicas (40). Teniendo en cuenta, la ubicación genómica, se les identifica como miRNAs intrónicos en la unidad de transcripción codificante, miRNAs intrónicos en la unidad de transcripción no codificante, miRNAs exónicos en la unidad de transcripción codificante, y miRNAs exónicos en la unidad de transcripción no codificante (40).

La transcripción de los miRNAs es llevada a cabo por la RNA polimerasa II (44), y un pequeño grupo (aproximadamente 50 miR's) depende de la acción de la polimerasa III (45). En el núcleo estas polimerasas sintetizan un RNA primario (pri-miARN) que suele tener entre 60-70 ribonucleótidos de largo con algunas regiones de doble hebra, el cual es modificado por el complejo microprocesador, compuesto por la proteína de unión al RNA DGCR8 (de DiGeorge syndrome critical region in gene 8) y la RNasa de tipo III Drosha, para obtener el precursor pre-miRNA, de aproximadamente 30 ribonucleóidos de largo, el cual se exporta al citoplasma acompañado del complejo Ran / GTP / Exportin 5. Una vez en el citoplasma los pre-miRNAs son procesados por la enzima RNase III Dicer y convertidos en un fragmento dúplex de ~ 20-22 ribonucleótidos. Posteriormente, se une a proteínas tipo Argonaute (AGO), y entonces una hebra del miRNA se degrada, mientras que la otra permanece unida a AGO y luego son reconocidas por las proteína DICER, TRBP y PACT , que en conjunto forman un complejo de silenciamiento miRISC que se une sobre el mRNA que será objeto de regulación (Figura 4) (36,40).

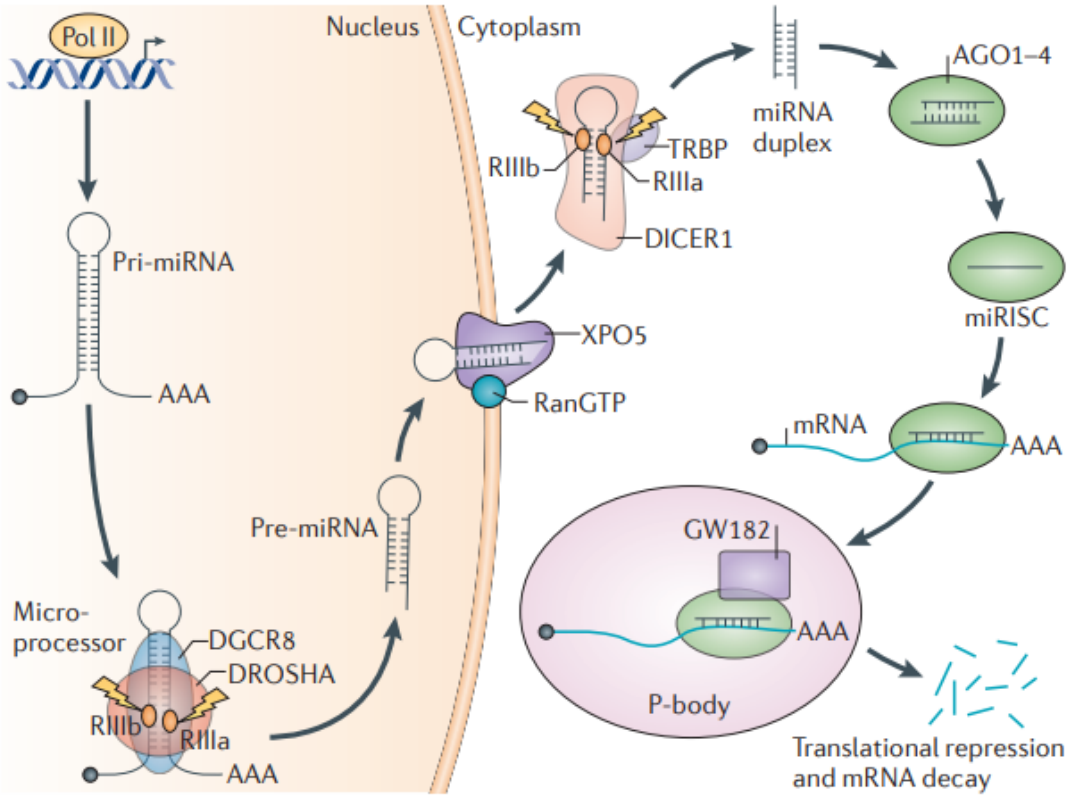

Figura 5. Biogénesis de los miRNAs. Figura tomada de Lin S, et al. *Nat Rev Cancer*. 2015 (46)

Por otro lado, se ha reconocido que los miRNAs no reguladores se integran con el complejo de inicio de la traducción eIF4F, que está compuesto por las subunidades eIF4A, eIF4E y eIF4G. Cuando el miRISC se une a los mRNA diana, un alto grado de complementariedad de miRNA-mRNA facilita la degradación catalizada por AGO de las secuencias de mRNA a través de los mecanismos de escisión del mRNA. La represión de la traducción se puede dar por cualquiera los siguientes cuatro mecanismos: (a) los miRISCs se unen al mRNA diana y evitan el reconocimiento por parte de la subunidad menor del ribosoma o (b) el ensamblaje de la subunidad mayor (c) miRISC puede evitar que el mRNA circularice (estructura que facilita la traducción), o (d) la unión de miRISC a los mRNA diana también facilita la separación prematura de los ribosomas, que reprimen la traducción durante la elongación (40). Otro posible mecanismo, consiste en la capacidad de miRISC para contribuir a la acumulación del mRNA diana en cuerpos de

procesamiento (P bodies). Los P-bodies carecen de cualquier maquinaria de traducción, por lo tanto, el aumento en la acumulación de mRNA sin ribosomas causan represión de la traducción (40,47).

6.6. Desregulación de la expresión de miRNAs en el cáncer

Como se mencionó anteriormente los miRNAs regulan la expresión de genes a través de mecanismos pre y post-transcripcionales. La desregulación de los miRNAs en cáncer puede ser efecto de alteraciones en cromosomas donde se encuentran clústeres de miRNAs, expresión aberrante de factores de transcripción, o cambios en la vía de procesamiento de los miRNAs. Estas alteraciones pueden generar aumento o disminución de la expresión de diferentes miRNAs con diferentes implicaciones moleculares (48,49).

En el año 2002, Croce CM y cols (50) publicaron el primer estudio que mencionó un vínculo entre la desregulación de miRNAs y cáncer, en el cual se mostró que el clúster miR15a / 16-1 se pierde con frecuencia en la leucemia linfocítica crónica, por lo que les atribuyeron una actividad supresora de tumores. En el año 2004, nuevamente el grupo de Croce CM, encontró que una gran cantidad de miRNAs se encontraban ubicados en regiones genómicas previamente asociadas a cáncer (51). Posterior a estos estudios se han descrito una gran cantidad de miRNAs sobre expresados y reprimidos en tumores sólidos como en hematopoyéticos. Para la detección de estos miRNAs se han usado diferentes técnicas como por ejemplo microarreglos de miRNAs, PCR cuantitativa, secuenciación de miRNAs, Northern blot, y NanoString (52).

Se ha descrito que la expresión aberrante de factores de transcripción por sí solo, o asociado a alteraciones cromosómicas (pérdida de la heterocigosidad, amplificación, translocaciones y deleciones) en loci de miRNAs pueden contribuir a la carcinogénesis. Un ejemplo de esto es la amplificación de ch13q31 en linfomas de alto grado (53). Esta alteración aumenta los niveles de expresión del miR17-5p y el miR-20a induciendo baja regulación del factor de transcripción E2F1, esto asociado a la activación del oncogén MYC por parte del clúster miR-17/92, lo que produce

un aumento en la tasa proliferación e induce la angiogénesis (49). Otro ejemplo es la estimulación de la transcripción del gen miR-34 vía p53, la cual fue evidenciada en tumores de ovario con pérdida de p53, en los que se observó disminución de la expresión de miR-34 (54). Otros eventos descritos comprenden alteraciones epigenéticas como por ejemplo en cáncer de mama, la inhibición de la histona deacetilasa clase I y II genera un aumento y disminución de los niveles de expresión de los miRNAs (55), también se ha descrito la represión epigenética de miRNAs que actúan como supresores de tumores. En CP y cáncer de vejiga se ha encontrado que la demetilación del DNA y la inhibición de la deacetilación de histonas activa la expresión de miR-127, el cual actúa como un miRNA supresor de tumores (56). Alteraciones en genes miembros de la maquinaria de la biogénesis de los miRNAs, como por ejemplo Drosha y Dicer, también se han asociado con la generación del cáncer. El 39% de los cánceres de ovario tienen disminuida la expresión de estas dos enzimas (57).

A nivel molecular múltiples miRNAs están implicados en diferentes procesos fundamentales de la oncogénesis al interactuar con blancos relevantes para la proliferación celular (ej.: miR-221/222, miR-663, miR-545), la apoptosis (ej.: miR-17-92, miR-34, miR-122), la angiogénesis (ej.: miR-210, miR-20, miR-519c, miR-424) , la senescencia (ej.: miR-34, let-7a-d, let-7i, mir-15b-16-2, mir-106b-25), la invasión y metástasis tumoral (ej.: miR-155, miR-200, miR-203, miR-9, miR-212) (36,46,58).

Los miRNAs cuya expresión está incrementada se les denomina oncomiRNAs, o en caso de estar disminuida serán los miRNAs supresores de tumores, o con función dual dependiendo del subtipo tumoral (59). El término oncomiRNAs hace referencia a la capacidad que tienen los miRNAs para actuar como oncogenes, es decir que la sobre expresión de estos miRNAs contribuye a la formación de tumores estimulando la proliferación, angiogénesis y/o invasión (60,61). Mientras que los miRNAs supresores de tumores actúan de manera contraria, es decir que si su expresión es baja o nula se favorece la carcinogénesis(60). Entre los miRNAs sobre expresados con relevancia en cáncer se han descrito los siguientes: miR-21, miR-221, miR-181b, miR-200a/b, miR-10b, y miR-196. Y de los que se ha evidenciado pérdida de función con actividad supresora de

tumores son: miR-221/222, miR-181c, miR-31, miR-34, miR-200c, miR-107, miR-126, miR-96, miR-196, y let-7 (48).

El interés por estudiar los miRNAs en oncología ha aumentado en los últimos años, tanto por su rol en la patogénesis como por ser candidatos a biomarcadores con impacto en el diagnóstico, tratamiento y pronóstico (36,46), de hecho se desarrollan ensayos clínicos con numerosos miRNAs como por ejemplo el miR-34a, miR10b, miR-29b, en diferentes tipos de tumor como el de mama, hígado, cerebro, ginecológicos, y hematológicos, entre otros (62). Los miRNAs se conservan relativamente bien en tejidos incluidos en parafina y fijados con formalina, esto se debe a su pequeño tamaño y posiblemente a un microentorno protegido, esto le da ventaja práctica sobre el mRNA (63) para el desarrollo de trabajos de investigación sobre ellos.

6.7 miRNAs en cáncer de próstata

En los últimos años una gran cantidad de miRNAs han sido identificados sobre expresados y/o reprimidos en CP (Tabla 2) (64–66). Estos miRNAs participan en diferentes eventos celulares que están alterados en el cáncer, como por ejemplo el ciclo celular (miR-221/222, miR-4534, miR-650, miR-34a, miR-135-a-1, miR-30a, Let-7), la apoptosis (miR-32, miR-106b/25, miR-125b, miR-34a, miR-204-5p), la regulación de la transición epitelio-mesénquima (miR-21, miR-145, miR-200b, miR-205), la adhesión y movilidad en el sentido que favorecen la metástasis (miR-21, miR-141, miR-145, miR-375, miR-18a, 224,27a) (65). En CP al igual que en los otros tumores, los miRNAs pueden actuar tanto como oncomiRNAs, y miRNAs supresores de tumores, o tener función dual (65). En la Tabla 3 se indica la etapa de la carcinogénesis de próstata en la que se ha identificado la acción de algunos oncomiRNAs y la pérdida de función de miRNAs supresores de tumores.

Tabla 2 Lista de miRNAs con cambio de expresión en tumores prostáticos reportados en diferentes estudios (64–66).

miRNAs sobre expresados	miRNAs reprimidos
let-7a, let-7b, let-7c, let-7d-3p,let-7d-5p, let-7f, let-7i, 7-5p,7b-5p, 9, 10b, 15a, 15b, 16, 17-5p, 18a, 18b, 19a, 19b, 20a-5p, 20b, 21-3p, 24, 25-3p, 26-a1/2, 26b, 27a, 29a, 29b-2-5p, 29c, 30b, 30c, 30d-3p, 31, 32, 34a, 34b, 34c, 92a-3p, 92b-3p, 93-3p, 95, 96-5p, 99b, 100, 101-1, 103a-3p, 106a, 106b, 107, 122, 124a-1, 125a, 125b, 126a-1, 128a, 130b-3p, 133b, 134, 135a, 135b, 141, 142-3p, 143, 145, 146b-5p, 148a, 148b, 149, 151-5p, 153, 174b, 181a, 181b, 181c, 182-5p, 183-3p, 183-5p, 184, 187, 188-5p, 191-5p, 193a-5p, 193b, 194-1/2, 195, 196-1, 196b-5p, 197, 198, 199a-1, 199a-2, 200b-3p, 200b-5p, 200c-3p, 202, 203, 206, 210, 214, 215, 218-2, 221, 222, 223, 296, 297, 301a, 301b, 302b, 320, 326, 329, 331-3p, 339-3p,342-5p, 345, 363, 365, 370, 372, 373, 374a, 374b, 375, 378, 421, 423-3p, 425, 432, 423-5p, 425-5p, 449, 451, 484, 489, 498, 501-3p, 503, 512-3p, 516a-3p, 519c-5p, 520d-5p, 548a-3p, 548c-3p, 551b, 562, 574-3p, 583, 615-3p, 625, 663a, 663b, 664a-3p, 801, 874, 875-5p, 892b, 939, 1224-5p, 1225-5p, 1248, 1249,1274b, 1260a, 1915, 2110, 4534, k12-3, SNORD78, U17b, U78_S, U78_x, UL70-3p	Let-7a, let-7b, let-7c, let-7d, let-7g, 1, 10b, 15a-5p, 16-5p, 19a, 19b-3p, 22-3p, 23a-3p, 23b-3p, 24-3p, 26a-5p, 26b, 27a-3p, 27b-3p, 29a-3p, 29b-3p, 30a-3p, 30a-5p, 30c-5p, 30e-3p, 30e-5p, 31-5p, 33a-5p, 34a-5p, 34b, 34c-3p, 34c-5p, 92, 99a-3p, 99a-5p, 100, 101-3p, 103, 125a,125b-2-3p, 125b-5p, 126, 127-3p, 128a, 130a-3p, 132-3p, 133a, 133b, 136-5p, 137, 139-5p, 141, 142-3p, 143-3p, 145-5p, 146, 149-5p, 150, 152, 154-5p, 155-5p, 181a-2, 181a-5p, 181b-5p, 181d, 184, 187, 193b, 195-5p, 199a-3p, 199a-5p, 199b-5p, 200b-5p, 204, 205-5p, 214-5p, 218-5p, 220, 221-3p, 221-5p, 222-3p, 223-3p, 224, 320, 328, 329, 331-3p, 335-5p, 338-3p, 340, 342-5p, 345, 346, 361-3p, 362-3p, 363-3p, 374a, 374b, 376a-3p,376c-3p, 378, 409-5p, 410, 424-5p, 451a, 455-3p, 455-5p, 483-3p, 485-3p, 487, 490, 494, 497-5p, 499, 502-3p, 505, 520, 551b, 623, 660-5p

Tabla 3. Rol de los OncomiRNAs y miRNA's supresores tumorales identificados en cáncer de próstata

OncomiRNAs	**Acción sobre la carcinogénesis y mantenimiento del cáncer de próstata**
miR-21	Promueve la invasión a otros tejidos e induce el fenotipo resistente a la castración.
miR-18a	Promueve la progresión tumoral.
miR-25/106	Facilita la progresión tumoral.
miR-32	Inhibe la apoptosis y promueve la proliferación.
miR-125b	Aumenta la proliferación celular e inhibe la apoptosis.
miR-141	Contribuye a mantener el fenotipo resistente a la castración, por dependencia del tumor a los andrógenos y también promueve la metástasis.
miR-221/222	Favorece la proliferación, invasión y sobrevida tumoral. Incrementa la clonogenicidad y la tumorigenicidad *in vivo*.
miR-650	Suprime la expresión del gen celular stress response 1 (CSR1) con rol de supresor tumoral.
miR-4534	Induce la aparición de características pre-cancerosas en líneas celulares no cancerosas.
miRNAs supresores de tumores	
Let-7	Promueve la proliferación celular, la migración celular y la transición epitelio-mesenquimática.
miR-17-92a	Disminuye la expresión de proteínas regulatorias del ciclo celular.
miR-27a	Suprime MAP2K4 en células tumorales de CP promoviendo la progresión.
miR-30a	Reduce la expresión de la proteína Ciclina E2.
miR-34a	Evita la detención del ciclo celular, la activación de senescencia y apoptosis. Promueve la invasión celular.
miR-133/146a	Suprime la progresión tumoral por la vía EGFR.
miR-135-a-1	Inhibe la proliferación celular, progresión del ciclo celular, migración, e invasión.

miR-145	Inhibe la invasión, migración y detiene el ciclo celular.
miR-200b	Inhibe el crecimiento de células tumorales y la invasión.
miR-205	Está involucrado en la transición epitelio-mesenquimal regulando la expresión de represores de la e-cadherina como ZEB1 y ZEB2.
miR-204-5p	Promueve la apoptosis por la vía BCL2.
miR-224	Inhibe la invasión, migración y detiene el ciclo celular
miR-372	Inhibe la proliferación, migración e invasión de celular tumorales de CP
miR-382	Inhibe la proliferación, migración, invasión, y metástasis de celular tumorales de CP

Tabla modificada de referencia (65)

6.8 miRNAs en cáncer de próstata de inicio temprano

En la literatura científica muy pocos estudios han evaluado el rol de los miRNAs en el cáncer de próstata de inicio temprano y lo han comparado con el cáncer de próstata de inicio tardío. Weischenfeldt J y cols. (67) se centraron en el análisis de los miRNAs que participan en la vía PTEN, dónde encontraron algunos miRNAs regulados positiva y negativamente. Entre los primeros Ding y cols (68), encontraron varios con expresión desregulada, sin embargo, el análisis bioinformático que reportaron estuvo centrado en los mRNAs y vías moleculares. De los regulados negativamente determinaron que tenían las regiones promotoras hipermetiladas, (hsa-miR-106b-5p, hsa-miR-93-5p, hsa-miR-25-3p, hsa-miR-141-3p).

7. OBJETIVOS

7.1. Objetivo general

- Determinar los perfiles de expresión de miRNAs en cáncer de próstata de inicio temprano y sus posibles genes blancos implicados en la carcinogénesis del cáncer de próstata

7.2. Objetivos específicos

- Comparar el perfil de expresión de miRNAs en tumores de próstata de inicio temprano con los de inicio tardío.
- Comparar los miRNAs hub en tumores de próstata de inicio temprano con los de inicio tardío.
- Identificar miRNAs con cambios en la expresión, las posibles vías moleculares en las que participan y su rol en el desarrollo de cáncer de próstata de inicio temprano.
- Determinar la expresión de uno de los genes que se hallen asociados a lesiones tumorales de próstata de inicio temprano.

8. INTRODUCCIÓN A LOS CAPÍTULOS

El cáncer de próstata de inicio temprano (CPITe) se define en función de la edad de los pacientes en el momento de confirmar el diagnóstico de CP. La edad de corte no está establecida, varios autores han considerado como inicio temprano si el paciente es menor de 50 años al momento del diagnóstico, sin embargo otros estudios lo han establecido en menores de 60, 55 y 45 años de edad (69). La incidencia de CPITe ha aumentado en los últimos años lo que ha llevado a considerarlo un problema emergente de salud pública (9,10). En Estados Unidos en el año 2012 cerca del 10% de los hombres con CP (241.740 personas) fueron diagnosticados antes de los 55 años (70).

En los últimos años se ha generado un gran interés en comprender el comportamiento clínico y las características moleculares de los pacientes con CPITe. Por tal motivo **el primer artículo del primer capítulo** (71) es una revisión narrativa de la literatura sobre la evidencia actual de las características del CPITe en donde se destaca que las características clínicas del CPITe y del CP inicio tardíos (CPITa) son similares, pero en pacientes jóvenes con alto riesgo de desarrollar la enfermedad el tumor es más agresivo. También se describen diferentes características moleculares entre los tumores de pacientes jóvenes y viejos.

Como se mencionó anteriormente, el CPITe se ha convertido en un problema emergente de salud pública (9,10). El aumento del número de casos a lo largo de los últimos años puede ser por los programas de detección temprana del cáncer o por el aumento de factores de riesgo para el desarrollo de la enfermedad en etapas tempranas. En Colombia no existen datos de la prevalencia de CPITe, por tal motivo en el **segundo artículo del primer capítulo** (72) fueron determinadas la prevalencia de CP y de CPITe mediante un análisis demográfico de los datos del Registro de Salud Nacional de Colombia durante los años 2009 y 2019.

En la última década los miRNAs han despertado gran interés para la biología tumoral por su rol tanto en diferentes eventos celulares alterados en cáncer (proliferación celular, apoptosis,

angiogénesis, senescencia, invasión, metástasis) como por ser candidatos a biomarcadores con posible impacto en el diagnóstico, tratamiento y pronóstico de diferentes neoplasias (36,46).

Debido a la relevancia de los miRNAs en la biología tumoral y a los pocos estudios publicados de miRNAs en CPITe (67,73) extrajimos miRNAs de tejido prostático incluido en parafina y fijados con formalina de pacientes con CPITe que ingresaron al Instituto Nacional de Cáncer (NCI) de Estados Unidos (Bethesda). La extracción de los miRNAs se realizó mediante microdisección de tejido prostático. En la **sección de anexos** se copia el artículo aceptado (74) sobre las ventajas y las desventajas de realizar extracción de ácidos nucleicos de tejido prostático por microdisección comparado con la técnica de punch.

En el **primer artículo del segundo capítulo** (75) publicamos los resultados obtenidos al comparar el perfil de expresión de miRNAs de tejido benigno y tejido maligno proveniente de pacientes con CPITe y CPITa. Como resultado final observamos miRNAs diferencialmente expresados entre los grupos, en algunos de estos determinamos que estaban asociados con características clínico-patológicas de mal pronóstico.

Continuando con el objetivo de identificar miRNAs hub, vías moleculares y posibles genes involucrados en CPITe se hizo una revisión sistemática de bases de datos moleculares (GEO, TCGA) para buscar artículos que evaluaran la expresión de miRNAs y mRNA de tumores provenientes de pacientes con CPITe y CPITa. En el **segundo artículo del segundo capítulo** (76) se presentan los datos con el respectivo análisis bioinformático en donde identificamos miRNAs hub característicos de pacientes con CPITe y CPITa. Adicionalmente observamos que las vías moleculares de los dos grupos en la mayoría de los casos fueron similares. Sin embargo, destacamos vías moleculares exclusivas de los tumores provenientes de pacientes jóvenes que son un hallazgo clave que aporta al entendimiento de la patogénesis de estos pacientes. Cuando se analizaron las redes de coexpresión de miRNAs se obtuvo para los jóvenes una red exclusiva mientras que en los viejos hubo dos. Finalmente, con el objetivo de identificar genes blancos correlacionados con miRNAs hub, mediante el uso de herramientas bioinformáticas se

encontraron genes involucrados en diferentes vías, tales como inflamación, MAPKs, metaloproteinasas, entre otros. Adicionalmente el análisis estadístico mostró que algunos genes coexpresados con miRNAs fueron exclusivamente expresados en los tumores provenientes de pacientes con cáncer de próstata de ascendencia africana.

Considerando los datos obtenidos de posible similitud de los pacientes con cáncer de próstata jóvenes y pacientes con cáncer de próstata de ascendencia africana, realizamos un estudio bioinformático similar al del **segundo artículo del segundo capítulo** (76) intentado buscar vías moleculares similares. Para esto hicimos un análisis bioinformático utilizando la base de datos de TCGA para comparar las poblaciones de ascendencia africana y no africana. Se analizaron mRNA, ARN largos no codificantes (lncRNAs), y miRNAs. En el **primer artículo del tercer capítulo** (77) mostramos los resultados obtenidos del análisis de redes y de vías moleculares en donde hay evidencia de expresión diferencial entre los dos grupos y adicionalmente se mostró que la vía de las MAPK estuvo estadísticamente sobrerrepresentada en los pacientes con ascendencia africana, la cual también mostró serlo para los pacientes con CPITe de diferentes ascendencias.

Para lograr el último objetivo de la tesis doctoral acerca de validar experimentalmente la expresión de un gen con rol en la carcinogénesis de próstata de inicio temprano, elegimos el gen que se coexpresó con miRNAs hub de CPITe que participa en las vías moleculares de enriquecimiento. El gen evaluado fue CCR7, que codifica para un receptor de quimiocina necesario para el tráfico y localización de las células T, células B, células asesinas naturales (células NK) y células dendríticas maduras (78). La expresión de CCR7 ha sido poco estudiado en CP, con eventual participación en la promoción de eventos de invasión EMT, angiogénesis y metástasis en varios tipos de tumores, como mama, pulmón, páncreas y esófago entre otros (79), también participa en eventos de metástasis linfáticas y su expresión se relaciona con mal pronóstico (80). Mediante la técnica de inmunohistoquímica se determinó la expresión de CCR7 en tejido prostático de pacientes colombianos con CPITe. Además, tomando información de la base de datos de TCGA (The Cancer Genome Atlas) evaluamos la asociación entre la expresión CCR7 y la presencia de metástasis nodal. En el **primer artículo del cuarto capítulo** reportamos la

expresión de CCR7 en el 72.7% de los tumores provenientes de los pacientes más jóvenes (≤ 50 años) y en los de mayor grado histológico (≥ grade grupo 3). Adicionalmente observamos que todos los pacientes con metástasis presentaron expresión de CCR7 en el tejido las células tumorales. Estos resultados se complementaron con el análisis que se hizo de datos tomados de TCGA de cáncer de próstata en donde se halló que la detección de CCR7 se asoció con la presencia de metástasis (FC: 2.6, p: 0.03).

8.1. CAPÍTULO I

Cáncer de próstata de inicio temprano

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Artículos de Revisión

Early onset prostate cáncer. A new entity?

Cáncer de próstata de inicio temprano. ¿Una nueva entidad?

Rafael Parra-Medina,[1,2,3*] Sandra Ramírez-Clavijo.[2]

Abstract

Prostate cancer (PC) is the second most common type of cancer and the fifth leading cause of death in men worldwide. It has been proposed that PC in young people is a different entity from late-onset PC. The cut-off point to define early-onset PC has not been established, but the term is used when at the time of diagnosis the patient is under 50 years old. In recent years, it has been observed that the clinical compartment may be similar to patients with late-onset PC, however, in high-risk patients it may be more aggressive. Additionally, it has been observed that the molecular mechanisms of oncogenesis are different between young and old. The aim of the present review is to gather the evidence for early-onset PC.

Keywords:
Prostate cancer, Early onset, Young

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Resumen

El cáncer de próstata (CP) es el segundo tipo de cáncer más frecuente y la quinta causa de muerte en hombres a nivel mundial. Se ha propuesto que el CP en jóvenes es una entidad diferente al de inicio tardío. No se ha establecido el punto de corte para definir CP de inicio temprano, pero con frecuencia se refiere al diagnóstico en pacientes menores de 50 años. Se ha observado que las características clínicas del CP de inicio temprano y de inicio tardío son similares, pero en pacientes jóvenes con alto riesgo, la enfermedad puede ser más agresiva. También se ha descrito que los mecanismos moleculares de oncogénesis son diferentes entre jóvenes y viejos. El objetivo de la presente revisión es reunir evidencia de las características del CP de inicio temprano.

Palabras clave:
Cáncer de próstata,
Inicio temprano,
Jóvenes

Introducción

El cáncer de próstata (CP) es el segundo tipo de cáncer más frecuente y la quinta causa de muerte en hombres a nivel mundial. El informe de GLOBCAN de 2018 mostró que la incidencia anual es de 1.3 millones y la mortalidad es de 359 000 casos anuales.[1] La etiología del CP al igual que otros tipos de cáncer es multicausal y se ha asociado con factores como edad avanzada, poseer un componente genético de susceptibilidad, exposición a agentes contaminantes y pertenecer a un grupo étnico. Esos factores son relevantes para calcular el riesgo.[2]

La presencia de CP se sospecha en pacientes con niveles elevados de antígeno prostático (PSA) en sangre y en pacientes con irregularidades en la prueba de tacto rectal. En esos casos se solicita tomar una biopsia para confirmar el diagnóstico por histopatología. El subtipo tumoral más frecuente es el adenocarcinoma convencional (aproximadamente el 99% de los casos), seguido del carcinoma ductal (0.14%), adenocarcinoma mucinoso (0.10%), carcinoma de célula pequeña (0.056%), y otros subtipos histopatológicos menos frecuentes.[3]

El CP se considera una enfermedad asociada a personas mayores con un pico de incidencia del 80% a los 65 años.[4] Sin embargo, desde 1990 se ha reportado un 2% de aumento anual de número de hombres con diagnóstico de CP entre los 15 y 40 años y en autopsias de cohortes occidentales se ha reportado que la prevalencia de CP en individuos con edades entre los 40 y 50 años es del 20 al 30%.[5–7]

El diagnóstico de CP en pacientes más jóvenes se ha relacionado de manera directa con el uso de la prueba para determinar los niveles de PSA como método de tamizaje y detección temprana de la enfermedad. Entre los años 2010 y 2020 la incidencia de CP en hombres menores de 50 años se incrementó cinco veces,[8] lo que llevó a considerar esta enfermedad un problema emergente de salud pública,[5,9,10] por tal motivo además de la detección temprana de la enfermedad también es relevante comprender los

mecanismos moleculares y fisiopatológicos de los pacientes con CP de inicio temprano y tardío. El objetivo del presente artículo fue reunir evidencia del CP de inicio temprano (CPITe).

