Detección y significado clínico de la enfermedad mínima residual o micrometástasis en tumores sólidos

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Los directores de esta tesis doctoral, D. Luis M. Antón Aparicio y Dña. Angélica Figueroa Conde-Valvís,

CERTIFICAN:

Que D. Manuel J. Valladares Ayerbes, Licenciado en Medicina y Cirugía por la Universidad de Sevilla, ha realizado en el Servicio de Oncología Médica del Complejo Hospitalario Universitario A Coruña y en el Grupo de Oncología clínica y de traslación del Instituto de Investigación Biomédica A Coruña (INIBIC), el trabajo ***"Detección y significado clínico de la enfermedad mínima residual o micrometástasis en tumores sólidos"***, el cual reúne todas las condiciones para ser presentado como Tesis Doctoral.

Y para que así conste, firman el presente certificado en A Coruña, a 21 de Septiembre de 2015.

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Director

Dra. Angélica Figueroa Conde-Valvís.
Directora

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RESUMEN

Resumen

La detección de células tumorales circulantes (CTC) y diseminadas (CTD) podría ser importante para evaluar el pronóstico en pacientes con cáncer. No se ha definido el perfil molecular asociado con diseminación tumoral. Los microRNAs desempeñan un papel clave.

En el primer artículo, se investiga la presencia de CTD en médula ósea en cáncer colorrectal (CCR), gástrico (CG) y pancreático y su asociación con el perfil de microRNAs. Los resultados muestran que las CTD y el incremento de miR-17-92 son potenciales marcadores pronósticos independientes.

A continuación, el objetivo fue identificar nuevos biomarcadores de mRNA y miRNA. En el segundo artículo, se analizó el valor diagnóstico y pronóstico de AGR2 y LGR5 en sangre en CCR. Los resultados indican que AGR2 y LGR5 pueden reflejar la presencia de CTC, incluyendo células progenitoras. Los incrementos de AGR2 y LGR5 se asociaron con peor pronóstico.

En los dos últimos artículos, se analiza si microRNAs circulantes de la familia miR-200s podrían constituir biomarcadores en CG y cáncer de mama. La familia miR-200s regula la migración y la invasión. Se encontró que el nivel de miR-200c circulante estaba desregulado en estos tumores en comparación con controles, siendo además, un factor pronóstico independiente en ambos tipos tumorales.

Resumo

A detección de células tumorais circulantes (CTC) e diseminadas (CTD) podería ser importante para avaliar a prognose en doentes con cancro. O perfil molecular asociado á diseminación tumoral, onde os microRNAs desenvolven un rol clave, aínda non foi definido.

No primeiro artigo foi investigada a presenza de DTC na médula ósea e a súa asociación co perfil de microRNA nos tumores colorrectais (CRC), gástricos (CG) e de páncreas. Os resultados indican que o incremento de CTD e miR-17-92 son potenciais marcadores prognósticos independentes.

A continuación, o obxectivo foi identificar novos biomarcadores de mRNA e microRNA. No segundo artigo, foi avaliado o valor diagnóstico e prognóstico de AGR2 e LGR5 no sangue en doentes con CCR. Os resultados indican que AGR2 e LGR5 poden reflectir a presenza de CTC, incluidas células proxenitoras. Os aumentos de AGR2 e LGR5 foron asociados cun peor prognóstico.

Nos dous últimos artigos, analizouse se os microRNAs circulantes da familia mir-200s poderían ser biomarcadores no CG e cancro de mama. A familia miR-200s regula a migración e a invasión. Encontrouse que o nivel de miR-200c circulante estaba desregulado nestes cancros en comparación cos controis, sendo ademais, un factor pronóstico independente en ámbolos dous tipos tumorais.

Abstract

Detection of circulating and disseminated tumor cells (CTC and DTC) could be important to evaluate the prognosis in cancer patients. However, the molecular profile associated with tumor dissemination has yet to be defined. MicroRNAs play key roles in cancer.

In the first article, we investigated in patients with colorectal, gastric and pancreatic cancer whether the presence of DTC in bone marrow (BM) were associated with microRNA tumor profile. Our results suggest the presence of BM-DTC and the up-regulation of miR-17-92 cluster are both significant but independent prognostic markers.

Next, we aim to identify novel mRNA and miRNA biomarkers. In the second article, we estimated the diagnostic and prognostic values of AGR2 and LGR5 mRNAs in blood in colorectal cancer. Our findings indicate AGR2 and LGR5 might reflect the presence of CTC, including stem cell CTC. Increased AGR2 and LGR5 were associated to poor prognostic.

In the last two articles, we hypothesized the quantitative PCR of the miR-200 family in the blood could be useful biomarkers for gastric and breast cancers. The miR-200s regulate invasiveness and migration. Circulating miR-200c levels were deregulated in cancer patients comparing to healthy controls. Furthermore, blood miR-200c levels were independent prognostic factors in gastric and breast cancers.

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Lista de abreviaturas

Ac anticuerpo. AcMo, anticuerpo monoclonal

AJCC "*American Joint Commitee on Cancer"*

AUC área bajo la curva, "area under the curve"

CCR cáncer colorrectal

Ct valor umbral del ciclo, "*threshold cycle value"*

CK citoqueratinas (del inglés "*citokeratins*")

CM cáncer de mama

CTC células tumorales circulantes

CTD células tumorales diseminadas

DDD "*Digital Differential Display*"

EMR enfermedad mínima residual

EMT, TEM transición epitelio mesenquimal

Ep-CAM "*Epithelial cell adhesion molecule*", molécula de adhesión de células epiteliales

HR razón de riesgo, "*hazard ratio*"

ICQ inmunocitoquímica

MO médula ósea

ncRNA RNA no codificante

ROC "*Receiver Operating Characteristic*", o Característica Operativa del Receptor

RT-qPCR, qRT-PCR transcripción reversa seguida de reacción en cadena de la polimerasa cuantitativa

SG supervivencia global

SLP supervivencia libre de progresión

SP sangre periférica

TNM "*Tumor, Lymph Nodes, Metastasis*"

UICC "*Union for International Cancer Control*"

ÍNDICE

1. INTRODUCCIÓN

1. INTRODUCCIÓN

En el año 2011 se produjeron en Europa 1.281.436 muertes por cáncer. En España, se estiman unos 108.000 fallecimientos por cáncer en ambos sexos, siendo de manera global los cánceres de pulmón (18.408) y de colon y recto (8.816) en el hombre y el cáncer de colon y recto (6.368) y de mama (5.952) en la mujer, las causas más frecuentes [1].

Más del 90% de las muertes en pacientes con tumores sólidos serán debidas al desarrollo de metástasis hematógenas. Durante este proceso, las células tumorales deberán desprenderse del tumor primario, invadir el torrente circulatorio y sobrevivir en él, migrando hasta órganos a distancia, en los cuales tras un proceso de extravasación, colonizarán este microambiente y proliferarán, constituyendo finalmente metástasis clínicamente relevantes [2]

El pronóstico de los pacientes con tumores sólidos malignos viene condicionado principalmente por su extensión anatómica y la presencia o no de enfermedad residual tras un tratamiento loco-regional, primariamente quirúrgico [3].

La extensión anatómica de los diversos tumores sólidos se establece habitualmente siguiendo las directrices de la clasificación TNM de la UICC y la AJCC. La clasificación en estadios de los tumores se basa en los hallazgos clínico-radiológicos (TNM clínico) y del estudio histopatológico del espécimen resecado quirúrgicamente (TNM patológico). Esta clasificación sirve de base habitualmente para establecer las indicaciones de un eventual tratamiento médico complementario o adyuvante.

En los pacientes con neoplasias sólidas malignas existe una diseminación sistémica precoz de células tumorales, bien en órganos a distancia como células tumorales diseminadas (CTD) o bien como células tumorales circulantes (CTC) en sangre [4]. La presencia de esta enfermedad metastásica subclínica, no detectable con los estudios de extensión habituales, puede conducir a la aparición clínica de metástasis y servir como factor indicador de un mayor potencial invasivo y un peor pronóstico.

Los estudios que se incluyen en el presente trabajo investigan la enfermedad micrometastásica en distintos grupos de pacientes con cánceres gastrointestinales y en pacientes con cáncer de mama (CM).

Los tumores del tracto gastrointestinal constituyen una de las causas principales de morbimortalidad en todo el mundo. En la Unión Europea en 2011 se estimaron unas

162.026 muertes por cáncer colorrectal, 69.304 por cáncer de páncreas y unas 62.340 por cáncer de estómago [1].

En España el cáncer colorrectal representa el segundo tumor más frecuente en ambos sexos siendo responsable del 12,6% y del 15,1% de las muertes por cáncer en hombres y mujeres respectivamente. Se calcula cada año una incidencia de 22.000 casos y 13.500 muertes. La supervivencia a 5 años, para los pacientes con cáncer colorrectal diagnosticados entre los años 2000 y 2002, fue del 61,5%. En los últimos años se ha registrado, no obstante, un aumento del 2% en la supervivencia global [5-7]

El cáncer gástrico se encuentra entre los cánceres más frecuentes en todo el mundo. Aunque las tasas de incidencia han ido disminuyendo en las últimas décadas, existen amplias variaciones entre países y regiones. La incidencia de adenocarcinomas de la unión gastroesofágica y de cardias se encuentra en aumento en los países occidentales [8]. En España, las tasas ajustados de mortalidad por cáncer gástrico fueron 13 por 100.000 varones y de 5.5 por 100.000 mujeres [9].

En 2011 el cáncer de páncreas fue la causa de muerte de 37.587 hombres y 37.120 mujeres, en la Unión Europea, suponiendo de manera global la cuarta causa de muerte por cáncer en ambos sexos [1]. En España, el número de muertes estimadas para 2011 fue de 2.779 y 2.380 en hombres y mujeres respectivamente, con un aumento de la mortalidad respecto a 2005, especialmente en el sexo femenino [10]..

El cáncer de mama (CM) es la principal causa de muerte por cáncer en la mujer en todo el mundo [11]. La supervivencia ha ido mejorando progresivamente especialmente en los países desarrollados en los últimos 25 años. En España se ha estimado para el año 2012 un total de 27.000 nuevos casos y continua siendo la primera causa de muerte en la mujer con 6.231 fallecimientos. La tasa de mortalidad ajustada por edad y para la población europea fue de 18 por 100.000 personas-año [12].

El desarrollo y la aplicación de métodos de detección de esta enfermedad micrometastásica o mínima residual puede ayudar a conocer con mayor exactitud el estadio de la enfermedad, mejorar la predicción del pronóstico y definir subgrupos de pacientes que podrían beneficiarse de terapia sistémica adyuvante o de nuevas estrategias de tratamiento en la enfermedad avanzada.

2. ANTECEDENTES Y ESTADO ACTUAL

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2.1. Micrometástasis y Enfermedad Mínima Residual. Conceptos

La nomenclatura utilizada para definir la enfermedad tumoral sistémica subclínica varía según distintos autores y a menudo se han usado diversas denominaciones. La presencia de células tumorales circulantes en sangre en pacientes con neoplasias sólidas avanzadas ya fue descrita por T.R. Ashworth en 1869 [13].

Los primeros trabajos con inmunohistoquímica e inmunocitoquímica se llevaron a cabo en la década de los 1980 [14] empleando estos autores por primera vez el término de "micrometástasis" en referencia a esta enfermedad tumoral subclínica detectada mediante anticuerpos (Ac) y tinción inmunoquímica.

En el sistema pTNM el término "micrometástasis" viene definido como la presencia en gánglios linfáticos o vísceras, de metástasis con una dimensión inferior o igual a 2 mm, detectadas en el estudio histopatológico convencional o más a menudo tras secciones seriadas [3]. P. Hermanek propone, en el sistema TNM, la categoría pM1(i), definida como el hallazgo de células tumorales aisladas en MO, mediante inmunocitoquímica (ICQ) con AcMo anti-citoqueratina (CK). Otros autores sostienen que la denominación "micrometástasis" requiere la presencia de acúmulos celulares de al menos 2-3 mm con una reacción del estroma en dónde se encuentran inmersos [15]. De esta forma, el diagnóstico sería exclusivamente histopatológico. No obstante el término "micrometástasis" es uno de los más empleados en trabajos basados en la detección ICQ de células tumorales epiteliales en médula ósea (MO) [16-19].

De igual forma se han añadido diferentes calificativos para hablar de "célula tumoral micrometásica oculta", "célula tumoral aislada o diseminada" del inglés "isolated (disseminated) tumour cell " [20-22]

En el desarrollo de nuestro trabajo usamos de forma equivalente los términos "células tumorales aisladas, diseminadas o micrometastásicas" y "micrometastásis".

Cuando la detección de esta enfermedad tumoral se efectúa en la sangre [23] se prefiere el término de "células tumorales circulantes". Distintos artículos han revisado recientemente los distintos métodos de detección, aislamiento y caracterización de estas CTC [24-26]. Esencialmente los métodos de detección directos se basarían en la demostración de las propias células tumorales en la sangre, incluyendo ICQ,

citometría de flujo, métodos de aislamiento inmunomagnético o por distintas propiedades físicas. Aquí se incluiría el sistema CellSearch, entre otros. Los diferentes métodos moleculares de detección, en general basados en RT-PCR, podrían considerarse métodos indirectos o subrogados, indicativos de la presencia de productos genéticos derivados de las CTC en médula ósea, en sangre, en subcomponentes como plasma, suero, microvesículas o exosomas.

El término "biopsia líquida" del inglés "*liquid biopsy*" fue empleado inicialmente para la detección de CTC en sangre [27-28]; actualmente se prefiere para definir el análisis de ácidos nucleicos en sangre, en las CTC aisladas y también en plasma o suero [29-30], incluyendo el estudio de potenciales dianas terapéuticas moleculares y alteraciones genéticas, especialmente mutaciones, relacionadas con la eficacia de los fármacos [31-33].

Finalmente, el concepto de "enfermedad minima residual" (EMR) hace referencia la presencia de células tumorales, no detectables clínicamente, tras un tratamiento. Desarrollado incialmente en las leucemias, pronto fue aplicado en distintos tumores sólidos [34]. Esta EMR tras la cirugía sería la responsable del posterior desarrollo de metástasis y justificaría la necesidad de tratamiento sistémico complementario [35]. Los distitntos métodos para la detección de células tumorales epiteliales micrometastásicas ayudarían a definir y caracterizar esta EMR.

2.2. Métodos de detección de micrometástasis

El número de células tumorales en la circulación sanguínea o en órganos dianas como la médula ósea es extremadamente reducido. Se estima que en sangre se encontraría, en pacientes con cánceres avanzados, una célula tumoral en $10^{5–7}$ células mononucleares de SP es decir < 10 CTC/mL [36]. Este número sería aún menor en pacientes con enfermedad locorregional o tras la cirugía. En médula ósea el número de células tumorales estaría en torno a $1 \times 10^{-5}–10^{-6}$ [16]. En pacientes con tumores en estadios más precoces su número sería aún menor. Por ejemplo se ha estimado que existirían en CM precoz unas 300 CTD [37]. Así, la detección de esta enfermedad metastásica subclínica con adecuada sensibilidad y especificidad representa un claro desafío metodológico y técnico. Los métodos más empleados para se basan esencialmente en el análisis de la expresión inmunoquímica de biomarcadores o de secuencias génica, en el aislamiento de las células tumorales

en función de sus características físicas específicas o por sus características funcionales.

Cada uno de estos métodos puede ofrecer ventajas potenciales y se han desarrollado en contextos clínicos distintos y en ocasiones complementarios.

De manera esquemática, los métodos basados en la detección y análisis de la expresión de biomarcadores específicos de células tumorales epiteliales en el seno de un tejido u órgano constituido esencialmente por células de estirpe mesenquimal (como la médula ósea o la sangre) más empleados en clínica se basan en la demostración de interacciones antígeno-Ac, como la ICQímica y el aislamiento inmunomagnético, o en la amplificación de secuencias génicas específicas, mediante PCR.

De manera general y aplicable a las técnicas ICQs y moleculares, las propiedades ideales que cualquier biomarcador debería presentar serían: i) una expresión constante y universal en las células tumorales; ii) ausencia de expresión en el resto de células normales del tejido a estudiar; iii) la presencia de dicho marcador debería indicar una información clínica relevante, basada en las características biológicas de esas células detectadas. Para cualquiera de los métodos de investigación de CTD es necesario evitar la presencia de falsos positivos (detección de células no tumorales debido a la expresión basal del biomarcador en tejidos normales) y falsos negativos (no identificación de células tumorales por falta de expresión del biomarcador en subgrupos de células neoplásicas).

Como apuntamos previamente existen excelentes revisiones de los distintos métodos empleados en la investigación de las CTD [25, 26]. En los últimos años se han desarrollado igualmente distintas plataformas, algunas de las cuales son ya disponibles comercialmente. Apuntaremos en esta introducción los principios básicos generales de los métodos inmunoquímicos y los métodos moleculares, que son los empleados en los trabajos de investigación que presentamos y suponen la bases esenciales en el desarrollo de las distintas plataformas. No entraremos a discutir el amplio campo de los estudios inmunohistoquímicos o moleculares en ganglios linfáticos, líquidos o lavados peritoneales.

2.2.1. Detección inmunocitoquímica

La inmunocitoquímica (ICQ) puede considerarse como el estudio e identificación de antígenos en células, mediante la demostración de interacciones específicas antígeno-Ac.

La ICQ ha constituido el método de referencia para la detección y análisis de CTD en la médula ósea. La sensibilidad de la ICQ se encuentra en torno a 1 CTD entre 10^5 a 10^6 células nucleadas de médula ósea [20].

Para la detección de CTD en MO se han utilizado AcMo frente a diferentes antígenos cuya expresión se ha considerado específica de diferenciación epitelial. Las CKs [38, 39] constituyen los marcadores más sensibles y específicos y más ampliamente empleados en la detección de CTD en diferentes tumores [15-17, 20, 23]. Se han establecido los criterios, recogidos en la tabla , para la evaluación de las preparaciones de MO, teniendo en cuenta aspectos inmunológicos, citomorfológicos y los distintos controles [40].

RESULTADO	TIPO CELULAR	CARACTERÍSTICAS	COMENTARIOS
POSITIVO PARA CÉLULAS TUMORALES: CT (+)	Célula tumoral	Morfología típica de célula tumoral. Agregados celulares ("*clusters*"). Núcleo aumentado o atípico	Estas características nunca se encuentran en los falsos positivos
POSITIVO (?) PARA CÉLULAS TUMORALES: CT (+?)	Probable célula tumoral	No presenta características típicas de células hematopoyéticas, pero ocasionalmente esta morfología aparece en falsos positivos. Tinción moderada o intensa irregularmente distribuida. Tinción citoplásmica que cubre parcialmente al núcleo. Cromatina finamente punteada o granulada.	Se clasificará como CT (+) sólo si estas células no aparecen en los correspondientes controles negativos
NEGATIVO PARA CÉLULAS TUMORALES: CT (-)	Células hematopoyéticas (CH), células escamosas de la piel, artefactos.	Células con tinción inmune positiva con morfología de CH: citoplasma microvacuolar, vacuolas, débil tinción homogénea, núcleo similar a las CH, límite celular con "bolsas" regulares, células plasmáticas. Células escamosas cutáneas Artefactos, elementos no evaluables.	

Tabla 1. Criterios para la categorización de CTD en médula ósea. Basado en [40].

Como principio general, se ha recomendado el uso de AcMo frente a determinantes antigénicos comunes a varias CKs o combinaciones de AcMo. El empleo de la combinación de Acs AE1/AE3, ha demostrado una adecuada especificidad, con aproximadamente 0,02 células reactivas en 10^6 células de médula ósea no tumoral suponiendo aproximadamente un 2% de especímenes de MO falsos positivos, especialmente cuando se utilizan criterios inmunológicos y morfológicos en la categorización de las CTD [41]. (Figura 1).

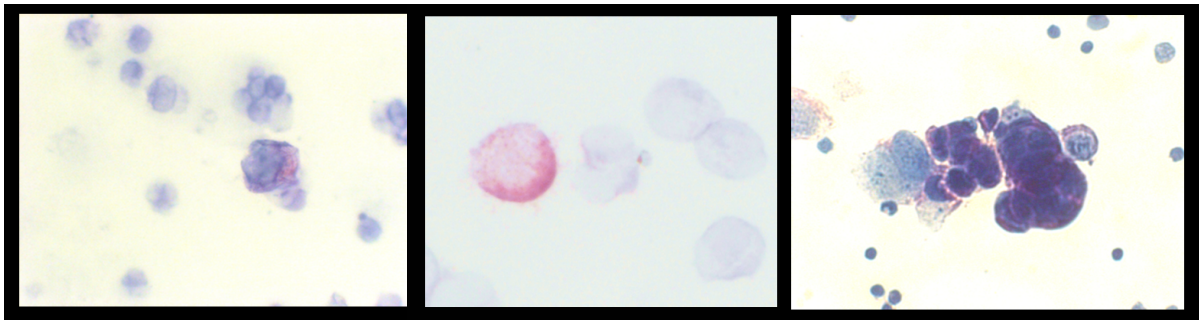

Figura 1. Células tumorales diseminadas en médula ósea en cáncer colorrectal. Inmunocitoquímica con AE1/AE3.

Los Acs AE1/AE3 son dos IgG1 kappa obtenidos mediante inmunización de ratones con queratinas humanas [39]. Identifican la mayoría de queratinas humanas y se emplean ampliamente para la demostración inmunohistoquímica de células procedentes de epitelios simples o estratificados. El Ac AE1 reacciona con un determinante antigénico presente en la subfamilia de queratinas A, incluyendo las CKs con la designación de Moll 10, 13, 14, 15 16 and 19 (con pesos moleculares de 56.5, 54', 50, 50', 48 and 40 kDa, respectivamente). El Ac AE3 reacciona con un determinante antigénico presente en la subfamilia de queratinas B, incluyendo las CKs 1 y 2, 3, 4, 5, 6, 7 y 8 (pesos moleculares de 65, 67, 64, 59, 58, 56, 54 y 52 kDa, respectivamente).

En CM distintos estudios han demostrado el valor pronóstico de la presencia de CTD detectadas mediante ICQ en MO [17, 42-44]. En un trabajo previo de nuestro grupo, empleando la tinción con AE1/AE3, se detectaron CTD en MO en un 24% de las pacientes con CM. La presencia de CTD se asoció con el grado histológico [45]. Esta misma metodología se ha empleado en el trabajo presentado referente a la detección de CTD en MO en cáncer digestivo (artículo 1).

En las publicaciones en cáncer digestivo, los resultados respecto al valor pronóstico de las CTD en MO han sido menos consistentes [46]. En cáncer colorrectal, en un meta-análisis con 36 estudios y 3094 pacientes, la demostración de CTD en MO no se asoció significativamente con la supervivencia global ni la supervivencia libre de recaída [47]. En general, los estudios son heterogéneos e incluyen un menor número de pacientes. Se han empleado distintos anticuerpos para la demostración de las CTD, bien frente a tipos únicos de CKs, como CK-18 y CK-20, bien frente a antígenos como Ep-CAM o mucinas y la evaluación de las preparaciones de MO se ha basado en algunos de los estudios en criterios sólo inmunológicos y no citomorfológicos [48-53]. En pacientes con cáncer gastroesofágico y de páncreas, algunos estudios demuestran una correlación entre la presencia de CTD en MO y un pronóstico adverso, con un mayor porcentaje de recaídas y peor supervivencia, aunque el resultado de los análisis multivariantes no siempre ha sido significativo [54-59].

2.2.2. Detección basada en amplificación de ácidos nucleicos (PCR)

Los métodos moleculares para la detección de EMR se basan en la amplificación, mediante reacción de la polimerasa en cadena (PCR), de secuencias de DNA específicas de las células neoplásicas o de genes expresados de forma diferenciada en las células tumorales o en el tejido del cual proviene el tumor.

La amplificación mediante PCR de ácidos nucleícos celulares o circulantes específicos o selectivos de tejido o de tumor es la herramienta más sensible para la detección de CTD o metástasis ocultas. [60-62].

En distintas neoplasias hematológicas se encuentran translocaciones cromosómicas específicas que pueden ser amplificadas y detectadas directamente a partir de DNA, como por ejemplo la translocación T(14;18) de los linfomas foliculares. En la leucemia mieloide crónica es característico el reordenamiento bcr/abl (translocación T(9;22) o cromosoma Philadelphia). Ambas estrategias han demostrado su utilidad en el diagnóstico de enfermedad residual en estas entidades hematológicas.

En los tumores sólidos, especialmente en los carcinomas, no existen o no están definidas estas translocaciones cromosómicas específicas. Las alteraciones genéticas más frecuentes son mutaciones que afectan a oncogenes (por ejemplo, *K-RAS*) o genes supresores (*TP53*) y alteraciones epigenéticas como metilación de

residuos CG [63]. Mediante métodos de transcripción reversa seguida de reacción en cadena de la polimerasa (RT-qPCR) se puede detectar la presencia de mRNA que se expresa de forma específica en una determinada población celular. De manera esquemática la RT-qPCR comprende en definitiva tres etapas: (i) la conversión del RNA en cDNA mediada por una enzima transcriptasa inversa, (ii) la amplificación del cDNA mediante PCR y, iii) la detección e idealmente cuantificación del producto amplificado.

La detección de la presencia del producto amplificado específico se pone de manifiesto mediante la electroforesis (resolución en geles, en la PCR convencional, o "*end-point PCR*") o mediante la detección y cuantificación de la señal fluorescente mediante PCR en tiempo real ("*real time PCR*"). Mientras que la primera permite la estimación cualitativa y sólo una aproximación semicuantitativa en la estimación de los resultados, la PCR en tiempo real ofrece la posibilidad de establecer una cuantificación del RNA diana.

La PCR en tiempo real o PCR cuantitativa con transcriptasa inversa (RT-qPCR en tiempo real) se ha convertido en el método de elección para cuantificar la expresión de genes específicos, por su sensibilidad y especificidad y por la accesibilidad del equipamiento específico [64]. La PCR en tiempo real permite generar amplicones muy pequeños (desde 60 pb) lo que la hace ideal para la cuantificación de niveles de mRNA en muestras de tejidos con RNA parcialmente degradado.

En la PCR en tiempo real el producto se mide al final de cada ciclo. La detección de la amplificación en cada uno de los ciclos puede efectuarse mediante sondas fluorescentes que se unen de manera específica al producto de la PCR (amplicón) o bien con marcadores fluorescentes inespecíficos como SYBR-Green, que se unen a la cadena doble de DNA del producto de la PCR.

Para la cuantificación se calcula el umbral y el valor umbral del ciclo ("*threshold cycle values*", Ct). El valor Ct está representado por el ciclo en el cual la producción de fluorescencia en la fase exponencial de la reacción de la PCR cruza un umbral establecido. El análisis de la curva de fusión ("*melt curve*") permite discriminar los productos no específicos amplificados de los amplicones específicos. (Figura 2).

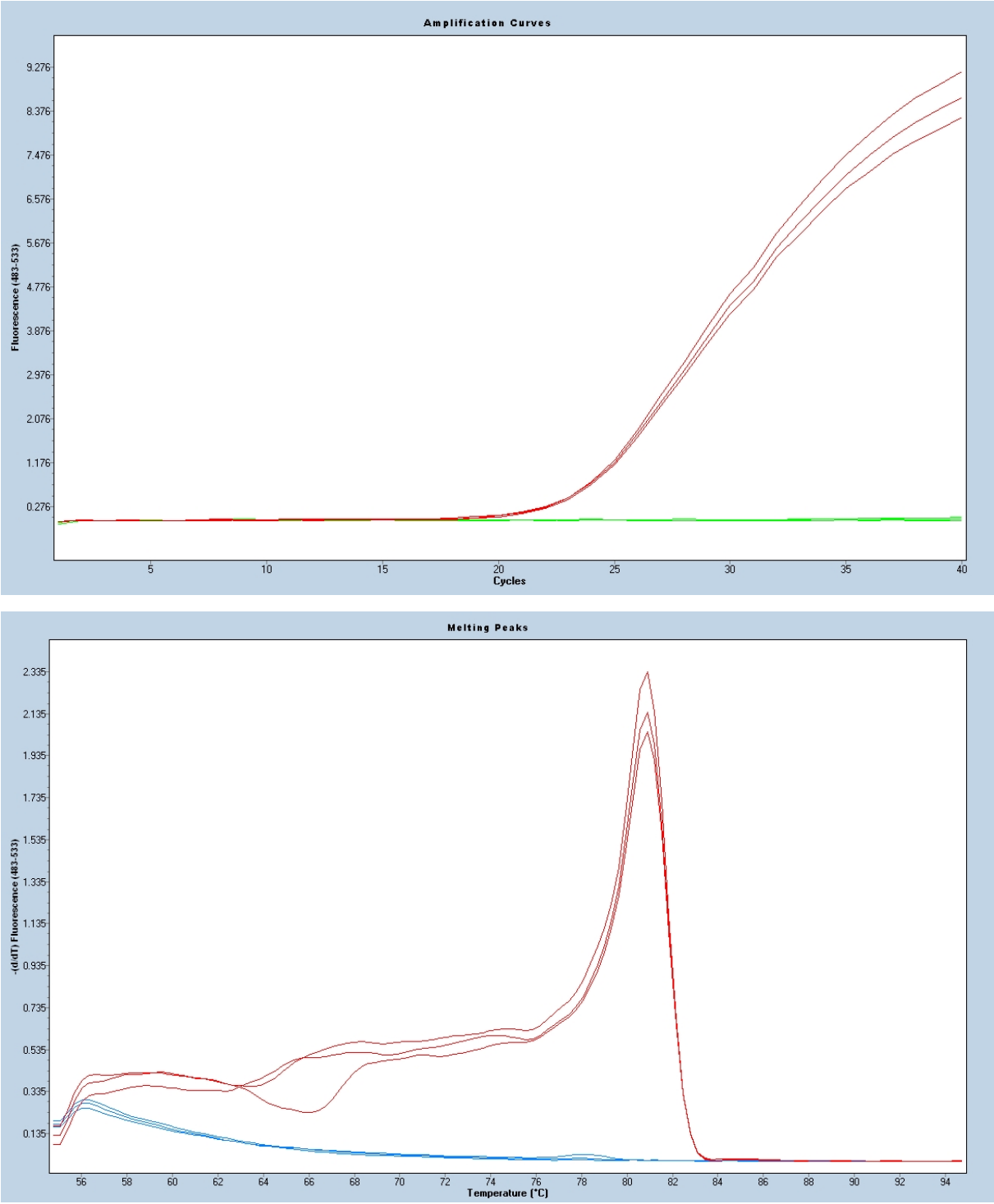

Figura 2. PCR cuantitativa con transcriptasa inversa (RT-qPCR en tiempo real) para miR-200c en sangre. Se muestra la curva de amplificación (arriba) y la curva de fusión (abajo).

Se han definido una serie de criterios y controles a la hora de diseñar ensayos basados en la PCR en tiempo real para la cuantificación de la expresión de genes.

Es necesario atender de manera cuidadosa los distintos pasos y procedimientos, desde la retrotrascripción hasta los controles endógenos empleados y las técnicas de normalización para obtener resultados consistentes y reproducibles [65]

En modelos experimentales la sensibilidad de la RT-qPCR es mayor que la sensibilidad analítica de las técnicas ICQ, estimándose en una célula tumoral entre 10^5 y 5 x 10^6 células hematógenas. La sensibilidad y especificidad depende en parte de los diversos mRNA empleados para la amplificación. En distintos estudios se han usado el antígeno carcinoembrionario (CEA), mucina-1, diferentes CKs, mamaglobina, la molécula de adhesión epitelial (Ep-CAM) o el receptor para el factor de crecimiento epidérmico ("*Epidermal Growth Factor Receptor"*, EGFR) y HER2, entre otros [47, 63, 66, 67].

Se han publicado numerosos trabajos que evalúan en estudios unicéntricos el potencial valor clínico y pronóstico de la detección molecular de CTC o CTD. En cáncer de mama precoz, la presencia de CK-19 en sangre se ha correlacionado con la supervivencia [68]. En cáncer de colon se ha encontrado en un meta-análisis [47] una asociación significativa de la presencia de CTC mediante técnicas moleculares con la recaída y una peor supervivencia.

Sin embargo tanto la presencia del mRNA marcador en tejidos hematopoyéticos como la existencia de pseudogenes y de transcripción ilegítima podrían limitar la especificidad de los diferentes biomarcadores [69-72]. Otro problema adicional sería la heterogeneidad en la expresión de un determinado mRNA en las diferentes células tumorales [73, 74]. Los marcadores moleculares de detección de células tumorales epiteliales tumorales en sangre o médula ósea, de manera idónea, deberían reflejar no solo la presencia de esta enfermedad tumoral subclínica sino también proporcionar una información acerca del potencial de estas células circulantes para sobrevivir en la microcirculación, extravasarse y crecer de forma secundaria en órganos a distancia. De esta forma, la detección de marcadores moleculares que reflejen la capacidad de las células tumorales de actuar como "células iniciadoras de tumores" o "*stem cells*" podría ser de especial relevancia [75, 76].

2.2.3. Otros métodos de detección de micrometástasis

Diferentes propiedades físicas de las CTD o circulantes en sangre permitirían, aunque no de manera absoluta, diferenciarlas de las células hemáticas normales. Entre estas características se incluye un mayor tamaño de la mayoría de células epiteliales, diferencias en la densidad, carga, capacidad migratoria y en su plasticidad.

El aislamiento de las células mononucleares de sangre y médula ósea y las eventuales CTC mediante centrifugación y gradientes de densidad permite un cierto enriquecimiento, esencial para las técnicas de ICQ. El método de aislamiento más utilizado se fundamenta en las diferentes densidades que presentan cada uno de los tipos celulares de la sangre. Para conseguir la separación, la muestra de sangre o del aspirado de médula ósea se deposita sobre un líquido de densidad 1.077 g/ml, (Lymphoprep, Ficoll-Hypaque) y se centrifuga para acelerar la separación. Las CTC migran con la fracción de células mononucleares [77].

El aislamiento de CTC en virtud de su mayor tamaño en comparación con los leucocitos se ha aplicado empleando diferentes sistemas de filtros [78, 79].

Se han descrito distintos sistemas que combinan métodos basados en el reconocimiento de antígenos celulares de las células tumorales (por ejemplo Ep-CAM en células epiteliales) con sistemas de captura de las células a través de partículas o nanopartículas cargadas magnéticamente, de captura sobre matrices sólidas o de filtrado.

En modelos experimentales los distintos sistemas de enriquecimiento ofrecen resultados prometedores, con una significativa especificidad, aunque la aplicación de estos en clínica requiere de estudios de validación.

Cualquiera de los métodos de aislamiento o enriquecimiento de CTC/CTD podría verse limitado por una pérdida de células tumorales durante el proceso, de manera que la sensibilidad del método se vea disminuida. Se considera que las células tumorales constituyen en torno al 0.1% de la población enriquecida.

El sistema CellSearch (Veridex) permite el análisis y cuantificación de CTC en sangre y su uso clínico en distintos tumores ha sido registrado por la FDA. Se basa en el enriquecimiento de las células que expresan Ep-CAM mediante partículas magnéticas recubiertas de Ac [82]. Las células se identifican mediante tinción nuclear (diamini-fenil-indol, DAPI), Acs frente a CKs (8, 18 y 19) con el fluorocromo

ficoeritrina y un Ac frente a CD-45 marcado con otro fluorocromo (aloficocianina) para identificar los leucocitos. Posteriormente la identificación y recuento de las células epiteliales (elementos nucleados, con tinción con DAPI, Ep-CAM y CKs positivas, CD-45 negativas) se realiza por microscopía de fluorescencia, mediante un sistema semiautomatizado. Se ha confirmado en distintos estudios la seguridad, precisión y reproductibilidad del recuento de CTC mediante el sistema CellSearch, con relevancia para el pronóstico, especialmente en pacientes con metástasis, en los que un recuento elevado de CTC se asocia con peor respuesta al tratamiento, progresión de la enfermedad y peor supervivencia [83-87].

Este sistema ha recibido en Estados Unidos la aprobación de la FDA para su uso en clínica, como test pronóstico y de seguimiento de los pacientes sometidos a tratamiento en tumores colorrectales, de mama y próstata.

Sin embargo, las CTCs representan, como se ha dicho, una población heterogénea de células con una combinación de diferentes características moleculares, algunas propias de las células epiteliales del tejido de origen del tumor primario, otras similares a las características moleculares típicas de células de linaje mesenquimal, y otras características similares a células progenitoras. Esta heterogeneidad poblacional y molecular que se engloba bajo el término de CTCs es una de las causas que subyacen a la dificultad para encontrar un método que sea capaz de detectarlas, aislarlas, cuantificarlas y caracterizarlas de forma precisa. Distintos métodos suelen tener una escasa concordancia [45, 88], por lo que el empleo de diferentes biomarcadores y metodologías en la detección de las CTC pueden resultar complementarios [28].

2.3. Biología de las metástasis en los tumores sólidos

Además de su potencial valor en la clínica, los estudios sobre las micrometástasis han permitido profundizar en la comprensión del proceso de diseminación tumoral. Paralelamente, es necesario comprender estos aspectos para diseñar nuevas estrategias de detección de la EMR y valorar las ventajas y potenciales limitaciones de cada una de las metodologías.

Se ha definido una serie de eventos y de características biológicas de las células tumorales, necesarias para completar el proceso de formación de metástasis clínicamente detectables [2, 4, 89]. La célula tumoral individual o un grupo de ellas

debe adquirir la capacidad de invadir y emigrar desde la masa tumoral, a través de los tejidos circundantes, entrar en la circulación linfática y sanguínea, y sobrevivir en la circulación (inhibición de la anoikis, es decir, inhibición de la apoptosis inducida por la pérdida de una adecuada interacción célula-matriz extracelular) resistiendo tanto los mecanismos inmunológicos como las fuerzas generadas en el flujo sanguíneo. Estas CTC deberán nuevamente adherirse al endotelio vascular, migrar a través de éste y anidar en el microambiente de nuevos órganos, como el hígado, el pulmón o la médula ósea [89-90].

En estos órganos a distancia, las CTD pueden eventualmente proliferar o permanecer en un estado de latencia (dormancia) incluso durante años [90-91]. La médula ósea se considera como un órgano de anidamiento de las células tumorales, no solo en tumores dónde las metástasis óseas son frecuentes como en el CM y próstata sino también en otros como adenocarcinomas de origen digestivo. Así mismo, podría actuar como fuente secundaria de nuevas CTC [92].

En modelos experimentales, millones de células tumorales alcanzan la circulación sanguínea, aunque sólo unas pocas células son capaces de completar todo el proceso de metástasis [93]. Se ha correlacionado en la clínica la aparición de metástasis con diferentes características genéticas y biológicas como patrones de expresión de genes específicos, mutaciones, alteraciones cromosómicas, metilación y expresión de RNA no codificantes [94, 95]. Distintos procesos biológicos pueden considerarse de especial relevancia para la detección de las CTC y las CTD y para entender su potencial valor predictivo y pronóstico.

Uno de los eventos moleculares cruciales en el proceso de metástasis es la plasticidad epitelio-mesenquimal [96-97]. (Figura 3). En una primera etapa se produce la transición epitelio-mesenquimal (TEM). La TEM es un conjunto de cambios moleculares y funcionales en las células epiteliales que incluye la disolución de las uniones celulares y la pérdida de la polaridad y que resulta en la pérdida de marcadores epiteliales, como E-cadherina, γ -catenina/plakoglobina, plakofilinas [98,99] y moléculas de adhesión como Ep-CAM [100], en cambios en el citoesqueleto y en la expresión de CKs y en la adquisición de capacidades migratoria e invasiva, propias de células mesenquimales. Este proceso [96-99] conlleva la expresión de marcadores mesenquimales como vimentina y N-cadherina. Las vías de señalización celular esenciales para la TEM son la vía TGF-beta, la vía

WNT y la vía Hippo . La expresión de E-cadherina se encuentra regulada a distintos niveles. Se han descrito distintos factores de trascripción [96] que actuarían como represores transcripcionales directos de E-cadherina, como SNAIL, ZEB1, ZEB2, ETS1, y FLT1. Otros actuarían de manera indirecta como Twist, FoxC2. Adicionalmente, distintos mecanismos post transcripcionales participan en la regulación de la plasticidad epitelio-mesenquimal, incluyendo las proteínas de unión al RNA [101] y los microRNAs [102, 103].

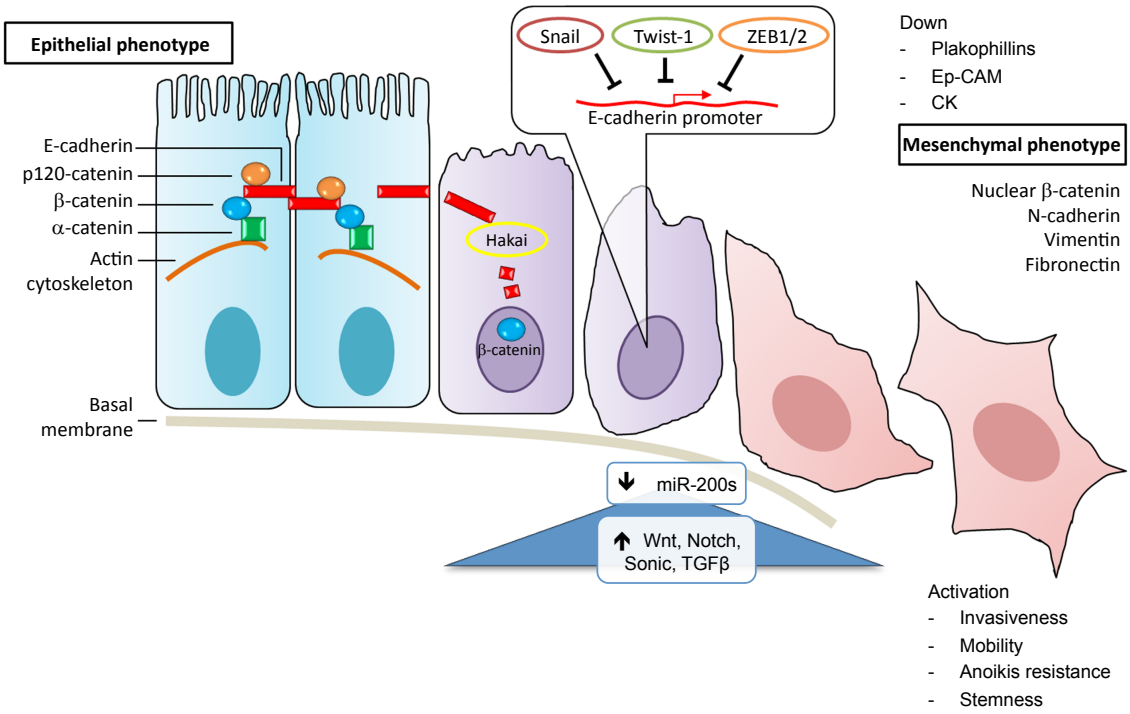

Figura 3. Plasticidad epitelio mesenquimal. Figura modificada, a partir de la original publicada por Aparicio LA y cols. [97].

Este proceso dinámico de plasticidad epitelio mesenquimal conduce a una expresión heterogénea en las CTC y CTD de los marcadores moleculares de origen epitelial (como CKs y Ep-CAM) empleados para su detección y caracterización. Así mismo, la identificación de CTC con diferenciación mesenquimal se hace más compleja en el seno de un tejido como el hematopoyético también de origen mesenquimal. El uso de marcadores moleculares presentes en las células sometidas a procesos de TEM

podría en este sentido aumentar la sensibilidad en la detección de CTD y CTC [76, 88, 100].

Una vez que las CTC alcanzan el lecho capilar de los órganos a distancia, deben adherirse al endotelio y migrar a su través para anidar (*"homing"*) en el tejido a distancia. Este proceso requiere adquirir de nuevo características epiteliales, en el proceso de transición mesénquima a epitelio. Habitualmente las células tumorales expresan de nuevo E-cadherina, CKs y proteínas de los desmosomas en las metástasis [96-97]. A su vez, el propio microambiente del órgano a distancia actuaría modificando el programa transcripcional de las CTD [104-105].

Todos estos procesos condicionan una marcada heterogeneidad de las CTD y CTC, con células con capacidades diferentes de invasión, proliferación y tumorogénesis. Teniendo en cuenta estos aspectos, puede plantearse la hipótesis que mediante el empleo de marcadores moleculares específicos podremos identificar diferentes subpoblaciones de CTC, incluyendo subgrupos de especial relevancia biológica y clínica [106]. Se puede considerar que sólo aquellas CTC y CTD con características de "células iniciadoras de tumores" podrán completar el proceso biológico completo de diseminación y desarrollaran metástasis clínicamente relevante [107].

Se ha demostrado como durante el proceso de plasticidad epitelio-mesénquima se generan poblaciones celulares con características de células progenitoras [108-115]. El programa de TEM, posibilitaría la diseminación de las células tumorales a partir del tumor primario y a su vez promovería la capacidad de auto renovación.

Los microRNA aparecen en este contexto como elementos reguladores claves. (Figura 4). Los microRNA (miRNA) son pequeñas moléculas, de 18 a 24 nucleótidos, que conforman una cadena simple de RNA no codificante [116-119]. Se ha descrito la participación de los microRNAs en procesos esenciales en el cáncer, incluyendo entre otros la diferenciación celular, la proliferación, la angiogénesis, la invasión y la plasticidad epitelio-mesenquimal [102, 103, 120]. Diferentes estudios han puesto de manifiesto una asociación entre los diferentes tumores con un perfil de expresión de microRNA específico [121-122]. Su abundancia, estimada en torno a 10^3 - 10^4 moléculas por célula (hasta 2 órdenes de magnitud mayor que para la mayoría de mRNAs) y su estabilidad [123-124], son características que aumentan el potencial de los microRNAs como biomarcadores en diferentes neoplasias.

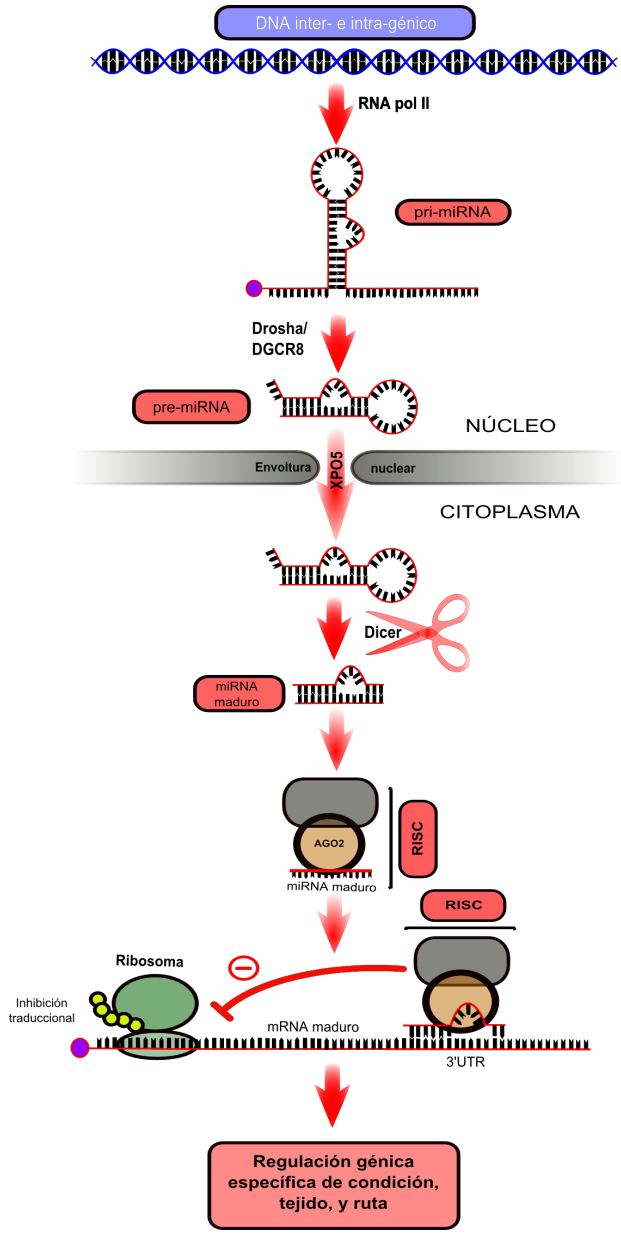

Figura 4. Biogénesis y función de los microRNAs. Los genes que codifican los miRNAs se transcriben en precursores (pri-miRNAs) que contienen una doble cadena y una estructura en horquilla. Los pri-miRNAs son procesados secuencialmente primero por Drosha, transportados al citoplasma por Exportina-5 y posteriormente por Dicer. Finalmente mediante una helicasa se producen dos cadenas, una de miRNA maduro y su miRNA*. La cadena madura se incorpora en el complejo efector RISC (RNA-induced silencing complex). Cada miRNA reconoce una secuencia específica de mRNA, a través de una complementariedad de bases imperfecta. Cada miRNA específico puede regular diferentes mRNA, mediando su degradación o reprimiendo la translación [116, 117].

3. HIPÓTESIS Y OBJETIVOS

3. HIPÓTESIS Y OBJETIVOS

3.1. Hipótesis

En los pacientes con cáncer epitelial existe una diseminación sistémica precoz de células tumorales, bien en órganos a distancia, como CTD, bien como CTC en sangre. La presencia de esta enfermedad metastásica subclínica, no detectable con los estudios de extensión habituales, puede conducir a la aparición clínica de metástasis y servir como biomarcador de un mayor potencial invasivo y un peor pronóstico.

La ICQ con Acs frente a CKs constituye el método de referencia para la detección de CTD en médula ósea. La presencia de CTD en médula ósea en pacientes con diferentes tumores constituye un factor pronóstico adverso. Sin embargo, no están bien definidas las características moleculares de los tumores primarios asociadas con la presencia de esta EMR.

Las técnicas de RT-PCR, especialmente la RT-qPCR cuantitativa en tiempo real, ofrecen ventajas potenciales para detectar biomarcadores de CTD y CTC, como una mayor sensibilidad y especificidad y con la posibilidad de caracterizar, en sangre, la expresión de moléculas implicadas en la progresión tumoral, incluyendo genes específicos de células progenitoras y RNAs no codificantes (ncRNA), como los microRNAs.