Cáncer de próstata de inicio temprano

El cáncer de próstata de inicio temprano (CPITe) se define en función de la edad de los pacientes en el momento de confirmar el diagnóstico de CP. El punto de cohorte de la edad no está establecido, pero se considera de inicio temprano si el paciente es menor de 50 años, sin embargo otros estudios lo han establecido en menores de 60, 55 y 45 años de edad.[11] En Estados Unidos en el año 2012 cerca del 10% de los hombres con CP (241 740 personas) fueron diagnosticados antes de los 55 años.[12] En un estudio realizado con los datos obtenidos de SEER (*The Surveillance, Epidemiology, and End Results*) que incluyó el análisis de 30 338 pacientes con CP durante 1998 y 2003, determinó que el 9% eran pacientes ≤55 años. La mayoría eran hombres de origen afroamericano, con menor probabilidad de tener tumores de alto grado y de tener la enfermedad confinada al órgano al momento del diagnóstico.[13] Estudios previos a la era del PSA sugirieron que los pacientes más jóvenes entre 15 y 40 años tenían seis veces más probabilidad de tener una enfermedad metastásica en el momento del diagnóstico que los hombres mayores.[5]

La sobrevida específica de los pacientes con CPITe puede variar según el *Gleason Score* (GS), se ha observado que los pacientes jóvenes con tumores GS 5-7 presentan características clínicas similares a los pacientes con CP mayores de 55 años. Sin embargo, los pacientes con CPITe que presentan la enfermedad localmente más avanzada o con GS 8-10 tienen la probabilidad de morir 3 veces más pronto que los pacientes mayores de 55 años.[13] Otros estudios han mostrado que los tumores de los pacientes con CPIT tienen un comportamiento biológico más agresivo y una puntuación GS más alta.[10,14–16] Mientras que otros estudios no reconocen diferencias significativas en la supervivencia entre los grupos de edad después de los tratamientos prostatectomía, braquiterapia y radioterapia.[10,17,18]

En un reciente meta-análisis que incluyeron 39 1068 pacientes con CP de Alemania, Australia, Brasil, Canadá, Corea, Italia, y Estados Unidos a los cuales se les hizo prostatectomía radical en la era posterior al uso de PSA, observó que la incidencia en menores de 50 años fue de 2.6% y 16.6% con una mediana de 8.3%. Los pacientes jóvenes mostraron una mejor favorabilidad en las variables clínico-patológicas con mejor pronóstico de recurrencia bioquímica. En el metaanálisis también encontraron que el pronóstico de la recurrencia bioquímica es mejor en los jóvenes (HR:1.38 (1.09-1.74)),[11] en este análisis se incluyeron tres estudios.[19–21] Sin embargo, en los jóvenes con CP de alto riesgo se observó asociación con peores desenlaces oncológicos.[11] En una cohorte retrospectiva de 28 039 pacientes de Taiwán con CP, se observó que los pacientes con CP ≤54 años tuvieron mayor riesgo de mortalidad (HR:1.22 (1.10-1.49)) principalmente los catalogados como CP de alto riesgo o con presencia de metástasis.[22]

Factores de riesgo asociados al cáncer de próstata de inicio temprano

Se han identificado diferentes factores de riesgo relacionadas con el desarrollo de CPITe: el

grupo étnico al que se pertenece, la historia familiar, factores genéticos y ambientales y obesidad.[23] Se ha descrito que la incidencia de CP es mayor en afroamericanos comparado con los caucásicos, y en los primeros al momento del diagnóstico se encuentran en estadios más avanzados de la enfermedad (GS alto) por tal motivo tienen una mayor mortalidad.[24–26] Con relación a la historia familiar la presencia de la enfermedad en familiares de primer grado aumenta 2-3 veces el riesgo a desarrollar CP, y el riesgo incrementa para hombres jóvenes con familiares de primer grado afectados (padre afectado HR: 6.14, hermano afectado HR 6.62, más de tres hermanos afectados HR 23).[27,28] También se ha determinado que si el padre tiene la enfermedad antes de los 60 años, el riesgo relativo (RR) para desarrollar la enfermedad aumenta 1.5 a 2.5 veces para sus hijos.[29,30]

Entre los factores genéticos, se han descrito polimorfismos de nucleótido simple (SNPs), estos han sido identificados a partir de la comparación de ADN de pacientes con CPITe y de pacientes con CP de inicio tardío (rs9364554, rs10486567, rs6465657, rs6983267, rs1447295, rs1571801, rs10993994, rs4962416, rs7931342, rs4430796, rs1859962, rs2735839, rs5945619). Estos polimorfismos se han asociado con un mayor riesgo para el desarrollo de CP clínicamente más agresivo.[31]

Por otro lado, las mutaciones en línea germinal, más comunes, asociadas con el desarrollo y mayor agresividad tumoral de CPITe son BRCA1 (RR: 1.8-4.5), BRCA2 (RR: 23) y HOXB13 (OR 6.6).[32] BRCA1 y BRCA2 son genes que se expresan en todas las células del cuerpo y su principal función es participar en mecanismos de reparación esenciales para el mantenimiento de la integridad del genoma. Estos genes forman parte de conjunto de componentes que reparan roturas de doble cadena en el ADN por recombinación homóloga (HRR), que utiliza como molde la cromátide hermana no dañada para llevar a cabo la reparación del ADN.[32] BRCA1 y BRCA2 también intervienen en la activación de puntos críticos de control del ciclo celular como en G1/S y S/G2. Esto lo hacen mediante la formación de distintos complejos como BRCA1/BRD1 en G1/S, en este punto se requiere la fosforilación de BRCA1 por ATM o ATR, para facilitar la fosforilación de p53,[33] la cual evita la degradación de p53 y facilita la detención del ciclo celular en G1/S mediante su acción indirecta sobre p21 (a member of the Ink4a/Cip1 family of cyclin-dependent kinase (CDK) inhibitors).[33] Por lo tanto, alteraciones en BRCA impiden que las células reparen el DNA, se evada el punto de control G1/S en el ciclo celular y se promueva la tumorogénesis en tejidos distintos, como la glándula mamaria, el ovario, próstata, páncreas, entre otros.[33–35] BRCA1 y BRCA2 son particularmente sensibles a los inhibidores de poli-ADP ribosa polimerasa (PARPi), como talazoparib y olaparib. Además, la deficiencia de HRR asociada a mal funcionamiento de BRCA (HRD) hace que los tumores sean particularmente sensibles a la quimioterapia basada en platino.[36]

En cuanto al gen HOXB13, este codifica para un factor de transcripción de la familia de los genes homeobox, los cuales están relativamente bien conservados en vertebrados y son esenciales para el desarrollo embrionario. En edad adulta también se ha visto que puede expresarse y tienen roles en la proliferación (ciclo celular), apoptosis, diferenciación y en mecanismos de migración celular.[37] La presencia de mutaciones en los genes BRCA1/2 en CPITe han sido asociadas con mayor agresividad del tumor mientras que mutación en

el gen HOXB13 lo han sido con un pronóstico favorable.[23] En general mutaciones en BRCA2 se han reportado en el 2% de los pacientes con CPITe.[38]

La infección por el virus del papiloma humano, la cual ha aumentado en los últimos años en los jóvenes,[5] se ha descrito como un factor biológico inductor de cáncer. También presentar obesidad es un factor de mayor riesgo de recurrencia bioquímica después de la prostatectomía, de una mayor agresividad y aumento de la mortalidad específica por cáncer de próstata, todo esto posiblemente debido a la baja cantidad de andrógenos secundario a la obesidad expresión de adipoquinas y a la hiperinsulinemia asociada con aumento de los niveles séricos de IL-17 y factor de crecimiento similar a la insulina 1 (IGF1), los cuales aumentan la expresión de genes proinflamatorios.[5,39,40]

Oncogénesis del cáncer de próstata de inicio temprano

Aunque no se conoce el mecanismo mediante el cual se originan las lesiones de CPITe, se ha propuesto que la etiopatogenia es diferente a los CP de viejos e incluso se ha propuesto que los pacientes con CPITe pueden tener un componente hereditario de susceptibilidad.[41] La carcinogénesis es causada por la compleja interacción de múltiples factores ambientales y hereditarios que mantienen estados de inflamación crónica, activación permanente del receptor de andrógenos (AR) y la inestabilidad genómica, eventos que se encuentran bajo el estricto control de vías de señalización celular.

En la oncogénesis de CP, participan tres vías de transducción de señal: vía del phosphatidylinositol 3-kinase PI3K/AKT, RAS/MAPK, y STAT3,[42] en las cuales se ha descrito la participación de diferentes oncogenes con función en el crecimiento y en la sobrevida de células tumorales. La activación de estos oncogenes cambia en relación con el estadio de la lesión, bien sea en la fase de iniciación, o en los casos de una neoplasia intraprostática (PIN), en el adenocarcinoma invasivo, en los casos de metástasis o en los tumores resistentes a la castración.[42]

La vía del PI3K/AKT también se relaciona con la resistencia a la castración. Esta vía se activa por acción de la unión de ligandos sobre receptores de membrana con actividad tirosina cinasa (RTKs), y sin actividad tirosina cinasa (no RTKs), que activan la cinasa PI3K, que a su vez actúa sobre el fosfatidilinositol 4,5-bisfosfato (PIP2), presente en la membrana y genera el segundo mensajero fosfatidilinositol (3-5) -trisfosfato (PIP3). PIP3 actúa como un ligando de alta afinidad para reclutar hacia la membrana proteínas que contienen el dominio de homología de pleckstrina (PH), incluidas las quinasas AKT/PKB. AKT sufre un cambio conformacional inducido por PiP3, que la hace blanco de fosforilación en la treonina 308, por la quinasa 1 dependiente de fosfoinosítido constitutivamente activa (PDK1) y en la serina 473 por la diana de mamífero del complejo de rapamicina 2 (mTORC2) o PDK2.[43,44] AKT fosforila diferentes proteínas inhibiéndolas (Caspasa 9, FKHR, BAD, GSK3b) o activándolas (Mdm2, IKKa). Esta vía es bloqueada por la fosfatasa lipídica y homólogo de la tensina (PTEN).[45] La pérdida de PTEN ocurre en aproximadamente el 40% de cánceres de próstata avanzados y no solo promueve directamente crecimiento del cáncer, sino que también promueve la inflamación e inmunosupresión.[45] En modelos murinos deficientes en PTEN en el epitelio prostático,

presentaron activación amplificada de AKT en lesiones tipo PIN y resistencia a la castración.[46] En hombres obesos se ha demostrado que AKT reduce la degradación proteasómica de IL-17 por lo que esta se acumula y se observa un aumento de la expresión de genes proinflamatorios, sobre los cuales ejerce un control transcripcional, incluso el de su propio receptor (IL-17RA).[47]

La vía de las proteínas cinasas activadas por mitógeno (MAPK), está compuesta por quinasas tipo serina-treonina, que se activan en cascada y fosforilan diversos sustratos. Está compuesta por tres rutas que controlan varios procesos celulares diferentes, como la expresión génica, el metabolismo, la motilidad, la proliferación y la apoptosis. En cada ruta el evento inicial ocurre en la membrana y se relaciona con proteínas monoméricas que hidrolizan GTP, la ruta que interpreta señales extracelulares y es regulada por cinasas (ERK 1/2) está asociada a Ras, la liderada por la cinasa c-Jun N-terminal (JNK) cuenta con la participación de Rac y Rho y la de la proteína p38 con Rac.[48] Alteraciones en los componentes de la vía, pueden causar el bloqueo, pérdida de la función o activación permanente de la vía, que pueden favorecer la oncogénesis. La activación de ERK1/2 promueve la metaplasia y el desarrollo de tumores, mediante la fosforilación de FOXO3a sobre las serinas 294, 344 y 425 y facilita la interacción FOXO3a-MDM2. Esta interacción mejora la degradación de FOXO3a a través de una ruta de ubiquitina-proteasoma dependiente de MDM2, lo que conduce al desarrollo de tumores. Entre las proteínas fosforiladas en esta ruta están la cinasa de cadena ligera de miosina, calpaína, quinasa de adhesión focal y paxilina promueve la migración de células cancerosas.[48]

Altos niveles de actividad de la ruta JNK en CP, se han relacionado con tumorigénesis, evasión de la respuesta inmune mediada por interferón-γ (IFN-γ) y el factor de crecimiento tumoral beta (TGFβ) en CP y movilidad celular que favorece la metástasis.[46] JNK en conexión con otras vías como la del receptor de andrógenos (AR), el factor nuclear kappa potenciador de las cadenas ligeras de células B activadas (NF-κB) y la proteína p38 desempeñan funciones vitales en la regulación de la apoptosis y supervivencia de las células cancerosas.[49] La ruta JNK y PTEN, cooperan para regular la progresión e invasión de del adenocarcinoma de próstata.[46]

Por otro lado, p38 se activa en las células en respuesta a múltiples señales como las causantes de estrés, exposición a radiación UV, aumento de la temperatura, choque osmótico, proinflamatorias (TNFα, IL-6 o IL-1) o citocinas antiinflamatorias (EGF, TGF-β). p38 fosforila y regula muchos factores de transcripción (incluidos ATF-2, NF-κB, Elk-1, Max, MEF-2, Mac, p53 o Stat1) y otros mediadores apoptóticos y del ciclo celular (por ejemplo, Cdc25A, Bcl-2).[46] En el epitelio prostático canceroso humano se ha encontrado a p38 sobreexpresada y se ha relacionado con aumento de la actividad del AR, y mayores supervivencia, clonogenicidad, movilidad e invasividad en células de cáncer de próstata.[50]

Las proteínas transductoras de señales y activadoras de la transcripción (STAT) se han asociado con la tumorigénesis de la próstata, en particular la proteína STAT3. La activación de esta proteína se da por fosforilación en la tirosina 705 y niveles aumentados de activación están relacionados con una puntuación de Gleason más alta, una menor supervivencia en los pacientes, tasas de supervivencia sin re-

cidiva más bajas. Se observaron niveles séricos elevados de IL-6, un activador conocido de la señalización de STAT3, en pacientes con CP metastásico en comparación con pacientes con formas benignas o no malignas. La activación de STAT3 juega un papel vital en la capacidad de autorrenovación y propagación de tumores de originados por células madre tumorales de próstata (PCSC). La proteína supresora de tumor p19ARF, que actúa como inhibidora del ciclo celular, es un blanco de STAT3, la interrupción de esta relación está asociada con mayor progresión hacia la metástasis en CP. [51]

The Cancer Genome Atlas Research Network (TCGA) es una red de instituciones que dirige su esfuerzo a explorar sistemáticamente todo el espectro de cambios genómicos que ocurren en el cáncer humano. En el año 2018 completó el análisis de 33 tipos de cáncer diferentes entre ellos el cáncer de próstata. Varios estudios en CP describen diferencias en reordenamientos estructurales a nivel genético, entre el CPITe y el CPITa. En el estudio de TCGA de CP se analizaron 67 pacientes diagnosticados con CPITe (≤55 años), se encontró que el 68% eran del subtipo molecular ERG, seguido del subtipo sin otras alteraciones moleculares (28%), ETV1 (9%), SPOP1 (8%), IDH1 (3%), ETV4 (1%), FOXA1 (1%), y FL1 (1%).[52] En otro estudio a 11 pacientes con CPITe se les practicó pruebas de RNAseq, metiloma del DNA, secuenciación de genoma completo, secuenciación transcriptómica, y expresión de miR'S,[53] Los principales hallazgos fueron alteraciones en la vía relacionada con la acción de los andrógenos y presencia de un gen de fusión ERG/TMPRSS2. Weischenfeldt *et al.*, encontraron aumento en la frecuencia del número de reordenamientos estructurales del gen de fusión TMPRSS2:ERG en CPITe.[53] En pacientes con CPITe de origen europeo y norteamericano se encontró mayor nivel de expresión del AR y de ERG en el 64% de los pacientes.[53–55] El gen de fusión TMPRSS2:ERG se ha asociado principalmente con GS≤7. Pérdida de la función de PTEN también se ha identificado en pacientes con CPITe principalmente con positividad para ERG.[56] La presencia de alteración de ERG se ha asociado con bajos niveles de PSA,[54] escala de GS bajo (≤ 7),[55,56] y mayor recaída bioquímica (aumento de los niveles de PSA).[55]

El mismo grupo de investigadores también identificó cuatro subtipos moleculares en los tumores de pacientes con CPITe que incluían uno particularmente agresivo con duplicaciones 8q22 recurrentes) y las relacionaron una mayor expresión de ESRP1.[57]

En el estudio de Ding *et al.*, en 49 pacientes con CP,[58] en donde incluyeron 25 pacientes con CP ≤45 años, identificaron genes diferencialmente expresados en los jóvenes comparado con los viejos entre los cuales se encontró genes que participan en vías de inflamación y respuesta inmune, sobreexpresados significativamente como CTLA4 e IDO1/TDO2, los cuales también se asociaron con recurrencia bioquímica.

Otro estudio en donde incluyeron 1281 pacientes con CPITe (≤60 años) identificaron 23 genes únicos de reparación del ADN asociados con una mayor predisposición o riesgo de enfermedad agresiva. La expresión de cuatro genes (BRCA2, MSH2, ERCC2 y CHEK2_non-1100del) se asociaron con una enfermedad más agresiva.[59]

Todo lo anterior hace pensar que en el desarrollo de CP participan mecanismos moleculares diferentes, lo que podría establecer diferencias entre la tumorigénesis del CPITe con respecto al CP de inicio tardío.

Los miRNAs en el cáncer de próstata de inicio temprano

Los miRNAs son moléculas pequeñas (~20-22 nucleótidos) de ARN no codificante con función regulatoria de la expresión génica en procesos biológicos tumorales y no tumorales.[60] El interés en los miRNAs y su relación con CP ha aumentado en los últimos años debido a que han sido considerados como buenos candidatos a actuar como buenos candidatos a actuar como biomarcadores de diagnóstico, pronóstico y posibles blancos de tratamiento.[61–63]

Estudios de expresión diferencial de miRNAs (microRNAs) entre el CPITe y de inicio tardío han identificado varios posibles biomarcadores de la enfermedad. Weischenfeldt *et al.*, centraron su análisis en los miRNAs implicados en la vía PTEN.[53] Detectaron algunos regulados positivamente y otros regulados negativamente, y algunos de estos con regiones promotoras hipermetiladas, particularmente genes supresores de tumores, exhiben expresión reducida (hsa-miR-106b-5p, hsa-miR-93-5p, hsa-miR-25-3p, hsa-miR-141-3p).

Ding *et al.*, encontraron varios miRNAs expresados diferencialmente sin embargo, centraron su análisis en los RNA mensajeros de genes que tienen un rol proinflamatorio.[58] Adicionalmente, Valera *et al.*, encontraron miRNAs diferencialmente expresados en tejido tumoral de pacientes con CPITe en comparación con los pacientes con inicio tardío, así como en tejido tumoral en comparación con tejido normal.[64] Adicionalmente identificaron expresión de miRNAs que fue asociada con una puntuación de Gleason alta, extensión extraprostática de la lesión e invasión linfática.

Recientemente en un análisis bioinformático en nuestro grupo observamos que los tumores de pacientes con CPITe expresan diferentes miRNAs en comparación o con los de CP de inicio tardío, de los cuales unos fueron exclusivos y otros centrales (hub).[65] Los miRNAs exclusivos en pacientes jóvenes (hsa-miR-3065, 146b, 676, 32, 96, 10a) fueron asociados con menor sobrevida o recaída tumoral. Los miRNAs centrales (hsa-miR-142-5p, 150-5p, 146b-3p) se coexpresan con genes asociados a severidad de la enfermedad (MMP9, CCR7, CCL21, IL7R, entre otros), que además han sido previamente identificados en tumores de pacientes afroamericanos.

Hallazgos histopatológicos en tumores de próstata de inicio temprano

En las pocas series que han sido publicadas de pacientes con CPITe se ha reportado que la mayoría presentan un adenocarcinoma de tipo convencional con un GS ≤ 7 (77% al 98%) además de alteraciones en la expresión de ERG y PTEN como se comentó previamente.[52,55,66] En pacientes con mutación en el gen BCRA2 se encontró que presentan generalmente adenocarcinoma acinar prostático asociado a carcinoma intraductal prostático en el 42%,[67] mientras que en los casos con mutación de HOXB13 se asoció principalmente con adenocarcinoma acinar prostático de bajo grado y la presencia de la variante histopatológica pseudohiperplásica (45%).[66]

Tratamiento

Por ahora, no hay consenso para diferenciar el tratamiento de los pacientes jóvenes y viejos. En los jóvenes con la enfermedad confinada al

órgano, la prostatectomía radical se ofrece cada vez más que las alternativas basadas en la radioterapia.[5] Las técnicas de conservación de nervios para preservar la continencia y la función sexual son posibles. La cirugía robótica puede necesitar más consideración en los jóvenes, aunque toleran los procedimientos quirúrgicos abiertos mejor que los hombres mayores.[5]

Teniendo en cuenta que los últimos estudios muestran que los pacientes con CPITe con alto riesgo o con metástasis tienen peor pronóstico que los viejos, estos pacientes deberían tener una aproximación terapéutica más agresiva.[11,22]

Conclusión

La presente revisión de la literatura ofrece elementos que señalan el aumento en la prevalencia de CPITe en los últimos años, probablemente por la búsqueda activa de la enfermedad. Lo que ha despertado el interés por dilucidar las características moleculares de la enfermedad en pacientes con CPe, a pesar de que el comportamiento clínico es similar al de pacientes con cáncer de próstata de inicio tardío, sin embargo, cuando el CP es de alto riesgo el comportamiento clínico es más agresivo. La evidencia muestra que desde el punto de vista molecular, la enfermedad puede ser diferente al cáncer de inicio tardío lo cual abre muchos interrogantes para continuar con el estudio de los mecanismos moleculares que intervienen en la tumorigénesis, respuesta al tratamiento y pronóstico de esta enfermedad.

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Prevalence and Demographic Characteristics of Prostate Cancer Patients in Colombia: data from the National Health Registry from 2015 to 2019

Prevalencia y características demográficas de pacientes con cáncer de próstata en Colombia: datos del Registro de Salud Nacional de 2015 a 2019

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Abstract

Background and Objective Prostate cancer is a multifactorial disease and is among the top five causes of death in men worldwide. The Colombian Ministry of Health has adopted the Integrated Information System on Social Protection (Sistema Integrado de Información de la Protección Social, SISPRO, by its Spanish acronym) registry to collect comprehensive information from the Colombian health system. The system provides close to universal coverage (around 95%). We aimed to establish the prevalence of prostate cancer in Colombia and to describe its demographics, based on data provided by SISPRO, openly available for scientific analysis.

Methods Using the SISPRO data from 2015 through 2019, we analyzed the prevalence and demographic characteristics of patients diagnosed with prostate cancer.

Results We identified a total of 43,862 patients with prostate cancer in the 5-year period and estimated a prevalence of 4.54 cases per 1,000 habitants, using as denominator males over 35 years old. We calculated a prevalence of early-onset prostate cancer (i.e., 35–54 years) of 0.14 per 1,000 habitants (791 cases in 5 years). The highest prevalence was observed in patients > 80 years (33.45 per 1,000 habitants). The departments with the highest prevalence were Bogotá, Valle del Cauca, Risaralda, and Boyacá, and the region with the lowest prevalence was Amazonas.

Keywords
- prostate cancer
- prevalence
- Colombia
- Latin America
- early-onset

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Conclusion We describe the prevalence and demographics of prostate cancer in Colombia using the national healthcare system database. We observed that the prevalence has been increasing over time, and the distribution is variable according to regions, which may be related to racial or environmental causes, or access to the urologist. These factors should be addressed in further studies.

Resumen

Antecedentes y Objetivo El cáncer de próstata es una enfermedad multifactorial, y se encuentra entre las cinco principales causas de muerte en hombres a nivel mundial. El Ministerio de Salud de Colombia ha adoptado el Sistema Integrado de Información de la Protección Social (SISPRO) para la recopilación de la información integral del sistema de salud colombiano. El sistema proporciona una cobertura casi universal (alrededor del 95%). El objetivo de este estudio fue establecer la prevalencia del cáncer de próstata en Colombia y describir su demografía, con base en los datos proporcionados por el SISPRO, disponibles de forma abierta para el análisis científico.

Métodos Utilizando los datos del SISPRO de 2015 a 2019, se analizaron la prevalencia y las características demográficas de los pacientes diagnosticados con cáncer de próstata.

Resultados Se identificó un total de 43,862 pacientes con cáncer de próstata en el período de 5 años, con una prevalencia de 4,54 casos por cada mil habitantes, utilizando como denominador hombres mayores de 35 años. La prevalencia de cáncer de próstata de inicio temprano (es decir, paciente de 35 a 54 años) fue de 0.14 por mil habitantes (791 casos en 5 años). La mayor prevalencia se observó en pacientes >80 años (33,45 por mil habitantes). Los departamentos con mayor prevalencia fueron Bogotá, Valle del Cauca, Risaralda, y Boyacá. Y la región con menor prevalencia fue Amazonas.

Conclusión Describimos la prevalencia y la demografía del cáncer de próstata y el cáncer de próstata de inicio temprano en Colombia utilizando la base de datos del sistema nacional de salud. Observamos una distribución desigual de la prevalencia entre las regiones, que puede estar relacionada con factores raciales, ambientales, o de acceso, que justifican más estudios.

Palabras clave
- cáncer de próstata
- prevalencia
- Colombia
- América Latina
- inicio temprano

Introduction

Prostate cancer (PCa) is a multifactorial disease that is considered a public health issue. According to the Global Cancer Observatory (GLOBOCAN), 1,276,106 new cases of PCa were registered worldwide, representing 7.1% of all cancers in men.[1,2] Age is a major risk factor and a median age of diagnosis of 66 years has been reported. Other well-established non-modifiable risk factors are genetic susceptibility, black ethnicity, and family history. Its association with other modifiable factors, such as chemical contaminants, diet, and medications, among others, is not yet clear.[3]

The annual incidence of PCa has been increasing worldwide thanks to better cancer surveillance programs. The screening for and management of early PCa are some of the most challenging and controversial issues in medicine.[4] In the last years, several countries in Latin America, including Brazil, Chile, Costa Rica, Mexico, Peru and Colombia, made PCa a public health priority in their national cancer plans; guidelines for early detection, diagnosis, treatment and follow-up have been developed.[5,6] In Colombia, PCa is the most frequent cancer in men and the second cause of mortality in the male population, with a mortality rate of 12 per 100,000 people.[1]

The present study aimed to establish the prevalence of PCa in Colombia and to describe its demographics, based on data provided by the Integrated Information System on Social Protection (Sistema Integrado de Información de la Protección Social, SISPRO, by its Spanish acronym). This system is the official administrative registry of the Colombian Ministry of Health.

METHODS

Database Search

Colombia has one of the widest health coverages in Latin America: 95.97% of the 50.5 million inhabitants as of April 2020, according to official data from the Colombian Ministry of Health.[7] The Ministry developed an information database called SISPRO, which stores and processes the basic

data that the system requires for its regulation and control processes. Demographics and clinical data are grouped in the Individual Health Services Delivery Registry (Registro Individual de Prestación de Servicios de Salud, RIPS, by its Spanish acronym), which are collected by medical staff during each outpatient or inpatient medical contact. This consolidated registry receives input from different sources: both private and public health providers, insurers, and other sources, such as RIPS, the Unified Affiliate Registry (Registro Único de Afiliados, RUAF, by its Spanish acronym), the System of Public Health Surveillance (Sistema de Vigilancia en Salud Pública, SIVIGILA, by its Spanish acronym), high-cost account, among others. These databases are publicly available for scientific analysis (http://www.sispro.gov.co/); the information to carry out this study was retrieved from the online dynamic tables. We have described searching methods previously.[8–11] Due to recent updates on the database, the currently available information comprises only the period between 2015 and 2019. Briefly, we obtained the information for the whole country, for the period between January 1st 2015 and December 31th 2019. We analyzed the RIPS database using the International Classification of Diseases code for PCa (ICD-10 code: C61). ICD-10 codes: D075 (In situ carcinoma of the prostate) and D400 (tumor of uncertain or unknown behavior of the prostate) were not included to avoid bias. Following the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines, we analyzed the distribution in 5-year age groups, according to the data from the most recent census, which was performed in 2018 with results published in the second semester of 2019.[12]

Prevalence per 1,000 inhabitants was calculated using as numerator the number of males diagnosed with PCa (counted once). The denominator was the number of inhabitants reported by the National Administrative Department of Statistics (Departamento Administrativo Nacional de Estadística, DANE, by its Spanish acronym) in each age group or geographical area. To calculate the prevalence, we included only men older than 35 years. We selected 35 years as the age threshold, as PCa diagnosis below this age is extremely rare; thus, the recorded cases under this age are probably due to codification errors, and their inclusion would imply that the prevalence calculation would be based on a larger denominator that could underestimate the real prevalence. Besides, we determined the prevalence of early-onset PCa (EO-PCa), defined as PCa diagnosis in patients < 55 years (but older than 34 years), as mounting evidence supports differential pathophysiology that warrants further research.[13] Data were recorded and analyzed using Microsoft Excel (Microsoft Corp., Redmond, WA, USA).

RESULTS

After removing cases under 35 years of age ($n = 51$), a total of 43,862 cases of patients with a primary diagnosis of PCa (both from outpatient and hospital services) were attended at some point during the 5 years (2015–2019). ►**Table 1** shows the number of cases per year divided by age group. The unadjusted prevalence in the 5-year period was 4.54 cases per 1,000 habitants. The highest prevalence was observed in patients > 80 years (33.45 per 1,000 habitants). The prevalence of EO-PCa was 0.14 cases per 1,000 habitants. ►**Fig. 1** shows the number of cases by age group and by year, with the highest being in 2019.

Regarding the distribution of PCa by departments, the prevalence ranged from 0.42 to 6.45 per 1,000 habitants. The departments with the highest prevalence were Bogotá (capital city of Colombia), Valle del Cauca, the Colombian coffee trail (Risaralda) and Boyacá. The lowest prevalence was observed in the region of Amazonas (Amazonas, Vaupés, Guaviare, and Vichada) (►**Table 2** and ►**Fig. 2**).

DISCUSSION

Colombia has a national health system that serves 63,000 new cases of cancer annually.[14] The most prevalent malignancies in

Table 1 Patients with a main diagnosis of prostate cancer according to age between 2015 and 2019

Age group (years)	2015	2016	2017	2018	2019	5-year total cases	x1,000
35–39		4	5	7	9	22	0.01
40–44	2	6	11	9	17	37	0.03
45–49	17	20	21	47	63	123	0.09
50–54	65	84	146	278	384	609	0.47
55–59	250	278	492	805	1,079	1,759	1.55
60–64	719	796	1,090	1,805	2,349	4,002	4.42
65–69	1,321	1,381	1,835	3,044	3,751	6,612	9.61
70–74	1,832	1,900	2,430	3,974	4,674	8,495	17.25
75–79	2,092	2,119	2,404	3,825	4,426	8,506	26.18
80–older	4,205	3,890	3,765	5,371	5,700	13,697	33.45
Total	10,503	10,478	12,199	19,165	22,452	43,862	4.54

Note: Numbers do not add up since patients might be included in more than one cell.

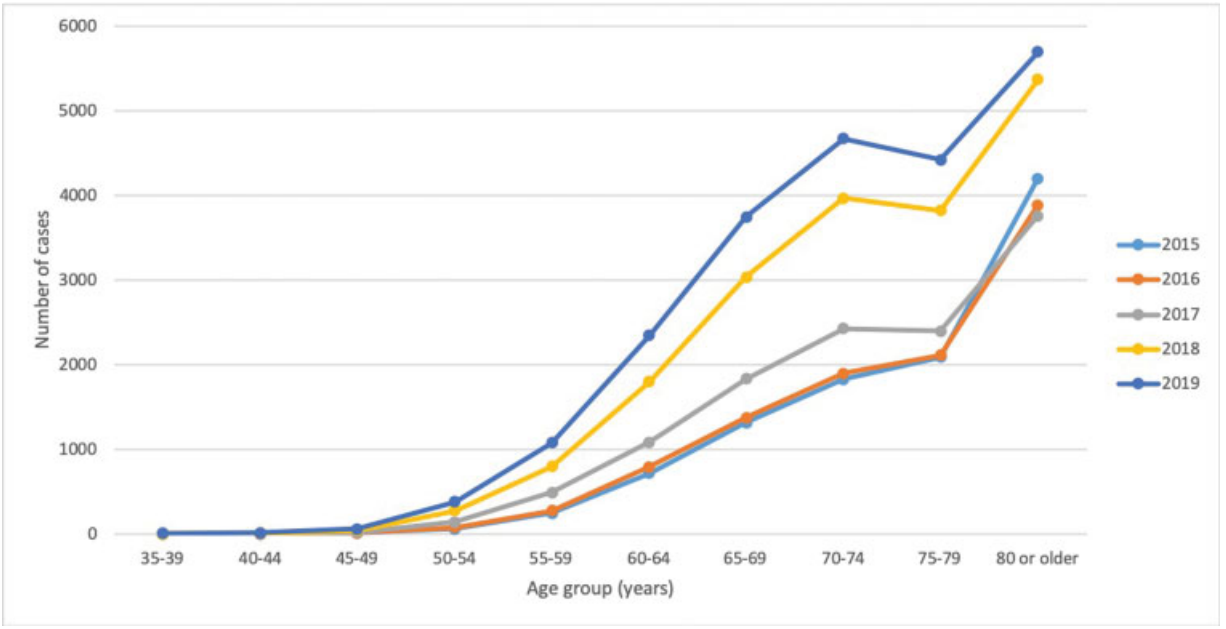

Fig. 1 Number of cases per age group (x 1,000 inhabitants).

men are prostate, stomach, colon, and lung.[1,15] Based on the present study, the prevalence of PCa in 5 years was 4.54 cases per 1,000 habitants, and it has been increasing over time (►**Fig. 1**). The incidence rate reported in 2013 varies between 40.5 to 45.9 per 100,000 habitants.[6] Data from Cali (southwest, capital city of Valle del Cauca) described that the age-standardized rate (ASR) changed from 22.3 per 100,000 males (1962–1966) to 64.8 per 100,000 males (2001–2005).[16] Between 2003 and 2007, the ASR in Colombia was 54.4 per 100,000 males.[5] According to data from high cost account (data present in SISPRO), in 2019, 37.7% of the PCa patients had a Gleason score (GS) of 6 (3 + 3), 20.5% GS 7 (3 + 4); 15.8% had a GS of 8, 15.2% GS 7 (4 + 3), and 10.8% had a GS of 9 or 10. And the most frequent clinical stage was II (40.6%) followed by stages I (27%) and IV (20.1%).[17]

The population in Latin America is heterogeneous regarding race, life expectancy, diet, and socioeconomic and cultural

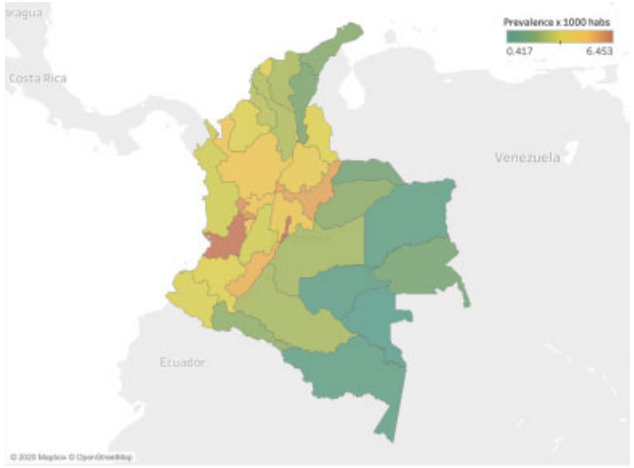

Fig. 2 Prevalence per Colombian department (x 1,000 inhabitants).

levels.[3] These factors may explain the differences in PCa epidemiology among countries. For instance, Brazil has the highest reported ASR (91.4 per 100,000 inhabitants) in the region,[5] probably due to its high proportion of black race population, which stands out as a non-modifiable risk factor.[18] In contrast, Peru has the lowest rate (34.6 per 100,000 inhabitants),[5] probably due to the small proportion of people over 50 years old (12%).[3]

Colombia is a country with several ancestry backgrounds. According to the 2005 census (DANE), 49% of the population is mestizo (mixed European and Amerindian ancestry), 37% is of European ancestry (predominantly Spaniard), 10% is of African ancestry, and 3.4% identify themselves as Amerindian.[19] Interestingly, one of the regions with the highest PCa prevalence is Valle del Cauca (southwest). According to the 2018 census (DANE),[12] this region has one of the largest proportions of Afro-Colombian inhabitants. It is noteworthy to point out that prevalence is also influenced by a high population density and a high number of available urologists. According to the Colombian Ministry of Health, the departments with the larger number of urologists are Bogotá (capital city of Colombia), Antioquia, Valle del Cauca, and Santander,[6] a fact that is consistent with our data. On the other hand, the population density and urban development in the Amazonas regions is low (Amazonas, Vaupés, Guaviare and Vichada), which implies less access to specialists, and, therefore, a lower probability of obtaining an accurate diagnosis; this would explain the lower prevalence in this region. This phenomenon is supported by the fact that the registered cases in SISPRO are dependent on the official report made by the treating physician. Thus, patients from rural and less developed areas usually travel to larger cities to obtain a diagnosis, and, therefore, each case would be registered in that city; this implies that the calculated prevalence in less developed regions is underestimated.

Table 2 Prevalence per Colombian department

Department	Prevalence x1,000 habitants
Bogotá, D.C.	6.45
Valle del Cauca	6.34
Risaralda	5.73
Boyacá	5.48
Huila	5.22
Quindio	4.59
Caldas	4.51
Antioquia	4.44
Cundinamarca	4.29
Santander	4.14
Norte de Santander	3.65
Cauca	3.58
Córdoba	3.47
Atlántico	3.47
Nariño	3.39
Chocó	3.26
Tolima	3.24
Sucre	2.91
Bolívar	2.78
Magdalena	2.65
Caquetá	2.58
Meta	2.55
Archipiélago de San Andrés	2.34
Putumayo	2.19
Casanare	2.12
La Guajira	2.03
Cesar	1.88
Guainía	1.60
Arauca	1.51
Vichada	0.76
Amazonas	0.69
Guaviare	0.64
Vaupés	0.42

On the other hand, the prevalence of EO-PCa, defined as PCa under 55 years, was 0.14 cases per 1,000 habitants or (791 cases in 5 years). The incidence of EO-PCa has been increasing over the last years. For example, in the United States, it changed from 5.6 to 32 cases per 100,000 person years (confidence interval [CI] 95% CI 5.0–6.7), making EO-PCa an important emerging issue for public health.[20,21] In 2012, 10% (241,740 cases) of men $\leq$ 55 years old were diagnosed with PCa.[22] Different risk factors have been associated, such as family history, ethnicity, and genetic factors,[23] and different studies have described differences in the molecular characteristics and the clinical behavior when compared with classic PCa.[24–26]

One of the limitations of our study is the possible underreporting by physicians in the medical records, especially because there are many occasions in which patients cannot access or continue with the medical assistance, especially in remote regions of the Colombian territory. In addition, the prevalence would be underestimated in underserved areas, as the formal diagnosis would be registered in larger cities.

In conclusion, to the best of our knowledge, this is the first study that describes the prevalence and demographics of PCa and EO-PCa in Colombia using the national healthcare registry. The prevalence has been increasing over time and it varies between the regions; the departments with the highest prevalence were Bogotá, Valle del Cauca, Risaralda, and Boyacá, and the region with the lowest prevalence was the Amazonas. This behavior may be related to racial and environmental causes as well as to access to appointment with the urologist, among others. These factors should be addressed in further studies.

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Conflict of Interests
The authors declare no conflict of interests for this study.

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8.2. CAPÍTULO II

Perfil de expresión de microRNAs en cáncer de próstata de inicio temprano y vías moleculares relacionadas

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Research Paper

microRNA Expression Profiling in Young Prostate Cancer Patients

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Abstract

MicroRNAs (miRNAs) are small, non-coding RNA molecules with multiple roles in many biological processes. Few studies have shown the molecular characteristics in younger prostate cancer (PCa) patients. In this study, we performed miRNA profiling in young PCa (EO-PCa) cases compared with PCa arising in older men (LO-PCa).

Experimental Design: Formalin-fixed, paraffin embedded tissue was used. miRNA was extracted for PCR array and NanoString methods. Relative miRNAs expression levels were obtained by comparing young vs older men, and young PCa tumor samples vs normal epithelium.

Results: miRNA profiling showed a different expression pattern in PCa arising in younger men, and young PCa tumoral and its normal counterpart. Nine miRNAs (hsa-miR-140-5p, hsa-miR-146a, hsa-miR-29b, hsa-miR-9, hsa-miR-124-3p, hsa-let-7f-5p, hsa-miR-184, hsa-miR-373, hsa-miR-146b-5p) showed differences in the expression compared to LO-PCa. Fourteen miRNAs were significantly up-regulated (miR-1973, miR-663a, miR-575, miR-93-5p, miR-630, miR-600, miR-494, miR-150-5p, miR-137, miR-25-3p, miR-375, miR-489, miR-888-5p, miR-142-3p), while 9 were found down-regulated (miR-21-5p, miR-363-3p, miR-205-5p, miR-548ai, miR-3195, 145-5p, miR-143-3p, miR-222-3p, miR-221-3p) comparing young PCa tumoral tissue compared to normal counterpart. The higher expression of miR-600 and miR-137 were associated with high Gleason score, extraprostatic extension and lymphatic invasion.

Conclusion: These results suggest that PCa in younger patients has a different expression profile compared to normal tissue and PCa arising in older man. Differentially expressed miRNAs provide insights of molecular mechanisms involve in this PCa subtype.

Key words: MicroRNAs, prostate, early onset, young

Introduction

Prostate cancer (PCa) is the most common tumor in men and the fifth cause of cancer death. It is estimated that there were almost 1.3 million new cases of PCa and 359.000 associated deaths worldwide in 2018 [1]. The incidence of PCa has been increasing across the time. Between 1986 and 2008 the incidence of PCa in young men, defined as PCa arising in men under 55 years has been increased by 5.7-fold from 5.6 to 32 cases per 100.00 persons years (IC 95% CI 5.0–6.7), making PCa in young men an important emerging issue for public health [2,3]. In 2012, 10% of men (241.740 persons) with newly diagnosed PCa were 55 years old or younger [4]. Different risk factors as ethnicity, familiar history and genetic factors has been associated in this setting [5].

The genetic profile between young PCa and 'classical' or PCa arising in older men are different [3,6–9]. Previous studies also have revealed different

genetic alterations in young PCa, such as a greater number of single nucleotide polymorphism in the germinal DNA [6–8], different expressed genes involved in the inflammatory and immune-related pathways (CTL4, IDO1/TDO2) [9], and gene mutations in BRCA 1 and 2, and HOXB13 [5]. The relative risk to develop PCa in patients with BRCA 1 mutations has been reported as 1.8–4.5, while the relative risk reported in patients with mutations in BRCA 2 is 23, and patients with HOXB13 mutation have eightfold higher risk [5]. PCa in young man with mutations in BRCA 1 and 2 has been associated with unfavorable prognosis, in contrast to patients with HOXB13 mutations were the genetic alteration has been associated with a favorable prognosis [5].

The molecular pathway of PCa in younger men is unknown. Weischenfeldt, et al. [7] recognized the important role of androgen regulated transmembrane serine protease 2 (TMPRSS2) gene fusion with the ERG gene. This fusion is a very early event in prostate oncogenesis and might be driven by increased androgen stimulation. Young PCa patients have a significantly increased tumor androgen receptor levels and positive correlation with ERG rearrangements. The frequency of ERG rearrangements in younger PCa patients is approximately 64% [10,11]. TMPRSS2–ERG fusion positive cases are associated with loss of PTEN suppressor gene and TMPRSS2–ERG fusion negative cases with loss of 5q21 and 6q15 [12].

Differences in clinical behavior between young and older PCa patients are controversial [3]. Some studies report aggressive biological behavior and higher Gleason score in the young [3,11,13,14] while other studies report no significant difference in survival across age groups after prostatectomy, brachytherapy and radiation therapy [3,15,16]. Schaefer et al. [10] observed that ERG-positive status was associated with low-serum PSA and lower prostate volume, while Huang et al. [11] further confirmed the ERG-positive status was associated with Gleason score and higher biochemical relapse rate but not with presurgical PSA levels, tumor volume, pathological stage, surgical margin or lymphovascular invasion.

MicroRNAs (miRNAs) are small, non-coding RNA molecules with multiple roles in many biological processes. They can prevent protein expression through cleavage of specific target mRNAs or through inhibition of their translation [17]. Since the discovery of miRNAs, numerous studies have demonstrated their relevance in carcinogenesis of several cancers [18]. Recently, it has been demonstrated the relevance of miRNAs in PCa as biomarkers in diagnosis, treatment, and prognosis.

In this study, we investigated whether PCa arising on younger men has a different microRNA profile compared to PCA in older men in order to further characterize its potential role in tumorigenesis, tumor progression and disease prognosis.

Material and Methods

Patient Samples

Formalin-fixed, paraffin embedded (FFPE) tissue samples from younger (age <55 y.o) patients with PCa and older (>55 y.o) PCa were retrieved from the surgical archives of the Laboratory of Pathology, National Cancer Institute, Bethesda, MD, USA after IRB approval. Samples without sufficient tumor tissue were excluded. Hematoxylin and eosin (H&E) stained slides were reviewed to confirm the diagnosis. Tumoral and normal tissue adjacent to the tumor (epithelial cells from the prostate glands) were selected from the same patients. The clinicopathological features were reviewed, including Gleason score, extraprostatic extension, margins, seminal vesicle, perineural invasion, lymphatic invasion, and pTNM (pathological tumor-node, metastasis) staging.

microRNA Isolation

Isolation of total miRNA from FFPE specimens was performed as described previously [21,22]. In brief, tumoral and normal tissue were manually microdissected under light microscope followed by miRNA isolation using the RecoverAll™ Total Nucleic Acid Isolations Kit (Ambion by Life Technologies, Foster City, CA, USA). The concentration of all RNA samples was quantified using NanoDrop 2000 (Thermo Scientific, USA). RNA concentration of samples used for profiling was normalized at 33 ng/µl following the recommendations from NanoString Technologies.

microRNA expression profiling using PCR arrays

As we described previously [21], the extracted total RNA including miRNAs (10 ng/µl concentration) was first reverse transcribed into first strand cDNA using the RT2- miRNA First Strand Kit following manufacturer's recommendations (SA Biosciences, Rockville, MD). One µl cDNA per well was then mixed with SYBR Green qPCR Master Mix and placed into a 96-well PCR-array plate containing a panel of 88 mature miRNAs sequences. The arrays also contain appropriate small nucleolar RNA sequences that are used as housekeeping assays and quality controls. One µl was used in a 12 µl final volume reaction for Real-time PCR analysis on an Applied Biosystems Step-One Plus Real Time PCR system. Relative amounts were calculated by the ΔΔ

CT method. Samples without good RNA quality were excluded in the statistical analysis.

microRNA profiling using NanoString nCounter miRNA assay and data analysis

Total RNA samples were analyzed according to the manufacturer's instructions for the nCounter Human miRNA Expression Assay kit (NanoString Technologies, Seattle, WA). From each sample 100 ng from total RNA sample was used as input into the nCounter Human miRNA sample preparation. Hybridization was conducted for 16 h at 65°C. Subsequently, probes were purified and counted on the nCounter Prep Station. Each sample was scanned for 600 FOV (fields of view) on the nCounter Digital Analyzer. Data was extracted using the nCounter RCC Collector. The analysis was made as we described previously [22]. miRNAs raw data was normalized for lane-to-lane variation with a dilution series of six spike-in positive controls. The sum of the six positive controls for a given lane was divided by the average sum across lanes to yield a normalization factor, which was then multiplied by the raw counts in each lane to give normalized values. For each sample, the mean plus 2 times the standard deviation of the 8 negative controls was subtracted from each miRNA count in that sample. Only miRNAs with non-negative counts across all samples were retained for downstream analysis. The relative miRNA levels were indicated as median fold changes (tumor/normal tissue) and a cutoff of 1.5-fold-change (up or down) was used.

Assessment of prognostic significance of miRNAs associated with clinicopathological features

With PROGmir V2 we compared the overall survival, relapse-free survival, and metastasis-free survival of prostate adenocarcinoma patients with high and low expression of miRNAs associated with clinicopathological features. PROGmiR V2 is an online free tool that combines the prognostic data of miRNAs for different kinds of cancers [23].

Statistical Analysis

Only mature miRNAs that showed at least a 1.5-fold change in expression are reported. p-values were calculated for each miRNA between the normal and tumor samples using the biological replicates and genes. They were considered differentially expressed and statistically significant if their p value was < 0.05. To compare relative miRNA fold changes between Young PCa and older PCa patients we used Mann-Whitney and Kruskal-Wallis non-parametric tests. According to edgeR, t-tests were carried out to compare the two groups (tumor vs. normal), p value was adjusted for multiple comparisons with the calculation of the false discovery rate (FDR) (<0.05). To evaluate differences between miRNA expression and clinicopathological features (Gleason score was categorized in low grade (3+3; 3+4) and high grade (4+3; 4+4;5+5)) we used t test. Supervised and non-supervised hierarchical clustering was conducted based on the Euclidean distance of miRNAs in samples using the Pheatmap package in R3.5.1. Analysis was performed using STATA SE 15 and R3.5.1.

Results

Characterization of studied population

In all, ten cases of young PCa and nineteen cases of older PCa patients were included. The clinicopathologic characteristics of the cases are shown in Table 1. The median age of young PCa was 46 years (range 40-55), 7 had low or intermediate risk Gleason score (3 + 3 or 3 + 4) and 3 had high-grade Gleason score (4 + 5 and 5 + 5). Perineural invasion was observed in seven patients, extraprostatic extension in three patients (high-grade Gleason score). While the average age of older PCa patients was 63 years (range 58-71), nine were low-grade Gleason score and ten were high-grade Gleason score. Perineural invasion was observed in 12 patients, extraprostatic extension and positive margins in six, and lymphatic invasion in four.

microRNA expression profile comparing young PCa to older PCa patients

To determine if young PCa patients have a tumor specific pattern of miRNAs expression, the expression level of 88 mature miRNAs using PCR based assay was compared. Different expression was recognized in nine miRNAs between the groups (hsa-miR-140-5p, hsa-miR-146a, hsa-miR-29b, hsa-miR-9, hsa-miR-124-3p, hsa-let-7f-5p, hsa-miR-184, hsa-miR-373, hsa-miR-146b-5p) (Figure 1). Three were upregulated (Fold change >1.5) (hsa-miR-140-5p (p 0.008), hsa-miR-146a (p 0.01), hsa-miR-29b (p0.01)) in younger PCa patients and one (hsa-let-7f-5p (p 0.02)) was downregulated (Fold change <1.5) (Table 2).

microRNA expression profile between tumoral tissue to its normal counterpart in young PCa tumors

A panel of 800 miRNAs was analyzed in 6 young PCa patients. A t-test was performed to comparing the tumor versus its corresponding normal prostate epithelium. In total, 14 miRNAs were up-regulated ranging from 1.51-fold to 2.17 while nine miRNAs showed to be down-regulated ranging from -1.52-fold

to -12.42-fold change. Only two miRNAs showed FDR <0.05 (hsa-miR-205 (FDR: 0.002) and hsa-miR-21-5p (FDR: 0.02) (Table 3). Among the 14 miRNAs up-regulated (hsa-miR-1973, hsa-miR-663a, hsa-miR-575, hsa-miR-93-5p, hsa-miR-630, hsa-miR-600, hsa-miR-494, hsa-miR-150-5p, hsa-miR-137, hsa-miR-25-3p, hsa-miR-375, hsa-miR-489, hsa-miR-888-5p, hsa-miR-142-3p), two miRNAs (hsa-miR-1973 and hsa-miR-93-5p) were the most prominently up-regulated (p<0.05).

Table 1. Clinicopathological features of prostate cancer patients included in the study.

Variable	Young-PCa (N:10)	Older-PCa (N:19)	*p* value
Age, median (Years) (range)	46 (40-55)	63 (58-71)	
Gleason score groups			0.06
≤3+4	7	9	
≥4+4	3	10	
Extraprostatic extension			0.63
Yes	3	6	
No	7	13	
Positive margins			0.42
Yes	2	6	
No	8	13	
Seminal vesicle invasion			0.34
Yes	1	0	
No	9	19	
Perineural invasion			0.52
Yes	7	12	
No	3	7	
Lymphatic invasion			0.66
Yes	2	4	
No	8	15	
T Stage			0.24
pT2a ≤ pT2C	8	13	
pT3a≥ pT3	1	6	
N Stage			0.003
N0	8	4	
N1	2	15	
M Stage			1.000
M0	10	19	
M1	0	0	

Table 2. MicroRNAs differentially expressed in young PCa patients versus older PCa patients.

miRNA	Young PCa (mean, range)	Older-PCa (mean, range)	*p* Value
hsa-miR-140-5p	1.95 (1.69-2.15)	-2.28 (-5.53- -0.3)	0.008
hsa-miR-146a	4.78 (2.07-7.3)	0.79 (-3.21- 7.3)	0.01
hsa-miR-29b	5.77 (3.76-9.54)	0.8 (-1.66-2.8)	0.01
hsa-miR-9	1.21 (-3.38-4.11)	-4.84 (-9.63 – 1.9)	0.02
hsa-miR-124-3p	0.11 (-0.15-0.34)	2.55 (-7.03-8.61)	0.01
hsa-let-7f-5p	1.27 (0.43-2.67)	5.84 (1.88-9.73)	0.02
hsa-miR-184	0.09 (-1.14-1.27)	4.13 (-4.31-10.14)	0.02
hsa-miR-373	-1.43 (-4.48-0.41)	2.65 (-5.93-9.24)	0.03
hsa-miR-146b-5p	-0.69 (-1.32-0.03)	1.92 (-3.77-9.95)	0.04

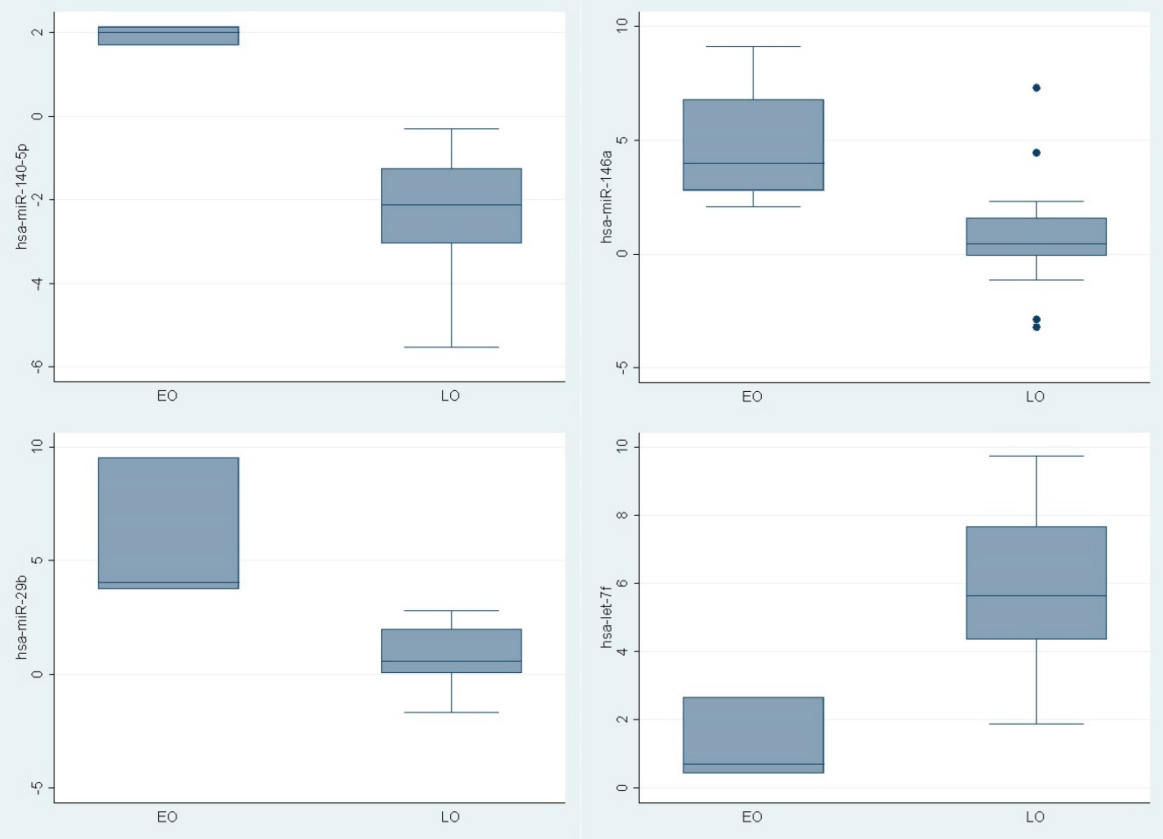

Figure 1. ΔΔ CT of miRNAs differentially expressed between young PCa and older PCa tumoral tissue

Among the nine miRNAs down-regulated, three miRNAs (hsa-miR-21-5p, hsa-miR-363-3p, hsa-miR-205-5p) were the most prominently down-regulated ($p<0.05$).

Supervised hierarchical clustering of the miRNAs down-regulated with FDR <0.05 was made based on miRNAs normalized expression, showing two groups separating normal and tumor epithelium mainly based the miR-205 expression, indicating a cancer specific expression pattern (Figure 2).

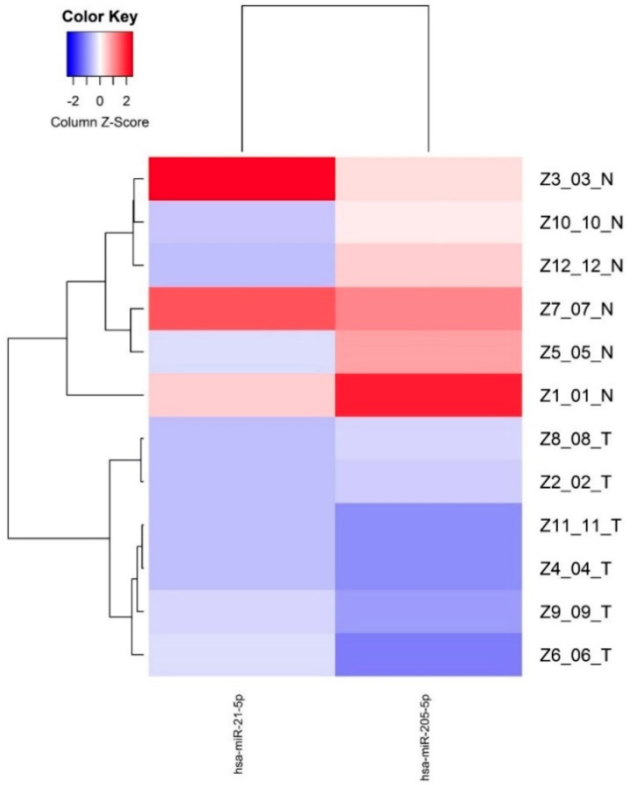

Figure 2. Differentially expressed miRNAs (FDR<0.05) between normal epithelium and tumor tissue from young PCa patients.

microRNA expression and clinicopathological features

Using the expression profile data, we also evaluated the possible correlation between clinicopathological features and the expression of deregulated miRNAs in tumoral tissue. We analyzed the group of Young PCa patients for expression profiling regarding the Gleason score grade (low or high), extraprostatic extension, margins, seminal vesicle invasion, perineural invasion, lymphatic invasion, and pTNM stage. We found association between high levels of hsa-miR-575, hsa-miR-663a, hsa-miR-600, hsa-miR-137 with high grade Gleason score and presence of extraprostatic extension, as well the low levels of hsa-miR-143 (Table 4). In contrast, low levels of hsa-miR-221 was associated with low grade Gleason score and absence of extraprostatic extension. High levels of hsa-miR-137 and hsa-miR-600 were associated with presence of lymphatic invasion, while high levels hsa-miR-663, and low levels of hsa-miR-221 and hsa-miR-143 were associated with absence of lymphatic invasion. Low levels of hsa-miR-143 and high levels has-miR-1973 were associated with absence of perineural invasion (Table 4).