Estudios recientes han puesto de manifiesto la participación de los microRNAs en procesos esenciales en el cáncer, incluyendo entre otros, la diferenciación celular, la proliferación, la angiogénesis, la capacidad de invasión y la plasticidad epitelio-mesenquimal. La detección en sangre periférica de microRNAs específicos y la cuantificación de esta expresión reflejaría la existencia de células tumorales circulantes en los pacientes con cáncer.

Por último, la expresión y la cuantificación de estos biomarcadores de enfermedad micrometastásica deberían ser informativas respecto al pronóstico de los pacientes.

Así, se plantea como hipótesis global:

I) La detección de enfermedad mínima residual en pacientes con cáncer de estirpe epitelial (ejemplificados en los trabajos presentados en cánceres gastrointestinales y de mama) puede servir en la clínica como biomarcador pronóstico.

II) La presencia de EMR se asocia a las características clinico-patológicas y a la desregulación de la expresión de microRNAs oncogénicos en el tumor.

III) La selección de nuevos marcadores de EMR en sangre periférica, basados en RNA (mRNA, microRNA) implicados en la progresión tumoral aportaría una información de utilidad diagnóstica y para la estratificación del pronostico.

3.2. Objetivos

3.2.1. Investigar la asociación de la presencia de CTD en MO, en cánceres gastrointestinales (colorrectal, gástrico y páncreas), detectadas mediante ICQ con Acs anti-CK, con los parámetros clínicos y patológicos establecidos.

3.2.2. Investigar la asociación de la presencia de CTD en MO, en cánceres gastrointestinales, con el perfil molecular de expresión de microRNA en los tumores primarios.

3.2.3 Como objetivo exploratorio, se analizará el valor pronóstico, tanto de la presencia de CTD en MO como de la expresión de microRNA en el tumor.

3.2.4 Identificar y evaluar nuevos biomarcadores (mRNA) de células tumorales circulantes en cáncer colorrectal. Analizar la asociación entre la expresión en sangre de marcadores de CTC y de marcadores de células progenitoras.

3.2.5 Establecer la correlación entre los niveles de expresión sanguínea de estos marcadores de CTC y de células progenitoras con las características clínicas y el pronóstico de los pacientes.

3.2.6 Identificar microRNAs biomarcadores de células tumorales circulantes en cánceres gastrointestinales y en cáncer de mama.

3.2.7 Validar la utilidad potencial de los microRNAs seleccionados como marcadores de CTC mediante RT-qPCR. Los estudios de validación se han diseñado como análisis prospectivos de casos-controles, estimando la seguridad diagnóstica.

3.2.8 Como objetivos exploratorios secundarios, se analizará la asociación de los diferentes biomarcadores de microRNA con los parámetros clínicos y patológicos de valor establecido en cáncer gástrico (como modelo de cáncer gastrointestinal) y en cáncer de mama.

3.2.9 Finalmente, estudiar el impacto de los microRNAs circulantes en sangre en la progresión de la enfermedad y la supervivencia, en dos cohortes de pacientes, con cáncer gástrico y con cáncer de mama.

4. INVESTIGACIÓN Y RESULTADOS

4. INVESTIGACIÓN Y RESULTADOS

4.1. Resumen de las publicaciones

Impacto pronóstico de las células tumorales diseminadas y de la desregulación de la familia de microRNA-17-92 en el cáncer gastrointestinal.

La presencia de células tumorales en la médula ósea (MO) podría ser relevante para la identificación de pacientes con cáncer gastrointestinal y un mayor alto riesgo de progresión de la enfermedad y de muerte. Por otro lado, el perfil molecular del tumor que se asocia con la presencia de CTD en MO aún no se ha definido. Los microRNA (miRNA) juegan un papel clave en los procesos celulares implicados en el cáncer. Se investigó en 38 pacientes con cáncer colorrectal, gástrico o pancreático si la presencia de CTD en MO se asocia con un perfil específico de miRNA en el tumor y se analizó su potencial impacto pronóstico. Las CTD se detectaron mediante ICQ y Acs anti-CK en 42,1% de los pacientes. Los miRNAs se cuantificaron mediante RT-qPCR en muestras de tumores, fijado en formol e incluidos en parafina. No se hallaron asociaciones significativas entre la detección de CTD y desregulación de los miRNAs. Las curvas de Kaplan-Meier demostraron una reducción significativa de la supervivencia libre de progresión (SLP) y de la supervivencia global (SG) en los pacientes con CTD. Aunque miR-21 se sobreexpresó en el 90,6% de los tumores, no se encontraron asociaciones con la presencia de CTD ni el pronóstico. Se encontró un aumento de la expresión de miR-17 y miR-20a (ambos pertenecientes a la familia de miRNA-17-92) en el 33,3 y 42,4% de los tumores, respectivamente. Existió una correlación de los niveles de ambos miRNAs y ambos se encontraron elevados en el 30,3% de los tumores. El análisis univariante demostró que el aumento de los valores de miR-20a se asoció significativamente con la reducción de la SLP (HR 1,022, p = 0,016) y SG (HR 1,027, p = 0,003). En los modelos multivariados de Cox, la positividad para CTD (HR 4,07; p = 0,005) y la sobreexpresión de miR-17 (HR 2,11; p = 0,003) se asociaron significativamente con un mayor riesgo de progresión de la enfermedad. La presencia de CTD en la MO (HR 3,98; p = 0,010) y la sobreexpresión de miR-17 (HR 2,62; p <0,001) también se asociaron con el riesgo de muerte. Nuestro estudio sugiere que la presencia de CTD en MO y el aumento de la expresión de los miRNAs de la familia miR-17-92 en el tumor son marcadores

pronósticos significativos pero independientes en pacientes con cáncer gastrointestinal.

Evaluación del gen asociado a adenocarcinomas AGR2 *y del marcador de células progenitoras intestinales* LGR5 *cómo biomarcadores en cáncer colorectal.*

Nuestro objetivo es estimar el rendimiento diagnóstico de AGR2 y LGR5 en sangre periférica (SP) como biomarcadores de mRNA en el cáncer colorrectal (CCR) y explorar su significado pronóstico. Se empleó la PCR en tiempo real para analizar AGR2 y LGR5 en 54 pacientes con CCR en estadios I-IV y en 19 controles. La expresión de ambos mRNAs fue superior significativamente en la SP de los pacientes con CCR en comparación con los controles. El área bajo las curvas COR fueron 0.722 (p = 0.006), 0.376 (p = 0,123) y 0,767 (p = 0,001) para AGR2, LGR5 y para la combinando de AGR2 / LGR5, respectivamente. El análisis de AGR2/LGR5 mostró una sensibilidad del 67,4% y una especificidad del 94,7%. AGR2 se correlacionó con pT3-pT4 y con tumores de alto grado. LGR5 se correlacionó con la presencia de metástasis, cirugía R2 y tumores de alto grado. La supervivencia libre de progresión (SLP) de los pacientes con alta expresión de AGR2 se redujo (p = 0,037; HR, 2,32), también en el subgrupo de pacientes con estadios I-III (p = 0,046). La expresión en SP de LGR5 se asoció con mal pronóstico, con respecto tanto a la SLP(p = 0,007; HR, 1,013) y como a la supervivencia global (p = 0,045; HR, 1,01). Los niveles elevados de AGR2/ LGR5 se asociaron a una peor SLP (p = 0,014; HR, 2,8) en el análisis multivariado. Nuestros resultados indican que la evaluación de AGR2 y LGR5 en SP podría reflejar la presencia de células tumorales circulantes, incluyendo CTC con características de células progenitoras en el CCR. Los incrementos de AGR2 y LGR5 están asociados con peor resultado clínico.

MiR-200c circulante como biomarcador diagnóstico y pronóstico en cáncer gástrico

En los tumores sólidos existe una desregulación en la expresión de los microRNAs. Esta expresión anómala se correlacionan con la génesis y la progresión tumorales. La familia miR-200 caracteriza el fenotipo epitelial de las células neoplásicas y participa en la regulación de la capacidad invasiva y la migración celulares. Por tanto, se plantea como hipótesis que la detección y cuantificación de microRNAs de la familia miR-200 como microRNAs epiteliales específicos en la sangre, podrían constituir biomarcadores útiles en clínica en pacientes con cáncer gástrico (GC).

Inicialmente validamos la expresión de miR-200a, 200b, 200c y 141 en líneas celulares de GC (n = 2) y en la sangre de controles sanos (n = 19) utilizando transcripción inversa con PCR cuantitativa en tiempo real (RT-qPCR). Los perfiles de expresión de la familia de miR-200 en 160 muestras pareadas de mucosa gástrica no tumoral y GC fueron descargados a través de ArrayExpress y analizados. Mir-200c fue seleccionado para la validación clínica. Se realizó la evaluación prospectiva de miR-200c utilizando 67 muestras de sangre (52 pacientes con GC en estadios I-IV y 15 controles); Se estimó el área bajo la curva ROC (AUC-ROC). Se utilizaron las pruebas de Kaplan-Meier y Breslow-Wilcoxon para evaluar la correlación de miR-200c con la supervivencia global y libre de progresión (SLP y SG). El análisis multivariante se realizó con modelos de Cox.

Los niveles de expresión de miR-200C en sangre en pacientes con GC fueron significativamente más altos que en los controles normales (p = 0,018). La AUC-ROC fue 0,715 (p = 0,012)., Se observaron tasas de sensibilidad, especificidad y exactitud de 65.4%, 100% y 73,1% respectivamente. Los niveles de miR-200c en la sangre por encima del límite definido por la curva ROC se encontraron en el 17,6% de los pacientes con estadios I-II, en el 20,6% de los pacientes en estadio III y en el 67,7% de los pacientes en estadio IV (p <0,001). Los niveles de expresión de miR-200C no se asociaron con características clínicas ni patológicas o con la cirugía reciente. Hubo una correlación (p = 0,016) con el número de ganglios linfáticos metastásicos. El aumento de los niveles de expresión de miR-200c en sangre se asoció significativamente con peor supervivencia global (mediana de SG, 9 vs 24 meses; p = 0,016) y con la SLP (mediana de la SLP, 4 vs 11 meses; p = 0,044). El análisis multivariado confirmó que la sobreexpresión de miR-200c en la sangre se asoció con la SG (HR = 2,24; p = 0,028) y la SLP (HR = 2,27; p = 0,028), independientemente de covariables clínicas.

Estos datos sugieren que el aumento de los niveles de miR-200C se detectan en la sangre de pacientes con cáncer gástrico. Mir-200c tiene el potencial de ser un predictor de progresión y peor supervivencia.

MiR-200c y miR-141 circulantes en cáncer de mama y resultados

Tanto en el tumor primario como en la sangre se ha descrito una desregulación de la expresión de microRNAs. Esta expresión diferencial de microRNAs podría se de utilidad para indicar la presencia de cáncer y predecir el pronóstico. En este trabajo

planteamos la hipótesis basada en cómo la desregulación de miR-200c y de miR-141 en la sangre puede identificar a pacientes con cáncer de mama y cómo la expresión en sangre de estos microRNAs podría constituir una firma indicativa del pronóstico.

La expresión de miR-200c y miR-141 se examinó en sangre (57 pacientes con CM, en estadios I-IV y en 20 controles femeninos, emparejados por edad) mediante PCR cuantitativa y transcripción inversa. Se analizaron las asociaciones de los microRNAs circulantes con las características clínicas y patológicas. Sus efectos sobre la supervivencia fueron analizados por el método de Kaplan-Meier y las regresiones de Cox.

Mir-200c se encontró en niveles inferiores (P <0,0001) en la sangre de las pacientes respecto a los controles con un área bajo la curva (AUC-ROC) de 0,79 (90% de sensibilidad, especificidad 70,2%). Loa niveles circulantes de miR-141 no fueron discriminatorio. En las pacientes con CM los niveles de mir-200c y miR-141 en la sangre se correlacionaron inversamente (P = 0,019). Los niveles de miR-200C fueron numéricamente superiores en el estadio IV y en los tumores con menor MIB-1. MiR-141 fue significativamente más alto en la sangre de pacientes con estadio I-III, metástasis en los ganglios linfáticos y los tumores HER2 negativos. La alta expresión sanguínea de miR-200c y/o la baja expresión de miR-141 se asociaron con una supervivencia global desfavorable (razón de riesgo, 3,89; [IC del 95%: 1,28 a 11,85]) y la supervivencia libre de progresión (3,79 [1,41-10,16]) de manera independiente de la edad, el estadio y los receptores hormonales.

Los niveles circulantes de miR-200c y de miR-141 fueron desregulados en las pacientes con CM en comparación con los controles. Por otra parte, la expresión de miR-200c y de miR-141 fueron factores pronósticos independientes, asociados con un diferentes resultados clínicos en las pacientes con CM.

4.2. Publicaciones

4.2.1 *ARTÍCULO 1. Valladares-Ayerbes M, Blanco M, Haz M, Medina V, Iglesias-Díaz P, Lorenzo-Patiño MJ, Reboredo M, Santamarina I, Figueroa A, Antón-Aparicio LM, Calvo L.* ***Prognostic impact of disseminated tumor cells and microRNA-17-92 cluster deregulation in gastrointestinal cancer. Int J Oncol. 2011; 39: 1253-64.***

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4.2.2 *ARTÍCULO 2. Valladares-Ayerbes M, Blanco-Calvo M, Reboredo M, Lorenzo-Patiño MJ, Iglesias-Díaz P, Haz M, Díaz-Prado S, Medina V, Santamarina I, Pértega S, Figueroa A, Antón-Aparicio LM.* ***Evaluation of the adenocarcinoma-associated gene AGR2 and the intestinal stem cell marker LGR5 as biomarkers in colorectal cancer. Int J Mol Sci. 2012; 13: 4367-87.***

DOI: 10.3390/ijms13044367.

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4.2.3*. ARTÍCULO 3. Valladares-Ayerbes M, Reboredo M, Medina-Villaamil V, Iglesias-Díaz P, Lorenzo-Patiño MJ, Haz M, Santamarina I, Blanco M, Fernández-Tajes J, Quindós M, Carral A, Figueroa A, Antón-Aparicio LM, Calvo L.* ***Circulating miR-200c as a diagnostic and prognostic biomarker for gastric cancer. J Transl Med. 2012; 10: 186.***

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4.4.4. *ARTÍCULO 4. Antolín S, Calvo L, Blanco-Calvo M, Santiago MP, Lorenzo-Patiño MJ, Haz-Conde M, Santamarina I, Figueroa A, Antón-Aparicio LM, Valladares-Ayerbes M.* ***Circulating miR-200c and miR-141 and outcomes in patients with breast cancer. BMC Cancer. 2015; 15: 297.***

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Prognostic impact of disseminated tumor cells and microRNA-17-92 cluster deregulation in gastrointestinal cancer

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Abstract. The presence of tumor cells in the bone marrow (BM) could be relevant to identifying high risk of disease progression and death in gastrointestinal cancer. However, the molecular profile associated with disseminated tumor cells (DTCs) homing to the BM has yet to be defined. MicroRNAs (miRNA) play key roles in cellular processes implicated in cancer. Thus, we investigated in 38 patients with colorectal, gastric or pancreatic cancer whether the presence of BM-DTCs is associated with a specific miRNA tumor profile and analyzed their potential prognostic impact. DTCs were detected by immunocytochemistry and anti-cytokeratin antibodies in 42.1% of the patients. miRNAs were isolated from formalin-fixed, paraffin-embedded tumors. qRT-PCR was used for miRNA profiling. No significant associations were found among DTC detection and miRNA deregulation. Kaplan-Meier curves demonstrated significantly reduced progression-free survival (PFS) and overall survival (OS) in the DTC-positive patients. Although miR-21 was upregulated in 90.6% of the tumors, no associations with outcomes were found. miR-17 and miR-20a (miRNA-17-92 cluster) were upregulated in 33.3 and 42.4%, respectively. Upregulation of both was correlated and found in 30.3%. Univariate analysis shows that increasing values for miR-20a were significantly associated with reduced PFS (HR 1.022; p=0.016) and OS (HR 1.027; p=0.003). In multivariate Cox models, DTC positivity (HR 4.07; p=0.005) and miR-17 overexpression (HR 2.11; p=0.003) were significantly associated with a higher risk of disease progression. The presence of DTCs in the BM (HR 3.98; p=0.010) and a miR-17 overexpression (HR 2.62; p<0.001) were also associated with a risk of death. Our study suggests that the presence of BM-DTCs and the upregulation of the miR-17-92 cluster in tumors are both significant but independent prognostic markers in gastrointestinal cancer patients.

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Key words: gastrointestinal cancer, disseminated tumor cells, microRNA, prognostic, real-time PCR

Introduction

Cancers of the gastrointestinal (GI) tract are a leading cause of cancer-associated morbidity and mortality across the world. The predicted numbers of deaths in 2011 in the European Union (1) due to GI cancer are 162,026 deaths for colorectal cancer, followed by 69,304 deaths for pancreatic cancer and 62,340 deaths for stomach cancer. Although multimodal therapies, including improved local treatments, chemotherapy and molecular-targeted agents, have recently been introduced in the clinical care of GI cancer patients, better staging and prognostic factors to guide treatment decisions are clearly required.

Well-characterized biomarkers are needed to personalize therapy and to predict metastatic progression. Tumor seeding is considered an early event in the process of metastasis formation. Therefore, the detection of these disseminated tumor cells (DTC) in distant organs such as the bone marrow (BM) could be important to identify patients at a high risk of disease progression and death and might indicate the need for further therapeutic approaches (2). Although significant associations with relapse and survival have been reported (3-12), the prognostic relevance of the detection of BM-DTC in GI cancer patients is controversial (13-15). However, the biological characteristics and proliferative potential of this DTC are poorly understood (16). The identification and characterization of molecules that control cancer cell spread in distant organs is critical to our understanding of cancer dissemination. The molecular profile that links the biologic characteristics of primary GI tumors with the presence of epithelial cells homing to bone marrow has yet to be defined.

Tumor progression and metastasis development are complex processes that involve the activation of oncogenes, functional loss of tumor suppressor genes and microRNA (miRNA) deregulation (17). Mature miRNAs are single-stranded, non-coding RNAs that are involved in many biological pathways. miRNA regulation plays key roles in various cellular processes commonly implicated in cancer, such as differentiation, cell growth, angiogenesis, epithelial-to-mesenchymal transition (EMT) and invasion. An increasing number of studies analyzing

miRNA expression profiles in gastrointestinal tumors and their potential clinical relevance have been reported (18-25).

miR-21 is upregulated in different tumor types, including lung, breast, prostate, pancreatic, and colorectal cancer (17). In colorectal cancer, high expression of miR-21 is correlated with poor survival, poor therapeutic outcome (19), and development of distant metastasis (20). Overexpression of miR-21 has been included in a seven-miRNA signature that was able to predict relapse-free survival and overall survival (OS) in gastric cancer patients (21). In pancreatic cancer, high miR-21 expression is associated with shorter survival (22) and can predict outcomes in resectable patients treated postoperatively (23).

miR-20a and miR-17 belong to the miR-17-92 miRNA cluster, a family of oncogenic miRNAs commonly deregulated in cancer (24). miR-20a and miR-17 are upregulated in colorectal (18,19,25), gastric (21,25), and pancreatic (25) adenocarcinomas. However, miR-17-92 miRNA cluster deregulation in GI cancer and its relationship with prognosis are poorly defined.

In the present study, our aims were to investigate whether the presence of BM micrometastasis, detected by a standardized immunocytochemical method, is associated with a specific miR-21, miR-20a and miR-17 tumor profile, which could serve as a prognostic factor in gastrointestinal cancer patients.

Patients and methods

Patient data. Consecutive patients with GI cancer from the Medical Oncology Unit at University Hospital in La Coruña (Spain) were prospectively included in the study. Inclusion criteria were as follows: confirmed pathological diagnosis of invasive adenocarcinoma of the GI tract, including colorectal, gastric and pancreatic tumors; stage I-III cancer with no prior systemic therapy for GI cancer; stage IV cancer without previous systemic therapy or with confirmed cancer progression after such treatment; and provision of written informed consent. Exclusion criteria were defined as follows: any other previous malignancy, coagulation disorders, platelet count less than $20.0x10^9$ L^{-1}, and any previous systemic therapy for cancer except stage IV patients with progressive disease confirmed at the time of BM sampling.

The diagnostic work-up included a clinical examination, blood sampling with CA 19.9 and CEA serum determination, an endoscope (when clinically indicated), a chest X-ray and computed tomography (CT) scan of the abdomen and pelvis. Chest CT was performed on the upper digestive tract, rectal tumors and stage IV patients. Patients were followed to observe disease progression, with imaging every 6-12 weeks.

Serum CEA (with an upper limit of normal of 5 ng/ml) and CA 19.9 (with an upper limit of normal of 37 U/ml) levels were determined using enzyme immunoassay test kits (Advia Centaur, Siemens Healthcare Diagnostics), according to the manufacturer's instructions.

After informed, written consent was obtained from each patient, BM aspiration was performed under local anesthesia, just before systemic treatment for pathological confirmed GI cancer. In patients who first underwent surgery as loco-regional treatment for primary disease, BM aspirate was obtained after the operation. Otherwise, BM samples were obtained before neo-adjuvant chemotherapy or in the presence of active metastatic disease. BM was aspirated from anterior or posterior iliac crest unilaterally. A skin incision was made to avoid contamination with epidermal cells. The study was approved by the Institutional Review Board of the Ethics Committee of Clinical Investigation of Galicia (Spain), and written informed consent was obtained from all patients.

Pathological analysis. Primary tumors, regional lymph nodes and tissues collected during surgery were processed on a routine diagnostic basis. Histological tumor type, depth of tumor invasion and nodal involvement were analyzed, and the disease was staged and graded according to the TNM/UICC system (26). Vascular and perineural invasion were analyzed. When surgery was not performed, pathologic diagnosis was obtained using endoscope- or radiological-guided biopsy.

Residual disease status at the time of BM aspiration was classified as R0 when no residual disease was present after surgery, R1 when microscopic residual disease was found, and R2 in the presence of macroscopic disease. Patients from whom BM was obtained before the start of neo-adjuvant systemic treatment were categorized as R2.

Preparation of the bone marrow. Unilateral BM aspiration was performed from anterior or posterior iliac crest under local anesthesia and transferred into heparinized tubes. Mononuclear cells (MNCs) were separated by density-gradient centrifugation using Lymphoprep (Nycomed, Oslo, Norway). MNCs were collected from the interphase layer and washed twice in PBS with 10% FCS. Cytospins were prepared ($5x10^5$ MNCs/slide) on polylysine-coated slides in a Hettich cytocentrifuge. The cytospins were air-dried at RT overnight before freezing at -80˚C or immunostaining.

Immunocytochemical staining. Immunocytochemistry (ICC) was performed as described previously (27) using the Vectastain ABC-AP kit (Vector), according to the manufacturer's instructions. Slides ($5x10^5$ BM MNCs) were incubated with the anti-cytokeratin (CK) monoclonal antibodies AE1/AE3 (Dako). At least two slides were incubated with a negative control antibody of the same immunoglobulin isotype (IgG1). The visualization stage included use of a Vector Red alkaline phosphatase substrate kit. Endogenous alkaline phosphatase activity was inhibited by addition of levamisole. The slides were counterstained with Gill's hematoxylin to visualize nuclear morphology. The slides were manually screened by light microscopy by two pathologists (PID, MLP) with no knowledge about clinical or follow-up data. All of the stained cells were closely evaluated. Categorization of CK-positive cells was performed according to the recommended guidelines (28). The presence of DTC was recorded as positive when at least one stained cell exhibited typical tumor cell morphology or when this immunostained cell lacked hematopoietic characteristics and was not found in negative controls.

microRNA isolation and qRT-PCR in tumor tissue. microRNA-enriched total RNA was isolated from formalin-fixed, paraffin-embedded (FFPE) tumor and normal colonic mucosa samples using the Recover All Total Nucleic Acid Isolation Kit for FFPE Tissues (Ambion/Applied Biosystems, USA), following the manufacturer's instructions.

The mirVana qRT-PCR miRNA Detection Kit and the corresponding mirVana qRT-PCR primer sets (Ambion/Applied

Biosystems, USA) were used to detect and quantify miR-17 and miR-20a. Results were normalized using 5S rRNA and U6 snRNA (mirVana qRT-PCR Primer Sets for Normalization; Ambion/Applied Biosystems).

miR-21 was detected and quantified using a miRCURY LNA First-Strand cDNA Kit, miRCURY LNA SYBR Green Master Mix and specific miRCURY LNA Primer Sets (Exiqon, USA). Mircury LNA Endogenous Control Primer Sets for 5S and U6 (Exiqon) were used as reference genes.

miRNAs were amplified and detected in a LightCycler 480 (Roche, Germany) real-time thermal cycler, using SYBR Green dye. Melting curves were generated using the LightCycler analysis software to determine whether there were spurious amplification products (29).

The relative expression software tool (REST) was used to analyze the relative miRNA expression in each tumor and control sample (non-tumor colonic tissue) and to determine the fold-difference for miR-17, miR-20a and miR-21 (30). The expression levels of target miRNAs were standardized by an index containing 5S rRNA and U6 snRNA. The REST program is based on the correction for exact PCR efficiencies and the mean crossing point deviation between sample group(s) and control group(s). Subsequently, the expression ratio results of the investigated transcripts were tested for significance by a pair-wise fixed reallocation randomization test and plotted using standard error (SE) estimation using a complex Taylor algorithm. miRNA analyses were performed with no knowledge about clinical or follow-up data.

Study design and statistical analysis methods. Differences in the distribution of variables between patient groups according to the presence of DTC in BM were assessed by Pearson's χ^2 test, Fisher's exact test or the Kruskal-Wallis test. Non-normality of the distribution of miRNA expression values was confirmed by the Kolmogorov-Smirnov test. Thus, non-parametric statistics (Mann-Whitney and Kruskal-Wallis tests) were used to analyze the potential correlation between miRNA expression and the clinical and pathological features of the study subjects.

Logistic regression analyses were used to assess the effect of tumor miRNA levels on the risk of DTC detection in BM. Odds ratios (OR) and 95% confidence intervals (CI) were estimated.

Progression-free survival (PFS) was measured as the time between the baseline BM sampling for DTC analysis and the documentation of first tumor progression based on clinical and radiological studies, second tumor or death (events). OS was measured from the time baseline BM was obtained to the date of death from any cause or date of last follow-up. Patients who were alive and progression-free at the time of analysis were censored by using the time between the BM assessment and their most recent follow-up evaluations. The distribution of time-to-event end points, namely PFS and OS, were estimated using the Kaplan-Meier method and compared using the log-rank test.

Multivariate survival analyses (PFS and OS) were performed with Cox proportional hazard regression models. We estimated hazard ratios (HRs), 95% CI and p-values. All statistical tests were two-sided, and p-values <0.05 were considered statistically significant. SPSS software (version 16.0) was used for data analysis.

Table I. Patients and clinicopathological characteristics.

	N	%
Age (years)		
Mean (SD, range)	62.5 (7.9, 45-76)	
<65 years	21	55.3
≥65 years	17	44.7
Gender		
Female	13	34.2
Male	25	65.8
Primary tumor site		
Colon or rectum	28	73.7
Stomach	7	18.4
Pancreas	3	7.9
Stage		
I-II	6	15.8
III	9	23.7
IV	23	60.5
pT		
pT1-pT2	5	13.1
pT3	22	57.9
pT4	8	21.1
pTx	3	7.9
pN0	14	36.8
pN1	12	31.6
pN2	7	18.4
pNx	5	13.2
M		
M0	15	39.5
M1	23	60.5
R status		
R0-R1	13	34.2
R2	25	65.8
Location of metastasis		
Liver	8	34.8
Extra-hepatic metastasis	8	34.8
Liver and extra-hepatic	7	30.4
Grade		
Low grade	15	39.5
High grade	23	60.5
Vascular/perineural invasion		
Yes	18	47.4
No	11	28.9
Unknown	9	23.7

Results

Patient characteristics. From February 2002 to January 2003, 38 GI cancer patients were included. Clinical characteristics are shown in Table I. Surgery was performed in 30 patients before

Table II. Distribution of clinicopathological parameters and disseminated tumor cells in bone marrow.

		DTC positive	
Parameter	N	%	P-value
Age (years)			0.917
<65	9	42.9	
≥65	7	41.2	
Gender			0.715
Male	10	40	
Female	6	46.1	
Primary tumor site			0.267[a]
Colon or rectum	10	35.7	
Stomach/pancreas	6	60	
Stage			0.683[b]
I-II	2	33.3	
III	3	33.3	
IV	11	47.8	
pT			0.141[a]
pT1-T2	4	80	
pT3-T4	11	57.9	
pN			0.142
Node negative	8	57.1	
Node positive	6	31.6	
R status			0.743
R0-R1	5	38.5	
R2	11	44	
Location of metastasis			0.675[b]
None	5	33.3	
Liver	7	46.7	
Non-liver metastasis	4	50	
Grade			0.071
Low grade	9	60	
High grade	7	30.4	
Vascular/perineural invasion			1.000
No	5	45.4	
Yes	7	38.9	
Ca 19.9			0.850
≤37	10	41.7	
>37	5	38.5	
CEA			0.461
≤5	10	45.4	
>5	5	33.3	

P-value computed by Pearson's χ^2 test. [a]P-value computed by Fisher's exact test. [b]P-value computed by Kruskal-Wallis test.

BM sampling. Median time from surgery to BM aspiration was 7 weeks (mean 26.8; standard error: 7.2; range 3-162 weeks).

BM aspiration was obtained after R0 or R1 surgery in 13 patients. In this subgroup, 69.2% of the patients suffered PFS events. In 25 patients, BM samples were obtained before neo-adjuvant chemotherapy or in the presence of active metastatic disease, both of which were categorized as R2 at the time of BM aspiration. In this subgroup, PFS events were found in 92% of patients (Fisher's exact test, p=0.154).

All patients were followed up until death or the end of the study. The mean follow-up time was 153 weeks (standard error 21.4; median 108.5 weeks; range 2-388 weeks). Progression events were detected in 32 patients (84.2%); 27 patients died (71.1%). The median PFS was 37 weeks (standard error 9.2; 95% CI: 18.9-55.1). The median global survival was 137 weeks (standard error 44.5; 95% CI: 49.9-224.1).

Cytokeratin immunocytochemistry. Isolated tumor cells in BM were detected using anti-CK monoclonal antibodies AE1/AE3 and standardized morphological criteria (28). At least $2x10^6$ BM MNC cells were screened per patient. DTC was found in BM in 16 patients (42.1%, standard error: 0.08). The numbers of tumor cells detected were: 1 (five patients), 2 (seven patients), 3 (two patients), 15 (one patient) and 22 (one patient).

Previous surgery was not related to the presence of DTC in BM (Fisher's exact test, p=0.698). To explore the possible influence of the time elapsed from the most recent surgery on the presence of DTC, we analyzed the detection of DTC according to time interval from the operation and BM sampling. The median time from surgery to BM was 26.8 weeks ± 7.2 weeks (SEM) (median, 7 weeks; range, 3-162). The 25th percentile was 5 weeks. There was no significant difference in DTC detection between time intervals (≤5 or >5 weeks) from the last surgery (50 and 36.4%, respectively, Fisher's exact test, p=0.678).

Bone marrow DTC and correlations with clinicopathology. Clinical and pathologic characteristics of the patients and associations with the detection of DTC in BM are shown in Table II. The detection of DTC was not associated with any of the parameters analyzed.

However, Kaplan-Meier survival analyses demonstrated significantly reduced PFS and OS among the DTC-positive patients (Fig. 1). The median PFS was 40 weeks (95% CI: 12.4-67.6) for the patients without DTC versus 29 weeks (95% CI: 0-60.4) for the subgroup positive for DTC in BM (log-rank; p=0.026). The median OS was 165 weeks (95% CI: 93.9-236 weeks) in patients without DTC. In contrast, OS was significantly reduced (median 75 weeks; 95% CI: 22.8-127.2 weeks) in those patients with CK-positive cells in BM (log-rank test; p=0.045. When only stage I-III patients were considered, the presence of DTC in BM defined a subgroup with a lower median survival (137 weeks vs. not reached; log-rank; p=0.038).

miRNA expression in tumors. microRNA-enriched total RNA was retrieved from 33 FFPE-tumor samples for miR-17 and miR-20a analysis and from 32 specimens for miR-21 analysis. Expression of miR-17, miR-20a and miR-21 showed a right-skewed distribution over the patient population (Fig. 2). Mean relative expression levels were 10.1 (SEM 3.4; range 0-89.4) for miR-17, 12.7 (SEM 4.2; range 0-110.3) for miR-20a and 24.3

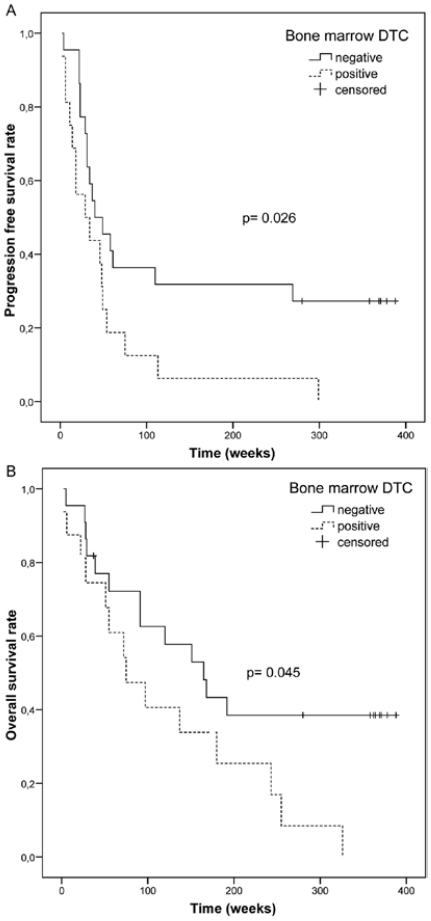

Figure 1. Kaplan-Meier curves depicting progression-free survival (A) and overall survival (B) depending on the detection of disseminated tumor cells in bone marrow. p-values were calculated with the log-rank test.

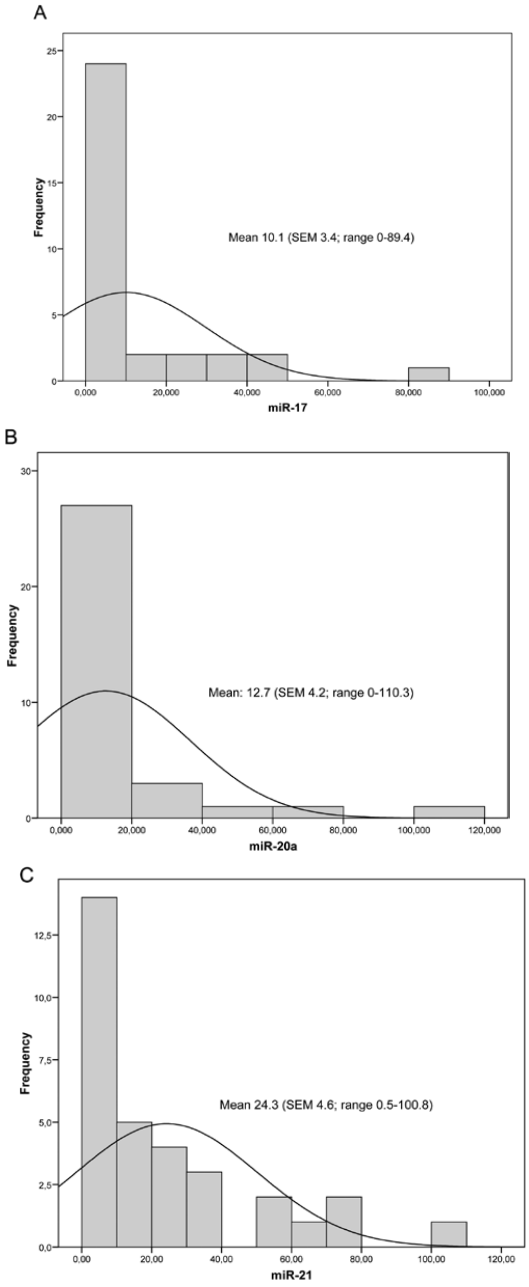

Figure 2. Distribution of (A) miR-17, (B) miR-20a and (C) miR-21 expression in tumors. Frequencies of expression are plotted as a function of the expression values. A continuous line was added to indicate the normal distribution. Mean, SEM and range of miRNA expression values are indicated.

(SEM 4.6; range 0.5-100.8) for miR-21. In tumors, miR-17 and miR-20a were highly co-expressed (Spearman correlation coefficient 0.650; p<0.001). No correlations were found between miR-21 and miR-17 (p=0.476) or miR-20a (p=0.362) expression.

Two different strategies were used to categorize relative expression levels for each miRNA in every tumor sample. First, a miRNA was considered up-regulated when the relative expression level was higher than the mean value in the tumor cohort. Using this approach, miR-17 and miR-20a were overexpressed in 27.3% (9/33) and 21.2% (7/33) of tumors, respectively. miR-21 was overexpressed in 40.6% of patients (13/32).

In the second strategy, REST was used as described in Patients and methods (30). The REST output tells the user if the expression ratio results of the investigated targets are up- or down-regulated in the sample group (tumor) in comparison with the control group (non-tumor colonic tissue). Differences in expression between tumor samples and controls were assessed for statistical significance at p-values ≤0.01 by a randomization test. Using REST, miR-21 was up-regulated in 90.6% (29/32) of the tumors. The REST analysis indicated that miR-17 and miR-20a were up-regulated in 33.3% (11/33) and 42.4% (14/33) of tumors, respectively. Up-regulation of both miR-17 and miR-20a was found in 10 patients (30.3%; Fisher's exact test, p<0.001).

Table III. MiRNA expression according to the clinical and pathologic characteristics of the patients.

		miR-17			miR-20			miR-21		
	N	Mean	SEM	P-value	Mean	SEM	P-value	Mean	SEM	P-value
Age (years)				0.605			0.334			0.317
<65 years	19	13.69	5.59		18.46	6.87		28.98	7.07	
≥65 years	14	5.22	2.49		4.9	2.08		16.93	5.15	
Gender				0.474			0.837			0.876
Male	21	10.83	4.8		14.92	6.32		23.7	5.05	
Female	12	8.81	4.51		8.84	3.22		25.3	9.12	
Primary tumor site				0.983			0.799			0.883
Colon or rectum	23	10.54	4.25		9.79	3.63		23.34	5.53	
Stomach/pancreas	10	9.07	5.99		19.44	11.11		24.22	8.53	
Stage				0.386[a]			0.963[a]			0.449[a]
I-II	6	10.87	7.21		8.80	6.34		13.85	6.29	
III	8	4.51	3.97		6.80	3.41		21.56	7.62	
IV	19	12.20	5.30		16.43	6.82		28.63	6.84	
pT				0.415			0.559			0.686
pT1-pT2	5	14.69	9.03		15.19	7.61		38.24	19.26	
pT3-pT4	25	8.44	3.85		12.41	5.24		23.64	4.52	
pN				0.459			0.080			0.464
Negative	13	3.78	2.44		2.78	1.47		32.67	9.02	
Positive	15	12.21	6.25		20.12	8.33		24.07	5.84	
M				0.203			0.827			0.388
M0	14	7.23	3.76		7.66	3.21		17.99	4.94	
M1	19	12.2	5.3		16.43	6.82		28.63	6.84	
Location of metastasis				0.439[a]			0.966[a]			0.474[a]
None	14	7.23	3.76		7.66	3.21		17.99	4.95	
Liver	11	16.46	8.81		15.88	7.29		34.36	10.19	
Non-liver metastasis	8	6.36	3.24		17.20	13.40		20.74	8.16	
R status				0.052			0.911			0.173
R0-R1	12	3.00	2.66		13.65	9.1		29.56	7.93	
R2	21	14.15	5,00		12.18	4.21		21.15	5.58	
Grade				0.747			0.311			0.527
Low grade	13	7.1	3.21		5.70	2.36		19.75	6.42	
High grade	20	12.03	5.27		17.27	6.59		27.69	6.35	
Vascular/perineural invasion				0.845			0.429			0.38
No	10	6.51	3.75		17.3	10.71		27.38	10.12	
Yes	14	10.28	6.57		10.18	5.76		31.70	6.66	
Serum tumor markers										
Ca 19.9 >37	13	11.56	5.26	0.116	18.97	10.01	0.487	12.18	3.22	0.071
CEA >5	15	21.82	8.67	0.017	28.95	10.85	0.137	19.58	7.45	0.155

Mean values and standard error (SEM) for miRNA expression in subgroups are shown. Mann-Whitney test, 2-sided. [a]Kruskal-Wallis test.

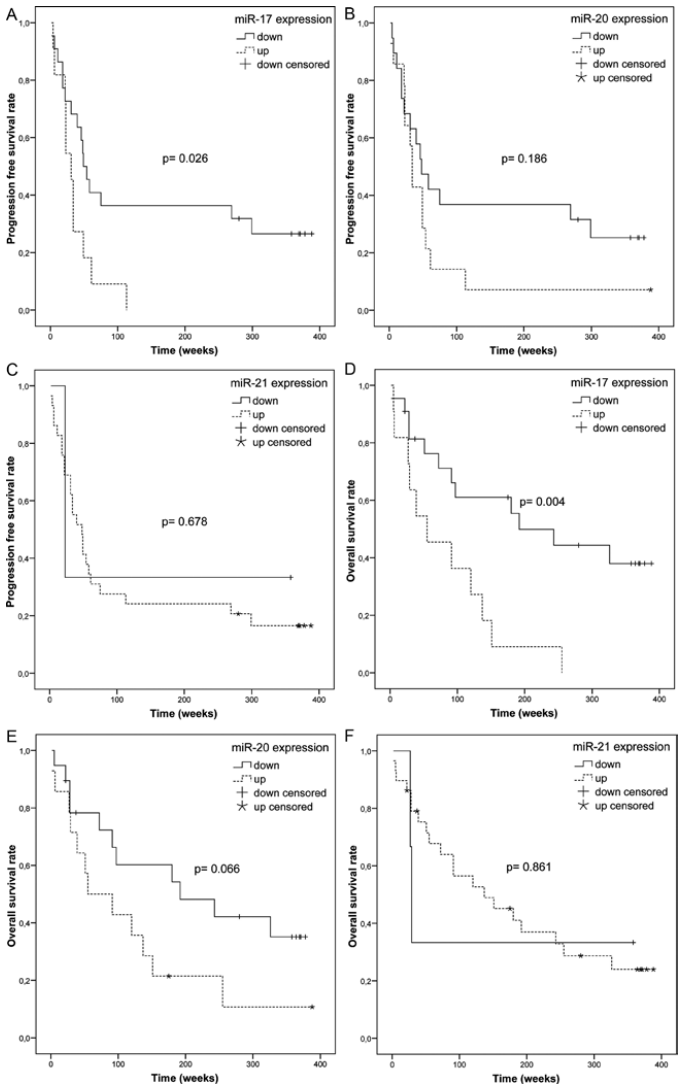

Figure 3. Kaplan-Meier curves depicting progression-free survival (PFS) and overall survival (OS) depending on the tumor miRNA expression. PFS according miR-17 expression (A). PFS according miR-20a expression (B). PFS according miR-21 expression (C). OS according miR-17 expression (D). OS according miR-20a expression (E). OS according miR-21 expression (F). p-values were calculated with the log-rank test.

Associations of deregulated miRNAs with clinical outcome. miRNA expression according to clinical and pathologic characteristics is listed in Table III. Only increased serum CEA level was associated with high miR-17 tumor expression (p=0.017). Otherwise, no significant associations were found among miRNA tumor expression and any of the clinical or pathologic parameters analyzed. A trend was observed between high expression of miR-17 and macroscopic residual tumor (R2) at the time of BM sampling (p=0.052), suggesting a more aggressive clinical presentation. Also, a trend was observed between miR-20a expression and lymph node metastasis (p=0.08). Likewise, a trend between miR-21 tumor expression and serum CA 19.9 level was found (p=0.071).

To verify the association between miRNA expression and survival (PFS and OS), the miRNA expression data were dichotomized into clearly defined high and low expression groups. This cutoff was set based on REST as described above. Mean values (with SEM) in the low and high expression groups, respectively, for each miRNA were as follows: 0.09 (0.05) and 30.1 (7.24) for miR-17 (p<0.001); 0.37 (0.33) and 29.45 (7.97) for miR-20a (p<0.001); 0.8 (0.23) and 26.74 (4.82) for miR-21 (p=0.005).

Kaplan-Meier curves for patients categorized according to miRNA expression are shown in Fig. 3. The median PFS for the group with high miR-17 expression and the other group without miR-17 up-regulation were 31 weeks and 49 weeks, respectively (Fig. 3A; p=0.026). In addition, the differences in median OS

Table IV. Relations of disseminated tumor cells in bone marrow with tumor miRNAs expression: logistic regression analysis.

		mir-17			miR-20			miR-21		
Groups	DTC positive N (%)	OR	95% CI	P-value	OR	95% CI	P-value	OR	95% CI	P-value
Global	16 (42.1)	0.947	0.879 1.020	0.148	1.026	0.961 1.095	0.438	0.998	0.976 1.020	0.832
Stage										
I-III	5 (33.3)	1.042	0.847 1.282	0.700	0.970	0.769 1.224	0.800	0.943	0.874 1.019	0.137
IV	11 (47.8)	0.848	0.680 1.058	0.143	1.035	0.903 1.187	0.618	1.020	0.983 1.058	0.289
Lymph node										
Negative	8 (57.1)	0.218	0.013 3.567	0.285	16.961	0.090 3202.2	0.290	1.022	0.980 1.066	0.315
Positive	6 (31.6)	0.962	0.797 1.160	0.681	1.117	0.876 1.425	0.373	0.874	0.729 1.049	0.148
Primary tumor site										
Colon or rectum	10 (35.7)	0.996	0.884 1.122	0.944	0.981	0.847 1.136	0.798	0.997	0.967 1.028	0.849
Stomach/pancreas	6 (60)	0.711	0.308 1.642	0.424	1.285	0.588 2.809	0.529	1.003	0.964 1.044	0.870

Table V. Multivariate Cox proportional hazard regression analyses for progression-free survival and overall survival.

		Progression-free survival			Global survival		
Variable	Subset	Hazard ratio	(95% CI)	P-value	Hazard ratio	(95% CI)	P-value
Disseminated tumor cells	Negative/positive	4.07	1.54 10.73	0.005	3.98	1.39 11.41	0.010
miR-17 expression	Down-/up-regulated	2.11	1.29 3.45	0.003	2.62	1.55 4.49	0.000
Stage	I-III/IV	5.30	1.59 17.70	0.007	3.97	1.08 14.56	0.037
Surgical resection	R0-1/R2	1.10	0.60 2.00	0.766	1.15	0.50 2.21	0.681
Primary tumor	Colorectal/non-colorectal	0.16	0.06 0.45	0.000	0.17	0.06 0.53	0.002

according to miR-17 values (55 weeks for the up-regulated group and 192 weeks for the low miR-17 group) were also significant (Fig. 3D; p=0.004). However, Kaplan-Meier estimates of PFS and OS were not significantly correlated with up- or down-regulation of miR-20a and miR-21 in tumors (Fig. 3).

HRs for PFS and OS were estimated considering the actual values of every miRNA as a continuous variable in the Cox regression model. Across the entire cohort, increasing values for miR-20a were associated with PFS events (HR 1.022; 95% CI: 1.004-1.040; p=0.016) and reduced OS (HR 1.027; 95% CI: 1.009-1.046; p=0.003). When adjusting for TNM/UICC stage as covariate in the Cox model, increasing values for miR-20a and miR-17 were associated with the risk of progression and death in stage I-III patients. The estimated HRs for miR-20a levels were 1.063 (95% CI, 1.002-1.127; p=0.043) and 1.065 (95% CI, 1.003-1.130; p=0.040) for PFS and OS, respectively. For miR-17, increased values were also significantly associated with risk of progression (HR 1.056; 95% CI, 1.007-1.107; p=0.024). The estimated HR for miR-17 values and OS was 1.065 (95% CI, 0.999-1.102; p=0.052).

No significant associations were found between actual miR-21 values for the entire cohort and PFS (HR 1.007; 95% CI, 0.993-1.022; p=0.346) or OS (HR 1.0; 95% CI, 0.983-1.017; p=0.988). Moreover, in stage I-III patients, hazard ratios for increasing miR-21 values and PFS (HR 1.005; 95% CI, 0.966-1.045; p=0.821) or OS (HR 0.985; 95% CI, 0.939-1.032; p=0.52) were not significant.

miRNA deregulation in tumors and bone marrow DTC. We analyzed whether the tumor expression levels of miR-17, miR20a and miR-21 were associated with the presence of DTC in BM. In DTC-positive patients, the mean miR-17 value in tumors was 4.89 (SEM 2.8), which was not significantly different (p=0.43) from those who were DTC-negative (13.9; SEM 5.5). The mean value of miR-20a in tumors from the patients positive for DTC in BM was 13.2 (SEM 7.8) and 12.4 (SEM 4.6) in those patients without DTC (p=0.91). There was no significant difference (p=0.92) in mean miR-21 value between DTC-positive (26.4; SEM 8.8) and DTC-negative patients (22.9; SEM 4.97).

Furthermore, to assess independent relationships of DTC in BM with tumor miRNA expression, logistic regression analysis was performed. OR was adjusted for other covariables, including primary tumor site (colorectal vs. non-colorectal), stage (I-III vs. IV) and lymph node metastasis (negative vs. positive). However,

the presence of DTC in BM was, again, not associated with miRNA expression (Table IV).

Prognostic significance of DTC and miRNA expression: Cox models. We used multivariate Cox regression analysis to investigate whether the presence of DTC and miRNA expression was an independent predictor of progression-free survival and overall survival in gastrointestinal cancer patients. The stage, extent of surgical resection and location of the primary tumor were used as covariates (Table V).

According to Cox multivariate regression analysis, the factors associated with a higher risk of PFS events were DTC positivity (HR 4.07; p=0.005), miR-17 overexpression (HR 2.11; p=0.003) and stage IV cancer (HR 5.3; p=0.007). The risk of progression was lower in colorectal cancer patients (HR 0.16; p<0.001).

The presence of DTC in BM was also associated with poor overall survival (HR 3.98; p=0.010) in the multivariate model. Moreover, the higher expression of miR-17 resulted in a 2.62-fold increase in the risk of death (p<0.001). In addition, stage IV (HR 3.97; p=0.037) was also associated with poor OS. The risk of death was lower in colorectal cancer patients (HR 0.17; p=0.002).