Table 3. MicroRNAs differentially expressed in tumoral young PCa tissue versus normal epithelium.

miRNA	Fold change	*p* Value
hsa-miR-93-5p	1.87	**0.006**
hsa-miR-1973	2.17	**0.03**
hsa-miR-25-3p	1.6	0.07
hsa-miR-137	1.6	0.05
hsa-miR-575	2.01	0.09
hsa-miR-150-5p	1.61	0.09
hsa-miR-375	1.6	0.10
hsa-miR-663a	2.03	0.11
hsa-miR-142-3p	1.51	0.12
hsa-miR-630	1.83	0.16
hsa-miR-600	1.74	0.16
hsa-miR-888-5p	1.55	0.16
hsa-miR-489	1.6	0.17
hsa-miR-494	1.73	0.29
hsa-miR-205-5p	-4.81	**2.48E-06**
hsa-miR-21-5p	-12.42	**6.43E-05**
hsa-miR-363-3p	-5.47	**0.0089**
hsa-miR-145-5p	-1.61	0.05
hsa-miR-222-3p	-1.55	0.07
hsa-miR-3195	-1.87	0.08
hsa-miR-548ai	-2.83	0.09
hsa-miR-143-3p	-1.55	0.13
hsa-miR-221-3p	-1.52	0.12

Table 4. Correlation between clinicopathological features and miRNA expression levels

Gleason score			
	High	**Low**	**P-value**
hsa-miR-575	465.43	144.34	0.01
hsa-miR-663	91.95	26.56	0.04
hsa -miR-600	50.41	26.89	0.02
hsa -miR-137	88.25	68.24	0.03
hsa-miR-143	1356.32	532	0.01
hsa -miR-221	44.36	112.32	0.04
Extraprostatic extension			
	Yes	**No**	
hsa-miR-575	465.43	144.34	0.01
hsa-miR-663	91.95	26.56	0.04
hsa -miR-600	50.41	26.89	0.02
hsa -miR-137	88.25	68.24	0.03
hsa-miR-143	1356.32	532	0.01
hsa -miR-221	44.36	112.32	0.04
Perineural invasion			
	Yes	**No**	
hsa-miR-973	29.38	80.94	0.03
hsa -miR-143	824.02	1596.64	0.04
Lymphatic invasion			
	Yes	**No**	
hsa -miR-137	88.82	68.24	0.03
hsa -miR-600	50.41	26.89	0.02
hsa -miR-663	91.95	26.56	0.04
hsa -miR-221	44.36	112.32	0.04
hsa -miR-143	532.05	1356.32	0.01

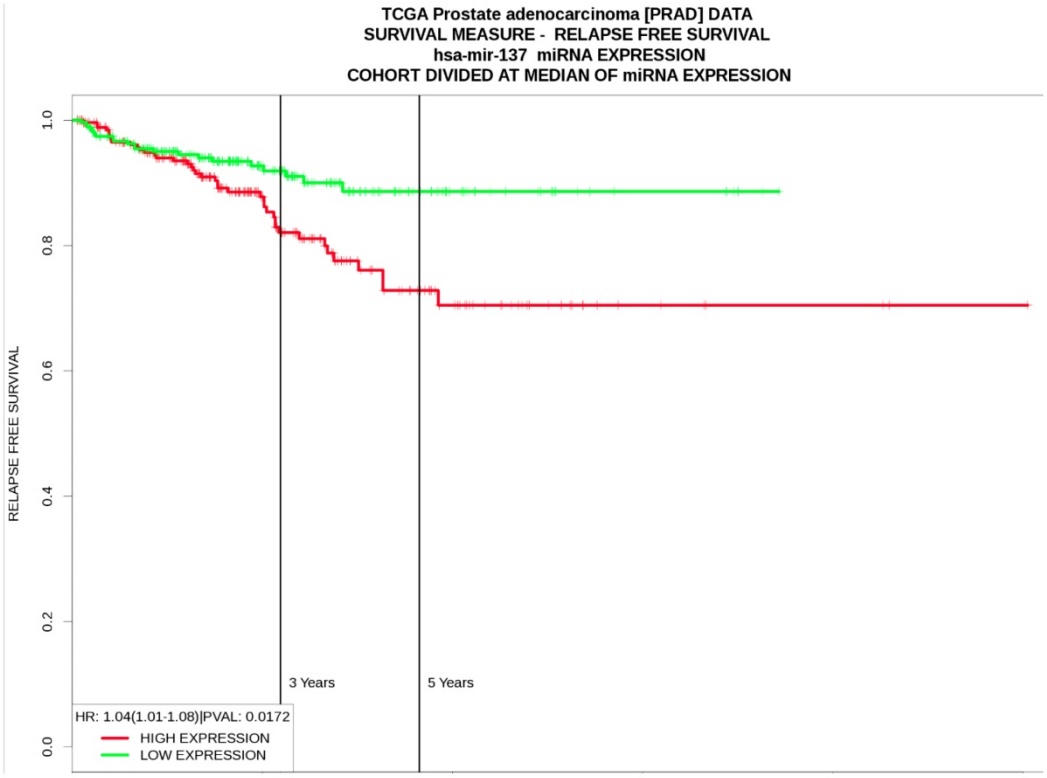

Figure 3. Relapse-free survival curves based on hsa-miR-137 expression levels in prostate cancer from PROGmiR V2.

Assessment of prognostic significance of miRNAs associated with clinicopathological features from PROGmir V2

According to the results from PROGmir V2, prostate cancer patients with high expression levels of hsa-miR-137 had significant poor relapse-free survival (HR: 1.04 (1.01-1.08, p=0.01) (Figure 3). In contrast, high levels of hsa-miR-143 was associated as protective factor for relapse-free survival (HR= 0.64 (0.48-0.84, p=0.001), same as hsa-miR-221 (HR= 0.68 (0.51-0.89, p=0.005) (Table 5).

Table 5. Prognostic significance of miRNAs with clinicopathological associations

miRNA's	Overall survival	Relapse-free survival	Metastasis-free survival
hsa -miR-137	HR: 0.98 (0.91-1.05)	HR: 1.04 (1.01-1.08)	HR: 1.05 (0.9-1.22)
hsa-miR-143	HR: 0.53 (0.27-1.06)	HR: 0.64 (0.48-0.84)	HR: 1.94(0.27-13.69)
hsa -miR-221	HR: 0.52 (0.27-1.02)	HR: 0.08 (0.51-0.89)	HR: 0.65 (0.19-2.3)
hsa-miR-663	HR: 0.96 (0.88-1.04)	HR: 0.97 (0.94-1.01)	HR: 0.91 (0.78-1.07)

Discussion

In the present study, we observed a different expression profile of miRNAs in young PCa compared to older PCa patients (Table 2) and compared tumoral to normal tissue (Table 3), suggesting a cancer-specific miRNAs expression profile for Young PCa. Due to lack of studies about the miRNAs in young PCa, the current knowledge about its biology is limited. In the study by Diung et al [9] they found the different miRNAs expression between young PCa with GS 7 (3+4) and PCa in older patients. Like them, we observed differences in the expression of hsa-miR-146a, hsa-miR-9, hsa-miR-124-3p, hsa-miR-146b-5p. Other study tested the miRNA's expression in eleven young patients with PCa. They measured the expression of genomic alterations in younger PCa and found rearranged genes in androgen pathway. They focused on finding miRNAs that had as target to PTEN and identified 13 miRNAs (hsa-miR-17-5p, hsa-miR-19a-3p, hsa-miR-19b-3p, hsa-miR-20a-5p, hsa-miR-92a-3p, hsa-miR-106b-5p, hsa-miR-93-5p, hsa-miR-25-3p, hsa-miR-141-3p, hsa-miR-214-3p, hsa-miR-494, hsa-miR-222-3p, hsa-miR-21-5p) differentially expressed(> 1.5-fold change). In our study we also observed upregulation of hsa-miR-93-5p, hsa-miR-25-3p and hsa-miR-494, and downregulation of hsa-miR-222-3p when we compare tumoral vs normal tissue. Unlike of them, we found downregulation of hsa-miR-21-5p.

Among the miRNAs with differences between young and older PCa patients, we observed nine miRNAs with different levels of expression between them (hsa-miR-140-5p, hsa-miR-146a, hsa-miR-29b, hsa-miR-9, hsa-miR-124-3p, hsa-let-7f-5p, hsa-miR-184, hsa-miR-373, hsa-miR-146b-5p). Three were upregulated (Fold change >1.5) (hsa-miR-140-5p (p 0.008), hsa-miR-146a (p 0.01), hsa-miR-29b (p0.01)) in

young PCa patients and one was downregulated (Fold change <1.5) (hsa-let-7f-5p (p 0.02)). This in in agreement with published work that shows that hsa-miR-140-5p and hsa-let-7f-5p in PCa are upregulated [19,20,24] while hsa-miR-29b is downregulated [19,20]. High expression levels of hsa-miR-140-5p has been observed mainly in metastatic PCa [24]. hsa-miR-146a has shown to modulate androgen-Independent prostate cancer cells apoptosis through regulation of ROCK/Caspase 3 pathway [25]. Furthermore, hsa-miR-29b has been observed downregulated in PCa [19,20] and may be involved in the epithelial-mesenchymal transition by the interaction with different targets such as e-cadherin, MMP-2, snail and twist [26].

Several miRNAs were observed dysregulated when we compared tumoral tissue versus tissue in Young PCa. Fourteen miRNAs were upregulated (Table 3). hsa-miR-93-5p has been reported overexpressed in PCa [27] and may are involved in cell proliferation, migration, invasion, block cell cycle, and promote the early apoptosis [27]. In a previous study, it was found that hsa-miR-93 with hsa-miR-106b and miR-375 may downregulate CIC-CIC-CRABP1 and promote the progression of PCa [28]. In young PCa, Weischenfeldt et al. found hypomethylation in the promotor region (R: -0.682, p<0.001) [7]. On the other hand, hsa-miR-25-3p is part of a cluster with hsa-miR-93, hsa-miR-25 and hsa-miR-106b [29]. This miR-106b-25 cluster promotes cell-cycle progression and hyperproliferation due to relationship with several actors in different oncological pathways such as PTEN, E2F1, and p21/WAF1 [30–32]. In PCa, the expression of this cluster is high and has been associated with tumor progression and metastasis [21,33]. In a previous study, we had already observed upregulation of miR-25 in tumor cells versus normal epithelium [21]. In young PCa the hypomethylation of the hsa-miR-25-3p promotor region (R: -0.625, p<0.001) has also been observed [7].

Among the miRNAs without previously reported association and whose biological functions have not been characterized in PCa are hsa-miR-1973, hsa-miR-575, hsa-miR-630 and hsa-miR-600. hsa-miR-575 and the hsa-miR-630 are proposed oncomiRNAs in other tumors such as gastric cancer, lung cancer, renal cell carcinoma, hepatocellular carcinoma, and breast cancer [34–40]. hsa-miR-630 has different gene targets such as BCL-2, MTDH, YAP-1, SNAI2 [37–39] involved in PCa oncogenesis [41–44]. hsa-miR-575 has as a target BLID (BH3-like motif containing, BRCC2). BLID is a tumor-suppressor gene involved in DNA repair and gene integrity [40]. In breast cancer, BLID inhibited cancer cell growth and metastasis via downregulating AKT pathway [40]. In prostate cancer cell lines, it has been demonstrated that BLID induce a caspase-dependent mitochondrial pathway of cell death [45]. Expression of hsa-miR-1973 has noted in other tumors as breast cancer, Hodgkin lymphoma and ovarian cancer [46–48]. Other miRNA without previously reported in PCa is hsa-miR-600, this has been involved in breast cancer, lung and colorectal cancer [49–51].

Among the differentially expressed miRNAs, some were associated with Gleason score, extraprostatic extension and lymphatic invasion. high expression levels for hsa-miR-137 and hsa-miR-600 were associated with high Gleason score, presence of extraprostatic extension, and lymphatic invasion. hsa-miR-137 also was associated with poor relapse-free survival (HR= 1.04). In the present study, we found that hsa-miR-137 is upregulated in young PCa, while in the literature has been reported downregulated and associated with recurrence following prostatectomy [52]. hsa-miR-137 has a function as an androgen regulated suppressor of androgen signaling by modulating expression of an extended network of transcriptional coregulators [53].

Conclusion

The present study supports the hypothesis that young PCa have a different miRNAs signature compared with normal tissue and older PCa patients. We present new miRNAs that may be involved in PCa pathogenesis (e.g. hsa -miR-1973, hsa-miR-575, hsa-miR-630, hsa-miR-3195), and present miRNAs with different expression (high or low) compared with previous studies (e.g hsa -miR-494, 150-5p, hsa -miR-137, hsa -miR-548ai, hsa -miR-21). In addition, we show that low expression of miR-600 and miR-137 were associated with clinicopathological features with poor prognosis and with poor relapse-free survival. The identification of these miRNAs may provide insights into understanding this subtype of PCa and these miRNAs may prove to be useful as a biomarker in the diagnosis, prognostic as well in the development of new therapeutic approaches. More studies with larger sample size are needed to confirm the results presented in this study and to correlate them with clinicopathological outcomes in young patients with PCa.

Competing Interests

The authors have declared that no competing interest exists.

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Identification of candidate miRNAs in early-onset and late-onset prostate cancer by network analysis

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The incidence of patients under 55 years old diagnosed with Prostate Cancer (EO-PCa) has increased during recent years. The molecular biology of PCa cancer in this group of patients remains unclear. Here, we applied weighted gene coexpression network analysis of the expression of miRNAs from 24 EO-PCa patients (38–45 years) and 25 late-onset PCa patients (LO-PCa, 71–74 years) to identify key miRNAs in EO-PCa patients. In total, 69 differentially expressed miRNAs were identified. Specifically, 26 and 14 miRNAs were exclusively deregulated in young and elderly patients, respectively, and 29 miRNAs were shared. We identified 20 hub miRNAs for the network built for EO-PCa. Six of these hub miRNAs exhibited prognostic significance in relapse-free or overall survival. Additionally, two of the hub miRNAs were coexpressed with mRNAs of genes previously identified as deregulated in EO-PCa and in the most aggressive forms of PCa in African-American patients compared with Caucasian patients. These genes are involved in activation of immune response pathways, increased rates of metastasis and poor prognosis in PCa patients. In conclusion, our analysis identified miRNAs that are potentially important in the molecular pathology of EO-PCa. These genes may serve as biomarkers in EO-PCa and as possible therapeutic targets.

The incidence of patients under 55 years old diagnosed with prostate cancer (PCa) (Early onset, EO-PCa) in the United States has increased during recent years. Between 1986 and 2008, the incidence of EO-PCa was from 5.6 to 32 cases per 100.00 persons years (IC 95% CI 5.0–6.7)[1,2]. In 2012, PCa was diagnosed in 241,740 men (10%) < 55 years old in the United States[3]. Thus, PCa in young patients is an emerging issue for public health[1,2]. Interest in understanding the molecular and clinical behavior of EO-PCa has been increased[4]. Several risk factors are associated with diagnosis: family's medical background, ethnicity, and genetic factors, such as single nucleotide polymorphisms and mutations in BRCA1, BRCA2, and HOXB13[5,6]. Different single nucleotide polymorphisms in germinal DNA[7] and rearranged genes in the androgen receptor axis (e.g., TMPRSS2-ERG, PTEN, and AR) have been identified EO-PCa[8]. Additionally, abnormal expression of genes involved in inflammatory and antitumoral immune-related pathways (CTL4, IDO1/TDO2) was detected[9]. A recent analysis of 1281 EO-PCa cases (≤ 60 years) identified 23 unique DNA repair genes associated with an increased predisposition or risk of aggressive PCa disease, and four genes (BRCA2, MSH2, ERCC2, and CHEK2_non1100del) were associated with more aggressive disease[10]. Other recent studies identified four molecular subgroups that included a particularly aggressive subgroup with recurrent duplications (8q22) associated with increased ESRP1 expression[11].

MicroRNAs (miRNAs) are small (~ 20–22 nucleotides), noncoding RNA molecules that are well conserved among different species of organisms and play multiple roles in several biological processes. miRNAs can interact with the RNAm of their target gene to exert its biological regulatory effect on gene expression by inhibiting the translation process[12]. This effect is achieved by binding to the cognate sequence 3′ UTR of RNAm to promote its degradation or inhibit the translation process[13]. Transcription activation is a non-canonical mechanism of miRNA action that was recently described[14]. In addition, miRNAs regulate expression of up to 30% of human

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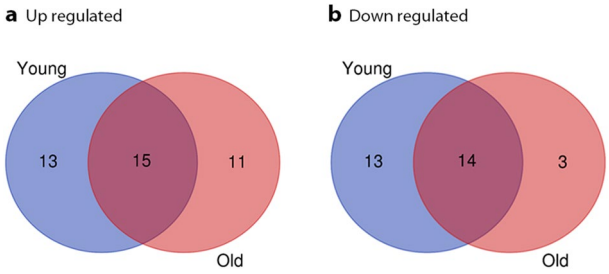

Figure 1. Venn diagram of DE-miRNAs. A. Upregulated DE-miRNAs; B. Downregulated DE-miRNAs.

genes with a proven impact in significant parts of different molecular pathways, including cell proliferation, differentiation and apoptosis[13]. In addition, miRNA expression in human cancer is dysregulated as a result of chromosomal disorder (amplification, translocations, and deletions), the presence of single-nucleotide polymorphisms (SNPs), the induction of epigenetic changes, deficiency in its biogenesis machinery, and modification of the expression of transcription factors necessary to control miRNA gene transcription[15].

Different miRNAs may have similar expression profiles when the miRNAs are functionally related or modulate the same pathway[16]. Based on the previous assumption, the Weighted Gene Coexpression Network Analysis (WGCNA) has been used to calculate the level of correlation among miRNA expression and identify clusters of coexpressed miRNAs in biological samples. A cluster of coexpressed miRNAs could be involved in the same pathway or biological process[17]. Additionally, using network analysis of coexpressed miRNA clusters, it is possible to identify its most central point within a cluster, namely, the hub genes, that could play the most important role or function in the initial step in the deregulation of other miRNAs.

In PCa, numerous miRNAs involved in different process related to PCa oncogenesis, such as cell cycle, apoptosis, epithelial-mesenchymal transition, DNA replication/repair, migration, androgen receptor suppression, metastasis, and treatment resistance, have been described[18]. Therefore, the miRNAs are studied as promising candidates that can be detected using minimally invasive diagnostic techniques and prognostic biomarker tools[19]. Several miRNAs involved in tumor growth are upregulated and downregulated in recurrent PCa compared to nonrecurrent PCa samples[20]. In EO-PCa patients, miRNA expression has been evaluated in a few studies[8,9,21] and differences in expression profiles have been observed compared to LO-PCa (Late onset-PCa). Weischenfeldt et al.[8] focused the analysis on miRNAs involved in the PTEN pathway. Some upregulated and downregulated miRNAs were detected, and some of genes with hypermethylated promoter regions, particularly tumor suppressor genes, exhibit reduced expression (hsa-miR-106b-5p, hsa-miR-93-5p, hsa-miR-25-3p, hsa-miR-141-3p). Ding et al.[9] found several miRNAs with deregulated expression (miRNAs DE); however, the analysis focused on mRNAs and found genes mainly involved in inflammation pathways. Recently, Valera et al.[21] found miRNAs DE in EO-PCa tumor tissue compared to LO-PCa as well as tumor tissue compared to normal tissue. They also identified miRNAs associated with high Gleason score, extraprostatic extension and lymphatic invasion. In these studies, deeper miRNA expression analyses were not performed. Therefore, we employed a systems biology analysis to identify fundamental miRNAs with transcriptional alterations, their target genes and coexpressed mRNAs that can explain the early appearance of PCa as well as the increased aggressiveness and different responses to treatment noted in these tumors.

Results

Data selection. Database analyses identified 3623 articles, of which 506 were duplicates. In total, 5 full-text articles were assessed for eligibility, and one paper met the inclusion criteria: GSE89193[9]. The pathological stage of all tumors was T2 (T2a and T2c), and the Gleason score was 7 (3 + 4). Among these patients, 67% had PSA ≤ 10.0. In total, 58% and 76% of young and old patients, respectively, had PSA ≤ 10.0 In both groups, 88% (n = 22) were white, 4% (n = 1) African-Americans, 4% (n = 1) Hispanics and 4% (n = 1) Asians.

Total RNA was extracted from the primary tumor tissue and matched control normal tissue samples, which were obtained from formalin-fixed paraffin-embedded tissue blocks from prostatectomies. The small RNA profile was generated using the Illumina Human Whole-Genome DASL (cDNA-mediated annealing, selection, extension, and ligation), while the miRNAs were sequenced on the Illumina HuSeq 2500 platform.

Identification of differentially expressed miRNAs. The comparison between transcriptomes of tumor and normal prostate samples employed stringent criteria of a fold change (FC) greater than 2 and less than -2 and a false discovery rate (FDR) less than 0.01. In the LO-PCa group, 43 miRNAs were identified as differentially expressed, including 26 upregulated and 17 downregulated miRNAs. In the EO-PCa group, 55 miRNAs were identified as differentially expressed, including 28 upregulated and 27 downregulated miRNAs. Subsequently, the two lists of differentially expressed miRNAs included 69 miRNAs DE in total with 29 miRNAs in common, and further analyses were conducted (Fig. 1 and Supplementary Table 1).

Functional enrichment analysis of miRNAs with dysregulated expression. KEGG pathway enrichment analysis was successively predicted by miRNet and aimed to validate that these miRNAs DE are involved in the prostate cancer pathway. This analysis revealed that in EO-PCa samples, 23 and 44 pathways

Hub miRNAs in young					Hub miRNAs in old				
Rank	Name	Score MCC	logFC	adj.P.Val	Rank	Name	Score MCC	logFC	adj.P.Val
1	hsa-miR-31-5p	7716	− 2.021	0.00001	1	hsa-miR-32-5p	182	1.372	0.00007
2	hsa-miR-224-5p	7705	− 1.052	0.00000	2	hsa-miR-96-5p	169	1.216	0.00006
3	hsa-miR-3065-3p	744	− 1.082	0.00051	3	hsa-miR-182-3p	168	1.007	0.00942
4	hsa-miR-205-5p	6858	− 2.804	0.00002	4	hsa-miR-183-5p	160	0.986	0.00000
5	hsa-miR-205-3p	6738	− 2.283	0.00003	5	hsa-miR-375	156	1.297	0.00000
6	hsa-miR-3545-3p	6368	− 2.509	0.00000	6	hsa-miR-183-3p	126	0.886	0.00454
7	hsa-miR-224-3p	5544	− 1.052	0.00000	7	hsa-miR-224-3p	90	− 1.066	0.00039
8	hsa-miR-676-3p	5424	− 1.249	0.00024	8	hsa-miR-205-5p	80	− 2.327	0.00000
9	hsa-miR-135b-5p	1680	− 1.951	0.00161	9	hsa-miR-224-5p	72	− 1.142	0.00000
10	hsa-miR-452-3p	1560	− 0.761	0.00090	10	hsa-miR-31-5p	60	− 1.813	0.00018
11	hsa-miR-488-3p	846	− 1.368	0.00358	11	hsa-miR-3545-3p	48	− 1.829	0.00012
12	hsa-miR-1911-5p	762	− 2.578	0.00027	12	hsa-miR-452-5p	42	− 1.014	0.00000
13	hsa-miR-1912	732	− 2.022	0.00228	13	hsa-miR-32-3p	36	1.101	0.00160
14	hsa-miR-509–3-5p	258	− 2.235	0.00032	14	hsa-miR-1298	24	− 1.855	0.07505
15	hsa-miR-31-3p	246	− 1.557	0.00157	15	hsa-miR-10a-3p	24	− 0.391	0.29418
16	hsa-miR-452-5p	244	− 0.974	0.00000	16	hsa-miR-5096	24	1.181	0.00334
17	hsa-miR-150-5p	26	1.120	0.00005	17	hsa-miR-1911-5p	24	− 1.032	0.19284
18	hsa-miR-142-5p	26	1.140	0.00008	18	hsa-miR-1912	24	NS	NS
19	hsa-miR-146b-3p	25	1.568	0.00000	19	hsa-miR-205-3p	24	− 1.942	0.00006
20	hsa-miR-514a-3p	24	− 2.516	0.00008	20	hsa-miR-944	18	− 1.988	0.00000

Table 1. Top 20 of hub miRNAs in young and old obtained from CytoHubba analysis.

were present in upregulated and downregulated miRNAs, respectively. In LO-PCa, 10 and 33 pathways were upregulated and 33 downregulated, respectively (Fig. 2). Genes in the PCa pathway as annotated by KEGG were overrepresented as targets in upregulated and downregulated miRNAs in EO-PCa and LO-PCa. The detected pathways are dysregulated by upregulated and downregulated miRNAs in EO-PCa and by downregulated miRNAs in LO-PCa. These pathways had roles in carcinogenesis, such as increasing the cellular proliferation rate, reducing cellular focal adhesion, and alteration of signaling pathways, such as MAPK, p53, Jak-STAT, neurotrophin, Wnt and ErbB. These results are similar to previous reports in renal cell carcinoma and thyroid cancer. The identified pathways dysregulated by upregulated miRNAs in LO-PCa included cellular proliferation, p53 signaling pathway, protein processing in the endoplasmic reticulum, adherens junction formation, and amino acid lysine degradation. Supplementary File 1 shows the target genes present in each of the dysregulated pathways.

Weighted coexpression networks. To capture most biological differences in the compared groups, we used less stringent criteria to select differentially expressed miRNAs: FC greater than 1.5 or less than − 1.5 and FDR less than 0.05. With those parameters, we identified in total 157 miRNAs differentially expressed (DE), 102 miRNAs DE in EO-PCa, 121 miRNAs DE, and 66 miRNAs DE in common. Based on the expression levels of the 157 miRNAs DE, two coexpression networks were calculated. To generate comparable Weighted Coexpression Networks (WGCA), we used the same list of miRNAs in each of the two networks generated. First, the 24 samples from young patients were used, and the similarity threshold was calculated using the maximum local method. A Pearson correlation coefficient greater than or equal to 0.57 differentiates the distributions of the correlations between the miRNAs and those of a random population. In total, 35 miRNAs had a correlation coefficient greater than the threshold. A similar process was performed with the 25 samples from the oldest patients using the same list of miRNAs. In this case, a Pearson correlation coefficient greater than or equal to 0.62 differentiates the distributions of the correlations between the miRNAs and those of a random population. In LO-PCa, 33 miRNAs were coexpressed over the threshold and were used for the following analysis.

miRNA-miRNA interactions to detect hub miRNAs. The networks calculated were analyzed using Cytoscape. The top 20 nodes ranked by the metrics MCC implemented CytoHubba were chosen[22]. In young and elderly samples, two well-defined networks composed of downregulated and upregulated miRNAs were observed (Fig. 3 and Table 1). In EO-PCa, the network had 35 nodes, one connected component, a clustering coefficient of 0.695, network centralization of 0.250, and an average of 6.97 several partners of neighbors. On the other hand, in LO-PCa, the network had 33 nodes, two connected components, a clustering coefficient of 0.740, network centralization of 0.180, and an average of 5.394 several partners of neighbors. The score of the top 10 hub miRNAs was from 1560 to 7716 in the young network and from 60 to 182 in the old network. In the EO-PCa top 10 miRNAs, all miRNAs were downregulated. However, in the LO-PCa network, the top 6 miRNAs were upregulated, and the following 4 were downregulated. In the comparison between the top 10 and the top group, hsa-miR-3065-3p and hsa-miR-676-3p were exclusively identified in young patients, whereas these genes did not exhibit statistical significance in the elderly population. Additionally, in the top 20, hsa-miR-488-3p was

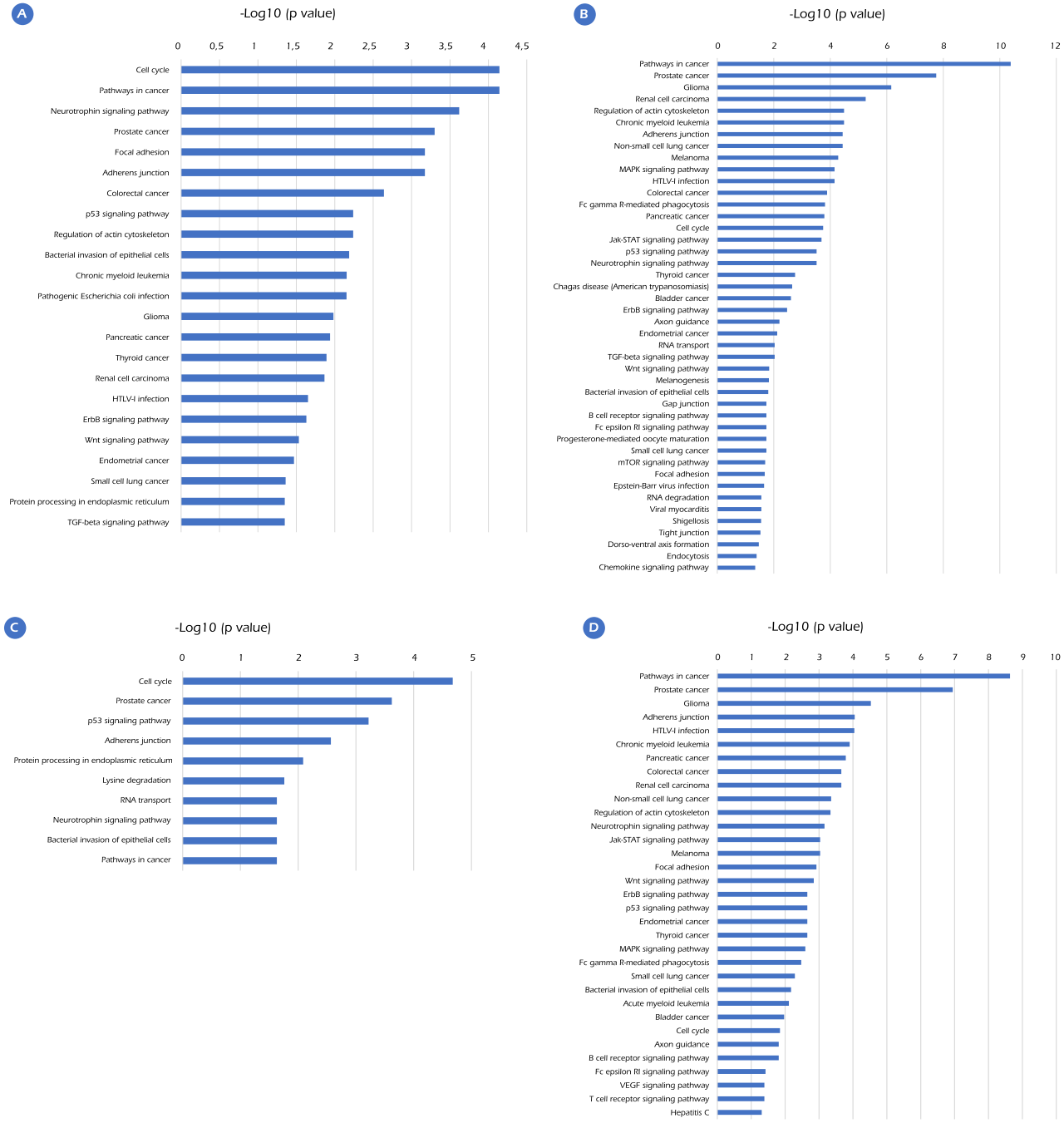

Figure 2. Pathway enrichment analysis for the predicted target genes of potential DE-miRNAs (p value < 0.05). (**A**). Enriched KEGG pathways for target genes of upregulated DE-miRNAs in EO-PCa. (**B**). Enriched KEGG pathways for target genes of downregulated DE-miRNAs in EO-PCa. (**C**). Enriched KEGG pathways for target genes of upregulated DE-miRNAs in LO-PCa. (**D**). Enriched KEGG pathways for target genes of downregulated DE-miRNAs in LO-PCa.

exclusively identified in young patients, but this gene was not statistically significant in elderly patients. In the top 20 miRNAs, nine downregulated miRNAs were present in the two groups (hsa-miR-31-5p, hsa-miR-224-5p, hsa-miR-205-5p, hsa-miR-205-3p, hsa-miR-3545-3p, hsa-miR-224-3p, hsa-miR-1911-5p, hsa-miR-1912, and hsa-miR-452-5p). In addition, only three miRNAs were upregulated in young patients (hsa-miR-150-5p, hsa-miR-142-5p, and hsa-miR-146b-3p). This result is in contrast to elderly patients, where eight miRNAs were upregulated (hsa-miR-32-5p, hsa-miR-96-5p, hsa-miR-182-3p, hsa-miR-183-5p, hsa-miR-375, hsa-miR-183-3p, hsa-miR-32-3p, and hsa-miR-5096). The exclusively upregulated and downregulated hub miRNAs in the young patients were not statistically significant in the elderly patients.

To identify the relevance of the hub miRNAs in the EO-PCa network we used miRNet to perform a KEGG pathway enrichment analysis with the predicted transcriptional targets of the 20 hub miRNAs. The analysis

Figure 3. Network analysis identified hub miRNAs using cytoHubba plug-in ranked by MCC. (**A**) EO-PCa. (**B**) LO-PCa. miRNAS with high centrality are noted in red. miRNAS with high-moderate centrality are noted in orange. miRNAS with low-moderate centrality are noted in yellow. miRNAS with low centrality are noted in blue.

showed that 32 genes in the Prostate Cancer pathway were potentially regulated by the selected miRNAs (Supplementary File 1).

Assessment of prognostic significance of EO-PCa cohort. The prognostic significance of the 20 hub miRNAs of the EO-PCa network were analyzed via PROGmir V2, which used the PRAD dataset[23]. Of the 20 hub miRNAs, four had prognostic value using survival data from general PCa. The hub miRNAs that are exclusively dysregulated in EO-PCa were hsa-miR-3065 (Hazard ratio (HR): 1.3) and hsa-miR-146b (HR: 1.34), which are associated with poor relapse-free survival. In addition, miR-676 (HR: 1.84) was related to poor overall survival. On the other hand, two miRNAs exclusively identified in old patients, namely hsa-miR-32 and hsa-miR-96 (HR: 1.46), were associated with poor relapse-free survival. In addition, hsa-miR-10a (HR: 2.31) was related to poor overall survival. Figure 4 presents the Kaplan-Meir survival plots and the number of events in each analysis.

Correlated mRNA genes with hub miRNAs from the EO-PCa coexpression network. To molecularly explain the effects of dysregulation of those miRNAs in EO-PCa, we identified genes that were coex-

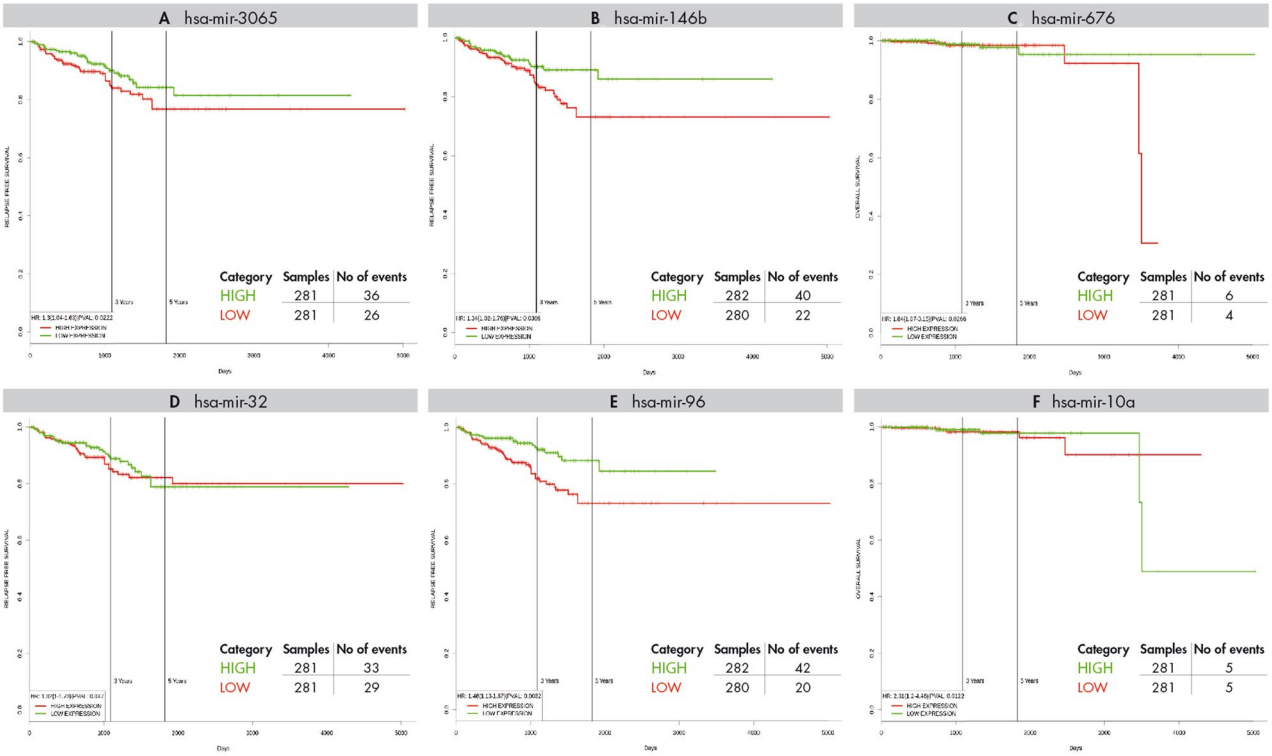

Figure 4. Kaplan–Meier survival plots for overall survival related to hub miRNAs exclusively identified in young patients. The X and Y axes represent survival time (days) and recurrence-free survival (**A** and **C**) or percent survival (**B**), respectively. The analysis was made in PROGmiR V2.

pressed with the hub miRNAs. We used mRNA microarray data generated using the same samples from the same patients who were used to generate the miRNA expression data.

After normalization and batch effect correction of the microarray dataset, six samples were identified as outliers, three in EO-PCa patients and three in LO-PCa patients. Those samples were not included in the additional analysis.

The Pearson coefficients of the correlation of the 20 hub miRNAs in EO-PCa with the genes in the microarray were calculated for the EO-PCa samples and similarly for the LO-PCa samples. A permutation test revealed that correlation coefficients greater than or less than ± 0.614 were statistically significant for EO-PCa samples and correlation coefficients greater than or less than ± 0.673 were statistically significant for LO-PCa samples.

Different numbers of coexpressed genes were identified for each hub miRNAs with numbers ranging from 0 to 167 genes. Supplementary file 2 presents the numbers, names and correlation coefficients of the coexpressed genes for EO-PCa and LO-PCa samples. The most remarkable result was that EO-PCa upregulated hub miRNAs had more coexpressed genes than downregulated miRNAs, and most of these miRNAs exhibited positive correlation coefficients (Supplementary file 2).

To provide a biological meaning of the lists of coexpressed genes, a pathway analysis with the statistically significant coexpressed genes for each hub miRNA was performed. We performed an overrepresentation analyses against the 21 cancer prostate pathways that we collected from Molecular Signatures Database (MSigDB)[24] and the list of DEGs detected by Ding et al. (Supplementary file 3). We found that the three upregulated miRNAs (hsa_miR_142_5p, hsa_miR_146b_3p and hsa_miR_146b_3p) were coexpressed with the DEGs in EO-PCa versus normal tissue (Ding Early onset prostate cancer 2016)[9]. Two of the upregulated miRNAs were coexpressed with DEGs upregulated in the more aggressive prostate cancers of African-Americans compared with the less aggressive prostate cancers of European-American patients (WALLACE PROSTATE CANCER RACE UP)[25] (Table 2). Among the downregulated miRNAs, hsa_miR_3545_3p and hsa-miR-224-5p were significantly coexpressed with genes in LIU PROSTATE CANCER DN[26](Table 2). This pathway was obtained from microarray analysis of 31 PCa samples. The Gleason score was variable, and information on patient age was not provided. Table 3 shows all the genes with significant correlation coefficients for Ding Early onset prostate cancer 2016 and WALLACE PROSTATE CANCER RACE UP.

A similar analysis was performed using the LO-PCa data, demonstrating that none of the miRNAs had more coexpressed genes in the pathways of early onset prostate cancer or more aggressive cancer in African-Americans than expected by chance. Four miRNAs (hsa_miR_31_5p, hsa_miR_205_5p, hsa_miR_224_3p, and hsa_miR_3545_3p) were significantly coexpressed with genes in the pathway LIU PROSTATE CANCER DN[26] (Supplementary Table 2).

Assessment of prognostic significance of genes coexpressed with hub miRNAs. Using GEPIA[27], which employed the PRAD dataset, the genes correlated with hub miRNAs that exhibited statistical signifi-

hub miRNA	Gene set	Size	Expect	Ratio	Overlap	FDR
hsa_miR_142_5p	WALLACE PROSTATE CANCER RACE UP	277	3.64	4.12	15	1.0889E−06
hsa_miR_150_5p	WALLACE PROSTATE CANCER RACE UP	277	2.32	3.88	9	8.83E−04
hsa_miR_142_5p	Ding Early onset prostate cancer 2016	158	2.08	5.78	12	1.0889E−06
hsa_miR_150_5p	Ding Early onset prostate cancer 2016	158	1.32	6.81	9	1.46E−05
hsa_miR_146b_3p	Ding Early onset prostate cancer 2016	158	6.89	4.93	34	3.12E−13
hsa_miR_3545_3p	LIU PROSTATE CANCER DN	473	5.65	2.30	12	0.015

Table 2. Over-representation analysis of co-expressed genes with hub miRNA using EO-PCa data. Only statistically significant associations are shown. Size means the number of genes in the pathway. Expect means the expected number of genes in the pathway co-expressed by chance with the miRNA. Ratio means the additional number of times that there are more genes co-expressed with the miRNA compared with the expected number. Overlap means the number of co-expressed genes that are part of the pathway. FDR is the false discovery rate of the overlap.

Ding Early onset prostate cancer 2016				WALLACE_PROSTATE_CANCER_RACE_UP		
ID_REF	hsa-miR-142-5p	hsa-miR-150-5p	hsa-miR-146b-3p	ID_REF	hsa-miR-142-5p	hsa-miR-150-5p
ADAMTS1	NS	NS	− 0.639	ADAMDEC1	0.724	0.715
APOE	NS	NS	0.648	**CCR7**	0.695	0.680
C4A	NS	NS	0.664	CD28	0.668	NS
CCDC74B	NS	NS	− 0.694	**CD3D**	NS	NS
CCL19	NS	NS	0.660	CD48	0.657	NS
CCR7	0.695	0.680	0.746	CXCL9	0.677	NS
CD3D	NS	NS	0.644	DOCK10	0.681	0.693
CD3E	NS	0.651	0.685	GZMK	0.707	0.709
CD3G	0.684	0.684	0.644	IDO1	0.657	NS
CD6	0.641	0.644	NS	IL2RG	0.723	0.712
CD84	0.654	NS	0.675	**IL7R**	0.647	0.663
COL2A1	NS	NS	0.658	ITGB2	0.705	0.640
CP	NS	NS	0.648	**ITK**	0.685	0.659
E2F2	NS	NS	0.681	**MMP9**	0.640	NS
EOMES	0.650	NS	0.673	PLEK	0.655	NS
ERG	NS	NS	0.653	**PTPRC**	0.675	0.689
HIST1H2AI	NS	NS	0.723			
HIST1H2BM	NS	NS	0.701			
HLA-DMB	NS	NS	0.705			
IKZF1	NS	NS	0.716			
IL7R	0.647	0.663	0.702			
ITK	0.685	0.659	0.681			
LEPREL1	NS	NS	− 0.770			
LTB	0.639	NS	0.661			
MMP7	NS	NS	0.687			
MMP9	0.640	NS	0.650			
PDE3B	NS	NS	0.667			
PLP1	NS	NS	− 0.705			
PTPRC	0.675	0.689	0.687			
PYHIN1	0.680	0.683	0.649			
SERPINA3	NS	NS	0.709			
SLAMF6	0.726	0.672	0.647			
SLC35F1	NS	NS	− 0.654			
TMEM178	NS	NS	0.671			
UBD	NS	NS	0.767			

Table 3. Genes with coefficient of correlation statistically significant for Ding Early onset prostate cancer 2016 and WALLACE_PROSTATE_CANCER_RACE_UP with upregulated hub miRNAs in EO-PCa. Genes in bold are common genes between both pathways.

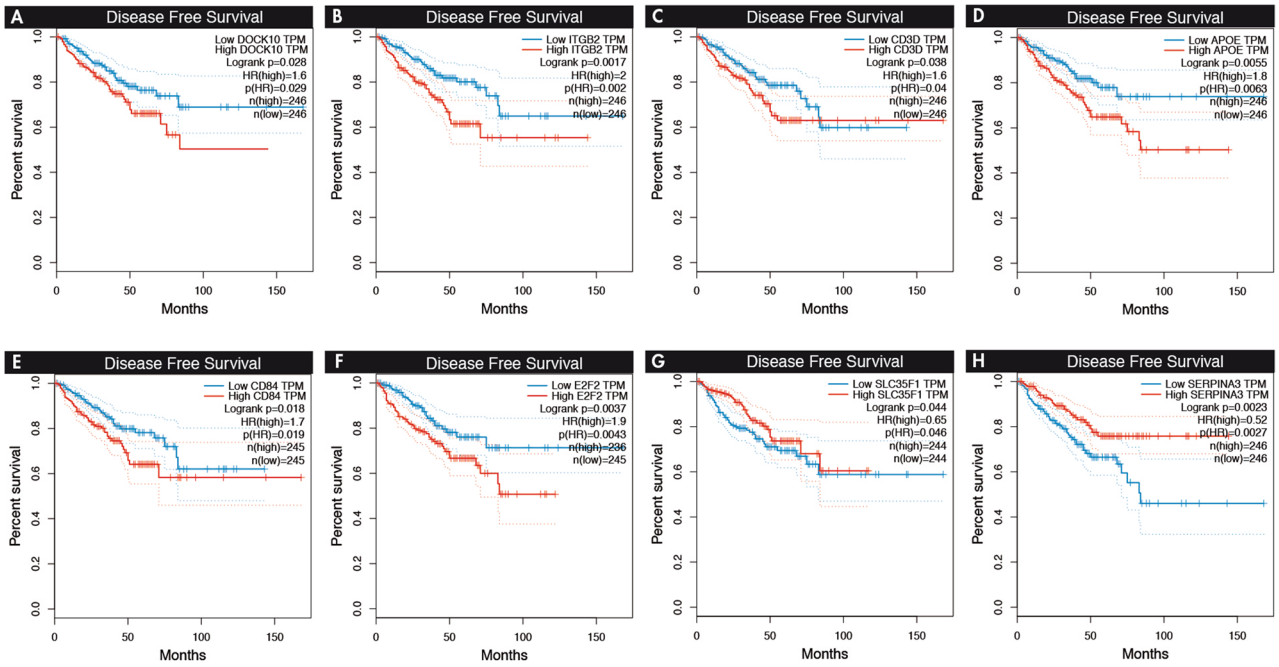

Figure 5. Kaplan–Meier survival plots for overall survival related to target genes correlated with hub miRNAs. The X and Y axes represent survival time (months) and disease-free survival, respectively. The analysis was made in GEPIA.

cance were analyzed (Table 3). Among the upregulated hub miRNAs, two genes that exhibited high expression (DOCK10: HR: 1.6, $p = 0.02$; ITGB2: HR: 2, $p = 0.001$) (Fig. 5) in the Wallace_Prostate_Cancer_Race_Up database were associated with poor disease-free survival. In the Ding_PlosGenetics2016, the high expression of four genes (CD3D: HR: 1.6, $p = 0.03$; APOE: HR: 1.8, $p = 0.005$; CD84: HR: 1.7, $p = 0.01$; E2F2: HR: 1.9, $p = 0.003$) was associated with poor disease-free survival, and the low expression of two genes (SLC35F1: HR: 0.65, $p = 0.04$; SERPINA3: HR: 0.52, $p = 0.002$) served as protective factors. In the hub of downregulated miRNAs, the low expression of FBXO17 served as a protective factor (HR: 0.63, $p = 0.03$) of disease-free survival.

Discussion

EO-PCa is a subtype of PCa, which is currently receiving high interest due to its impact on clinical behavior and pathobiological differences with the "classical" or elderly PCa (LO-PCa). In this study, novel data analysis was performed using transcriptomic data from patients with EO-PCa who were < 45 years old and LO-PCa who were 71 to 74 years old. The data analyzed were generated by Ding et al. using 49 PCa patients[9]. The tumor samples were GS 7 (3 + 4) and grade T2 (T2a or T2c). Samples were obtained from different ethnic groups, including 88% whites, 4% African-Americans, 4% Hispanics and 4% Asians. We identified 55 miRNAs DE in EO-PCa, including 28 upregulated and 27 downregulated. In addition, 26 of these genes were exclusively dysregulated in EO-PCa. Using an overrepresentation analysis with the predicted targets genes of the miRNAs DE, we identified several pathways commonly dysregulated between EO-PCa and LO-PCa. These pathways are related to adherence junctions, cell cycle and p53 signaling. In addition, the neurotrophin signaling pathway was identified as dysregulated, and members of this pathway are expressed in PCa, i.e., trk receptors and neurotrophins (NGF, BDNF, and/or NT-3).[28]. Strikingly, in upregulated miRNAs in elderly patients, the lysine degradation pathway exhibited statistical significance. Lysine modification is associated with carcinogenesis in different types of tumors[29].

Among the hub miRNAs exclusively deregulated in EO-PCa without significance in LO-PCa, three were downregulated (hsa-miR-3065-3p, hsa-miR-676-3p, and hsa-miR-488-3p), and three were upregulated (hsa-miR-150-5p, hsa-miR-142-5p, and hsa-miR-146b-3p). The role of hsa-miR-3065-3p and hsa-miR-676-3p in PCa is unknown, but reduced expression of these genes is observed in tumors, such as esophageal squamous cell carcinoma[30], hsa-3065-3p in clear cell renal cell carcinoma[31], and hsa-miR-676-3p in breast cancer cell lines[32]. In survival analysis, hsa-miR-3065 was associated with poor relapse-free survival, and hsa-miR-676 was related to poor overall survival. In the PCa cell lines (LNCaP and C4-2B), hsa-mir-488-3p inhibits the androgen receptor, blocks proliferation and induces apoptosis[33]. Among the exclusively upregulated hub miRNAs in EO-PCa, hsa-miR-142-5p and hsa-miR-150-5p had been described as deregulated[34–37]. The last may act as antitumor miRNA targeting SPOCK1[35]. hsa-miR-150 has been reported as upregulated with a role in proliferation and invasion by targeting p53[36], and its expression is associated with poor overall survival (HR: 1.87, CI: 1.19–2.94)[37]. In addition, Ding et al.[9] found that hsa-miR-146b-3p exhibited the highest level of overexpression in young PCa patients. However, the specific role of hsa-miR-146b-3p in PCa its unknown, it was associated with poor relapse-free survival in the survival analysis (Fig. 4). hsa-miR-146b-3p is member of the miR-146 a/b family. hsa-miR-146a

is dysregulated in PCa and other tumors. In androgen-independent PCa, its downregulation is involved in apoptosis through regulation of the ROCK/Caspase 3 pathway[38,39].

To understand how dysregulation of hub miRNAs modulates the normal behavior of prostatic tissue, we searched the targets genes of each hub miRNAs. The number of potential target genes identified was 112 for hsa-miR-3065-3p, 51 for hsa-miR-676-3p, 131 for hsa-miR-488-3p, 534 for hsa-miR-150-5p, 226 for hsa-miR-142-5p, and 56 for hsa-miR-146b-3p. These hub miRNAs are annotated as members in different KEGG pathways that are relevant in PCa biology. For example, regulatory targets of hsa-miR-3065-3p, such as GSK3B (Glycogen Synthase Kinase-3), are involved in apoptosis, cell cycle, DNA repair, tumor growth, invasion, and metastasis pathways. In recent years, it has become a targeted gene for therapy[40]. In the progression to androgen-independent PCa, GSK3B may act with PTEN[41] as a positive regulator of androgen receptor transactivation and growth independent of the Wnt/β-catenin pathway[42]. hsa-miR-3065-3p and hsa-miR-488-3p are related to p53, which is a very relevant gene that is mutated in greater than half of all cancers and is associated with progression in PCa[43]. hsa-miR-3065-3p is repress by mutant p53[44], and hsa-miR-488-3p activates the p53 pathway through suppressing ZBTB2[45]. On the other hand, C-terminal Binding Protein 1 (CTBP1) is a target gene of hsa-miR-676-3p. This gene is a transcriptional corepressor of tumor suppressors genes involved in cell death, and dysregulated expression of this gene is associated with PCa progression[46]. Platelet-derived growth factor receptors-β (PGDFR-β) is regulated by hsa-miR-488-3p and hsa-miR-146b-3p), which are key regulators of cell growth and division[47]. In PCa, PGDFR-β is expressed in the early stage of the disease[48]. Its activation is associated with the loss of PTEN[49], and high PGDFR-β expression is associated with prostate cancer recurrence[50].

Among the upregulated hub miRNAs, several target genes involved in the KEGG PCa pathway were identified. The targets of hsa-miR-150-5p in this pathway include CDK2, EP300, and TP53. CDK2 is a key regulatory protein involved in cell cycle arrest upon DNA damage[51]. It is upregulation is associated with PCa progression, and it is a probable novel target gene in treatment[52]. hsa-miR-142-5p is modulator of important genes involved in the pathogenesis of PCa, including Cyclin D1 (CCND1), MAPK1, and PTEN. CCND1 is associated with aggressiveness[53]. MAPKs are serine/threonine kinases that mediate intracellular signaling associated with a variety of cellular activities, such as cell proliferation. In PCa, MAPKs are involved in apoptosis, survival, metastatic potential, and androgen-independent growth[54]. Additionally, PTEN is the most commonly lost tumor suppressor gene in primary disease. In most cases with PTEN loss, the gene is lost by genomic deletion. The loss of PTEN is associated with prostate tumor aggressiveness, progression, and poor prognosis (reduced disease-specific survival)[55].

Given the limited number of studies on the effects of the hub miRNAs in the pathogenesis of PCa, we performed a correlation analysis based on the expression of hub miRNAs and all the genes in the genome in patients with EO-PCa. The overrepresentation analysis of the genes with significant correlations among hub miRNAs in the EO-PCa network revealed that the three upregulated hub miRNAs were significantly coexpressed with the Ding Early-onset prostate cancer 2016 pathway: two of the upregulated hub miRNAs were coexpressed with Wallace_Prostate_Cancer_Race_Up and one downregulated hub miRNA was coexpressed with LIU PROSTATE CANCER DN (Table 2). In the primary analysis of the data used in the present study, Ding et al.[9] reported differential expression of genes annotated in immunological pathways in the age:tissue interaction analysis (B Cell Development, iCOS-iCOSL Signaling in T Helper cells, CD28 Signaling in T Helper Cells, Primary Immunodeficiency Signaling, Calcium-induced T Lymphocyte Apoptosis), including genes such as complement family genes, immune-cell surface antigens, chemokines, interleukin receptors, natural killer cells and extracellular matrix remodeling genes. Moreover, the Wallace_Prostate_Cancer_Race_Up[25] dataset was generated from the comparison of gene expression profiles of PCa from 33 African-American patients with 36 European-American patients. The genes in this pathway are involved in immune response, stress response, cytokine signaling, and chemotaxis pathways. Several known metastasis-promoting genes, including autocrine mobility factor receptor, CXCR4 (chemokine (C-X-C motif) receptor 4), and MMP9, were more highly expressed in tumors from African-Americans than European- Americans. The expression profiles of two upregulated hub miRNAs, namely hsa-miR-150-5p and hsa-miR-142-5p, were correlated with the Wallace_Prostate_Cancer_Race_Up dataset.

The genes shared in Wallace and Ding pathways and statistically correlated with upregulated hub miRNAs (hsa-miR-150-5p and hsa-miR-142-5p) are genes involved in the immunology response, such as CCR7, IL7R, ITK, PTPRC, MMP9, APOE, CCL19, and CD3D. These genes were upregulated in PCa of African-American patients and in EO-PCa[25]. CCR7 and MMP9 are genes associated with PCa progression and metastases[56–58]. MMP-9 is involved in several hallmarks of PCa progression, such as proliferation, angiogenesis, epithelial to mesenchymal transition, apoptosis, and metastasis[59]. MMP-9 expression is associated with the risk of PCa (OR = 7.91; 95% CI: 5.27–11.89; $P < 0.00001$)[60]. A primary PCa cell line derived from an African–American patient (E006AA) exhibited increased MMP9 expression compared to other studied cell lines (LNCaP, C4-2, and MDAPCa2b)[61]. CCR7 is a chemokine receptor that is associated with lymph node metastasis in other tumors, such as breast cancer[62], non-small cell lung cancer[63], and gastric carcinoma[64]. The CCR7 ligand CCL21 is expressed selectively in high endothelial venules at the entry point into the lymph node and promotes cancer progression[56,62]. Polymorphisms in CCR7 (rs3136685) are present in African–American PCa patients[65]. IL7R and IL7 are highly expressed in PCa and are associated cancer cell invasion and migration probably by activating the AKT/NF-κB pathway and upregulating MMP-3 and MMP-7 expression[66].

All the coefficients of correlation among the upregulated hub miRNAs and the mRNAs genes were positive. It could be counterintuitive that the upregulation of a specific miRNA causes the up-regulation of a specific mRNA. We proposed that it may explained by the activation of the transcription of target genes by binding of miRNAs with reverse complementary sequences in promoter regions of genes[14]. For example, overexpression of hsa-miR-205 increases IL-35 and IL-24 expression[67]. In PCa cells (DU145 and PC3), overexpression of miR-3619-5p induces CDK1N1 gene expression via direct interaction with the promoter region[68]. On the other hand, one gene target can be targeted by several miRNAs[69]. Therefore, the targets of this gene are potentially regulated by miRNAs DE. Thus, it is necessary study the regulation of this gene's targets.

In the analysis of overall survival of genes with high expression in Wallace_Prostate_Cancer_Race_Up[25], high expression of DOCK10 and ITGB2 was associated with poor prognosis. Both genes are coexpressed with hsa-miR-150-5p and hsa-miR-142-5p. The roles of these genes in PCa are unknown. In cancer, DOCK10 (Dedicator Of Cytokinesis 10) is involved in the regulation of the epithelial to mesenchymal transition[70]. ITGB2 (Integrin beta-2 (CD18)) combines with integrin alpha to form the integrin lymphocyte function-associated antigen-1 (LFA-1). This gene is involved in tumor growth and metastasis[71].

A limitation of this study was the high homogeneity of the patients, who all had Gleason scores of 7 (3 + 4). Patients with low-grade PCa have different molecular characteristics, clinical behavior and treatment[72]. However, the expression profile of miRNAs in low-grade PCa does not exhibit significant differences. Walter B et al.[73] compared miRNA expression in 26 patients with low-grade and 15 patients high-grade PCa. The results did not reveal any specific signatures to differentiate the two groups. Another limitation involves the survival analyses of DE miRNAs and DEGs because the data were obtained from TCGA database. In this analysis, we could not perform separate evaluations for EO-PCa and LO-PCa.

In conclusion, this is the first study that analyzed the expression of miRNAs in EO-PCa and LO-PCa patients using network analysis. Connections among miRNA expression, target genes, and molecular pathways for EO-PCa and LO-PCa were identified. Furthermore, specific miRNAs with clinical significance in young patients may explain molecular differences, and the different biological processes in young and elderly patients were identified. In addition, we found coexpression of genes and hub miRNAs that play important roles in PCa progression and metastasis and genes associated identified in Afro-American PCa patients. Most of these genes are involved in the immunology response. As a recommendation, the constructed network of biomarkers should be further assessed in EO-PCa, and candidate miRNAs and gene targets should be validated for patient diagnosis and prognosis.

Materials and methods

Data selection. In June 2019, an advance search was performed to identify studies that analyze miRNA expression in EO-PCa patients. The sources used included PubMed and the National Center for Biotechnology Information (NCBI) GEO database (https://www.ncbi.nlm.nih.gov/geo/). The keywords 'young OR early-onset AND prostate cancer' were used in the search. The results were limited to *Homo sapiens* as the organism and expression profiles were determined using array dataset types.

The inclusion criteria for the systematic review were (1) miRNA expression was assessed in prostate tissue of young and elderly PCa patients in the same dataset, (2) raw data were available, and (3) data passed quality control. Two reviewers performed an eligibility assessment by screening titles and abstracts from the publications. Subsequently, the articles that did not meet the eligibility criteria were rejected. Additionally, we searched The Cancer Genome Atlas (TCGA); however, we could not use these data because the database only contained matched tumor and normal data from three patients diagnosed with PCa under the age of 55 years.

Preprocessing and identification of differentially expressed miRNAs. Raw counts of miRNAs from 49 patients with prostate cancer diagnoses were downloaded from GSE89193 and deposited in the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE89193). The data included 25 elderly men (ages 71–74 years) and 24 young men (ages 38–45 years). For each patient, the tumor and a standard region of the prostate were analyzed. Samples were sequenced on the Illumina HiSeq 2.500 platform (https://www.illumina.com/systems/sequencing-platforms/hiseq-2500.html).