Discussion

In our study, we examined the presence of DTCs in the BM of patients with colorectal, gastric and pancreatic cancer and their clinical outcomes. We then investigated the existence of any correlation between these findings and the tumor expression of selected miRNA. We found that CK-positive bone marrow cells and upregulation of the miR-17-92 microRNA cluster in the tumor were both significant prognostic markers in gastrointestinal cancer patients. Nevertheless, the tumor expression of miR-17, miR-20a and miR-21 was not associated with the presence of disseminated CK-positive cells in the BM.

Although the prognostic relevance of disseminated tumor cells in bone marrow is widely accepted in breast cancer (31), the clinical and biological significance of finding such micrometastasis in GI cancer patients remains controversial. However, the clinical value as a prognostic factor of DTC detection in BM using CK-based immunocytochemistry in GI cancer patients has been suggested in previous studies analyzing colorectal (4,10) and gastric cancer (7,8).

Independent prognostic values for progression-free survival and global survival were found in our study of DTC detection using Kaplan-Meier estimates and multivariate analysis. Furthermore, the presence of DTC in BM defines a subgroup of stage I-III patients with a significantly lower median overall survival.

Although the inclusion of patients with different primary GI tumors and stages could be considered a limitation of this study, we suggest that this pragmatic design accurately reflects the patients that attend the oncology clinic. Thus, the prognostic value of DTC detection and miRNA quantification has been estimated in a cohort of patients truly representative of those found in the clinical setting. In fact, there were no significant differences among the miRNAs relative expressions levels according to location of primary tumor (colorectal or non-colorectal cancer). Furthermore, prognostic impact of DTC detection and miRNA quantification remain significant in multivariate model adjusting for primary tumor site.

In different studies that used immunocytochemistry, CK-positive cells were detected in the BM of 16-64% of colorectal (13-15), 25-66% of gastric (5-8,13) and 21-61% of pancreatic cancer patients (9,32). In the present report, DTC were found in 42.1% of patients (35.7% of colorectal and 64.3% of gastric and pancreatic cancer patients). Interestingly, and in agreement with the results of previous publications (13), the detection of DTC was not associated in our study with TNM/UICC stage, pathological grade, vascular or perineural invasion or the extent of surgical resection. Therefore, we hypothesized that the presence of cancer cells in the bone marrow may reflect the distinct biological properties of a tumor. These unique biological characteristics of the primary tumors may influence the ability of cancer cells to disseminate in certain distant organs and subsequently trigger metastasis formation. Thus, combined analyses of the primary tumor histopathology, as well as their genetic and transcriptomic backgrounds and DTC status, will increase our understanding of invasion and overt metastasis development in GI cancer.

At present, little is known about the characteristics of primary gastrointestinal tumors that might have a role in the early shedding of tumor cells into the bloodstream and subsequent homing to bone marrow. The presence of disseminated carcinoma cells in bone marrow in patients with various types of epithelial tumors, including colorectal, gastric and pancreatic cancer, is not associated with *TP53* gene mutations in the primary tumor (33). Markers of tumor angiogenesis, such as microvessel counts and VEGF-A expression in primary gastric cancer, have been correlated with the presence of DTC in bone marrow (34,35). A significant association between CXCR4-positive expression of cancer cells in the primary tumor and the presence of CK-positive cells in the bone marrow has been described in patients with squamous cell carcinoma or adenocarcinoma of the esophagus (36). Tumor expression of the neural cell adhesion molecule L1 (CD171) is associated with micrometastatic spread and poor outcome in colorectal cancer (37).

Recently, attention has focused on the role of miRNA regulation in essential mechanisms for cancer progression and metastasis, including invasion, proliferation, cell migration, EMT, angiogenesis and apoptosis (17,18,24). Therefore, we investigated whether the occurrence of DTC in bone marrow was associated with miRNA tumor expression and the related potential prognostic value. Furthermore, the stability of miRNAs in formalin-fixed, paraffin-embedded tissue and the suitability for real-time PCR-based assays, as a gold-standard method for quantification, are clearly advantageous for biomarker research (38).

Here, tumor overexpression of the miR-17-92 cluster emerged as a compelling prognostic indicator in GI cancer. This was independently confirmed by a multivariate Cox proportional hazard model, which defined miR-17 upregulation as a statistically significant independent predictor of poor PFS and OS. Furthermore, Cox regression models showed that increasing continuous values for miR-20a and miR-17 were both associated with the risk of progression and death in stage I-III patients.

Nonetheless, deregulated expression of the miRNAs was not associated with any of the clinicopathological tumor parameters investigated or with the detection of CK-positive

cells in the BM. A significant association was observed only between high expression of miR-17 and CEA level.

Preclinical and translational studies have established the oncogenic role of the miR-17-92 cluster in several hematological and solid tumors (24,25). All six members of the mir-17-92 polycistron on chromosome 13 (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a-1) are some of the highest-overexpressed miRNAs in several cancer types (25,29).

It appears reasonable to assume that the prognostic impact of the up-regulation of miR-17 and miR-20a in GI cancer is attributable in part to the mechanistic relevance of their target genes (40). mir-17 and miR-20a down-regulate the activating members of the *E2F* family of transcription factors (*E2F1, E2F2*, and *E2F3*) and the cyclin-dependent kinase inhibitor CDKN1a/p21, which are regulators of the G1-to-S-phase transition in the cell cycle. Furthermore, the apoptosis inducer *BIM* and the proto-oncogene *LRF*, implicated in senescence, are also under the control of the miR-17-92 cluster (40). A recent report has identified hypoxia-inducible factor (HIF)-1A as a novel direct target of miR-17-5p and miR-20a (41). Furthermore, overexpression of the miR-17-92 cluster markedly inhibits hypoxia-induced apoptosis in colon cancer cell lines through the regulation of p53-mediated transcriptional repression (42). Recent data suggest that miR-17-5p and miR-20a directly control the expression of the type II TGFβ receptor in colorectal cancer progression, inhibiting the transcription of individual TGFβ-responsive genes and indirectly stimulating angiogenesis through inhibition of a wide repertoire of anti-angiogenic factors (43).

Nevertheless, studies on miR-17-92 cluster deregulation in GI cancer in the clinical setting and its potential prognostic impact are limited. Altered expression of miR-17-5p is associated (44) with vascular invasion and LOH in the *TP53* region in a series of colorectal cancer patients. A trend between poor disease-free survival and upregulation of miR-17-5p has also been observed, but only in stage I-II patients. In that series, downregulation of its paralog miR-106a was independently associated with DFS and OS (44). Contradictory results in relation to miR-106a have been reported elsewhere (19). In that study, in an analysis designed to identify whether any of the overexpressed miRNAs in colorectal cancer identified by microarray experiments are associated with poor survival, up-regulation of miR-20a, miR-21, miR-106a, miR-181b and miR-203 was significant in the training cohort. However, only miR-21 retained the prognostic impact in the validation cohort. Tumors with high expression of miR-21 were associated with poor survival outcome and poor response to adjuvant chemotherapy independent of staging and other clinical covariates (19). Likewise, in gastric cancer patients, miR-21 was overexpressed and selected in the progression signature but not in the survival signature (45).

In our study, increased miR-21 level was found in 90.6% of tumors, consistently with previous studies investigating patients with colon (25,27,39), gastric (21,39,45,46) and pancreatic (22,23,29) adenocarcinomas. However, we did not find any significant associations between miR-21 level and the clinical and pathologic characteristics, in line with recently reported results (47). Likewise, in our series, expression of miR-21 was not associated with any of the clinical outcomes analyzed.

miR-21, acting as an oncogene (48), targets the products of several genes relevant to cancer progression and metastasis, including *PDCD4, SPRY, PTEN* and *TPM1*. Recent data suggest that miR-21 induces a paradoxical negative regulation of cell cycle progression in hypoxic colon cancer models, through a Cdc25a protein phosphatase-dependent mechanism (49). Interestingly, *in situ* hybridization results indicate that miR-21 is located primarily in the stromal compartment of the tumors (50). These data suggest a set of connections between tumor cells and their microenvironment, where the net effect of a particular miRNA might be variable, in a cellular context-dependent manner.

Furthermore, technical differences could explain apparently contrasting results. In fact, in the study reported by Schepeler *et al* (51), there was no correlation between miR-21 expressions data obtained with real-time RT-PCR as compared to data obtained with microarray with probes designed against the mature form. Also, the limited numbers of patients included in the studies, including ours, make the data more susceptible to stochastic effects.

When we considered the different reports about the potential prognostic relevance of miRNA expression in GI cancer, a considerable degree of inter-study heterogeneity was noted. Differences in the detection and quantification methods (microarrays, qRT-PCR), types and numbers of miRNAs evaluated (pre-miRNA or mature form, expression profile, single marker), and sample source (FFPE, deep-frozen, specimen microdissection), as well as in the clinicopathological data of the included patients, ought to be considered as potential sources of heterogeneity.

In conclusion, our study suggests that the presence of CK-positive cells in the bone marrow and the upregulation of the miR-17-92 cluster in the tumor were both significant but independent prognostic markers in gastrointestinal cancer patients. These results will require validation in independent and large sample sets before firm conclusions can be reached.

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Article

Evaluation of the Adenocarcinoma-Associated Gene *AGR2* and the Intestinal Stem Cell Marker *LGR5* as Biomarkers in Colorectal Cancer

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Abstract: We aim to estimate the diagnostic performances of anterior gradient homolog-2 (*AGR2*) and Leucine-rich repeat-containing-G-protein-coupled receptor 5 (*LGR5*) in peripheral blood (PB) as mRNA biomarkers in colorectal cancer (CRC) and to explore

their prognostic significance. Real-time PCR was used to analyze *AGR2* and *LGR5* in 54 stages I-IV CRC patients and 19 controls. Both mRNAs were significantly increased in PB from CRC patients compared to controls. The area under the receiver-operating characteristic curves were 0.722 (p = 0.006), 0.376 (p = 0.123) and 0.767 (p = 0.001) for *AGR2*, *LGR5* and combined *AGR2/LGR5*, respectively. The *AGR2/LGR5* assay resulted in 67.4% sensitivity and 94.7% specificity. *AGR2* correlated with pT3–pT4 and high-grade tumors. *LGR5* correlated with metastasis, R2 resections and high-grade. The progression-free survival (PFS) of patients with high *AGR2* was reduced (p = 0.037; HR, 2.32), also in the stage I-III subgroup (p = 0.046). *LGR5* indicated a poor prognosis regarding both PFS (p = 0.007; HR, 1.013) and overall survival (p = 0.045; HR, 1.01). High *AGR2/LGR5* was associated with poor PFS (p = 0.014; HR, 2.8) by multivariate analysis. Our findings indicate that the assessment of *AGR2* and *LGR5* in PB might reflect the presence of circulating tumor cells (CTC) and stem cell like CTC in CRC. Increased *AGR2* and *LGR5* are associated with poor outcomes.

Keywords: colorectal cancer; real-time PCR; circulating tumor cells; prognostic markers; stem cells; anterior gradient homolog-2; leucine-rich repeat-containing G-protein-coupled receptor 5

1. Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-associated morbidity and mortality across the world. The predicted number of deaths in 2011 in the European Union due to CRC was 162,026 [1]. The stage at diagnosis and the possibilities for curative surgery remain the most important prognostic factors.

The development of blood-borne metastasis is ultimately responsible for most CRC-related deaths. Sensitive methods to detect circulating tumor cells (CTC) could serve as prognostic or predictive tools to identify patients at a high risk of disease progression who could be selected for additional treatment [2].

CTC are identified mainly by using antibodies against epithelial antigens or molecular approaches. The PCR amplification of tissue- or tumor-specific mRNA is commonly used to detect circulating or occult metastatic cells. Systematic reviews, meta-analyses and prospective studies [3–7] provide coherent evidence that the molecular detection of CTC in the peripheral blood (PB) is of strong prognostic significance in patients with CRC.

Our study aimed to evaluate promising CRC-specific mRNAs for multi marker detection of CTC in PB. We previously [8,9] identified anterior gradient homolog-2 (*AGR2*) and plakophilin-3 as potential CTC markers in gastrointestinal cancer through an in silico profile of gene expression and quantitative real-time reverse-transcription PCR (qRT-PCR). Moreover, *AGR2* has been included in the molecular signature that defined CTC in metastatic breast, prostate and colorectal cancers [10,11].

AGR2 encodes a 17 kDa secreted protein, homologue of the Xenopus cement gland gene *XAG-2* [12]. Although its functions in humans are poorly understood, recent reports indicate that

AGR2 can induce cellular transformation and tumor growth, promote cell survival through inhibition of p53, enhance tumor cell adhesion to the substratum and enhance cell migration [13–15].

Recent data [2,10,11,16,17] suggest that CTC encompass a heterogeneous cell population with different tumorigenic capabilities and include cells characterized by an epithelial-mesenchymal plasticity (EMP) with transient loss of epithelial markers. In that sense, the use of different mRNA biomarkers will yield better results in the identification of CTC and rare cell subsets of biological relevance. Thus, it has been hypothesized that only CTC with tumor-initiating properties will eventually complete the metastatic cascade and will develop clinically relevant metastases [18].

The leucine-rich repeat-containing G-protein-coupled receptor 5 (*LGR5*) also known as G-protein-coupled receptor 49 (*GPR49*), has been recently reported as a marker for stem cells (SC) in the small intestine and colon [19]. Recently [20,21] it was shown that the *LGR5* gene and protein were markedly over expressed in the majority of advanced CRCs and in CRC cell lines derived from metastatic tumors. Moreover, high *LGR5* expression has been associated with poor progression-free survival for CRC patients [22].

Thus, we hypothesized that *LGR5* mRNA expression in PB of CRC patients could indicate the presence of circulating tumor cells with stem cell properties.

The primary aims of our study were to estimate prospectively the diagnostic accuracy and usefulness of *AGR2* mRNA in PB as a surrogate biomarker of CTC and to explore its prognostic significance. Additionally, the blood expression of the intestinal stem-cell (ISC) marker *LGR5* was evaluated for correlations with *AGR2* and clinical parameters. Our findings revealed that molecular assessment of *AGR2* and *LGR5* can serve as a marker of CTC and ISC-like CTC in CRC patients, which underscores their potential clinical relevance as predictors of disease outcome.

2. Results and Discussion

2.1. Results

2.1.1. Patients and Clinical Data

Starting in July 2004, 54 patients with histological proven CRC and 19 controls were consecutively recruited for this study. This sample size allowed us to estimate an expected area under the ROC curve of 0.70 with a standard error of 0.065. Ninety per cent of the subjects were included within the first two years. The clinical characteristics of the patients are shown in Table 1.

Table 1. Patient baseline and clinical characteristics.

		Mean (SD)	**Range**
Age (years)		62.7 (9.6)	31–80
		N	**%**
	<60 years	20	37.0
	≥60 years	34	63.0

Table 1. *Cont.*

Gender		
Female	21	38.9
Male	33	61.1
Stage		
I-II	6	11.1
III	12	22.2
IV	36	66.7
pT		
pT1-pT2	6	14.1
pT3	36	66.7
pT4	8	14.8
pTx	4	7.4
pN		
pN0	13	24.1
pN1	26	48.1
pN2	11	20.4
pNx	4	7.4
M		
M0	18	33.3
M1	36	66.7
Residual disease status		
R0-R1	16	29.6
R2	38	70.4
Number of Metastatic Sites		
0	18	33.3
1	25	46.3
≥2	11	20.4
Location of Metastasis		
None	18	33.3
Liver Only	23	42.6
Liver and Other	11	20.4
Non-liver Metastasis	2	3.7
Grade		
Low Grade	10	18.5
High Grade	44	81.5
Vascular/Perineural Invasion		
Unknown	6	11.1
No	24	44.4
Yes	24	44.4

The mean age was 62.2 years (SEM 1.84; median, 62 years; range, 43 to 74 years) in the control group and 62.7 (SEM 1.30; median, 62.5; range, 31 to 80 years) in the patient group (*t* test, $p = 0.847$). The ratio of males to females was similar in the controls (men 63.2%) and the patients (men 61%) (χ^2 test, $p = 0.875$).

PB samples were obtained after R0 or R1 surgery in 16 patients. In 38 patients, blood samples were obtained before neo-adjuvant chemotherapy or in the presence of active metastatic disease, both of which were categorized as R2. In patients with node-negative disease and R0 resection, the mean number of lymph nodes analyzed was 12.8 (SEM 2.7; range 7–21).

Patients with metastatic CRC (n = 38) were grouped into high- (19.4%), intermediate- (36.1%) and low-risk groups (44.4%) using performance status, number of tumor sites, alkaline phosphatase and white blood cell count, as suggested by Köhne *et al*. [23] Median overall survival (OS) and progression-free survival (PFS) were 98 and 39 weeks, 56 and 26 weeks, and 59 and 14 weeks for the low-, intermediate- and high-risk groups, respectively. The median OS tended to be higher (log-rank p = 0.061) in the low-risk group (98 weeks; 95% CI, 43.1 to 152.9) compared to the combined intermediate/high-risk group (56 weeks; 95% CI, 47.2 to 64.8).

All patients were followed up until death or the end of the study. Disease progression events occurred in 39 patients (72.2%). There were three relapses among stage I–III patients and 36 progressions of metastatic disease. The median PFS was 44 weeks (95% CI, 24.8 to 63.2 weeks). The median OS was 132 weeks (95% CI, 84.4–179.6 weeks), and 34 patients (63%) died of advanced disease. The mean (SEM) follow-up time for the patients still alive at the time of the analysis was 232 (17.8) weeks (median, 232.5 weeks; range, 67 to 335 weeks).

2.1.2. Expression of *AGR2* and *LGR5* mRNA Transcripts in Blood Samples

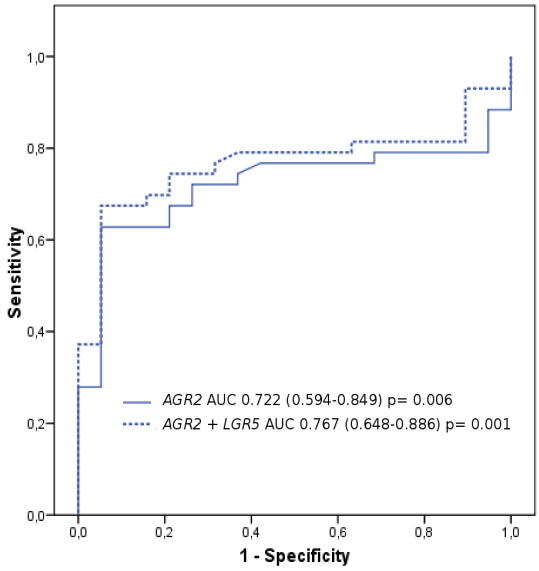
AGR2 mRNA was quantified in 62 blood samples (84.9%), including 43 samples obtained from patients with CRC and 19 from controls. The *LGR5* mRNA level was quantified in 67 blood samples (90.5%), 48 from CRC patients and 19 from controls. mRNA was insufficient or its quality was inadequate for qRT-PCR in 11 (15.1%) and 6 (8.2%) patients' samples for *AGR2* and *LGR5* respectively.

The mean relative *AGR2* mRNA expression was 29.1 (SEM 28.2; median 0.77; range, 0.21 to 536.7) in controls and 418.57 (SEM 84.4; median 191.2; range, 0.05 to 1989.5) in cancer patients (t test, $p < 0.001$). Likewise, the *AGR2* level was significantly increased (ANOVA, p = 0.007) in patients with stage IV CRC (mean 492.6; SEM 114) compared with stage I to III patients (mean 305.4; SEM 122.5) and non-cancer controls (mean 29.1; SEM 28.2).

The mean *LGR5* mRNA level was 0.21 (SEM 0.03; median 0.18; range, 0 to 0.4) in controls and 11.6 (SEM 4.9; median 0.08; range, 0.01 to 146.9) in patients (t test, p = 0.026). The *LGR5* level was significantly increased (ANOVA, p = 0.038) in patients with stage IV CRC (mean 18.40; SEM 7.70) compared with stage I to III patients (mean 0.20; SEM 0.06) and non-cancer controls (mean 0.21; SEM 0.03). There was no correlation between *AGR2* and *LGR5* blood levels in the patients group (Pearson correlation coefficient −0.009; p = 0.952).

ROC curves of circulating mRNAs were constructed in order to be able to discriminate different groups (Figure 1).

Figure 1. *AGR2* and *LGR5* ROC curves. mRNA relative levels were quantified in blood obtained from patients with colorectal cancer and from controls. Area under the curve (AUC), 95% confidence interval and *p*-values are shown.



Comparing the relative *AGR2* levels in patients and controls, the AUC was 0.722 (95% CI, 0.594–0.849; $p = 0.006$). According to the ROC curve, a relative level for *AGR2* mRNA in the blood of 1.65 was defined as the optimal cutoff value (Youden index) for differentiating patients with CRC from the controls. With this cutoff value for *AGR2*, the sensitivity and specificity of 62.8% (95% CI, 46.7 to 76.6) and 94.7% (95% CI, 71.9 to 99.7) respectively, were achieved. At this threshold value, *AGR2* positivity was associated with CRC diagnostic ($p < 0.001$).

The ROC curve for *LGR5* showed an AUC of 0.376 (95% CI, 0.233–0.520; $p = 0.123$). A relative blood level of 0.39 was defined as the optimal cutoff point for *LGR5*. With this cutoff value, the sensitivity and specificity for the *LGR5* mRNA assay were 18.8% (95% CI, 9.4 to 33.10) and 100% (95% CI, 79.1 to 99.5) respectively. At this cutoff value, *LGR5* positivity tended to associate with CRC diagnostic ($p = 0.052$).

In CRC patients, relative expression values for *AGR2* and/or *LGR5* in blood above these cutoff points, defined as the Youden index, were found in 16.7% of stage I–II, in 72.7% of stage III and in 76.9% of stage IV patients (χ^2 test; $p = 0.016$).

AGR2 and *LGR5* markers were analyzed in combination by logistic regression. The predicted probabilities of diagnosis generated a 'combination marker' ROC curve. The combination (*AGR2*/*LGR5*) had an AUC-ROC = 0.767 (95% CI, 0.648–0.886; $p = 0.001$) which was slightly improved [24] compared to *AGR2* alone ($p = 0.25$). The sensitivity and specificity of the combination were 67.4% (95% CI, 51.3 to 80.5) and 94.7% (95% CI, 71.9 to 99.7) respectively (Figure 1).

2.1.3. Clinic Pathological Characteristics and mRNA Markers in Blood

The clinical and pathological characteristics and the *AGR2* and *LGR5* mRNA expression in blood from cancer patients are shown in Table 2.

Table 2. Distribution of clinical and pathological parameters and levels of *AGR2* and *LGR5* mRNA in the blood.

Parameter	*AGR2*			*LGR5*		
	Mean	**SEM**	***p***	**Mean**	**SEM**	***p***
Age (y)			0.459			0.128
<60	497.0	142.2		22.5	10.0	
≥60	367.3	105.1		5.0	5.0	
Gender			0.075			0.203
Male	291.1	87.9		5.8	3.9	
Female	633.7	161.5		22.1	11.7	
Stage			0.137 *			0.204 *
I-II	1.1	0.2		0.3	0.05	
III	471.3	171.2		0.1	0.08	
IV	492.6	113.8		18.4	7.7	
pT			0.002 [a]			0.915
pT1-T2	82.1	57.5		10.7	10.5	
pT3-T4	453.7	92.4		12.5	5.8	
pN			0.063 *			0.309 *
Node Negative	306.9	162.3		0.26	0.05	
pN1	311.7	80.4		13.1	7.01	
pN2	795.2	266.3		23.9	16.4	
M			0.283			0.024 [a]
M0	305.4	122.5		0.18	0.06	
M1	492.6	113.8		18.4	7.7	
R Status			0.671			0.024 [a]
R0-R1	363.2	156.1		0.13	0.03	
R2	442.6	101.7		40.3	7.01	
Number of Metastatic sites			0.373 *			0.159 *
0	305.4	122.5		0.18	0.06	
1	407.7	145.0		21.5	10.6	
≥2	628.4	184.7		12.3	9.3	
Grade			0.023 [a]			0.024 [a]
Low grade	183.0	71.9		0.1	0.04	
High grade	480.9	102.8		14.6	6.1	
Vascular/Perineural Invasion			0.751			0.269
No	385.8	100.6		6.6	6.5	
Yes	441.2	146.1		18.6	8.5	

AGR2 and *LGR5*, mean relative expression levels, arbitrary units; SEM: standard error of the median, *t*-test; * ANOVA; [a] *p* values of less than 0.05.

A significant higher relative level of *AGR2* blood expression was found in pT3-T4 tumors ($p = 0.002$) and high-grade lesions ($p = 0.023$). There was a tendency ($p = 0.063$) to higher *AGR2* levels associated with lymph node metastasis. Increased *LGR5* expression was found in patients (Table 2) with stage IV ($p = 0.024$), R2 resections ($p = 0.024$) or high-grade tumors ($p = 0.024$).

Carcinoembryonic antigen (CEA) and carbohydrate antigen 19.9 (CA 19.9) serum levels were increased above the upper limits of normal in 46.3% and 38.9% of the patients, respectively. There were no correlations between *AGR2* or *LGR5* mRNA levels with CEA or CA 19.9 in serum (Pearson −0.172, −0.155, 0.021 and −0.063 respectively).