Raw count miRNA data were normalized using the trimmed mean of the M-value (TMM) method through the EdgeR package[74]. Because the original data were sequenced in two batches[9], this nonbiological variability source was corrected using nonparametric Bayesian statistics methodology using the sva package.[75]. The extent of the batch effect correction was assessed by principal component analysis.

The samples were assigned to two different experimental groups to identify the differentially expressed miRNAs in LO-PCa (71–74 years old) and EO-PCa (38–45 years old). miRNA expression levels were compared between tumor and normal samples in each group. The statistical significance level for this study was calculated using the Limma statistics package (Linear Models for Microarray and RNA-Seq Data)[76]. Limma uses a linear modelling to detect differentially expressed genes. The fold change was calculated, and the statistical significance was adjusted for multiple comparisons (False Discovery Rate (FDR)).

Weighted gene coexpression networks analysis (WGCNA). The total levels of differentially expressed miRNAs between normal and tumor samples in older and young patients were collected in one list. Two coexpression networks were developed using this gene list. The first list was generated for the young samples, and the second list was generated for the older group. First, the similarity matrix was calculated by identifying the Pearson correlation coefficients of the expression levels for the samples based on all possible gene pairs. Then, the similarity threshold was calculated with the adjacency function, which was established according to the unique characteristics of each similarity matrix[77]. The method developed by Elo was used to select the threshold[78]. This method compared the tau values for the network grouping coefficient (Co) with that expected for a random network (Cr). It uses the clustering coefficient of the real graph in comparison to a rando graph. The threshold for significant similarities is chosen so that the obtained real graph is scale free. Finally, the adjacency matrix (2 × 2) of the network was established and allowed the representation of binary relationships. In this case, a pair of genes that exhibit coordinated gene expression activity (coexpression) is indicated by (1); otherwise, a (0) is reported. All WGCNA analyses were performed in an R unique environment using statistical functions (https://www.r-project.org/).

Detection of hub miRNAs. The hub miRNAs were identified through network analysis using Cytoscape and its plugin (CytoHubba). This plugin accurately identifies hub genes by 12 topological analysis methods. For this study, the Maximal Clique Centrality (MCC) method proposed by CytoHubba was used; recently, this method exhibits improved performance to capture essential targets in the top rank list in both high- and low-grade PCa[22]. In addition, MCC helped to identify the top 20 hub miRNAs. On the other hand, the network analyzer plugin was used to recognize the network parameters.

Functional annotations of hub miRNAs. The miRNet database to identify target genes (https://www.mirnet.ca/) was used to facilitate the interpretation of biological mechanisms related to hub miRNAs. This tool integrates data from eleven different miRNA databases: TarBase, miRTar-Base, miRecords, miRanda, miR2Disease, HMDD, PhenomiR, SM2miR, PharmacomiR, EpimiR, and starBase[79]. The following information was provided for miRNet analysis: organism name (*H. sapiens*), ID type (miRBASE ID), and tissue origin (Not specified). No degree or betweenness filter was used for network visualization. Additionally, miRNet was applied to identify biological pathways and processes, molecular functions, and cellular components that are statistically enriched for the corresponding miRNA target genes. For the functional evaluation of the miRNAs, the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were conducted in mirNet. Only statistically significant annotation categories (P value < 0.05) were retained.

Survival analyses of miRNAs with dysregulated expression. PROGmiR V2 is an online free tool and is available at https://www.compbio.iupui.edu/progmir. This program combines the prognostic data of miRNAs for different types of cancers from TCGA dataset. This tool was used to compare the overall, relapse-free, and metastasis-free survival of prostate adenocarcinoma patients with DE of miRNAs in young and old cohorts. It also divides samples based on high and low expression and calculates the hazard ratio (HR) with relative confidence intervals (CI) and P values for the proportional hazards model[23].

Determination of mRNA expression levels of all genes in normal and tumor tissues from EO-PCa and LO-PCa for the diagnosis of prostate cancer. The level expression of mRNAs from cancer and normal tissues from EO-PCa and LO-PCa patients were downloaded from the same dataset GSE88808 available in the GEO OMNIBUS database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE88808).

These data were generated in parallel with the miRNA dataset. The same patients and same tissues were used to identify the level expression of mRNAs and miRNAs. A detailed description of the RNA obtention and determination of mRNA expression level are provided in the primary paper[9]. In summary, total RNA was extracted from approximately 5 mg of unsectioned formalin-fixed paraffin-embedded core samples using the RecoverAll Total Nucleic Acid Isolation kit (Life Technology, Inc.). The Illumina HumanHT-12 WG-DASL V4.0 expression beadchip was used for mRNA expression profiling of 29,000 genes in the human genome. The levels of intensity were normalized using the quantile normalization method[80]. The batch effects secondary to different times of hybridization were corrected using the empirical Bayes methods as is implemented in the ComBat in sva package[81].

Identification of outliers was performed using the Pearson correlation measurements of the level expressions of all genes in the microarray between all the samples. Samples with correlation coefficients less than 0.9 compared with the other samples were excluded from additional analyses.

Analysis of the correlation of the expression of hub miRNAs with the expression of genes involved in the pathogenesis of prostate cancer. The hub miRNAs in the EO-PCa coexpression network were included in additional correlation analyses to determine whether they were coexpressed with genes involved in prostate cancer.

First, we collected the 183 differentially expressed genes (DEGs) identify by Ding et al.in the primary analysis of the mRNA expression data[9]; they identified differences in tumor vs. normal tissues between samples from young and old patients. We refer to this list of genes as Ding Early-onset prostate cancer 2016.

Second, the Molecular Signatures Database (MSigDB)[24] was interrogated to collect the pathways related to prostate cancer. In total, 22 different pathways were identified (Supplementary file 3). This collection of pathways represents the state of knowledge about transcriptomic modifications between tumor tissues compared with normal tissues from prostate cancer patients (19 pathways) and tumor samples from African-American compared with European-American patients with primary prostate cancer[25].

Third, Pearson correlation coefficients were calculated among the expression profiles of selected hub miRNAs and mRNA expression levels from all the genes in the Illumina microarray. We initially performed correlation analysis only for young or old samples. Using a permutation test, the confidence intervals were calculated. Correlations coefficients with P values less than 0.002 were selected as statistically significant.

Finally, overrepresentation analyses were performed using the hypergeometric test as implemented in WebGestalt[82]. For each selected hub miRNA, statistically significant coexpressed genes were interrogated against the genes in the 22 prostate cancer pathways to identify whether more (overrepresentation) genes coexpressed with miRNAs are present than expected by chance.

Survival analyses of genes coexpressed with hub miRNAs. Gene Expression Profiling Interactive Analysis (GEPIA; https://www.gepia.cancer-pku.cn)[27] was used to calculated disease-free survival and overall survival between DEGs coexpressed with hub miRNAs. The lower and upper 50% of gene expression levels were

set as the standard for analysis. The confidence interval was 95%. High and low expression genes are represented in red and blue, respectively. Log-rank test results with $P < 0.05$ were regarded as statistically significant.

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Author contributions

Conceptualization, R.P.-M., S.R.-C., C.P.-G.; Methodology, R.P.-M., L.L.-K., S.R.-C., C.P.-G.; Validation, R.P.-M., L.L.-K., S.R.-C., C.P.-G.; Investigation, R.P.-M., S.R.-C., C.P.-G.; Writing—original draft preparation, R.P.-M., C.P.-G.; Writing—review and editing, R.P.-M., L.L.-K., S.R.-C., C.P.-G.; Project administration, R.P.-M., L.L.-K., S.R.-C., C.P.-G.; Funding acquisition, R.P.-M., S.R.-C.

Competing interests

The authors declare no competing interests.

Additional information

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8.3. CAPÍTULO III

Comparación del perfil de coexpresión de lncrRNA-mRNAs en pacientes con cáncer de próstata de ascendencia africana

Research Article

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Coexpression network analysis identified lncRNAs-mRNAs with potential relevance in African ancestry prostate cancer

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Aim: This study aims to investigate similarities and differences using lncRNA and mRNA coexpression network analysis in African ancestry (AA) and European ancestry (EA) among prostate cancer (PCa) patients. **Methods:** We performed weighted gene coexpression network analysis of the expression from 49 of AA and 49 of EA to identify lncRNAs-mRNAs. **Results:** 27 lncRNAs and 36 mRNAs were highly expressed in patients of AA. Two mRNAs and their antisense lncRNAs were expressed. Additionally, seven mRNAs were DE or coexpressed and had an impact on survival. **Conclusion:** We present a list of lncRNAs and mRNAs that were DE and coexpressed when comparing patients of AA and EA, and these data are a resource for future studies to understand the role of lncRNAs.

Lay abstract: This research demonstrated differences among AA and EA PCa patients. 27 lncRNAs and 36 mRNAs were highly expressed in patients of AA. 24 mRNAs were coexpressed with at least one lncRNAs. Seven mRNAs (CENPN, DNAAF3, DNASE1L2, PCDHB2, PCSK6, RTN4RL2, THBS4) were upregulated, respectively. In addition, the pathway enrichment analysis revealed two interesting signaling pathways (MAPK and Wnt) that are involved in oncogenesis and are exclusively involved in patients of AA. In summary, this study provides a resource for future investigation of the role of lncRNAs and coexpressed genes in patients of AA and EA.

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Keywords: African • ancestry • coexpression • lncRNA • mRNA • prostate cancer

Prostate cancer (PCa) is the most common tumor in men and the fifth-leading cause of cancer-related death around the world [1]. The incidence, clinical presentation and mortality of PCa vary according to race/ethnicity [2]. Globally, an increased number of PCa cases has been observed in men of African ancestry (AA) [2,3]. In the USA, African–American men are more likely to present at an earlier age, have developed advanced or metastatic PCa at diagnosis and have suboptimal outcomes after standard treatment [4,5]. Aspects related to patients, such as knowledge of the disease, low education, healthcare utilization, socioeconomic factors and genetic predisposition, have been associated with this elevated incidence [4,6].

Genetic alterations, such as mutations, changes in copy number, fusion of genes, changes in gene expression and abnormal splice variants, have been reported in PCa patients of AA [7]. In a recent study performed by Yuan *et al.* [8], through an integrative comparison of genomic and transcriptomic differences, significant differences were found between AA and European Ancestry (EA) patients. The most relevant differences were SPOP mutations (20.3% AA patients vs 10.0% EA patients), TMPRSS2-ERG fusions (29.3% AA patients vs 39.6% EA patients), PTEN deletion/losses (11.5% AA patients vs 30.2% EA patients), significant enrichment of eQTL target genes between patients of AA and EA and high expression of genes associated with immune-related pathways and PTEN/PI3K signaling in patients of AA compared with patients of EA.

FUTURE SCIENCE

The products of the human transcriptome are coding RNAs or noncoding RNAs (ncRNAs). Coding RNAs are templates used to organize the amino acids that constitute proteins during the translation process, and their structure and function are well known. Furthermore, based on their sizes, ncRNAs are classified as small ncRNAs (ncRNAs; <200 bp) and long ncRNAs (lncRNAs; >200 bp). Small ncRNAs are 20 to 30 bp in length, and based on structure/functionality, they are subdivided into *cis* regulatory (internal ribosome entry site [IRES], Leader, Riboswitch), gene (tRNA, rRNA, snRNA/snoRNA, miRNAs, among others) and intron sequences (Groups I and II) [9,10]. In medicine, miRNAs are of great interest because their expression constitutes a molecular fingerprint of different types of tumor cells, and they are possible candidates for treatment targets. miRNAs also play roles in gene expression by activating transcription [11] or inhibiting translation [12].

On the other hand, lncRNAs are classified as circular (circARN) or linear according to their structure. circRNAs are a type of single-stranded RNA that are generated by the formation of a loop in the primary pre-mRNA transcript due to back-splicing of an exon and/or intron, and the circle is closed by a covalent bond that makes it less susceptible to exonucleases. circRNAs regulate the expression of target genes during transcription and splicing as inhibitors of miRNAs, and circRNAs modulate the function of proteins by acting as decoys and encoding functional peptides [13]. In addition, linear lncRNAs are classified based on genomic localization relative to protein-encoding genes. These groups are sense, antisense, bidirectional, intronic, intergenic 3′ overlapping, macro ncRNA, process transcripts and enhancer lncRNAs [9,10]. LncRNAs are involved in different cellular processes, such as maintaining nuclear structure integrity, positively or negatively regulating gene expression by recruiting transcription factors and/or chromatin remodelling complexes to DNA targets, regulating RNA splicing, acting as decoys to sequester RNA-binding proteins (RBPs) and sequestrating miRNAs (miRNA sponges) [14,15].

Dysregulation of miRNAs and lncRNAs is widely observed in the pathogenesis of different cancer types, and these RNAs are mainly involved in tumor growth, invasion and metastasis [16–18]. LncRNAs are easily obtained for study from tissues and body fluids due to their resistance to degradation. These two types of ncRNAs are good candidate biomarkers with effects on the clinical diagnosis, prognosis and treatment of cancer. In recent years, numerous preclinical and early clinical studies have explored lncRNAs as new therapeutics [19].

In PCa, numerous miRNAs are involved in different processes that promote prostate oncogenesis, such as the cell cycle, apoptosis, epithelial–mesenchymal transition (EMT), DNA replication/repair, migration, androgen receptor suppression, metastasis and treatment resistance [20–22]. lncRNAs have also been shown to be dysregulated and associated with the pathogenesis and progression of PCa [14,18,23]. In a recent review published on the past 30 years, Ramnarine *et al.* [23] found 109 lncRNAs associated with PCa. These lncRNAs are involved in the migration and proliferation of tumoral prostate cells in different stages of disease, such as localized disease, metastatic disease or castration-resistant PCa. The molecular mechanisms targeted by these lncRNAs in PCa pathogenesis were grouped into transcriptional regulation (epigenetic modification, transcriptional activation, transcriptional inhibition, RNA decoy), post-transcriptional regulation (regulation of antisense RNA, translational regulation, mRNA stabilization, miRNA host, miRNA sponge) and posttranslational regulation (subcellular structure, protein transport, protein complex mediation). In PCa, several lncRNAs have been proposed as biomarkers because they favor both carcinogenesis and tumoral progression [24]. In the present study, a systems biology analysis was conducted to identify similarities and differences using lncRNA and mRNA coexpression network analysis.

Materials & methods

Data collection

The data provided by TCGA and processed for reuse by recount2 [25] were used as a primary source for data analysis. Gene counts for all prostate cancer and normal prostate tissues were downloaded. Samples annotated as having African and European ancestry by Yuan *et al.* [8] were selected. Finally, we kept all tumor samples from patients of AA, and we selected the same number of samples from patients of EA. These samples were randomly matched 1:1 (AA vs non-AA), taking into account the Gleason score obtained from the clinical information registered in The Cancer Genome Atlas Program (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga) as a parameter.

Data preprocessing

The transcriptomic data (mRNA and lncRNA) available in recount2 [25] were already processed with Rail-RNA as described in recount2 [25]. We performed quality control of all datasets to identify that the samples we selected had a similar distribution after and before normalization by the use of density plots and box plots. Additionally, we

plotted a multidimensional plot, and we performed a visual evaluation of the localization of the samples in the first four dimensions.

Identification of differentially expressed RNAs

The gene count data of each kind of RNA in patients of AA and EA were analyzed independently on the Galaxy web platform [26]. Data were normalized by library sizes using the trimmed mean of M-values (TMM) method. Genes without more than 1 count per million mapped reads (CPM) in at least 50% of the samples were considered not expressed and were filtered out. The limma-voom method was used to identify the differentially expressed (DE) genes. The primary factor was normal versus tumor, and the second factor was the Gleason score of the samples. An mRNA or lncRNA was selected as DE if the linear fold change was higher than the absolute value of 2 and the false discovery rate (FDR) was lower than 0.01.

Functional enrichment analysis of mRNAs with dysregulated expression

DAVID (https://david.ncifcrf.gov/) was used to identify the functions of the selected differentially expressed genes (DEGs). The upregulated and downregulated genes in each list of DEGs were analyzed separately. The Kyoto Encyclopedia of Genes and Genomes (KEGG) was chosen for overrepresentation analysis. Pathways with p-values lower than 0.05 were selected as enriched.

Weighted gene coexpression networks analysis

The total levels of differentially expressed mRNAs and lncRNAs in tumor samples from patients of AA compared with normal samples were added to one list. Two coexpression networks were developed using this gene list. The first list was generated for patients of AA, and the second list was generated for patients of EA.

To build the coexpression networks, we followed a typical methodology. First, the similarity matrix was calculated by identifying the Pearson correlation coefficients of the expression levels for the samples based on all possible mRNA-mRNA, lncRNA-lncRNA and lncRNA-mRNA combinations. Then, the similarity threshold was calculated with the adjacency function, which was established according to the unique characteristics of each similarity matrix [27]. The method developed by Elo was used to select the threshold [28]. This method compared the tau values for the network grouping coefficient (Co) with those expected for a random network (Cr). It uses the clustering coefficient of the real graph in comparison to a rando graph. The threshold for significant similarities is chosen so that the obtained real graph is scale-free. Finally, the adjacency matrix (2×2) of the network was established and allowed the representation of binary relationships. In this case, a pair of genes that exhibit coordinated gene-expression activity (coexpression) is indicated by (1); otherwise, a (0) is reported. All the weighted gene coexpression networks analysis (WGCNA) analyses were performed in an R unique environment using statistical functions (https://www.r-project.org/).

Analysis of WGCNA

The WGCNA was plotted through network analysis using Cytoscape [29]. The identification of the differences between the AA and EA networks was calculated using the advanced network merge interface function implemented in Cytoscape.

Survival analyses of differentially expressed genes among AA & EA & genes coexpressed with lncRNAs

Gene Expression Profiling Interactive Analysis (GEPIA;) [30] was used to calculate disease-free survival and overall survival between the DEGs coexpressed with lncRNAs. The lower and upper 50% of gene-expression levels were set as the standard for analysis. The confidence interval was 95%. High- and low-expression genes are represented in red and blue, respectively. Log-rank test results with $p < 0.05$ were regarded as statistically significant.

Results

Patient characteristics

In total, 98 PCa tissues, 49 in each group (AA and EA), and 44 normal prostate tissues (7 AA patient tissues and 37 EA patient tissues) were included. The mean age of the AA patients was 56.9 years, the mean age of the EA patients 58.8 years and the mean age of the patients from whom normal prostate tissue collected was 59.3 years. Each group included the same number of patients according to the Gleason score (GS). Nine patients had GS

Table 1. Number and direction of the change of differentially expressed mRNA and lncRNA in African ancestry and European ancestry.

	Total	Up	Down
mRNA			
TumorAA vs normal tissue	2419	702	1717
TumorEA vs normal tissue	2296	600	1696
TumorAA vs tumorEA	8	5	3
lncRNA			
TumorAA vs normal tissue	1563	708	855
TumorEA vs normal tissue	1304	593	711
TumorAA vs TumorEA tissue	22	11	11

AA: African ancestry; EA: European ancestry.

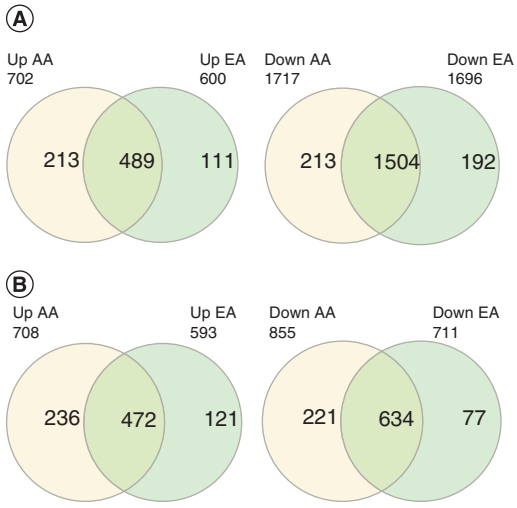

Figure 1. Venn diagrams show the number of (A) DE mRNAs in AA and EA. (B) lncRNAs in AA and EA.
AA: African ancestry; EA: European Ancestry.

3 + 3, 22 had GS 3 + 4, 11 had GS 4 + 3, 2 had GS 4 + 4, 2 had GS 4 + 5, 2 had GS 5 + 4, and 1 had GS 5 + 5. In the AA group, two patients were t2a, two were t2b, 22 were t2c, 14 were t3a, and 9 were t3b, while in the EA group, two patients were t2a, 29 were t2c, 11 were t3a, 5 were t3b, and two had no information. In the AA group, 7 were n1, 36 were n0, and 6 had no information; in the EA group, 5 were n1, 29 were n0, and 15 had no information. Only two patients died in each group. Supplementary Table 1 shows all clinical data information for the 98 PCa patients and 44 normal prostate tissues obtained from TCGA.

Identification of differentially expressed lncRNAs & mRNAs

All the samples had adequate quality control parameters, and all of them were used for additional analysis. A total of 2419 mRNAs were differentially expressed (DE) in prostate tumor tissue samples compared with normal prostate tissue samples in PCa patients of AA. Additionally, 2296 DE mRNAs were detected in PCa patients of EA. The same analysis was performed for the gene counts of lncRNAs, and 1563 and 1304 lncRNAs were detected as DE in the tissues from patients of AA versus the normal tissues and in the tissues from patients of EA versus the normal patients, respectively. Additionally, we compared the transcriptomes of tumor samples from AA versus EA patients. The number of DE mRNAs and lncRNAs was small in the direct comparison between tumor samples from AA versus EA patients. Table 1 summarizes the number of DEGs in both comparisons.

Comparison of differentially expressed mRNAs & lncRNAs in AA & EA cancer prostate patient samples

To obtain a general overview of the similarities and differences in the transcriptomic deregulation of AA and EA prostate cancer patient samples, we compared the lists of DE mRNAs and lncRNAs. Figure 1 shows the number of DE mRNAs and lncRNAs in both groups of patients. Common differentially expressed genes had the same direction of change.

Table 2. mRNAs and lncRNAs differentially expressed between prostate cancer samples from patients of African ancestry and European ancestry.

mRNA				lncRNA			
Gene ID	Gene symbol	logFC	Adjusted p-value	Gene ID	Gene symbol	logFC	Adjusted p-value
ENSG00000136883	KIF12	1.66	0.0024	ENSG00000274767	AC243829.1	2.08	0.0003
ENSG00000196436	NPIPB15	1.61	0.0092	ENSG00000259471	LINC01169	1.95	0.0016
ENSG00000244752	CRYBB2	1.50	0.0024	ENSG00000268181	AC073188.6	1.91	0.0038
ENSG00000182667	NTM	1.42	0.0031	ENSG00000269978	AL359881.1	1.64	0.0038
ENSG00000160973	FOXH1	1.01	0.0060	ENSG00000232283	HSD17B3-AS1	1.49	0.0088
ENSG00000104490	NCALD	-1.05	0.0083	ENSG00000275476	AC009318.4	1.43	0.0016
ENSG00000184058	TBX1	-1.28	0.0031	ENSG00000271959	AC100803.4	1.29	0.0073
ENSG00000198785	GRIN3A	-1.53	0.0083	ENSG00000262188	LINC01978	1.25	0.0062
				ENSG00000261159	AC112484.3	1.23	0.0003
				ENSG00000262877	AC110285.2	1.20	0.0046
				ENSG00000232850	PTGES2-AS1	1.20	0.0083
				ENSG00000253629	AP000426.1	-1.08	0.0029
				ENSG00000278936	AC244517.4	-1.08	0.0077
				ENSG00000277232	GTSE1-DT	-1.09	0.0016
				ENSG00000182366	FAM87A	-1.19	0.0005
				ENSG00000250604	AC098679.1	-1.19	0.0005
				ENSG00000261357	AC099518.2	-1.30	0.0069
				ENSG00000279068	AC244517.6	-1.31	0.0046
				ENSG00000249159	AC091965.1	-1.45	0.0088
				ENSG00000279472	AC244517.8	-1.76	0.0003
				ENSG00000278472	AC009268.2	-2.92	0.0080
				ENSG00000271314	AL161729.2	-3.00	0.0027

Most DE mRNAs and lncRNAs were shared between prostate tumors from patients of AA and EA. For example, of 702 upregulated mRNAs in AA patients, 489 (69.7%) were shared with the upregulated mRNAs in EA patients. A similar result was obtained when the lists of lncRNAs were compared, following the same example: of the 708 DE lncRNAs in the samples from patients of AA, 472 (67%) of the genes were DE in common with the samples from patients of EA. Interestingly, the overlap between the lists of downregulated mRNAs and lncRNAs was even higher than that of upregulated genes. A total of 87.6% of the mRNAs and 74.2% of the lncRNAs upregulated in patients of AA were simultaneously upregulated in patients of EA.

To clarify the potential transcriptomic differences between prostate cancer samples from patients of AA and EA, we performed a direct comparison of those tumor samples. Table 2 shows the number of DE mRNAs and lncRNAs. There were only 8 mRNAs and 22 lncRNAs differentially expressed in the mentioned comparison.

Functional enrichment analysis of differentially expressed mRNAs

KEGG pathway enrichment analysis was then performed with the DE mRNAs observed in patients of AA and EA. Both groups presented similarities in the pathways evaluated among up- and downregulated genes. Five pathways were overrepresented among the upregulated genes of patients of AA and four pathways were overrepresented in patients of EA, and the pathway most enriched in both groups was systemic lupus erythematosus. Twenty-eight pathways were overrepresented among the downregulated genes of patients of AA and 24 in patients of EA, 21 of which were shared between patients of AA and EA. Two interesting pathways exclusively overrepresented in patients of AA were the MAPK and Wnt signaling pathways (Figure 2).

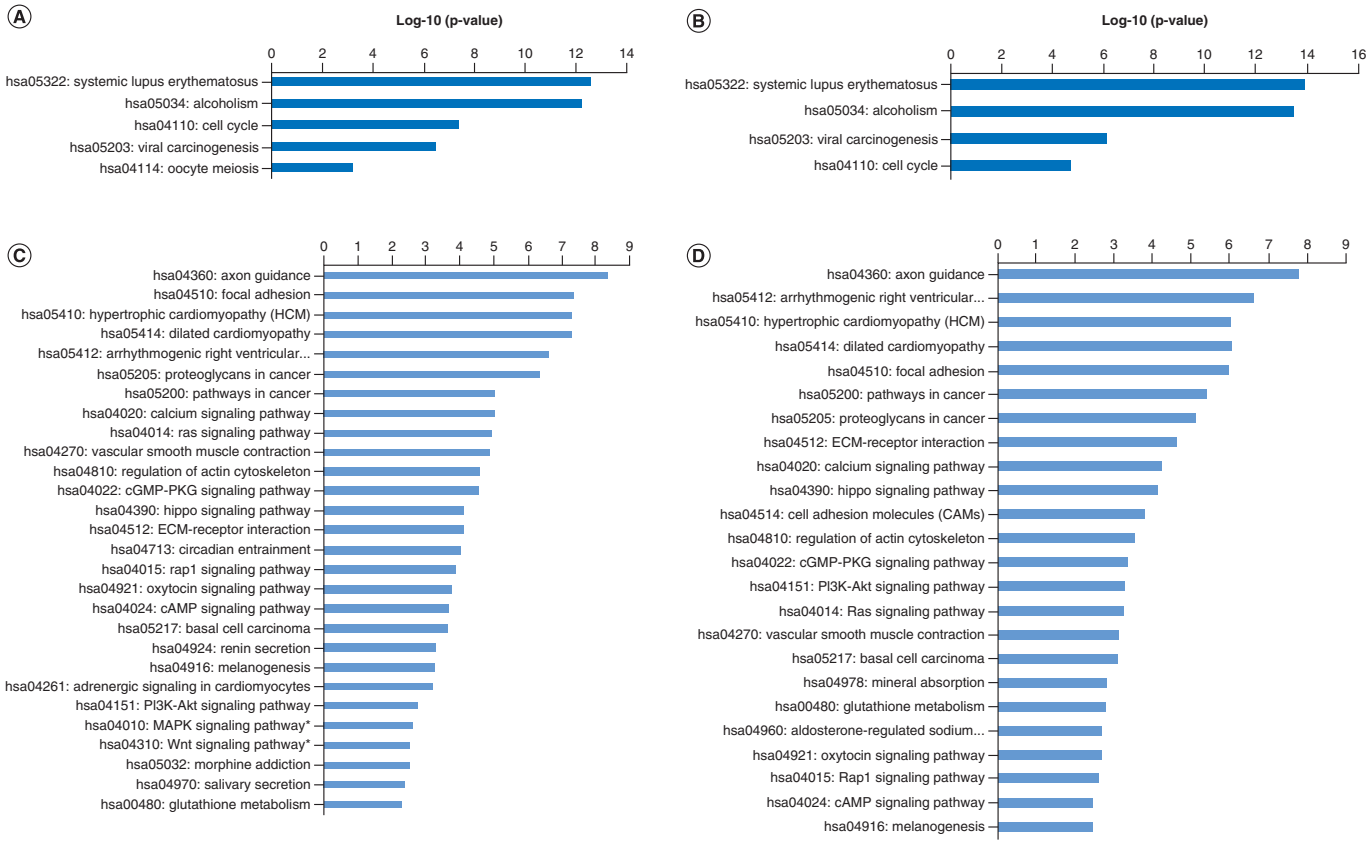

Figure 2. Pathway enrichment analysis for the differentially expressed between genes in patients of African ancestry and European ancestry ($p < 0.05$). (A) Enriched KEGG pathways for upregulated genes in AA. **(B)** Enriched KEGG pathways for upregulated genes in EA. **(C)** Enriched KEGG pathways for downregulated genes in AA (MAPK and WNT pathways). **(D)** Enriched KEGG pathways for upregulated genes in EA.
AA: African ancestry; EA: European Ancestry; KEGG: Kyoto Encyclopedia of Genes and Genomes.

Weight coexpression network analysis of DE mRNAs & lncRNAs in AA prostate cancer patient samples

Taking all of the previous results together, it was evident that there were few important differences in the transcriptomic deregulation of AA and EA. These differences are not sufficient to identify key molecular pathways related to the special clinical characteristics of AA prostate cancer patients.

We hypothesize that the combination of the lncRNA and mRNA expression profiles could be specific for each clinical group and that the differences could help to explain part of the differential clinical behaviour in prostate cancer patients of AA. Then, we performed a coexpression network analysis to identify critical networks of coexpressed lncRNAs-mRNAs in tumor samples from patients of AA.

First, we selected the DE mRNAs and lncRNAs in the comparison of tumor samples from patients of AA versus normal prostate samples. Second, using only the tumoral expression profiles, we calculated two coexpression networks, one for the 49 tumor samples from patients of AA and the other for the 49 tumor samples from patients of EA.