To explore the possible influence of recent surgery on the circulation of tumor cells, we analyzed *AGR2* and *LGR5* levels according to the time interval from operation and blood sampling. The mean time from surgery to blood sampling for mRNA quantification was 52.5 weeks (SEM 8.7 weeks; median, 18 weeks; range, 1 to 202 weeks). The 25th percentile was 6.75 weeks. There was no significant difference in *AGR2* and *LGR5* levels between time intervals (<6.75 or ≥6.75 weeks) from the last surgery.

In the group of patients with stage IV disease, *AGR2* and *LGR5* were analyzed according to the prognostic subgroups defined as described previously [19]. The mean (SEM) relative *AGR2* levels were 443.1 (229.6) and 518.8 (129.9) for low- and combined intermediate/high-risk groups, respectively (*t* test, $p = 0.759$). The median (SEM) relative *LGR5* levels were 15.5 (11.7) and 20.6 (10.4) for low- and combined intermediate/high-risk groups, respectively (*t* test, $p = 0.746$).

2.1.4. Prognostic Significance of *AGR2* and *LGR5* in Blood

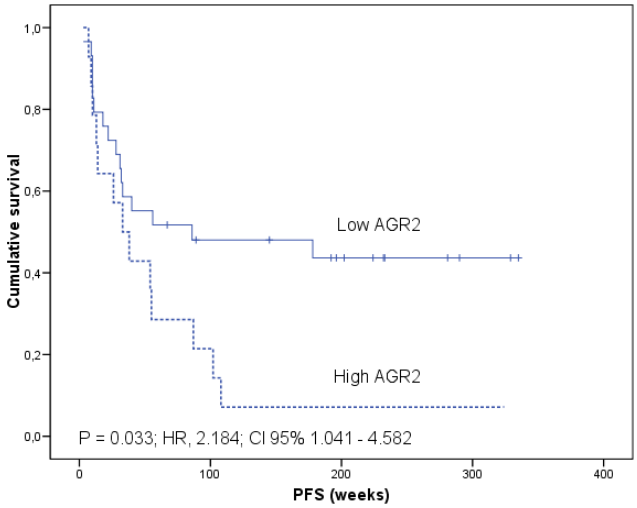
To analyze the relationships between biomarker expression and outcomes (PFS and OS) we estimated the hazard ratios associated with mRNA levels as continuous variables using Cox regression models [25]. There was a trend for a high risk of disease progression associated with increased *AGR2* relative blood expression (HR 1.0; 95% CI, 1.0 to 1.001; $p = 0.093$). There was no association with the risk of death (HR 1.0; 95% CI, 0.999 to 1.001; $p = 0.913$). However, in stage I to III patients, the risk of disease progression was higher with increasing *AGR2* level (HR 1.002; 95% CI, 1 to 1.004; $p = 0.046$).

Increasing relative blood expression of *LGR5* mRNA as a continuous variable was associated with a higher risk of disease progression (HR 1.013; 95% CI, 1.004 to 1.023; $p = 0.007$). The risk of death was also higher with increasing levels for *LGR5* mRNA in the blood (HR 1.01; 95% CI, 1 to 1.020; $p = 0.045$).

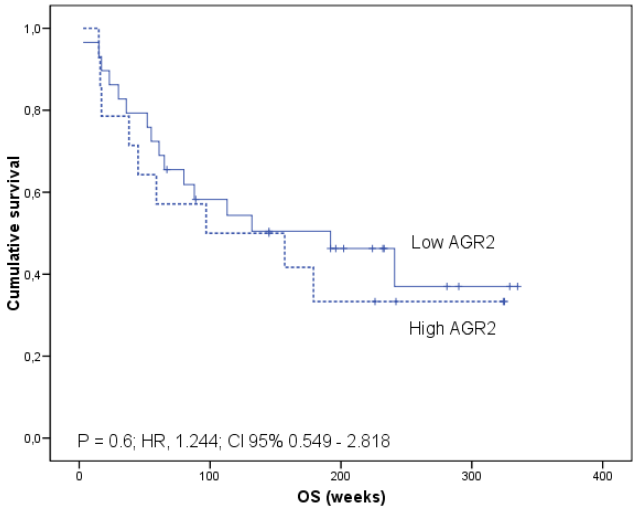
In addition, in order to generate survival curves, we converted continuous mRNAs expression levels measured on qRT-PCR to a dichotomous variable, using the mean levels of expression in the patients group as a threshold. Kaplan-Meier curves for patients categorized according to *AGR2* and *LGR5* mRNA expression in blood are shown (Figures 2–4).

The median PFS for the group with high *AGR2* blood expression were 33 weeks (95% CI, 11 to 55) compared with 86 weeks (95% CI, 0 to 305.1) in the group with low *AGR2* (log-rank test, $p = 0.033$). Patients with high *AGR2* showed worse OS (median 97 weeks; 95% CI, 0 to 262.9) compared with those with low *AGR2* expression (median 192 weeks; 95% CI, 56.6 to 327.4) although this difference was not statistically significant (log-rank test, $p = 0.6$) (Figure 2).

Figure 2. *AGR2* and survival analysis. Kaplan-Meier plots of (**a**) progression-free survival (PFS) and (**b**) overall survival (OS) in colorectal cancer patients according to *AGR2* mRNA expression in blood. Relative quantification of *AGR2* mRNA was calculated by the $2^{-\Delta\Delta Ct}$ method using HPRT as a reference gene. Continuous mRNA levels were converted to a dichotomous variable using the mean levels of expression as a threshold. p estimates by log-rank test. Hazard ratios (HR) were modeled using Cox proportional hazard regression analysis.



(**a**)

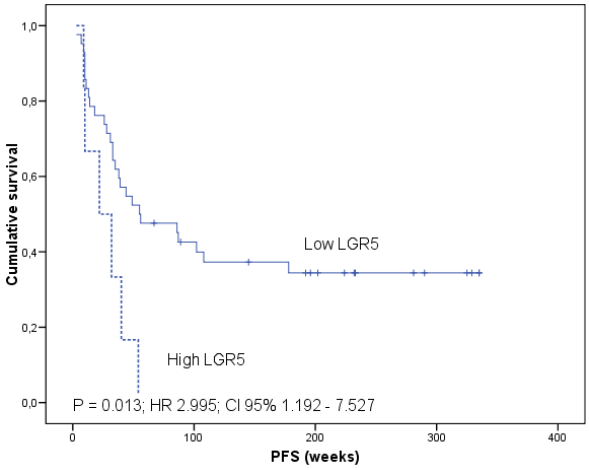


(**b**)

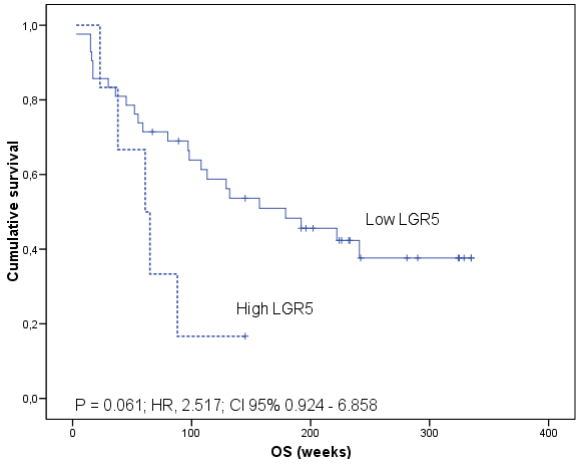
Analysis of the patients' outcome according to *LGR5* blood expression revealed that the high *LGR5* group exhibited significantly worse PFS (median 22 weeks; 95% CI, 0 to 48.4) compared with patients

in the low *LGR5* group (median 55 weeks; 95% CI, 5.1 to 104.9) (p = 0.013). Although non-significant, there was a trend (p = 0.061) for a better OS in the group of patients with low *LGR5* (median 179 weeks; 95% CI, 74.9 to 283.1) compared with the group with increased *LGR5* blood levels (median 61 weeks; 95% CI, 28.6 to 93.4) (Figure 3).

Figure 3. *LGR5* and survival analysis. Kaplan-Meier plots of (**a**) progression-free survival (PFS) and (**b**) overall survival (OS) in colorectal cancer patients according to *LGR5* mRNA expression in blood. Relative quantification of *LGR5* mRNA was calculated by the $2^{-\Delta\Delta Ct}$ method using HPRT as a reference gene. Continuous mRNA levels were converted to a dichotomous variable using the mean levels of expression as a threshold. p estimates by log-rank test. Hazard ratios (HR) were modeled using Cox proportional hazard regression analysis.



(**a**)

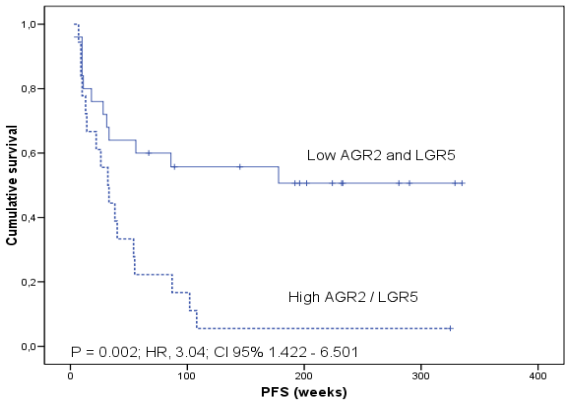


(**b**)

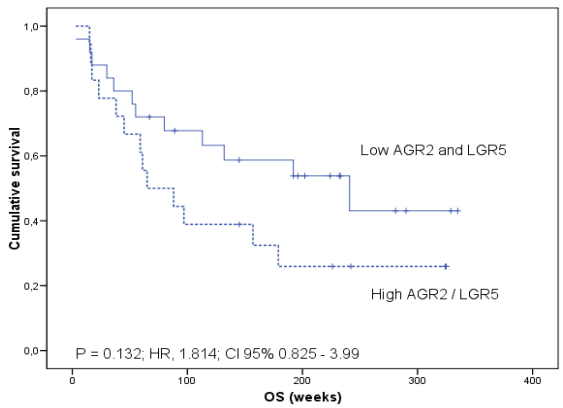
High mRNA in PB (combined *AGR2* and/or *LGR5* transcript above the threshold cutoff) was found in 0, 36.4% and 53.8% of stage I–II, III and IV patients, respectively (χ^2 test; $p = 0.05$).

Patients were divided into favorable mRNA profile (both *AGR2* and *LGR5* below the mean) and unfavorable mRNA profile (*AGR2* and/or *LGR5* above the mean). At the time of analysis, the mean and the median PFS in the favorable group were 190.8 weeks (95% CI, 131.2 to 250.4 weeks) and not reached in the unfavorable group. The mean and the median PFS were 54.7 weeks (95% CI, 21.2 to 88.1 weeks) and 32 weeks (95% CI, 17.5 to 46.6 weeks) in the unfavorable mRNA profile group (log-rank test $p = 0.002$) (Figure 4).

Figure 4. Combined *AGR2/LGR5* and survival analysis. Kaplan-Meier plots of (**a**) progression-free survival (PFS) and (**b**) overall survival (OS) in colorectal cancer patients according to combined *AGR2/LGR5* mRNA profile in blood. Relative quantification of mRNA was calculated by the $2^{-\Delta\Delta Ct}$ method using HPRT as a reference gene. Patients were divided into favorable mRNA profile (both *AGR2* and *LGR5* below the mean) and unfavorable mRNA profile (*AGR2* and/or *LGR5* above the mean). p estimates by log-rank test. Hazard ratios (HR) were modeled using Cox proportional hazard regression analysis.



(**a**)



(**b**)

Multivariate Cox regression analyses were performed to determine whether high mRNA in blood were independently statistically predictive of PFS or OS (Table 3).

Table 3. Progression-free survival and overall survival in relation to clinic and pathological characteristics and blood *AGR2*/*LGR5* mRNA: Multivariate Cox proportional hazard analysis.

		Wald	Hazard Ratio	95% CI		*p*
Progression free survival						
Depth of invasion	pT1-2/pT3/pT4	1.042	1.430	0.720	2.841	0.307
Lymph Nodes	Negative/Positive	0.834	1.714	0.539	5.445	0.361
Residual disease	R0-1/R2	8.047	5.824	1.724	19.68	0.005
AGR2/LGR5	Negative/Positive	6.025	2.803	1.231	6.385	0.014
Overall survival						
Depth of invasion	pT1-2/pT3/pT4	0.741	1.443	0.626	3.322	0.389
Lymph Nodes	Negative/Positive	0.020	1.085	0.348	3.384	0.888
Residual disease	R0-1/R2	7.041	7.338	1.683	31.99	0.008
AGR2/LGR5	Negative/Positive	1.158	1.594	0.682	3.724	0.282

AGR2/LGR5 negative in blood indicate both mRNA markers below the mean; a positive result indicates *AGR2* and/or *LGR5* above the mean.

In testing for the independent prognostic significance of high *AGR2*/*LGR5* expression in a model with pT depth of invasion, lymph node involvement and residual disease (R resection status), the R status (HR of recurrence, 5.8; 95% CI, 1.7 to 19.7; $p = 0.005$) and the high mRNA blood expression (HR, 2.8; 95% CI, 1.2 to 6.4; $p = 0.014$) remained associated with PFS (Table 3). In this model, the only factor that retained independent prognostic significance for OS was R2-residual disease (HR of death, 7.338; 95% CI, 1.683 to 31.985; $p = 0.008$).

2.2. Discussion

Highly sensitive detection of CTC and detailed molecular characterization of rare cancer cell subpopulations may not only provide insights into the biology of early metastatic spreading, but these tools can also potentially indicate substantial predictive or prognostic information. PCR amplification of tumor mRNA is a powerful analytical tool for surrogate detection and characterization of CTC. Real-time RT-PCR allows for quantification of the tumor cell load in the PB and, at least theoretically, the determination of cutoff values of mRNA expression of clinical relevance in cancer patients. However, the sensitivity and specificity of this approach both depend on the expression level of candidate biomarkers in tumor cells as well as their background expression in the blood [26,27].

Evidence is rapidly accumulating that cancers are composed of heterogeneous populations of cells. Thus, one would predict that CTC might be enriched in cancer cells that express those biomarkers indicating the greatest invasive and metastatic capacity, including cancer stem cells (CSC) markers. Hence, the selection of appropriate target mRNAs that may be useful for clinical detection of CTC and CSC remains an important outstanding issue.

The current study was intended to assess the diagnostic performance of quantitative RT-PCR detection of *AGR2* in the blood as a surrogate marker of CTC. We then hypothesized that a marker

indicative of the phenotype of colonic stem cells, such as *LGR5*, would improve the detection of biologically and clinically relevant CTC.

We found that *AGR2* mRNA was significantly elevated in the blood of patients with CRC compared to controls. ROC analysis suggested that at 94.7% specificity, *AGR2* achieved 62.8% sensitivity in distinguishing CRC blood samples from the control group. Furthermore, in CRC patients, blood *AGR2* mRNA levels correlated with different pathological prognostic factors, including pT3–pT4 depth of invasion and high-grade tumors.

These results are in line with the current evidence indicating that *AGR2* can promote cancer growth, cell survival, migration and anchorage-independent growth and cellular transformation [14,28]. In the clinical setting, *AGR2* protein expression in the primary tumor is an independent prognostic indicator of poor outcome in patients with breast [29] and prostate adenocarcinomas [30], and one recent study showed that increased *AGR2* protein in plasma is associated with ovarian cancer [31].

However, to the best of our knowledge, no comprehensive report has been published about the potential prognostic relevance of *AGR2* in colorectal cancer. Our findings indicate for the first time that the quantitative assessment of *AGR2* mRNA in blood might indicate a poor patient outcome in CRC. Remarkably, in stage I to III patients, the risk of disease progression was higher with increasing levels of *AGR2* in the blood. Likewise, in CRC patients with high *AGR2* blood expression, the PFS was significantly reduced, and there was a numerical but non-significant inferior OS.

A recent study [32] demonstrates that *AGR2* induces the expression of the growth-promoting EGFR ligand amphiregulin in human adenocarcinomas. This effect is mechanistically mediated through Yes-associated protein (YAP1) dephosphorylation. Interestingly, YAP1 is also implicated in the regulation of stem cell division through the repression of the Hippo pathway. These data and a previous report [14] show that proliferating and non-proliferating ISCs, as well as transit-amplifying cells from a secretory lineage express *AGR2* and suggest additional mechanisms for oncogenic actions for *AGR2*.

We next explored the expression of the ISC marker *LGR5* in the blood of our cohort of controls and CRC patients. We found that *LGR5* mRNA was significantly elevated in the blood of patients with colorectal carcinoma compared to controls. However, mean levels of *LGR5* mRNA were similar in controls and early stage CRC patients. Nevertheless, there was a significant increase of *LGR5* in blood obtained from metastatic CRC patients. When a cutoff point was defined based on the ROC curve, the *LGR5* assay achieved only 18.8% sensitivity but 100% specificity in distinguishing CRC and control blood samples. Conversely, *LGR5* mRNA in the blood showed a significant correlation with high-grade tumors, metastatic disease and R2 resections. Likewise, *LGR5* expression in the blood showed a prognostic value regarding both PFS and OS in CRC patients, as suggested by the Cox regression and Kaplan-Meier analysis. In that sense, our results suggested that *LGR5* is expressed only in a rare subset of CTC possibly including cancer stem-like cells. We could speculate that these circulating *LGR5*-expressing cells might contribute to cancer progression and therapeutic response.

The clinical and biological significances of *LGR5* expressing-cells in CRC are poorly understood. A primary tumor profile that encompasses known ISC markers, such as *LGR5*, has been strongly associated both with CRC stages and the occurrence of tumor relapse and metastasis [33]. *LGR5* protein expression had been associated with a poor PFS in CRC patients [22]. In contrast, in a recent

report [34] a gene signature defined by methylation silencing of the Wnt-driven ISC marker genes, including *LGR5*, in CRC tumors was associated with a poor prognosis.

A number of proposed CSC markers, such as CD44 and CD133, have been explored in CTC detection. Recently, Iinuma H. *et al*. [7] demonstrated in patients with Dukes' stage B and C CRC that the detection of CEA/Cytokeratins (CK) 19/20/CD133 mRNA in blood was useful for determining which patients were at high risk for recurrence and poor prognosis. However, in the CD133 single-marker analysis, no significant differences in OS and PFS were found [7]. In metastatic CRC, the transcriptional amount of CD133 in blood before resection of hepatic metastases resulted in a high risk of dying of recurrence after apparently curative liver surgery [35]. Nonetheless, CD133 and other putative markers for CRC stem cells such as CD44 are also expressed in a variety of cells including hematopoietic and/or endothelial cells (reviewed Hundt, S. in [27]), a factor that could diminish their specificity. The expression patterns of LRG5 and colon differentiation markers such as cytokeratin−20 are mutually exclusive [33] are of special interest for CTC detection. These facts strengthen the relevance of non-CK mRNA biomarkers for the detection of the most aggressive and specific subpopulations of CTC in CRC patients.

CTC in gastrointestinal cancer patients are increasingly detected when blood is obtained per- or intra-operatively [36]. However, the postoperative sampling time might reflect the most relevant CTC status [4,37]. In our study, blood samples were obtained several weeks after surgery. In order to explore the possible influence of recent surgery on the circulation of tumor cells, *AGR2* and *LGR5* levels were analyzed according to time intervals between surgery and blood sampling; conversely, no significant differences in biomarker mRNA levels between time intervals were found.

From a clinical perspective, assessment of baseline prognostic factors and CTC detection rates may be of interest. In previous studies [38,39] including patients with metastatic CRC, the number of CTC detected using the Cell Search System was associated with high LDH level, liver metastasis and poorer performance status. Hence, we performed an exploratory analysis in the subset of stage IV CRC patients, which showed no association between a positive mRNA result and baseline clinical prognostic subgroups categorized according to performance status, white blood cell count, alkaline phosphatase and number of metastatic sites. In addition, levels of *AGR2* and *LGR5* were not significantly different either.

The combined *AGR2* and *LGR5* assay resulted in an increased sensitivity (67.4%; AUC-ROC = 0.767; $p = 0.001$) to separate cancer patients and controls. Remarkably, and in spite of the limited number of patients, Cox multivariate analysis demonstrated that *AGR2*/*LGR5* mRNA detection was a significant prognostic factor for PFS (HR, 2.8; 95% CI, 1.2 to 6.4; $p = 0.014$). Thus, the transcriptional amount of *AGR2*/*LGR5* in the PB defined subgroups of CRC patients with significantly different risks of disease progression, improving the so-called biologic specificity [40] of CTC detection.

Our findings indicate a high sensitivity and specificity for *AGR2*/*LGR5* qRT-PCR for the surrogate detection of CTC in PB samples and it could be useful as a prognostic factor in patients with CRC. However, taking into account the design and sample size of the study, the outcome results could only be considered as generating a hypothesis. Additional possible limitations of this study must be considered. Although the inclusion of patients with different stages and residual tumor status could be considered limitations of the study, we suggest that this pragmatic design accurately reflects the patients attending the oncology clinic every day. Thus, the diagnostic performance of mRNA

quantification has been estimated in a cohort of patients truly representative of those found in the clinical setting. However, to adequately assess the prognostic role, if any, of *AGR2* and *LGR5* mRNA levels in the blood, a larger, more homogeneous cohort of patients is clearly needed. Furthermore, a comparative study with immunofluorescence-based methods such as the Cell Search System is warranted.

3. Experimental Section

3.1. Patients

Consecutive patients with CRC from the Medical Oncology Unit at the University Hospital in La Coruña (Galicia, Spain) were included in the study. Inclusion criteria were as follows: A confirmed pathological diagnosis of colorectal adenocarcinoma; stage I–III patients with no prior systemic therapy for cancer; or stage IV patients without previous systemic therapy or with confirmed cancer progression after such treatment. Exclusion criteria were defined as follows: Any other previous malignancy; coagulations disorders; platelet count less than 20.0×10^9 L^{-1}.

The diagnostic work-up included a clinical examination, blood sampling with CA 19.9 and CEA serum determination, endoscopy (when clinically indicated), thoracic radiograph and computed tomography (CT) scanning of the abdomen and pelvis. Chest CT was performed in patients with rectal tumors and stage IV patients. Patients were followed up with imaging every 8 to 12 weeks to monitor disease progression.

Serum CEA (with an upper limit of normal of 5 ng/mL) and CA 19.9 (with an upper limit of normal of 37 U/mL) levels were determined using enzyme immunoassay (Advia Centaur, Siemens Healthcare Diagnostics) according to the manufacturer's instructions.

PBs for qRT-PCR analyses were obtained after surgery, before neo-adjuvant chemotherapy or in the presence of active, clinically and radiological advanced progressive disease. At least the first 5 mL of blood obtained was discarded to avoid contamination with epidermal cells.

Controls were consecutively recruited from the patients' family and relatives. We only excluded controls with a previous history of malignant disease. Thus, controls with different chronic but stable diseases (e.g., hypertension, diabetes mellitus or heart disease) were eligible and consecutively recruited. Controls were selected to include a sex and age distribution that was comparable to the patient group.

This study was approved by the Ethics Committee of Clinical Investigation of Galicia (Spain), and written informed consents were obtained from all patients and controls prior to their inclusion in the study.

3.2. Pathological Analysis

Tumors and regional lymph nodes collected during surgery were processed on a routine diagnostic basis. Histological tumor type, depth of invasion and nodal involvement were analyzed, and the disease was staged and graded according to the TNM [41].

Residual disease status at the time of blood sampling was classified as R0 when no residual disease was present after surgery, R1 when microscopic residual disease was found, and R2 in the presence of macroscopic disease. Patients from whom the blood was obtained before the start of neo-adjuvant treatment were categorized as R2.

3.3. Processing of Blood Samples and mRNA Isolation

Peripheral venous blood (10 mL) was collected in EDTA-containing tubes. Samples were stabilized within 1 h after withdrawal in guanidinium-based RNA/DNA reagent (Roche, Germany) at 10% (v/v) without cell and plasma separation. An isolation reagent for blood and bone marrow (Roche, Germany) was used for mRNA extraction according to the manufacturer's protocol with minor modifications [10]. Purified poly(A) + RNA was further processed for qRT-PCR or stored at −80 °C until use.

The RNA concentration was determined based on UV absorption at 260 nm. The A260/A280 ratio was calculated to assess RNA quality and purity.

3.4. Reverse Transcription and Quantitative Real-Time PCR

Reverse transcription (RT) was performed on 0.02 µg of mRNA using the Superscript First-Strand Synthesis System (Invitrogen Life Technologies, Carlsbad, CA, USA) as described previously [10]. Real-time PCR analysis was performed using the following primers: *AGR2*-2F, CTGGCCAGAGATACCACAGTC; *AGR2-2R*, AGTTGGTCACCCCAACCTC; *LGR5*-F, CAGCGTCTTCACCTCCTAC; *LGR5*-R, TTTCCCGCAAGACGTAACTC. The *AGR2* and *LGR5* primers amplified 101 bp and 108 bp of the respective cDNAs. Primer pairs were chosen so that the sequences were located in different exons. *Hypoxanthine-guanine phosphoribosyl-transferase 1* (*HPRT1*) was selected as reference gene, as previously reported [8]. *HPRT1* (102 bp) was also used as an internal control to verify the RNA integrity and the efficacy of reverse transcription. Any specimen with inadequate *HPRT1* mRNA was excluded from the study.