The AA network contained 224 nodes, 65 of them were lncRNA and 159 were mRNA. There were 68 pairs, six trios, two quartets, one quintet and four networks of more than eight nodes of co-expressed genes. Figure 3 shows the coexpression networks with more than five nodes as calculated with AA samples. While the EA network contained 203 nodes, 54 of them were lncRNA and 149 were mRNA. There were 52 pairs, six trios, two quartets, one quintet and three networks of more than eight nodes of co-expressed genes (Supplementary Figure 1).

We calculated the differences in the former network of coexpression of patients of AA with respect to the network of coexpression of patients with EA with the same mRNAs and lncRNAs but with the tumor samples from patients of EA. The results of the network difference analysis indicated the specific relations of PCa patients of AA. The

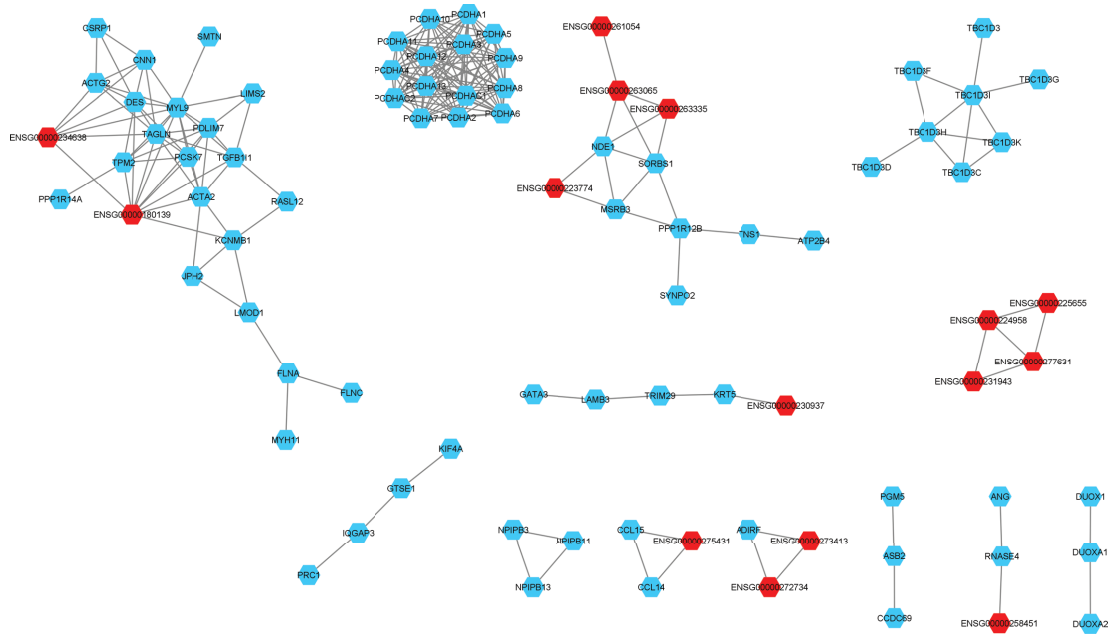

Figure 3. Co-expression network of DE mRNA and lncRNA in African ancestry tumor samples. lncRNA are marked in red and mRNA are marked in blue. Four networks with more than eight nodes of co-expressed genes are shown, one network co-expressed three lncRNA and another network co-expressed two lncRNA. Two tightly connected networks of the same family of genes (Protocadherin Alpha and TBC1 domain family member and 3 [TBC1D3]). In the quintet network one lncRNA was co-expressed. One quartets network was with only lncRNA co-expressed. Among the five trios networks, two had two lncRNA co-expressed and the other only one. Small networks with fewer than two nodes are not shown.

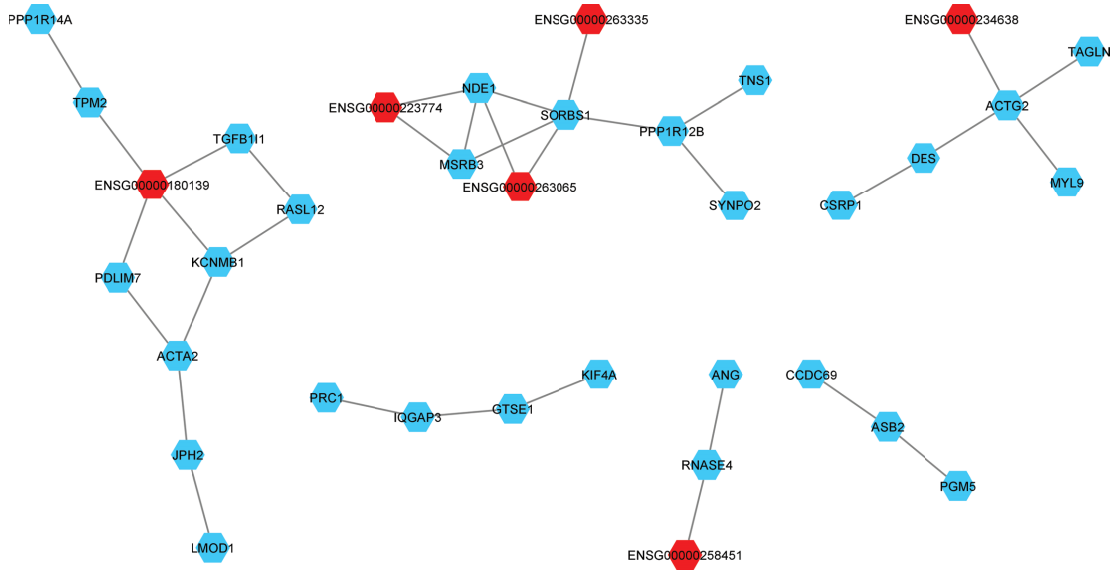

Figure 4. Co-expression network of the differences between African ancestry less European ancestry networks. lncRNA are marked in red and mRNA are marked in blue. One network co-expressed three lncRNA and three network co-expressed one lncRNA. Most of the small networks with fewer than two nodes are not shown.

instances in which patients of AA showed reduced expression compared with patients of EA included 63 nodes, 27 of which were lncRNAs and 36 of which were mRNAs. There were 29 pairs, one trio and four subnetworks with at least six nodes (Figure 4). 24 mRNAs were coexpressed with at least one lncRNAs. Seven mRNAs (CENPN,

Table 3. lncRNAs and mRNAs coexpressed in African ancestry.

Gene ID	lncRNA	logFC	Adjusted p-value		Gene ID	mRNA	logFC	Adjusted p-value
ENSG00000180139	ACTA2-AS1	-2.51	1.53E-08	Interact	ENSG00000145936	KCNMB1	-2.79	2.84E-08
ENSG00000180139	ACTA2-AS1	-2.51	1.53E-08	Interact	ENSG00000196923	PDLIM7	-1.74	3.82E-06
ENSG00000180139	ACTA2-AS1	-2.51	1.53E-08	Interact	ENSG00000140682	TGFB1I1	-2.28	4.33E-07
ENSG00000180139	ACTA2-AS1	-2.51	1.53E-08	Interact	ENSG00000198467	TPM2	-2.55	7.30E-09
ENSG00000223774	NA	-3.09	3.41E-10	Interact	ENSG00000174099	MSRB3	-2.35	6.16E-09
ENSG00000223774	NA	-3.09	3.41E-10	Interact	ENSG00000072864	NDE1	-2.94	1.19E-10
ENSG00000230729	NA	-2.34	0.000161077	Interact	ENSG00000168913	ENHO	-1.95	0.002818634
ENSG00000230937	MIR205HG	-4.12	3.32E-06	Interact	ENSG00000186081	KRT5	-3.65	1.64E-06
ENSG00000234405	NA	-2.86	3.36E-05	Interact	ENSG00000172476	RAB40A	-2.53	0.000185962
ENSG00000234477	NA	-3.42	1.81E-05	Interact	ENSG00000108244	KRT23	-3.11	0.000196113
ENSG00000234638	NA	-3.26	6.33E-10	Interact	ENSG00000163017	ACTG2	-3.19	7.14E-11
ENSG00000249825	**CTD-2201I18.1**	1.84	0.000143879	Interact	**ENSG00000113296**	**THBS4**	2.22	0.000308678
ENSG00000255301	**NA**	1.37	3.50E-05	Interact	**ENSG00000186907**	**RTN4RL2**	1.48	2.89E-06
ENSG00000258451	NA	-1.80	4.42E-08	Interact	ENSG00000258818	RNASE4	-1.55	6.29E-08
ENSG00000259018	NA	-2.68	7.19E-10	Interact	ENSG00000100842	EFS	-2.58	2.68E-08
ENSG00000259172	**NA**	1.27	0.000139358	Interact	**ENSG00000140479**	**PCSK6**	1.30	4.98E-05
ENSG00000259780	**NA**	1.97	2.91E-08	Interact	**ENSG00000167968**	**DNASE1L2**	2.00	3.32E-06
ENSG00000260213	**NA**	1.88	8.23E-07	Interact	**ENSG00000166451**	**CENPN**	1.78	7.19E-06
ENSG00000261707	NA	-1.29	7.90E-12	Interact	ENSG00000140876	NUDT7	-1.07	1.13E-08
ENSG00000263065	NA	-3.47	2.78E-09	Interact	ENSG00000072864	NDE1	-2.94	1.19E-10
ENSG00000263065	NA	-3.47	2.78E-09	Interact	ENSG00000095637	SORBS1	-2.52	3.83E-09
ENSG00000263335	NA	-3.38	6.01E-10	Interact	ENSG00000095637	SORBS1	-2.52	3.83E-09
ENSG00000267577	NA	1.95	5.17E-06	Interact	**ENSG00000167646**	**DNAAF3**	2.14	1.06E-06
ENSG00000267601	NA	-1.75	2.90E-07	Interact	ENSG00000178404	CEP295NL	-1.48	9.32E-05
ENSG00000271894	NA	-2.84	3.66E-10	Interact	ENSG00000055813	CCDC85A	-2.70	3.07E-07
ENSG00000279047	**NA**	2.15	0.00010411	Interact	**ENSG00000112852**	**PCDHB2**	2.27	7.26E-06

Genes in bold are upregulated.

DNAAF3, DNASE1L2, PCDHB2, PCSK6, RTN4RL2, THBS4) were upregulated. THBSA4 and PCSK6 were coexpressed with their antisense lncRNAs, ENSG00000249825 and ENSG00000259172, respectively (Table 3).

Assessment of the prognostic significance of differentially expressed genes among patients of AA & EA & genes coexpressed with lncRNAs

Using GEPIA [30], which employed the PRAD dataset, the DE genes between tumors from patients of AA versus tumors from patients of EA (Table 2) and mRNA coexpressed with lncRNA in the AA-EA coexpression network (Figure 3) were identified. Among the DE genes that were associated with poor disease-free survival were FOXH1 (HR: 2.3; p: 0.0002), NTM (HR: 1.8; p: 0.009), and NPIPB15 (HR: 1.7; p: 0.015) (Figure 5 A–C). Among the genes coexpressed in the network, patients of AA, to a lesser extent than those of EA, was associated with high expression of DNASE1L2 (HR: 2.2; p: 0.0003) (Figure 5 D). and poor disease-free survival, and low expression of RNASE4 (HR: 0.6; p: 0.019), NUDT7 (HR: 0.5; p: 0.011) and SORBS1 (HR: 0.65; p: 0.047) served as protective factors for disease-free survival (Figure 5 E–G).

Discussion

In the present study, we observed subtle differences among PCa patients of AA and EA when we compared transcriptomic dysregulation focused on individual genes. Few DE mRNAs and lncRNAs were obtained in the comparison between the two populations. In the pathway enrichment analysis, some differences between the groups were observed. Two pathways exclusive to patients of AA were the Wnt and MAPK signaling pathways. Both pathways have been associated with PCa oncogenesis due to cellular activities, including cell proliferation, differentiation, survival, death and transformation [31,32]. Interestingly, these two pathways are involved in the

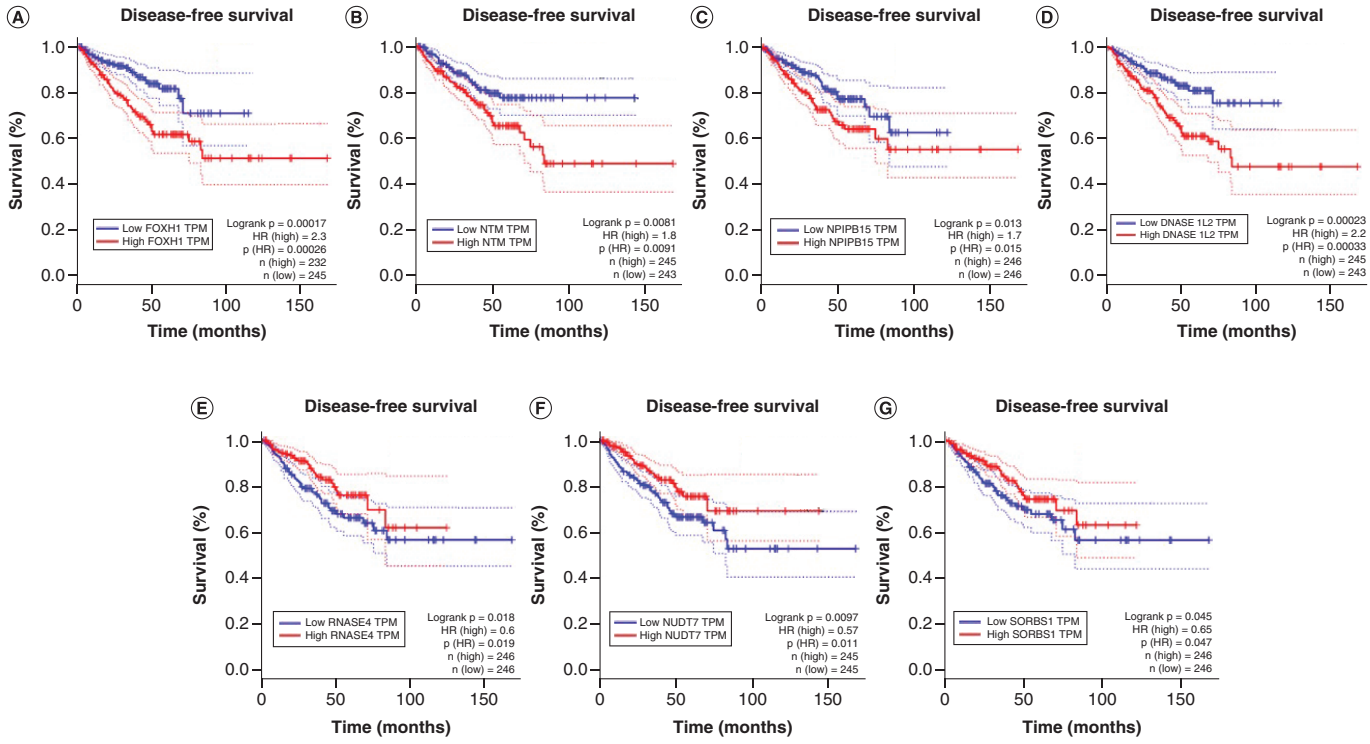

Figure 5. Kaplan–Meier survival plots for overall survival related to DE genes between tumors from patients of African ancestry versus tumors from patients of European ancestry and genes coexpressed with lncRNA in the African ancestry-European ancestry coexpression network. The X and Y axes represent survival time (months) and disease-free survival, respectively. The analysis was made in GEPIA.

immune-related pathway [31,32] and regulation of the AR (androgen receptor) pathway [33,34]. Yuan *et al.* [8] observed high expression of DE mRNA-associated immune-related pathways in patients of AA and found a strong regulatory role for the AR signaling pathway in DE-lncRNAs. Variants of genes of the enzymes involved in both biosynthesis and metabolism of androgen have been observed in patients of AA [35].

Among the DE genes obtained by comparing patients of AA and EA, two upregulated genes (FOXH1 and NPIPB15) in patients of AA associated with poor disease-free survival were associated with the AR signaling pathway. FOXH1 is a member of the forkhead-box (FOX) gene family of transcription factors and participates in mediating transforming growth factor-β/activin signaling through its interaction with the Smad2-Smad4 complex [36]. FOXH1 may act as a corepressor of AR [36]. On the other hand, NPIPB15 is a member of the nuclear pore complex-interacting protein family; however, its role in PCa is unknown. The nuclear pore complex promotes the progression of PCA by increasing POM121-driven E2F1, MYC and AR nuclear import [37].

We found 1563 DE lncRNAs expressed between tumoral tissue versus normal tissue in patients of AA. In the literature, the majority of DE lncRNAs are unstudied. Yuan *et al.* [8] also observed 1868 (11.7%) DE lncRNAs, and the majority were not studied. A total of 45.9% were classified as intergenic lncRNAs, which suggests that they are transcribed from protein-coding independent transcriptional units. Additionally, a high proportion of DE lncRNAs were observed in the nucleus, which indicates that they are probably involved in gene regulation at the transcriptional level.

Due to a few differences between patients of AA and EA, we constructed two coexpression networks oriented to identify critical networks of coexpressed lncRNAs-mRNAs in tumor samples from patients of AA. The results in the first network of DE RNAs in patients of AA showed two tightly connected networks of the same family of genes, TBC1 domain family member and 3 (TBC1D3) and protocadherin alpha (PCDHA). Members of the TBC1D3 family, such as TBC1D3A or PRC7 (prostate cancer gene 17 protein), have been associated with PCa [38]. PCDHA belongs to a subset of a group of cell adhesion molecules, a cadherin superfamily. Yang *et al.* [39] observed that PCDHA is involved in the EMT axis associated with miR-193a-5p and with p53/RBM25-circAMOTL1L. These interesting networks are also present in EA networks (Supplementary Figure 1).

In the second network, where only the coexpression of lncRNAs-mRNAs in patients of AA to a lesser extent than in patients of EA was analyzed, it was observed that mRNAs and lncRNAs were still preserved. Interestingly, two of the upregulated genes (THBSA4 and PCSK6) were coexpressed with their antisense lncRNAs. THBSA4 (thrombospondin 4) was coexpressed with its antisense lncRNA (THBS4-AS1). Liu *et al.* observed high levels of THBSA4 in PCA and mainly in patients with GS >7. Cell lines (PC-3 and DU145) were observed to demonstrate reciprocal regulation of the long noncoding RNAs THBS4-003 and THBS4 to control both migration and invasion processes [40]. A recent study demonstrated that the overexpression of THBSA4 promoted self-renewal and proliferation, inhibited the apoptosis of PCa stem cells and enhanced *in vivo* tumorigenicity, which was achieved by activating the PI3K/Akt pathway [41]. THBSA4 also plays a role in the carcinogenesis of gastric cancer [42] and hepatocellular carcinoma [43]. PCSK6 is another overexpressed gene and is coexpressed with its antisense lncRNA (AC023024.1). PCSK6 is a member of the proprotein convertase family that is involved in PCa carcinogenesis [44] and was recently associated with the proliferation, invasion and migration of breast cancer cells by disturbing cell cycle arrest via the mitogen-activated protein kinase pathway [45]. A coexpression network analysis described the TGFB1I1 mRNA which is a coactivator of the AR that is associated with PCa cell differentiation, and decreased gene expression was observed to be associated with tumor progression [46].

Interestingly, given its high expression in AA and clinical impact, the DNASEL1 gene was noted. This is a member of the DNase 1-like endonuclease family and possesses keratin cell-specific endonuclease activity with an important role in DNA degradation [47]. In a recent study, it was observed that DNASEL1 is upregulated in breast carcinoma, which impacts the EMT process and has relevance in poor overall survival [48].

Conclusion

In the present study, we observed that populations of AA and EA have many similarities in terms of DE lncRNAs and mRNAs and pathway enrichment. We provide a list of DE and coexpressed lncRNAs and mRNAs in both the AA and EA groups. The pathway enrichment analysis revealed two interesting signaling pathways (MAPK and Wnt) that are involved in oncogenesis and are exclusively involved in patients of AA. In the network analysis, lncRNAs and mRNAs were highly coexpressed in AA, and these RNAs have relevance in different pathways related to PCa oncogenesis and have been associated with survival. In summary, this study provides a resource for future investigation of the role of lncRNAs and coexpressed genes in patients of AA and EA.

Summary points

- We observed subtle differences among prostate cancer (PCa) patients of African ancestry (AA) and European ancestry (EA) when we compared transcriptomic dysregulation.
- The pathway enrichment analysis revealed that MAPK and Wnt are involved in oncogenesis and are exclusively involved in patients of AA.
- Two tightly connected networks of the same family of genes (TBC1 domain family member and 3 (TBC1D3) and protocadherin alpha) were expressed in AA. Members of these families have been associated with PCa.
- 24 mRNAs were coexpressed with at least one lncRNAs. Seven mRNAs (CENPN, DNAAF3, DNASE1L2, PCDHB2, PCSK6, RTN4RL2, THBS4) were upregulated. THBSA4 and PCSK6 were coexpressed with their antisense lncRNAs, ENSG00000249825 and ENSG00000259172, respectively.
- Genes differentially expressed or coexpressed as FOXH1, NTM, NPIPB15, DNASE1L2 were associated with poor disease-free survival. And low expression of RNASE4, NUDT7 and SORBS1 served as protective factors for disease-free survival.
- Further studies are required to evaluate a possible role of these mRNA-lncRNA in PCa.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2144/fsoa-2021-0076

Author contributions

Conceptualization: R Parra-Medina, C Payán-Gómez; Methodology, R Parra-Medina, L López-Kleine, S Ramirez-Clavijo, C Payán-Gómez; validation, R Parra-Medina, L López-Kleine, S Ramirez-Clavijo and C Payán-Gómez; investigation: R Parra-Medina and C Payán-Gómez; writing – original draft preparation: R Parra-Medina and C Payán-Gómez; writing – review and editing: R Parra-Medina, L López-Kleine, S Ramirez-Clavijo and C Payán-Gómez; project administration: R Parra-Medina, L López-Kleine, S Ramirez-Clavijo and C Payán-Gómez.

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that for investigations involving human subjects, informed consent has been obtained from the participants involved.

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8.4. CAPÍTULO IV. EXPRESIÓN DE CCR7 EN CÁNCER DE PRÓSTATA DE INICIO TEMPRANO

Expression of Chemokine (C-C motif) receptor 7 (CCR7) in young prostate cancer patients and in metastatic cancer cells

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Abstract

Chemokine (C-C motif) receptor 7 (CCR7) is a chemokine receptor involved in the carcinogenesis of several types of tumours due to its promoting action in epithelial-mesenchymal transition events, invasion, angiogenesis and metastasis. However, the role in prostate cancer (PCa) remains unclear. The objective of this study was to evaluate CCR7 expression by immunohistochemistry in tumours from young patients, and to determine the possible relationship with the clinicopathological characteristics. We retrospectively analyzed paraffin-embedded tissue sections from 23 young PCa (≤ 55 years old) patients and also evaluated the transcriptomic expression in the TCGA database. Expression of CCR7 was observed in 15 cases (65%). The tissue samples from younger patients (≤ 50 years) were mostly positive in 72.7% (8/11) of cases. And 71% (5/7) were high grade Gleason score (≥ grade group 3). The malignant cells present in lymph nodes were 100% CCR7 positive (4/4 cases). The bioinformatic analysis showed a high CCR7 expression associated with the presence of metastasis (FC: 2.6, p: 0.03) in the TCGA PCa cohort (PRAD). In conclusion, we showed that CCR7 expression in tumours from young patients is involved in the early onset of the disease and can also be related to lymph node metastasis.

Keywords: CCR-7, chemokine, Prostate cancer, Early onset, young

Introduction

Prostate cancer (PCa) is the most common tumour in men and the fifth most common cause of cancer death worldwide. It is estimated that there were almost 1.4 million new cases of PCa and 375.304 associated deaths worldwide in 2020 (1). Early-onset PCa (EO-PCa) has been defined as PCa in young patients under 50 years of age at the time of PCa diagnosis (2,3) However, other studies have set the age limit at 60, 55 and 45 years of age (2). Several risk factors are associated with EO-Pca, such as family medical background, ethnicity and genetic factors like single nucleotide polymorphisms (SNP) and mutations in BRCA1, BRCA2, HOXB13, MSH2, ERCC2 and CHEK2_non1100del (4–7), as well as rearrangements of genes in the androgen receptor axis (e.g., TMPRSS2-ERG, PTEN and AR) (8).

When a cell becomes cancerous, it exercises control over the surrounding environment, and affects neighbouring cells and the extracellular matrix. Several hallmarks have been described that identify changes in the activity, function, morphology and behaviour of the cancer cells, and which explain their admirable ability to bypass the cellular, tissue and systemic control of the organisms in which the cancer is established. One of the hallmarks is to promote inflammatory events in their environment (9), which are characterized by the recruitment of leukocytes, production of cytokines and chemokines, induction of angiogenesis, presence of epithelial-mesenchymal transition events (EMT), and the promotion of migration and metastasis (10,11). Several immune cells and chemokines influence PCa progression through action on the molecular pathways stimulating proliferation, angiogenesis, migration, invasion and metastasis events (11). In EO-PCa, Ding et al. (12) detected abnormal expression of genes involved in inflammatory and antitumour immune-related pathways (CTL4, IDO1/TDO2). In a previous study, we found dysregulated expression of mRNAs associated with inflammatory pathways in young PCa patients (13,14).

Chemokines are chemotactic cytokines that act by binding to their respective receptors. In cancer, some chemokines and their receptors have a promoting role in carcinogenesis, inflammation, immune surveillance and cancer progression events (10,11). One of these chemokine receptors is CCR7 which, along with its ligands CCL19 and CCL21, acts in the trafficking and homing of T cells, B cells, natural killer cells (NK cells) and mature dendritic cells (15). CCR7 is a G protein-coupled receptor and it has been demonstrated that the complexes CCR7-CCL21/CCL19 are involved with the promotion of EMT invasion, angiogenesis and metastasis events in several types of tumours, such as breast, lung, pancreatic and esophageal, among others (16). CCR7 expression often correlates with lymphatic metastases and poor prognosis (17). A meta-analysis showed that higher expression of CCR7 in different cancers is correlated with poorer overall survival and progression-free survival (18). CCR7 and its ligands have also been proposed as targets for therapeutic intervention due to their role in carcinogenesis (17).

Few studies have evaluated the role of CCR7-CCL21/CCL19 in PCa. It has been observed that CCR7 is associated with EMT invasion, metastasis (19,20) and enzalutamide resistance (21). As expressed above, the goal of this study was to evaluate the expression of CCR7 in young PCa and its possible relationship with the clinicopathological characteristics.

Material and methods

Patient Samples

Formalin-fixed, paraffin-embedded (FFPE) tissue samples from younger PCa patients (age <55) were retrieved from the surgical archives of the Laboratory of Pathology, National Cancer Institute, Bogotá, Colombia. Hematoxylin and eosin (H&E) stained slides were reviewed to confirm the diagnosis. The clinicopathological features were collected, including Gleason score, extraprostatic extension, margins, seminal vesicle, perineural invasion, lymphatic invasion and AJCC prostate cancer stage group 2018.

Immunohistochemistry study

Tumour tissue sections measuring 3 μm were kept at 60◦C for 2 hours, dewaxed by incubation with xylol for 10 minutes and rehydrated with ethanol in different grades. Heat-mediated antigen recovery was performed with EDTA 10X (Lab Vision™) at a 1/10 dilution in a "vaporizer" for 50 minutes. Next, the slides were immersed in a 1/10 hydrogen peroxide solution (UltraVision Hydrogen Peroxide Block) for 10 minutes at room temperature. Afterwards, they were incubated with primary Anti-CCR7 (ab 32527; abcam) at a 1/1000 dilution for 2 hours at room temperature in a wet chamber, before washing twice with TBS 1/10 (Dako Tris-Buffered Saline, pH 7.6). Later, the slides were incubated with biotinylated secondary antibody (Primary Antibody Amplifier) for 10 minutes at room temperature. They were subsequently buffered with DBA and counterstained with hematoxylin for two minutes. Negative controls were performed by omission of the primary antibody. Positive control slides included sections of tonsil.

Assessment of immunohistochemistry (IHC) staining

CCR7-positive staining was assessed by a single pathologist who was blinded to their clinical features. The staining pattern was catalogued as positive in cytoplasmic cells, or membranous in malignant cells. The intensity was classified as weak (+), moderate (++) or strong (+++).

Data synthesis and analysis

Univariate analysis was applied to determine distribution of clinical and pathological findings.

Chi-square test and Mann-Whitney test were employed to determine statistically significant differences between CCR7 and clinical and pathological findings. A p-value <0.05 was considered statistically significant. Statistical analysis was performed with the STATA 13.

RNA expression analysis

Data collection

The data obtained from The Cancer Genome Atlas (TCGA) and processed by recount2 (22) were used for data analysis. Gene counts for all prostate cancer cases were downloaded.

Data pre-processing

The data in recount2 was processed with Rail-RNA (22). Quality control with density plots and box plots was performed to identify that the selected samples had a similar distribution before and after normalization. Additionally, a multidimensional plot was performed to identify outlier samples.

Identification of differentially expressed genes

The gene count data of the mRNA were analysed on the Galaxy web platform (23). The data were normalized by library sizes using the trimmed mean of M-values (TMM) method. Genes without more than 1 count per million mapped reads (CPM) in at least 50% of the samples were considered not expressed and were filtered out. The limma-voom method was used to identify the differentially expressed (DE) genes.

With the TCGA transcriptomics data, three different comparisons were performed to identify the relevance of CCR7 expression in the molecular biology of prostate cancer. Firstly, the transcriptome of the tumour tissue from young people (83 samples, <55 years old) was compared against the transcriptome of normal tissue from people in the same age range (10 samples). Secondly, the transcriptome of tumours from older individuals (49 samples, > 65 years old) was compared against the transcriptome of normal tissue from people in the same age range (14 samples). Thirdly, the transcriptome of tumour tissue samples from people of any age with metastasis (79 samples) was compared against tumour tissue obtained from individuals of any age without metastasis (367 samples). Finally, FC and FDR values for CCR7 were extracted for all three analyses. A priori, it was defined that a gene is differentially expressed if the linear fold change is higher than the absolute value of 1.5 and the false discovery rate (FDR) is lower than 0.05.

Results

Characterization of the studied population

A total of 23 young PCa patients were included, of whom 20 were prostatectomies and 3 were biopsies. The demographic characteristics of our study population are summarized in Table 1. The median age was 50.8 years (range 46–55); nine were grade group (GG) 1, seven GG 2, four GG 3, two GG 4 and one GG 5. As for the AJCC prostate cancer stage group, 12 were stage IIB, six stage III, four stage IV and one stage IIA. Four patients were N1 (Table 1).

CCR7 expression in prostate tissue

The expression of CCR7 in malignant cells was observed in 15 cases (65%). The median age was 50.3 years old, versus 51.6 years in CCR7 negative samples (n:8), as younger (≤ 50 years old) patients' tissues were mostly positive for CCR7 staining (8/11; 72.7%). 12 cases had weak (+) intensity and three moderate (++) intensity. 71% (5/7) had high grade Gleason scores (≥ grade group 3). Compromised lymph nodes in 4 patients were also analyzed, and we found that the malignant cells of these lymph nodes were all positive cases (4/4 cases), two of which had moderate intensity (Figure 1).

RNA expression results

In the analysis of the TCGA database, we did not find differences among the CCR7 mRNA expression of tumour tissue vs. normal tissue in young patients (<55 years old) (FC: 0.21, p: 0.66), while in old patients (> 65 years old) we observed differences (FC: -0.88, p: 0.01) with lower expression in tumour tissue. In addition, we observed that high CCR7 expression was associated with the presence of metastasis (FC: 2.6, p: 0.03) in the TCGA PCa cohort (PRAD).

Discussion

In the present study, we observed CCR7 expression in 15 of 23 malignant prostate gland cells and in 100% (4/4) of the metastatic cases, suggesting that CCR7 is involved in the carcinogenesis of young PCa- patients. The upregulation (FDR 2.3) of CCR7 in young PCa patients was observed by

Ding et al. (12) in a molecular study which included 49 PCa patients, 24 of them young (38–45 years old) and 25 old (71–74), with the same Gleason score of 7 (3+4) and with T2a and T2c for the pathological stage. The PSA range was 1.9–15.4 ng/mL and the samples included different ethnic groups, comprising 88% whites, 4% African-Americans, 4% Hispanics and 4% Asians. They found that CCR7 and its ligands (CCL21 and CCL19) were age-related differentially expressed genes and they are involved in the inflammatory and immune related pathways. Also, it was observed in the molecular analysis that CCR7 is a target of several genes upregulated in younger PCa. In addition, Ding et al. (12) and we (13) found that mRNA hubs such as hsa-miR-142-5p, hsa-miR-150-5p and hsa-miR-146b-3p were correlated with CCR7 expression.

In the IHC study and in the bioinformatic analysis observed that CCR7 was present in malignant prostatic cells and non-tumoural cells. Ding et al. (12) also found the similar result. One explanation is that tumour-induced inflammation cannot be successfully resolved because it is persistent chronic inflammation (12,24). It is interesting that in the RNA expression results, the PCa patients over 65 years, a loss of CCR7 expression in malignant cells was observed compared to non-tumour cells (FC: -0.88, p: 0.01).

In our analysis, we observed that expression of CCR7 is associated with metastasis (FC: 2.6, p: 0.03) and it was observed in metastatic malignant cells through IHC (Figure 1). The mechanisms related to CCR7 and metastases are not fully understood. Studies have shown that CCR7 could be activated not only under pro-inflammatory mediators but also by other signalling pathways as well. It has been proposed that CCR7 is associated with complex chemokine–chemokine receptor interaction in the microenvironment and is involved in several steps during the process of metastasis (arrest, dissemination, extravasation, survival/proliferation) under the control of the immune system trafficking to the site of inflammation (25). CCR7 has an effect on tumour vascularization by increasing vascular endothelial growth factors (VEGF-A, VEGF-C, VEGF-D), which leads to angiogenesis and lymphangiogenesis (16).

In PCa cells, it has been observed that the expression of CCR7 is involved in their invasion and metastasis. Maolake et al. (20) found that TNF-a leads to the induction of CCR7 expression and the CCL21/CCR7 axis, which might increase migration to lymph nodes. Du et al. (19) observed that upregulation of Notch1 by CCR7 can be associated with EMT and participate in invasion and metastasis by activating MAPK and NF-κB signaling pathways (19). Bao-Jin et al. (26), on the other hand, detected that the silencing of CCR7 inhibits the growth, invasion and migration of PCa cells induced by VEGF-C and also this silencing may inhibit matrix metalloproteinase (MMP)-2 and MMP-9 protein expression. Finally, Youlin et al. (27) found that CCR7, along with MMP9 and Notch1, can be increased by Prostaglandin E2, a known member involved in immune responses inducing dendritic cell migration and homing to draining lymph nodes.

Chemokine receptors such as CCR7 activate intracellular signaling pathways in tumour cells like PI3K/akt, Jak/STAT and MAPK/ERK (17). As shown before, CCR7 may induce growth, invasion and migration of PCa cells through the MAPK pathway (19,26,27). MAPKs (mitogen activated protein kinases) comprise a family of protein-serine/threonine kinases that are involved in the different cellular process, such as cell proliferation, survival, death, differentiation, and transformation (28). In PCa, the MAPK pathway is involved in apoptosis, survival, metastatic potential and androgen-independent growth of PCa (28). Interestingly, we previously observed that the MAPK pathway is overrepresented in younger PCa patients and not in older PCa patients (71–74 years old) (13). Our previous study also found that mRNA hubs such as hsa-miR-142-5p, hsa-miR-150-5p and hsa-miR-146b-3p were overexpressed in younger PCa patients and these mRNA hubs were correlated with CCR7 expression, and hsa-miR-142-5p and hsa-miR-146b-3p were also correlated with MMP9 expression (13). MMP9 is involved in proliferation, angiogenesis, EMT, apoptosis and metastasis of PCa (29). As shown by Bao-Jin et al. (26) and Youlin et al. (27), CCR7 has an interaction with MMP9 and this may have a relationship with growth, invasion and migration of PCa cells

Moreover, hypoxia is a common phenomenon in human solid tumours and is associated with angiogenesis, invasion and metastasis (30). In PCa, hypoxia was associated with EMT, invasion,

metastasis and resistant to radiotherapy and chemotherapy (31–34). The key regulator under hypoxic conditions is stabilized hypoxia inducible factor-1 (HIF-1) alpha that it was observed upregulated in early stage of PCa and subsequent downregulation at later metastatic stages (34). In PCa, also it was observed the hypoxia increases CX3CR1 expression via HIF-1 and NF-κB (35). It has been observed that hypoxia induce CCR7 expression via HIF-1 alpha and HIF-2 alpha in different tumours such as breast (36), lung (37), head and neck (38) and ovary (39). This interaction among hypoxia-HIF1alpha-HIF2alpha-CCR7 was associated with migration and (37,38). This axis also was associated with the expression of pERK1/2 a member of MAPK/ERK pathway (37).

The major limitation of this study is the sample size of young PCa patients; therefore, additional studies with large sample sizes are needed to establish a relationship linking CCR7 expression with carcinogenesis and metastasis in PCa.

Conclusion

CCR7 is a chemokine receptor involved in the carcinogenesis of several types of tumours and associated with EMT invasion, angiogenesis, and metastasis (16,17). Few studies have evaluated its role in PCa. In the present study, we provide information that the youngest PCa patients had more CCR7 positivity in primary tumours, suggesting that CCR7 is involved in the early stage of the disease. In addition, we found that CCR7 expression was correlated with lymph node metastasis and was corroborated by immunohistochemistry. Taken together, the previous studies support our findings and suggest that CCR7 may participate in the metastasis of prostate tumour, especially in early-onset of the disease. This participation may associate with interaction that have CCR7 with endothelial growth factors, MAPK, NF-κB, hypoxia, and inflammatory pathways.

Table 1. Clinicopathological features of prostate cancer patients included in the study

Variable	CCR7 positive (n:15)	CCR7 negative (n:8)	Total (n:23)	p-value
Age, median (years) (range)	50.3 (46-55)	51.6 (49-54)	50.8 (46-55)	0.31
Grade group				0.35
1	4	5	9	
2	6	1	7	
3	2	2	4	
4	2	0	2	
5	1	0	1	
AJCC prostate stage				0.24
IIA	0	1	1	
IIB	7	5	12	
III	4	2	6	
IV	4	0	4	
Nodal Stage				0.25
N0	0	0	0	
N1	4	0	4	

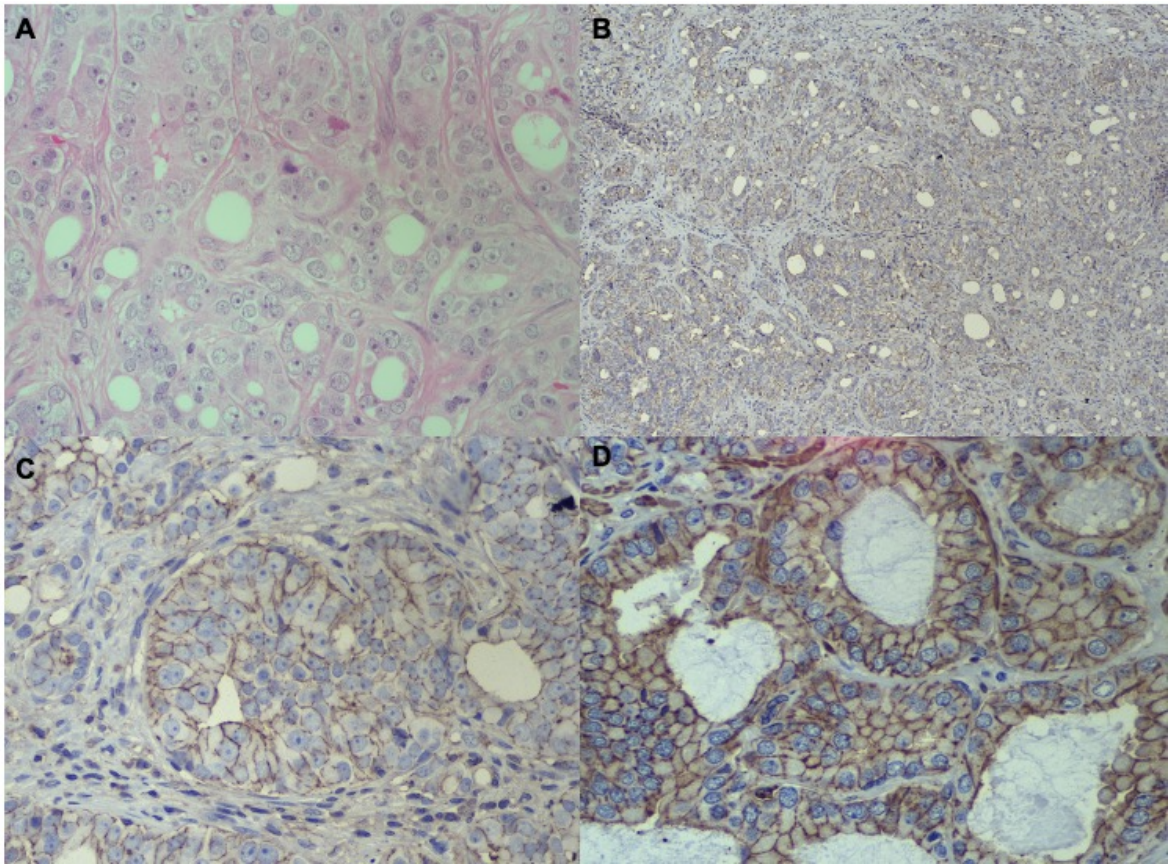

Figure 1. Gleason grade 4 prostate cancer (Hematoxylin and eosin) **A.** CCR7 protein expression in prostate malignant cells (+) (10x) **B.** and in metastatic malignant cells in lymph node (+) (40x) **C**. (++) (40x) **D**.

Ethics approval and consent to participate

This study was approved by the Research Ethics Board at the Instituto Nacional de Cancerología, E.S.E., Bogotá- Colombia, and it was designated as an exempt study for informed consent.

Conflicts of Interest

The authors declare that they have no conflicts of interest

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9. CONCLUSIONES

En esta tesis doctoral se identificó que las vías moleculares y la expresión de genes del CP pueden variar según la edad de presentación. Mediante la detección de miRNAs expresados diferencialmente, observamos miRNAs exclusivos entre los dos grupos, algunos de ellos con pronóstico en los estudios de sobrevida.

En la aproximación experimental para determinar el perfil de expresión de miRNAs en tumores de pacientes jóvenes y viejos con CP, y entre el tejido tumoral y el tejido normal. Entre los miRNAs observados encontramos algunos previamente reportados y otros no conocidos. Adicionalmente observamos que la expresión de algunos de estos se asoció con variables patológicas de mal pronóstico y con peor comportamiento clínico.

En el estudio de análisis de redes de coexpresión se encontró que los jóvenes tenían una red exclusiva mientras que en los viejos hubo dos. Los miRNAs se agruparon en las redes según su nivel de expresión (sobre expresados o reprimidos). El análisis de representación de vías permitió identificar vías exclusivas y compartidas entre los dos grupos evaluados. Finalmente, gracias al análisis de genes coexpresados con miRNAs se reconocieron genes involucrados en las vías de inflamación, MAPKs, metaloproteinasas, entre otros. Estos genes se asociaban con la expresión de miRNAs sobre expresados (hsa-miR-142-5p, hsa-miR-150-5p, hsa-miR-146b-3p), adicionalmente estos genes se encontraron asociado en las bases de datos de pacientes con CP de ascendencia africana.

Las vías moleculares de pacientes con CP de ascendencia africana tienen algunas diferencias cuando se comparan con no africanos. En nuestro estudio de análisis de redes de coexpresión con lncRNA y miRNA observamos que la vía WNT y MAPKs fueron exclusivas de los pacientes de ascendencia africana. Esta última vía también fue observada sobrerrepresentada en los pacientes con CPITe. En este estudio también se destaca que dos familias de genes (TBC1 Domain Family Member and 3 (TBC1D3) y Protocadherin Alpha (PCDHA)) estaban expresadas principalmente en jóvenes.

Finalmente, se eligió el gen CCR7 como gen de interés debido a que este se coexpresó con los miRNAs sobre expresados (hsa-miR-142-5p, hsa-miR-150-5p, hsa-miR-146b-3p) de pacientes con CPITe. CCR7 es un receptor de quimiocinas implicado en la carcinogénesis de varios tipos de tumores y asociado con la invasión, angiogénesis y metástasis. Pocos estudios han evaluado su papel en el CP. Nosotros observamos que los pacientes más jóvenes tenían más positividad para CCR7 en tumores primarios. Además, encontramos que la expresión de CCR7 se correlacionó con la metástasis en los ganglios linfáticos y fue corroborada por inmunohistoquímica. En conjunto, los estudios anteriores apoyan nuestros hallazgos y sugieren que CCR7 esta involucrado en el desarrollo de la enfermedad y su expresión puede observarse en las etapas iniciales de la enfermedad y en los pacientes cuyos tumores tienen probabilidad de desarrollar metástasis. Esta participación puede asociarse con la interacción que tienen CCR7 con factores de crecimiento endotelial, MAPK, NF-κB, hipoxia y vías inflamatorias.

En conclusión, en esta tesis doctoral fueron identificadas diferencias moleculares entre los tumores de pacientes con CP de inicio temprano y de inicio tardío. Se evidenció diferentes miRNAS, mRNAs y vías moleculares para cada grupo, lo que aporta nuevo conocimiento a las ciencias médicas a partir del cual se puede profundizar más en futuros trabajos. También fueron reveladas algunas similitudes entre los tumores de pacientes con CP de ascendencia africana y los pacientes jóvenes con CP independientemente de la ascendencia, esto genera una nueva línea de investigación sobre genes blancos que pueden estar involucrados en el desarrollo de CP con comportamiento clínico agresivo. Como se muestra en el documento es conocido que la manifestación clínica de los pacientes jóvenes con CP y pacientes con CP de ascendencia africana suele a ser mas agresiva en los pacientes con alto riesgo. En último lugar, el presente trabajo aporta valiosa información para que en futuras investigaciones se indague acerca de la posibilidad de validar si las moléculas aquí reveladas pueden llegar a ser biomarcadores con relevancia en el diagnóstico y pronóstico del CP.

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11. ANEXOS

Parra-Medina and Ramírez-Clavijo
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SHORT COMMUNICATIONS Open Access

Why not to use punch biopsies in formalin-fixed paraffin-embedded samples of prostate cancer tissue for DNA and RNA extraction?

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Abstract

Extraction of DNA and RNA from formalin-fixed paraffin-embedded (FFPE) tissue blocks is a critical process in molecular oncology testing. Using FFPE, it is possible to choose the portion of tissue to study, taking into account the cell morphology, storage stability and storage conditions at room temperature, and make retrospective studies with clinical and pathological information. In prostate cancer tissue, in contrast with macroscopic tumors, it is not easy to identify the tumor; therefore, it is very important to make a microscopic diagnosis. We do not recommend punching this tissue because it can choose normal tissue for molecular analysis. In the present article we review the differences between punch biopsy and microdissection.

Keywords: Prostate cancer; FFPE, Punch, Microdissection, Molecular

1 Introduction

One of the aims of molecular biology is the search for new biomarkers to determine the prognosis and response to treatment of patients in the era of personalized medicine. Tissue is a fundamental source of information in molecular biology. The tissue may be stored in fresh or in formalin-fixed paraffin-embedded (FFPE) tissue blocks. FFPE tissue may be stored in pathology laboratories or biobanks together with its clinical and pathological information, which allows retrospective molecular studies to be made.

Extraction of DNA and RNA from FFPE tissue is a critical process in molecular oncology testing. Several events to consider in the preanalytical phases are fundamental in order to get nucleic acids with high quality. These include tumor percentage in the tissue sample [1], age of the FFPE block [2], the method used for nucleic acid extraction [3] and quantity [4].

The results of prostate tumor tissue studies can be very different depending on the characteristics of the piece of tissue from which the nucleic acids were extracted. Procuring inadequate tissue may give wrong data. Therefore, the aim of this article is to review those aspects to consider in prostate tissue sampling and why we do not recommend punching FFPE blocks in those cases.

1.1 Advantages and disadvantages of FFPE use in molecular studies

The ideal tissue to get the best quantity and quality of nucleic acids is in fresh tissue. However, it is much more logistically difficult and expensive to collect fresh tissue than FFPE samples. Fresh tissue requires liquid nitrogen or dry ice for the freezing process and ultralow temperature freezers for storage [5].

FFPE is a very good option for molecular studies (genomics, proteomics, glycomics, metabolomics and lipidomics) [7]. Among the advantages that FFPE has are

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the possibility to choose the portion of tissue to study, taking into account the cell morphology, storage stability and conditions at room temperature, and make retrospective studies with clinical (clinical history, diagnosis, treatment history and response and outcome) and pathological (morphology, immunohistochemistry and immunofluorescence results) information. Fortunately, only small fragments of DNA and RNA are required to reconstruct a sequence of interest [7]. DNA isolated from FFPE blocks is usable for PCR amplification even in products up to 250 bp in length [6]. RNA is less stable than DNA; however, it has been observed that FFPE may be successfully used in gene expression analysis even in tissue more than 10 years old [8].

The disadvantages of FFPE are related to failures during the preanalytical phase related to issues such as cold ischemia, fixation, type of paraffin, high temperatures, the protocol used and archival times. The fixation process may cause modifications of biomolecules such as crosslinkage of nucleic acids with proteins, covalent modifications of both DNA and RNA, and fragmentation of RNA, making it challenging to extract nucleic acids of high quality from FFPE tissues [9]. Fixation should be carried out in 10% neutral buffered formalin and it is important that the formalin penetrates the whole specimen. The fixation time should be 24 to 72 h. Over fixation may have an impact on the molecular results [10, 11]. The storage time and conditions (temperature and humidity) of FFPE blocks may affect the nucleic acids. Studies observed that 3 years of storage cause changes in the DNA quality. However, it has been observed than 18 years of storage may have only a minor influence on sequence quality [5, 10, 11].

1.2 Why not to punch FFPE blocks of prostate cancer tissue

Two techniques have been proposed for obtaining tissue in order to extract nucleic acids to carry out molecular tests such as PCR, qPCR and next-generation sequencing (NGS), among others. They are punch biopsy and the microdissection method (manual or laser capture) [12, 13]. The goal of microdissection is to extract the previously captured area of interest at the discretion of the researcher.

The integrity of nucleic acids is fundamental to get the best results. Morlote et al [12] compared the DNA integrity following punch biopsy and a type of microdissection (Pinpoint Slide DNA Isolation System). They found less degradation in DNA from punched samples that those taken by the Pinpoint technique ($P < 0.0001$). They included samples with a high number of tumor cells such as colorectal adenocarcinoma, lung adenocarcinoma, melanoma and glioblastoma. In contrast with these samples, prostate cancer develops multifocally in anywhere from 60 to 90% of patients [14]. Unlike other tumors, prostate cancer does not cause a macroscopic tumor and the changes may be subtle (color and texture); therefore, the prostate is cut by serial section at 2- to 3-mm intervals from apex to base. Each serial section is put in a cassette [15]. Under microscopy, the foci may have different sizes, shapes and distributions within the prostate. The lack of a macroscopic tumor means that there is no guarantee that there will be tumor cells in the fresh frozen section; therefore FFPE is the best option.

In prostate cancer and samples with small target cells, we recommend the use of the microdissection method (manual or laser capture). Laser capture microdissection has an advantage in small samples such as prostate cancer [16]; however, special equipment is required that may cause limitations in terms of the special training, cost and space required [12, 17]. We do not recommend punching FFPE samples of prostate cancer because, as the foci of glands can be small and variable in size, they may be difficult to identify in the middle of the paraffin block. The punch method may also cause contamination of tumor cells with surrounding necrosis, mucin pools, inflammatory cells and non-tumor tissue. The presence of non-tumor tissue may cause wrong data (Fig. 1). It has been observed that the molecular profile of tissue adjacent to the tumor presents an intermediate state between normal and tumor tissue [18].

In the microdissection method (manual or laser capture), the paraffin blocks are cut (5–10 microns in thickness) to get unstained slides (five to ten slides). These slides are deparaffinized with xylene and ethanol. Afterward, the slides have to be stained with hematoxylin and eosin (H&E) for a few seconds and then the pathologist has to identify the tissue target (Table 1). In manual capture microdissection, the pathologist has to extract the tissue with a blade under microscopy (Fig. 2). Previous studies observed that H&E staining does not have effects on the DNA yield nor molecular oncology test results [19].

For best results, it is very important to use extraction kits with high performance to obtain enough quantity and good quality of nucleic acids. Studies published by Carlsson et al [9] and Patel et al [3] compared commercial nucleic acid extraction kits on prostate biopsies. The results showed that the extraction kit affects the quantity and quality of extracted products. Carlsson et al [9] found that nucleic acids extracted with RNeasy® FFPE and QIAamp® DNA FFPE Tissue kits had the highest quantity and good quality. Also, they found a strong association between good nucleic acid extraction and the amount of tumor taken from the tissue sample. Patel et al [3] observed that the AllPrep kit by Qiagen is the most suitable for FFPE.

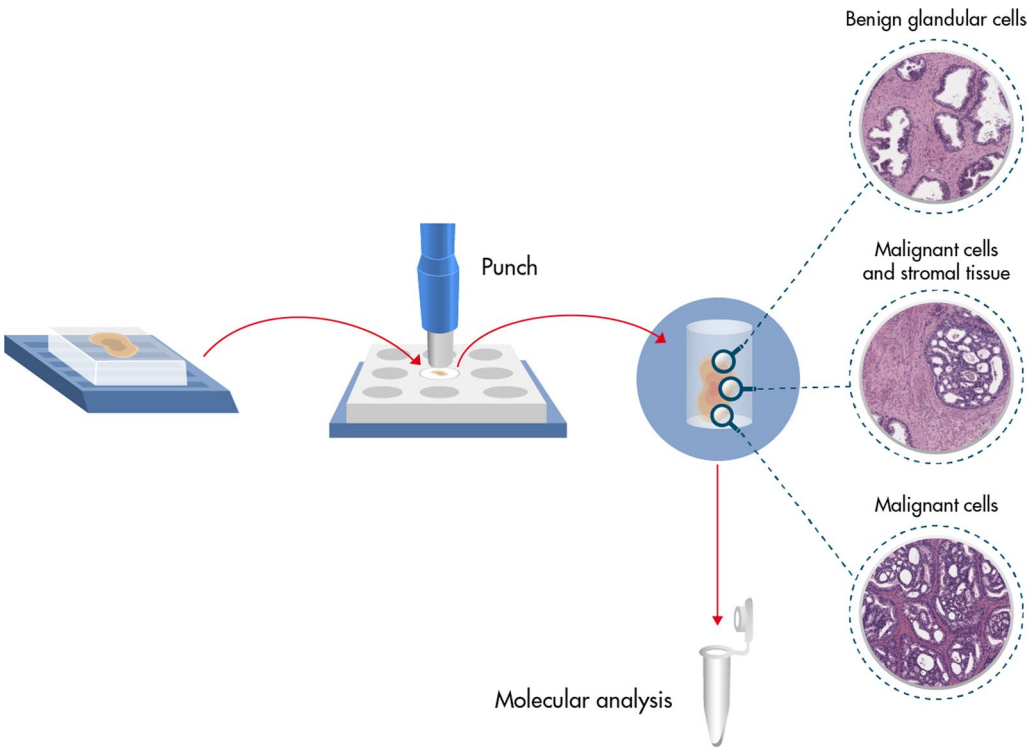

Fig. 1 Tissue obtained by punch biopsy. The tissue is on the FFPE blocks and is extracted by punch biopsy. The sample is sent to molecular analysis. The tissue may have malignant cells in the external part but inside, the paraffin may have benign glandular cells

Table 1 Protocol to deparaffinize the sections of FFPE tissue

1. Fresh xylenes (to deparaffinize the sections)-5 min	1
2. Fresh xylenes-5 min	2
3. 100% ethanol-30 sec	
4. 95% ethanol-30 sec	
5. 70% ethanol-30 sec	
6. Deionized water-30 sec	
7. Mayer's Hematoxylin (10%)- 30 sec	
8. Deionized water-rinse 15 sec (x 2)	
9. 70% ethanol-30 sec	
10. Eosin Y (10%) - 15 sec	
11. Deionized water-30 sec (x 2)	
12. 3% glycerol in deionized water-30 sec	
13. After removing the slide from the 3% glycerol step, shake the slide in the air to remove the layer of glycerol/water.	
14. The next 5–10 mins are the optimal time for microdissection. The tissue is dry, but retains a soft consistency. If the dissection takes more than a few minutes, the tissue will become increasingly brittle and the dissected fragments may be repelled as the needle is brought in proximity to the tissue. If the tissue becomes overly dry, re-soak in the 3% glycerol/water solution for 1–2 minutes.	

Table adapted from http://www.protocol-online.org/cgi-bin/prot/view_cache.cgi?ID=2411

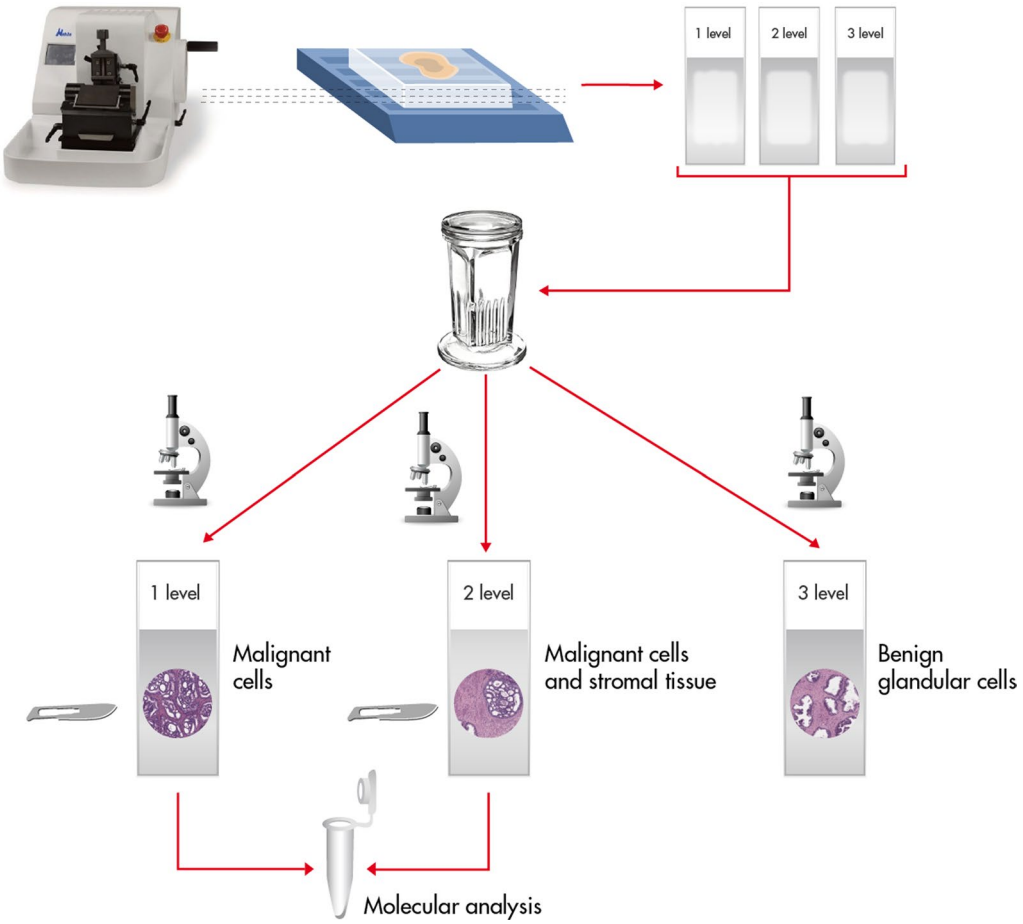

Fig. 2 Tissue obtained from microdissection. The tissue is cut with a microtome and unstained slides are obtained. These slides are deparaffinized (Table 1). The pathologist can choose the malignant cells and extracts the tissue with a blade under microscopy. For example, the whole slide in the first level, only glandular malignant cells in the second level. The third level has benign glandular cells. Therefore, only the tissue from first and second levels are sent for molecular analysis

Abbreviations
FFPE: Formalin-fixed paraffin embedded.

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R.P.-M. contributed to conceptualization; R.P.-M. and S.R.-C. contributed to methodology. R.P.-M. and S.R.-C. contributed to investigation. R.P.-M. and S.R.-C. contributed to writing—original draft preparation; R.P.-M. and S.R.-C. contributed to writing—review and editing. All authors read and approved the final version of the manuscript.

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