The PCR reaction consisted of 10 µL of 2× SYBR Green I Master Mix (Roche, Germany), 1.4 µL of forward (F) and reverse (R) primers at 5 µmol/L (Tib MolBiol, Germany), 4 µL of cDNA and PCR-grade water up to a final volume of 20 µL following the manufacturer's recommendations. Amplifications were performed in a Light Cycler 480 (Roche Applied Science, Penzberg, Germany).

The maximum number of cycles was 50. If after 40 cycles no fluorescent signal was detected on the amplification plots, the marker mRNA was assumed to be absent from the sample.

We verified that the amplifications and the size of each PCR product were specific by melting curve analysis. Data analysis was performed with Light Cycler 480 Relative Quantification software (Roche Applied Science, Penzberg, Germany). Relative levels of expression were calculated by the $2^{-\Delta\Delta Ct}$ method [42]. Each assay was done at least in triplicate. The average value of the replicates was used as the quantitative value for each sample.

Each assay included marker-positive, marker-negative and no-template controls. RNA analyses were performed with no knowledge about clinical or follow-up data.

3.5. Study Design and Statistical Analysis

This project was designed as a prospective early-phase, diagnostic case-control study. The primary aim was to estimate the diagnostic performances of *AGR2* and *LGR5* in blood as clinical biomarkers [43]. Receiver operating characteristic (ROC) curves were constructed by plotting sensitivity (*y*-axis) *versus* 1-specificity (false-positive rate; *x*-axis), and the area under the curve (AUC)

was calculated. The optimal cutoff for mRNAs expression level that separates cancer patients and controls was obtained at the point of the maximum Youden index. Binary logistic regression analyses were used to assess for diagnostic suitability of marker combinations.

Secondary aims included the evaluation of *AGR2* and *LGR5* mRNA blood levels in CRC patients according to the disease characteristics and clinical outcomes. Parametric tests were used to analyze the potential correlation between mRNA biomarker expression and clinical and pathological features of study subjects.

PFS was measured as the time between the baseline PB sampling for biomarkers analysis and the documentation of the first tumor progression based on clinical and radiological findings or death of any cause. OS was defined as the time from baseline blood sampling to death of any cause. Patients who were alive and progression-free at the time of analysis were censored by using the time between the baseline PB sampling and their most recent follow-up evaluation. The Kaplan-Meier method was used to estimate PFS and OS. Log-rank tests were used to assess the difference between the survival curves. Hazard ratios (HR) were modeled using Cox proportional hazard regression analysis.

All statistical tests were two-sided, with alpha levels lower than 0.05 considered statistically significant. PASW Statistics 18.0 for Windows (version 18.0; IBM Corporation: Armonk, NY, USA, 2010) was used for statistical analysis.

The study design and results are presented in accordance with the REMARK [44] and MIQE guidelines [45].

4. Conclusions

Our findings indicate that the quantitative molecular assessment of *AGR2* and *LGR5* can serve as a surrogate marker of CTC and ISC-like circulating tumor cells in CRC patients. Elevated *AGR2* and *LGR5* mRNA levels in the blood are associated with poor outcome in patients with CRC.

Acknowledgments

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RESEARCH Open Access

Circulating miR-200c as a diagnostic and prognostic biomarker for gastric cancer

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Abstract

Background: MicroRNAs are aberrantly expressed and correlate with tumourigenesis and the progression of solid tumours. The miR-200 family determines the epithelial phenotype of cancer cells and regulates invasiveness and migration. Thus, we hypothesised that the quantitative detection of the miR-200 family as epithelial-specific microRNAs in the blood could be a useful clinical biomarker for gastric cancer (GC).

Methods: We initially validated the expression levels of miR-200a, 200b, 200c and 141 in GC cell lines (n = 2) and blood from healthy controls (n = 19) using real-time quantitative reverse transcription PCR (qRT-PCR). The microarray expression profiles of the miR-200 family in 160 paired samples of non-tumour gastric mucosae and GC were downloaded through ArrayExpress and analysed. MiR-200c was selected for clinical validation. The qRT-PCR prospective assessment of miR-200c was performed using 67 blood samples (52 stage I-IV GC patients and 15 controls); the area under the receiver operating characteristic curve (AUC-ROC) was estimated. The Kaplan-Meier and Breslow-Wilcoxon tests were used to assess the correlation of miR-200c with overall and progression-free survival (OS and PFS). Multivariate analyses were performed using the Cox model.

Results: The miR-200c blood expression levels in GC patients were significantly higher than in normal controls ($p = 0.018$). The AUC-ROC was 0.715 ($p = 0.012$). The sensitivity, specificity and accuracy rates of 65.4%, 100% and 73.1%, respectively, were observed. The levels of miR-200c in the blood above the cutoff defined by the ROC curve was found in 17.6% of stage I-II GC patients, 20.6% of stage III patients and 67.7% of stage IV patients ($p < 0.001$). The miR-200c expression levels were not associated with clinical or pathological characteristics or recent surgical procedures. There was a correlation ($p = 0.016$) with the number of lymph node metastases and the increased expression levels of miR-200c in blood were significantly associated with a poor OS (median OS, 9 *vs* 24 months; $p = 0.016$) and PFS (median PFS, 4 *vs* 11 months; $p = 0.044$). Multivariate analyses confirmed that the upregulation of miR-200c in the blood was associated with OS (HR = 2.24; $p = 0.028$) and PFS (HR = 2.27; $p = 0.028$), independent of clinical covariates.

Conclusions: These data suggest that increased miR-200c levels are detected in the blood of gastric cancer patients. MiR-200c has the potential to be a predictor of progression and survival.

Keywords: Gastric cancer, MicroRNA, miR-200, Blood, Biomarker, Prognostic factors

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Background

Gastric cancer (GC) is among the most frequent types of cancer worldwide [1] with a total of 989,600 new cases and 738,000 deaths estimated in 2008. Although GC rates have decreased in recent decades, there are significant regional variations in incidence and the rates for the gastro-oesophageal junction and cardiac adenocarcinomas have increased in several Western countries. In Spain, the adjusted mortality rates were 13 per 100,000 males and 5.5 per 100,000 females. In Galicia, in the northwest of Spain, the mortality rates are even higher, reaching 16.14 per 100,000 in males. Global survival rates are poor, lower than 28% at 5 years [2].

The stage at diagnosis and the options for curative surgery remain the most important prognostic factors. However, distant and loco-regional relapses frequently occur in spite of resection and multimodality therapy. Well-characterised biomarkers are necessary for early diagnosis, to predict metastatic progression and to personalise therapy. Nevertheless, the currently available blood tumour markers are not recommended for the screening or diagnosis of GC, do not have independent prognostic value and are not recommended for prognosis or prediction [3].

Haematogenous tumour seeding is considered an early event in the metastatic process. Therefore, the detection of circulating tumour cells (CTC) could be useful to identify the patients at a high risk of disease progression and death and might indicate the need for further therapeutic approaches [4]. The PCR amplification of tissue or tumour-selective cellular and circulating nucleic acids (CNA) is the most powerful tool for the detection of CTC or occult metastases [4,5].

Mature microRNAs are single-stranded, noncoding RNAs that play key roles in various cellular processes commonly implicated in cancer, such as differentiation, cell growth, angiogenesis, epithelial-to-mesenchymal transition (EMT) and invasion. A large amount of data has revealed the correlation between specific tumours and differential miRNA expression profiles, thus providing a new class of disease-specific biomarkers [6-8]. An increasing number of studies analysing the miRNA expression profiles in gastrointestinal tumours, including GC and their potential clinical relevance have been reported [9,10]. The content for a given miRNA species is estimated at 10^3 to 10^4 molecules per cell, which is one to two orders of magnitude more than most mRNAs [11]. Both messenger and non-coding RNAs can be detected in blood and studies indicate that miRNAs are particularly stable and abundant [12-15]. Circulating miRNAs could be derived from passive leakage from apoptosis or necrosis of cancer cells but also from tissue damage or chronic inflammation. In addition, both cancer and nonmalignant cells, including immune cells, can actively release miRNAs, either microvesicles-associated or free, in a selective manner [16].

Developmental [17,18] and expression profiles studies [19,20] show an enrichment of the miR-200 family in differentiated epithelial tissues. It has been suggested that the miR-200 family is a powerful marker and an essential regulatory factor of the cancer cell epithelial phenotype [21-25]. The miR-200 family of miRNAs consists of five members: miR-200a, 200b and 429, located on chromosome 1p36; and miR-200c and 141, located on 12p13. MiR-200a and miR-141 share a seed sequence, while miR-200b, miR-200c and miR-429 also share a seed sequence, which differs from that of miR-200a/141 by one nucleotide. However, there is evidence that the different miRNAs could control different regulatory networks [26,27]. Previous reports have indicated that the levels of peripheral blood-derived exosomal miR-200c are increased in ovarian cancer patients [28] and the serum levels of miR-141 are specifically elevated in prostate cancer patients [13,29]. Both miR-200a and miR-200b are significantly elevated in the sera of pancreatic cancer and chronic pancreatitis patients compared with healthy controls [30].

Therefore, we hypothesised that the quantitative detection of the miR-200 family, as epithelial-specific miRNAs, in the whole blood could be useful as clinical biomarkers in gastric cancer patients. Therefore, the blood miR-200 cluster expression might correlate with GC diagnosis, staging and prognosis. Our results demonstrated that miR-200c expression levels were increased in the blood of GC patients. Likewise, the blood levels of miR-200c emerged as a compelling and independent prognostic indicator for the progression and survival of GC patients.

Methods

Participants

Consecutive GC patients from the Medical Oncology Unit at the University Hospital in La Coruña (Galicia, Spain) were eligible for the study. The inclusion criteria included a confirmed pathological diagnosis of gastric or gastro-oesophageal junction adenocarcinoma and no prior systemic medical therapy for cancer. The exclusion criteria included any other previous malignancy, coagulation disorders and a platelet count less than $20.0 \times 10^9\ L^{-1}$.

The diagnostic work-up included a clinical examination, blood sampling, endoscopy (when clinically indicated) and computed tomography (CT) scanning of the chest, abdomen and pelvis. The patients were followed up clinically with imaging every 8 to 12 weeks for the first 2 years and every 6 months thereafter to monitor disease progression.

In GC patients, peripheral venous blood (PB) for quantitative reverse transcription PCR (qRT-PCR) analysis was

obtained after surgery or in the presence of clinical and radiological disease when surgery was not indicated. The first 5 mL of collected blood was discarded to avoid contamination with epidermal cells. The PB (10 mL) was collected in EDTA-containing tubes. Then, the PB was frozen at −20°C in RNA*later* for storage until RNA extraction.

The controls were recruited from the patients' family and relatives. We only excluded subjects with a previous history of malignant disease. Thus, controls with different chronic but stable diseases (e.g., peptic disease, hypertension, diabetes mellitus or heart disease) were eligible and consecutively recruited. The control cohort was selected to include a sex and age distribution that was comparable to the patient group.

This study was approved by the Ethics Committee of Clinical Investigation of Galicia (Spain) and conducted in compliance with the Helsinki Declaration. Written informed consents were obtained from all the patients and the controls prior to their inclusion in the study.

Pathological analyses

Tumours and regional lymph nodes collected during surgery were processed on a routine diagnostic basis. Histological type, depth of invasion and nodal involvement were analysed and the disease was staged and graded according to the TNM and Laurent classification [31]. Residual disease status at the time of blood sampling was classified as R0 when no residual disease was present after surgery, R1 when microscopic residual disease was found and R2 in the presence of macroscopic disease. The patients from whom the blood was obtained before the start of neo-adjuvant treatment were categorised as R2. When surgery was not performed, the pathological diagnosis was based on endoscopic or radiological-guided biopsies.

Blood microRNA isolation and qRT-PCR

To isolate the miRNA fraction, the RiboPure-Blood Kit was used with the alternate protocol: isolation of small RNAs (Applied Biosystems, Foster City, CA, USA). The procedure was performed using 0.5 ml of whole blood per preparation. The absorbances at 260/280 and 260/230 were assessed using a NanoDrop™ 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The purified RNA was further processed using qRT-PCR or stored at −80°C until use.

Reverse-transcription (RT) PCR was performed with 25 ng (up to 6.6 μl) of total RNA using the mirVana™ qRT-PCR miRNA Detection Kit (Ambion, AM1558) with 2 μl 5X RT Buffer, 1 μl 1X RT Primer (Ambion, miR-200a, A30094; miR-200b, AM30095*; miR-200c, AM30096*; miR-141, AM2052*) and 0.4 μl of ArrayScript Enzyme Mix for a total volume of 10 μl.

For the PCR reaction, 10 μl of RT reaction and PCR Master Mix were used. The PCR Master Mix consisted of 5 μl 5X PCR buffer containing SYBR Green I, 0.2 μl SuperTaq 5 U/μl, 0.5 μl PCR primers and 9.3 μl of nuclease-free water for a total volume of 15 μl. Real-time PCR was performed on the LightCycler® 480 Instrument (Roche, Mannheim, Germany).

To control input variability and sample normalisation, primer sets specific for the small RNA species U6 snRNA (Ambion, AM30303) and 5S rRNA (Ambion, AM30302) were used. These primer sets were used not only as internal controls but also to verify the integrity of the RNA and the reverse transcription reaction. Any specimen with inadequate U6 snRNA or 5S rRNA expression would be excluded from the study.

For miR-141, miR-200b and miR-200c, the PCR cycling conditions and analysis were as follows: denaturation at 95°C for 8 seconds; cycling, 40 cycles of 95°C for 5 seconds, 60°C for 5 seconds and 72°C for 2 seconds; melting curve analysis, 1 cycle at 95°C for 5 seconds, 55°C for 1 minute 5 seconds and 95°C continuous; and finally, cooling at 40°C for 10 seconds. The conditions were identical for miR-200a, U6 snRNA and 5S rRNA, except the denaturation step was 1 cycle at 95°C for 6 seconds.

We verified that the amplification of each PCR product was specific using a melting curve analysis. The amplification efficiency was determined for both target and reference genes. Each assay was performed at least in triplicate. The quantification cycle (Cq) was performed using LightCycler 480 Quantification software (Roche, Mannheim, Germany). For further data analysis, only those miRNAs with a Cq value equal to or below 35, representing detection of one single-molecule template [32], were considered. Positive and negative controls were included in each experiment.

The Relative Expression Software Tool (REST) was used to analyse the relative miRNA expression in each sample and to determine the fold difference for every miRNA [33]. The expression levels of the target miRNAs were standardised using an index containing 5S rRNA and U6 snRNA.

miRNA analyses were performed with no knowledge of the clinical or follow-up data.

miR-200 cluster expression profiling

To analyse the expression of the miR-200 family in gastric cancer, the OE19 and MKN-45 human gastric cell lines were used. The cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) with high glucose and MegaCell™ RPMI-1640 medium (both provided by Sigma–Aldrich Química, Madrid, Spain) supplemented with 10% inactivated foetal calf serum, 1% penicillin, 1% streptomycin and 1% amphotericin at 37°C

in 5% CO_2. The cells were recovered with 1% trypsin–1% EDTA cell-dissociating reagent.

The isolation of total RNA (including miRNA) from the cell cultures was performed using the mirVana™ miRNA isolation kit (Ambion, Inc. AM1560). The procedure was performed using 10^7 cultured cells at 70% confluence.

The miR-200 expression profiles in paired samples (n = 160) of non-tumour gastric mucosa and GC were obtained using bioinformatic analysis of the data originally published by Ueda T, et al. [10]. The microarray expression was downloaded through the public repository ArrayExpress (experiment number E-TABM-341. http://www.ebi.ac.uk/arrayexpress/). Only the normalised expression values were used for subsequent analysis. The differential expression levels were calculated using a moderate *t*-test implemented in the Bioconductor *limma* package (R statistical software). The comparisons were performed using *t*-test and pairwise *t*-tests. The resulting *p* values were adjusted for multiple testing using Benjamini-Hochberg's adjustment [34,35].

Study design and statistical analyses

The primary aims were to estimate the diagnostic accuracy and usefulness of miRNA as measured by qRT-PCR in the blood of GC patients as a clinical biomarker and to determine its potential prognostic value. The study was performed following the proposed guidelines of the Early Detection Research Network [36]. The design and results are presented in accordance with the REMARK [37] and MIQE guidelines [38].

The receiver operating characteristic (ROC) curve was constructed by plotting sensitivity (Y-axis) *vs* 1-specificity (X-axis) and the areas under the curve (AUC) were calculated. The diagnostic performance including sensitivity, specificity, positive and negative predictive values and accuracy of miR-200c quantification was also estimated [36]. The potential correlation among blood miRNA levels and the clinical and pathological features of the study subjects were analysed. The normality of the distribution of miRNA expression was analysed using the Kolmogorov-Smirnov test. Thus, parametric or non-parametric statistics were used, as appropriate. The relationships between miR-200c levels and the quantitative clinical variables were analysed using the Pearson correlation.

Progression-free survival (PFS) was measured as the time between the baseline blood sampling for miRNA analysis and the documentation of first tumour progression, based on clinical and radiological findings, or death (events). Overall survival (OS) was measured from the time at which the baseline blood sample was obtained to the date of death from any cause or date of last follow-up. The patients who were alive and progression-free at the time of analysis were censored by using the time between the blood assessment and their most recent follow-up evaluations. The distributions of time-to-event end points, namely PFS and OS, were estimated using the Kaplan-Meier method and compared using the Breslow-Wilcoxon test.

Multivariate survival analyses (PFS and OS) were performed using Cox regression models. We estimated hazard ratios (HRs), 95% CI and *p* values. All statistical tests were two-sided and *p* values less than 0.05 were considered significant. SPSS Statistics 19.0 for Windows (IBM Corporation, Armonk, NY, USA, 2011) and Graph Pad Prism 5 (GraphPad Software, La Jolla, CA, USA, 2007) were used for data analyses.

Results

The miR-200 family of microRNAs was highly expressed in gastric cancer

To investigate the differential expression levels of the miR-200 cluster, we used real-time PCR to analyse the expression levels of miR-200a, 200b, 200c and miR-141 in total RNA extracted from the GC cell lines OE-19 and MKN-45. We compared the miRNA expression profiles, calculated using REST as described, with those of normal human blood (a control group consisting of pooled RNA obtained from 19 healthy donor blood samples). The relative expression ratios of every target miRNA were significantly higher in the GC cell lines compared with the control blood. In OE-19 cells, the miRNAs were upregulated by a mean factor of $6.61x10^5$, 9.99 $x10^3$, 4.47 $x10^5$ and 2.54 $x10^5$ for miR-200c ($p < 0.001$), 141 ($p = 0.018$), 200a ($p < 0.001$) and 200b ($p < 0.001$), respectively. In MKN-45 cells, the miRNAs were upregulated by a mean factor of 4.94 $x10^5$, 5.79 $x10^3$, 2.86 $x10^5$ and 1.30 $x10^5$ for miR-200c ($p = 0.033$), 141 ($p < 0.001$), 200a ($p < 0.001$) and 200b ($p < 0.001$), respectively. Thus, the highest fold-change observed in the GC cell lines relative to control blood was 5.78 $x10^5$ for miR-200c. In addition to the miRNA expression data analysis obtained by REST, we compared the raw Cq data for every miRNA in the control blood and gastric cancer cell lines. In the blood, the mean Cq was lower for miR-141 (Cq = 28) compared with miR-200a (Cq = 35), 200b (Cq = 35) and 200c (Cq = 35). These differences were significant (ANOVA, $p < 0.001$; Bonferroni *post hoc* test, $p < 0.001$) suggesting an increased background miR-141 expression in non-tumour blood relative to the other miR-200 family members. In the GC cell lines, the mean Cqs were 15.3, 16.7, 17.7 and 16.1 for miR-141, 200a, 200b and 200c, respectively, without significant differences (ANOVA, $p = 0.133$; Figure 1).

To ascertain whether the miR-200 cluster signature differs between GC and non-tumour mucosa and between

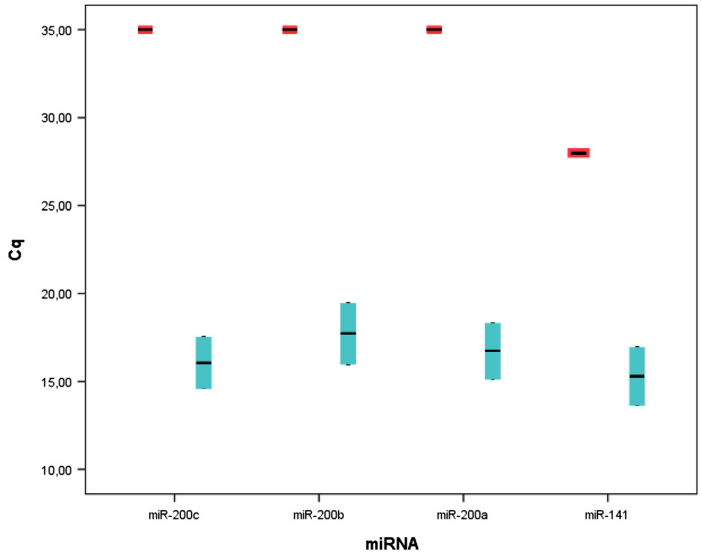

Figure 1 Real-time PCR of the miR-200 family in control blood and gastric cancer cell lines. The raw quantification cycle (Cq) data for the miR-200 cluster in the control blood samples (n = 19) and gastric cancer cell lines (OE-19 and MKN-45) are depicted. In the blood, the mean Cq was lower for miR-141 (Cq = 28) compared with those for miR-200a (Cq = 35), 200b (Cq = 35) and 200c (Cq = 35; ANOVA, $p < 0.001$; Bonferroni post hoc test, $p < 0.001$). In the GC cell lines, the mean Cqs were 15.3, 16.7, 17.7 and 16.1 for miR-141, 200a, 200b and 200c, respectively, without significant differences (ANOVA, $p = 0.133$). The red boxes indicate control blood samples, while the light-blue boxes indicate the gastric cancer cell lines.

the two histological subtypes of GC, the expression profiles were retrieved from Ueda et al. [10]. We observed that the miR-200 family was not differentially expressed in the paired non-tumour mucosa and cancer samples. Furthermore, miR-141, 200a, 200b and 200c were not differentially expressed between the GC histological subtypes (diffuse and intestinal). Additional file1: Figures S1, S2 and Additional File 1: Tables S1, S2.

Patients and clinical data

From November 2006 to July 2010, 52 patients with histologically proven GC were consecutively recruited for this study. The clinical characteristics of the patients are shown in Table 1. The control cohort included 15 cases. The mean age was 65.3 years (standard error of the mean [SEM], 1.9; range, 49 to 74 years) in the control group and 65.9 years (SEM, 1.32; range, 43 to 85) in the patient group (t test, $p = 0.82$). The ratio of males to females was similar among the controls and the patients (Yates-corrected χ^2, $p = 0.07$).

The blood was obtained after R0 surgery in 20 patients (38.5%). In 32 patients, the blood samples were obtained in the presence of residual or metastatic disease, both of which were categorised as R2 at the time of blood sampling. In the patients receiving surgery (71.2%; 37/52), the number of lymph nodes analysed was 19 (range, 0–76; St. D, 16.2). Chemotherapy was administered to 44 patients (84.6%).

All patients were followed until death or study completion. The last date of follow-up for the survivors was September 5, 2011. Disease progression events occurred in 38 patients (73.1%). The median PFS was 6 months (95% CI, 1.4 to 10.6 months). There were 7 relapses among stage I–III patients and 31 progressions of metastatic disease. The median OS was 15 months (95% CI, 11.1 to 18.9 months) and 35 patients (67.3%) died of advanced disease. Most of the PFS events (29/38; 76.3%) and OS (18/35; 51.4%) events occurred in the first 9 months of follow-up. The mean (SEM) follow-up time for the patients still alive at the time of the analysis was 26.3 (3.7) months (median, 24 months; range, 6 to 53 months).

Expression of miRNA in blood samples

As described above, we found that miR-200c was not only upregulated in GC cell-lines compared with control blood, it was expressed at the highest levels of all miR-200 family members. Thus, miR-200c was selected for clinical validation.

Real-time quantitative assessment of miR-200c was performed using 67 blood samples (52 patients and 15 controls). The mean relative miR-200c expression (Figure 2) was 16.2 (SEM, 5.6; CI 95%, 4.1 to 28.3) in controls, 90.3 (SEM, 17.4; CI 95%, 53.9 to 126.6) in stage I-III patients and 114.6 (SEM, 16.3; CI 95%, 81.4 to 114.9) in stage IV GC patients ($p = 0.018$; Kruskal-Wallis

Table 1 Patient characteristics (n = 52)

Characteristic	n	%
Median (range) age, yrs	65.9 (42–85)	
Gender		
Women	10	19
Men	42	81
ECOG		
0-1	37	71.2
2	10	19.2
Location		
Proximal, upper third	13	25
Distal	36	69.2
Multicentric	3	5.8
Stage		
I-II	9	17.3
III	12	23.1
IV	31	59.6
Lymph Nodes		
Negative	9	17.3
Positive	24	46.2
Histological type		
Intestinal	28	53.8
Diffuse	21	40.4
Mixed	3	5.8
R Status		
R0	20	38.5
R1-R2	32	61.5
Grade		
Low	21	40.4
High	27	51.9
Vascular / Perineural Invasion		
Unknown	22	42.3
No	11	21.2
Yes	19	36.5

Abbreviations: ECOG: Eastern Cooperative Oncology Group performance status. Residual Status (R): R0, no residual tumour; R1-2 microscopic or macroscopic residual tumour.

test. Bonferroni *post hoc* test: stage I-III *vs* control, $p = 0.018$; stage IV *vs* control, $p < 0.001$). The confidence interval with the alpha level of significance at 99% estimated using the Monte Carlo test was 0.015 to 0.022.

An ROC curve was constructed (Figure 3). Comparing the relative miR-200c levels in controls and patients, the AUC was 0.715 (95% CI, 0.597–0.833; $p = 0.012$). According to the ROC curve, the relative blood level of miR-200c of 62.4 was defined to be the optimal cutoff value for differentiating GC patients and controls (Youden's index). With this cutoff value for miR-200c, the sensitivity, specificity, positive and negative predictive values and accuracy values of 65.4% (95% CI, 50.8 to 77.7), 100% (95% CI, 74.7 to 99.4), 100% (95% CI, 87.4 to 99.7), 45.5% (95% CI, 28.5 to 63.4) and 73.1% (95% CI, 60.7 to

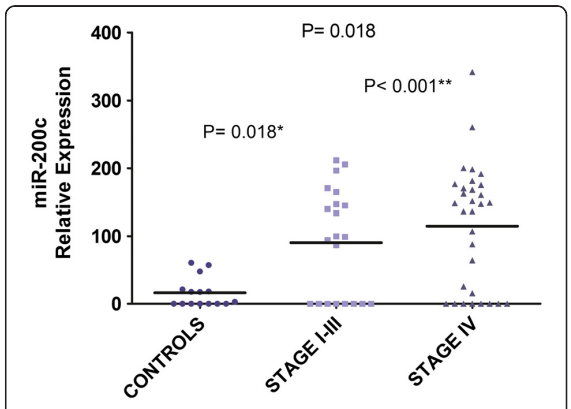

Figure 2 Real time PCR of miR-200c in blood samples. The graph depicts the increasing relative expression levels for the mean blood expression levels of miR-200c (Kruskal-Wallis test, p =0.018) from controls (n = 15) and gastric cancer samples (n = 52). Significant differences were observed between the blood expression levels of miR-200c in each TNM stage subgroup and the control group (Bonferroni *post hoc* test: stage I-III *vs* control, *$p = 0.018$; stage IV *vs* control, **$p < 0.001$). MiR-200c was measured in triplicate using qRT-PCR and normalised to U6 snRNA and 5S rRNA. The horizontal bar denotes the mean value for each group.

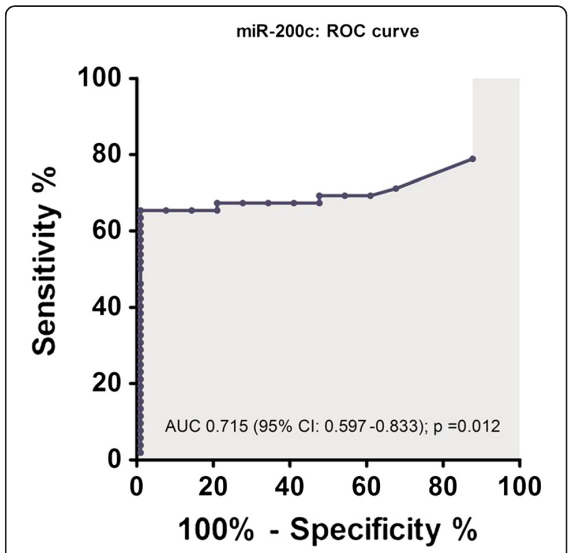

Figure 3 The role of blood miR-200c in gastric cancer diagnosis. The receiver-operating characteristic (ROC) curve analysis using blood miR-200c expression levels for discriminating gastric cancer (n = 52) and controls (n = 15) is shown. The area under the ROC curve is shown [AUC 0.715 (95% CI, 0.597–0.833); $p = 0.012$; cutoff value is 62.4; sensitivity, 65.4%; specificity, 100%].

82.9), respectively, were achieved. The relative expression values for miR-200c in blood above this cutoff point were found in 17.6% of stage I-II patients, in 20.6% of stage III patients and in 67.7% of stage IV GC patients ($p < 0.001$; exact test). These findings suggested that elevated blood miR-200c could be detected in the early stages of GC and therefore facilitate early disease detection.

Clinical and pathological characteristics and miR-200c levels in blood

The clinical and pathological characteristics and the miR-200c expression levels in the blood from cancer patients are shown in Table 2. The relative expression levels for miR-200c in the blood of GC patients were not associated with any of the parameters analysed. Furthermore, the percentage of patients with miR-200c levels above its mean value (mean, 104.8; SEM, 12) was not associated with clinical and pathological characteristics.

To explore the possible influence of recent surgical procedures on the circulation of miRNA, we analysed miR-200c levels according to the time interval from surgery and blood sampling. The median time from surgery to blood sampling for miRNA quantification was 6 weeks (mean, 19.1 weeks; SEM, 5.5; range, 2 to 155 weeks). There were no significant differences in miR-200c levels according to time intervals (< 6 or ≥ 6 weeks) from the last surgery adjusted for tumour stage (ANOVA, $p = 0.284$).

Prognostic significance of miR-200c levels in blood

The correlations of potential prognostic factors and miR-200c levels in the blood in gastric cancer patients are shown in Table 3. There was only a significant correlation (Pearson's r = 0.438, $p = 0.016$) between miR-200c levels and the number of lymph node metastases.

To generate survival curves, we converted continuous miR-200c expression levels measured using qRT-PCR to a dichotomous variable, using its mean levels of expression as a threshold (10). Using this approach, miR-200c was overexpressed in the blood of 53.8% (28/52) of patients. The mean values (with SEM) in the low and high expression groups for miR-200c were 23.9 (7.9) and 174.1 (8.5), respectively (Mann–Whitney test, $p < 0.001$). The percentage of patients with miR200c overexpression tended to increase with TNM stage: 33.3% (3/9) in stage I-II patients, 50% in stage III patients (6/12) and 61.3% (19/31) in stage IV patients ($p = 0.076$).

The Kaplan-Meier curves for patient OS and PFS categorised according to miR-200c expression levels in the blood are shown in Figure 4. The increased blood expression of miR-200c was significantly associated with a poor overall survival (Breslow-Wilcoxon text; $p = 0.016$). The median and mean OS for the group with high miR-200c expression levels were 9 months (95% CI, 1.7–16.3) and 17.4 months (95% CI, 11.2–23.6), respectively. In the group with low miR-200c blood expression levels, the median OS was 24 months (95% CI, 8.1–39.9) and the mean OS was 29.2 months (95% CI, 20.9–37.6).

With regards to PFS, the median estimate for those patients with low levels of miR-200c in the blood was 11 months (95% CI, 7.9 to 14.1). In contrast, the median PFS was 4 months (95% CI, 1.8 to 6.2) in patients with high miR-200c levels (Breslow-Wilcoxon test; $p = 0.044$).

The relative strength of blood expression levels of miR-200c as an independent prognostic factor was evaluated by performing a Cox multivariate analysis. The details of this analysis are listed in Table 4. With the inclusion of miR-200c expression levels in the model, the independent predictors of PFS were as follows: stage IV disease (HR = 5.52; $p = 0.005$), residual disease (HR = 4.29; $p = 0.023$); and miR-200c overexpression (HR = 2.27; $p = 0.028$). Similar results were achieved for the prediction of OS including stage IV disease (HR = 8.6; $p < 0.001$), weight loss higher than 10% (HR = 2.38; $p = 0.024$) and miR-200c overexpression (HR = 2.24; $p = 0.028$) in the model. Residual disease, Eastern Cooperative Oncology Group (ECOG) performance status and age were not independent prognostic factors for OS.

To further explore the relationship between miR-200c expression levels and outcomes, we estimated the hazard ratios associated with the miR-200c level as a continuous variable by performing Cox multivariate regression models. Concordant results were achieved for the prediction of PFS, considering miR-200c expression levels as a continuous variable (HR = 1.004; 95% CI; $p = 0.045$) in the multivariate Cox model including stage IV and residual disease. Likewise, the risk of death was higher with increasing miR-200c relative blood expression levels (HR = 1.007; 95% CI, 1.003 to 1.012; $p = 0.003$) independent of stage and weight loss.

Discussion

Accumulating reports have indicated that miRNAs are detectable in blood and that circulating miRNAs have the potential to be new biomarkers in patients with different diseases including cancer. Circulating miRNAs must demonstrate different hallmark characteristics to considered reliable biomarkers [15,39]: (i) stable and readily quantifiable in clinical samples; (ii) expressed by cancer cells at moderate or high levels; (iii) present at undetectable or very low levels in specimens from individuals without cancer; (iv) provide a predictive or prognostic clinical information; and (v) exhibit biological functions mechanistically linked to tumour progression.

Several studies have explored the use of miRNA expression levels in gastric tissues, sera and plasma samples to improve the diagnosis or prediction of GC [40-46]. Most reports focused on the diagnostic potential

Table 2 The distribution of clinical and pathological parameters and the levels of miR-200c in blood

Parameter	n	miR-200c mean (SEM)	*p* value	High miR-200c n (%)	*p* value
Age (y)			0.985		0.0895*
< 70	32	100.3 (14.1)		17 (53.1%)	
≥ 70	20	112 (21.9)		11 (55%)	
Gender			0.692**		1***
Male	42	107.3 (13.5)		23 (54.8%)	
Female	10	94.2 (26.8)		5 (50%)	
Location			0.742**		1***
Proximal, upper third	13	120.0 (31.1)		7 (53.8%)	
Distal	36	98.5 (12.8)		19 (52.8%)	
Multicentric	3	114.2 (58.3)		2 (66.7%)	
Stage			0.211		0.191*
I-III	21	90.3 (17.4)		9 (42.9%)	
IV	31	114.6 (16.3)		19 (61.3%)	
pT			0.683		0.693***
pT1-T2	18	93.2 (31.6)		13 (72.2%)	
pT3-T4	16	95.9 (17.6)		13 (81.3%)	
pN			0.516		0.259***
Node Negative	9	67.4 (28.4)		3 (33.3%)	
Node Positive	24	115.9 (18.6)		14 (58.3%)	
Histological type			0.179		0.246*
Intestinal	28	86.5 (15.1)		13 (46.4%)	
Diffuse	24	126.2 (18.5)		15 (62.5%)	
ECOG			0.263		0.481***
0-1	37	94.2 (15.5)		20 (54.1%)	
2	10	147.1 (56.2)		7 (70%)	
Residual disease (R)			0.113		0.312*
R0	20	89.7 (18.2)		9 (4%)	
R1-2	32	120.4 (27.9)		19 (59.4%)	
Number of Metastatic sites			0.551**		0.753***
0	23	95.4 (16.5)		11 (47.8%)	
1	21	113.2 (21.1)		12 (57.1%)	
≥ 2	8	109.8 (31.1)		5 (62.5%)	
Grade			0.405		0.146*
Low	21	73.6 (20.9)		8 (38.1%)	
High	27	116.9 (23.1)		16 (59.3%)	
Vascular / Perineural Invasion			0.914		0.705*
No	11	103.3 (22.8)		5 (45.5%)	
Yes	19	97.5 (22.8)		10 (52.6%)	
Neutrophils (10^{-9}/L)			0.705		0.696*
≤ 7.5	39	104.4 (13.1)		22 (56.4%)	
>7.5	12	114.9 (29.4)		6 (50%)	

The miR-200c relative expression levels (REL) are shown in arbitrary units. The levels of miR-200c were considered high when the REL was above the mean. Residual disease (R) was categorised as R0 when no residual disease was present and as R1-2 when microscopic or macroscopic residual disease was found. Mann–Whitney test. * Pearson $\chi 2$. **Kruskal-Wallis test. *** Fisher's exact test.

Table 3 The correlations of prognostic factors and miR-200c levels in the blood of gastric cancer patients

	n	r	*p* value
Weight loss (%)	51	0.082	0.568
Number of positive lymph nodes	30	0.438	0.016
LDH	52	- 0.023	0.872
Albumin	51	- 0.130	0.365
Alkaline Phosphatase	52	- 0.041	0.770
Neutrophil counts	51	0.132	0.356

Computed using the Pearson correlation test.

of quantifying miRNAs in blood; however, data regarding their possible prognostic role in solid tumours including GC are limited, as shown in Additional file 1, Table S3.

There are two main observations in the present study. First, miR-200c levels in the blood were significantly increased in GC patients compared with controls. These increased values were highly specific for a GC diagnosis and were associated with disease stage. Second, the blood expression levels of a single microRNA, miR-200c, provided prognostic information for patients with GC independent of a comprehensive panel of other established clinical predictors.

The miRNA-200 cluster has been shown to regulate the epithelial-mesenchymal plasticity that may be crucial at different stages of metastasis through direct targeting of the *ZEB–cadherin 1* axis [21-25]. However, *in vitro* and functional studies have yielded conflicting results regarding the net effect of miR-200 deregulation in the metastatic process [47-50]. Recent reports have indicated that tumour colonisation at metastatic sites might be enhanced by the expression of miR-200c. The xenograft model data have suggested that although miR-200 expression can hinder the intravasation of tumour cells, those that reach the circulation may be more proficient at colonising distant organs [47,48]. Our findings are consistent with these experimental data and with the clinical correlations observed between the up-regulation of miR-200c in tumours and poor prognosis in individuals with colorectal adenocarcinoma [51], oesophageal squamous cell carcinoma [52] and breast cancer [48,53].

In spite of the growing evidence highlighting its relevance in various cancers, very few studies have systematically explore the role of the miR-200 family in GC. MiR-141 was significantly down regulated in gastric cancer tissues compared with pair-matched adjacent non-tumour tissues [54,55]. Nevertheless, a recent report [56] found that miR-200a and miR-141 were significantly overexpressed in gastric cancer compared with those in normal gastric tissue. In addition, high miR-200a tumour expression was associated with a poor OS. Kurashige et al. have recently shown [57] that the downregulation of miR-200b in GC was associated with diffuse histologic type, depth of tumor, tumor size, lymph node metastasis, and lymphatic invasion. The upregulation of miR-200b was correlated with increased E-cadherin and low ZEB2. However, there were no differences in the tumour expression of miR-200c among histological types or other clinicopathological parameters.

To ascertain whether the miR-200 family expression profile can differ between GC and non-tumour mucosa and to analyse the association among miR-200a, 200b, 200c and miR-141 and histological characteristics, we used a large, public microarray database. The results of our *in silico* analyses demonstrated that the expression of miR-200a, -b, -c and miR-141 were similar in non-tumour gastric mucosae and gastric tumour tissue. Furthermore, miR-200a, -b, -c and miR-141 were not differentially expressed between intestinal and diffuse types of gastric carcinoma. In that sense, the miR-200 signature in GC was validated on an external data set. In our study, as shown in Table 2, there were no significant differences in the blood levels of miR-200c among histological types or other clinicopathological parameters. Similar data have been recently reported [57]. These findings suggest that elevated blood miR-200c levels can be detected throughout the wide spectrum of gastric adenocarcinomas and therefore underscore its potential role as a clinical biomarker.

However, tumour or cellular miRNA-expression patterns can differ from miRNA patterns released into the blood [58,59]. In addition, potential differences in the microRNAs expression profile between primary tumours and corresponding CTC or matching clinical metastases have not been systematically investigated. In that sense, the miR-200-a, -b and -c and miR-429 levels were increased in lung metastases compared to primary breast tumours [48]. Also, the expression of miR-200c/miR-141 cluster was significantly upregulated in liver metastasis from colorectal cancer, as compared with that in primary tumours [50]. Thus, circulating miRNAs may not always be directly associated with the changes occurring in primary tumor tissues.

When we considered the different reports regarding the potential diagnostic and clinical relevance of the blood-borne miRNA expression in cancer, a considerable degree of inter-study heterogeneity was noticed. Differences in the detection and quantification methods (microarrays, qRT-PCR and high-throughput sequencing technology), the types and numbers of miRNAs evaluated (pre-miRNA or mature form, expression profile or a single marker) and sample sources and timing (serum, plasma or blood cells obtained pre- or post-operatively), as well as in the clinical and pathological data of the included patients ought to be considered as potential causes of heterogeneity.

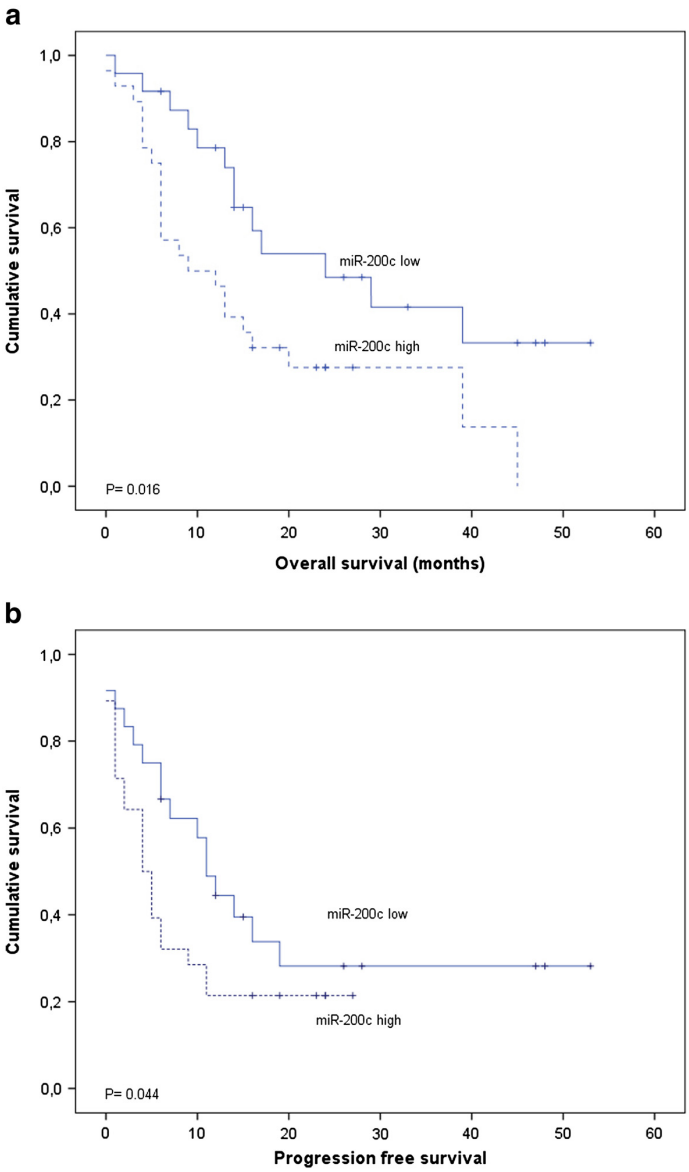

Figure 4 miR-200c expression levels measured in the peripheral blood are associated with poor prognosis in gastric cancer patients. Kaplan-Meier curves showing (**a**) the overall survival (OS) and (**b**) the progression-free survival (PFS) of 52 subjects with high or low blood expression levels of miR-200c. Continuous miR-200c expression levels measured using qRT-PCR were converted to a dichotomous variable using the mean level of expression as a threshold. The *p* values were computed using the Breslow-Wilcoxon test.

At present, there is no agreement on the most advantageous source from which to isolate circulating miRNA and the use of serum or plasma over whole blood for systemic miRNA analysis is debatable. One of the crucial problems is the efficient and reproducible extraction of small amounts of miRNA from plasma or serum. Therefore, higher yields of miRNAs have been consistently obtained from whole blood samples compared with matched serum or plasma samples and lower quantification cycles were performed in whole blood compared with matched serum and plasma samples in qRT-PCR experiments [60].

Recent reports have indicated that blood cells are major contributors of circulating miRNA [61]. Hence, one can hypothesise that increased levels of expression of epithelial-specific miRNAs in blood, including miR-

Table 4 Multivariate analyses (n = 52)

	Wald	*p* value	Hazard ratio	95% CI	
Progression-free survival (PFS)					
Stage IV disease	7.805	0.005	5.52	1.665	18.285
High miR-200c	4.835	0.028	2.27	1.093	4.712
Residual disease (R) status	5.195	0.023	4.29	1.226	14.993
Overall survival (OS)					
Stage IV disease	20.469	0.000	8.60	3.385	21.831
High miR-200c	4.827	0.028	2.24	1.091	4.614
Weight loss > 10%	5.074	0.024	2.38	1.119	5.048

The levels of miR-200c were considered high when the relative expression level was above the mean. Residual disease (R) was categorised as R0 when no residual disease was present and as R1-2 when microscopic or macroscopic residual disease was found.

200c, might indicate the circulation of tumour cells. However, the origins of circulating miRNAs are not yet clearly understood. In theory, analysis of miRNAs obtained from whole blood may be advantageous, detecting not only those miRNA derived from circulating blood cells comprising tumour cells but also those secreted in subcellular particles such as exosomes or those associated with RNA binding proteins and diverse tissues [62,63].

Any PCR-based technique still has the disadvantage of potentially detecting minimal amounts of miRNA expression in a non-disease-specific manner. Some of the proposed miRNA cancer biomarkers have been found to be highly expressed in one or more blood cell types and plasma levels of these miRNA have been correlated to blood cell counts [64]. Pritchard et al. reported that miR-200c was found in the blood and blood cells of controls, with the highest expression in neutrophils. However, patients with diverse metastatic cancer and severely ill conditions that could be considered as confounding factors were included in this study as "controls". Conversely, we did not find any correlation between miR-200c levels and neutrophil counts in our series. In addition, miR-200c levels did not differ in subgroups defined according to neutrophil counts.

From a clinical perspective, assessment of miRNAs in the PB obtained after definitive loco-regional treatment reflects the "minimal residual disease" status that might better predict the clinical behaviour and/or therapeutic response. Postoperative sampling time combines, in theory, the baseline level of CNA, the potential release of CTC due to the surgical manipulation and the rapid death of in transit cells within the blood stream but with reduced survival ability. Our study shows that increased miR-200c levels are detected even in patients with very low tumour burdens (i.e., early-stage disease and after potentially curative R0 surgical resections).

Remarkably, we found that levels of miR-200c measured in the PB of GC patients independently correlate with OS and PFS. A clear clinical association of the expression levels of a single circulating miRNA (miR-200c) with poor survival outcomes indicated by multivariate analysis has been demonstrated. However, large prospective and follow up studies will be necessary in the near future to confirm the clinical relevance of circulating miRNAs, including miR-200c, as independent prognostic indicators for cancer.

Conclusions

Beyond confirming initial reports, our study yielded the following evidence: (i) epithelial-derived miRNAs can be quantified in the whole-blood; (ii) the blood levels of a single epithelial and tumour-expressed miRNA, miR-200c, can distinguish, with significant specificity and sensitivity, patients with GC from healthy controls and (iii) remarkably, increased expression levels of miR-200c in blood were significantly associated with poor progression-free and overall survivals. Our study indicates unique results on its potential prognostic value that provide a firm basis for further investigation of miRNAs as blood-based cancer predictive and prognostic biomarkers.

Additional file

Additional file 1: Figure S1. Box plots of the miR-200 s family of microRNAs, miR-148a and miR-21 expressions in gastric cancer samples and normal gastric mucosae. Tissue miRNA concentrations were significantly lower for miR-148a ($p < 0.0001$) whereas miR-21 was significantly higher ($p < 0.0001$) in the gastric cancer samples compared to those in normal gastric mucosae. MiR-200 s were not differentially expressed in the paired non-tumour mucosa and cancer samples. MiR-148a and miR-21 were among the differentially expressed microRNAs in gastric cancer signature as defined by Ueda T, et al. The upper and lower limits of the boxes and the lines inside the boxes indicate the 75th and 25th percentiles and the median respectively. The upper and lower horizontal bars denote the 90th and 10th percentiles respectively. **Table S1**. MiR-200 s family of microRNAs, miR-148a and miR-21 expressions in the gastric cancer samples compared to those in normal gastric mucosae. **Figure S2.** Box plots of the miR-200 s family of microRNAs, miR-148a and miR-21 concentrations in gastric cancer samples according to histological type: diffuse or intestinal. Tissue miRNA concentrations were significantly higher for miR-148a ($p = 0.004$) and miR-21 ($p = 0.011$) in the diffuse type compared to intestinal type. MiR-200 s were not differentially expressed according to histological type. MiR-148a and miR-

21 were among the differentially expressed microRNAs in gastric cancer signature as defined by Ueda T, et al. The upper and lower limits of the boxes and the lines inside the boxes indicate the 75th and 25th percentiles and the median respectively. The upper and lower horizontal bars denote the 90th and 10th percentiles respectively. **Table S2.** MiR-200 s family of microRNAs, miR-148a and miR-21 expressions in the gastric cancer samples according to histological type: diffuse or intestinal. **Table S3.**. Studies assessing miRNAs expression in blood among gastric cancer patients.

Abbreviations

(GC): Gastric cancer; (CTC): Circulating tumour cells; (CNA): Circulating nucleic acids; (EMT): Epithelial-to-mesenchymal transition; (PB): Peripheral venous blood; PCR (qRT-PCR): Quantitative reverse transcription; (Cq): Quantification cycle; (REST): Relative expression software tool; (ROC): Receiver operating characteristic curve; (AUC): Area under the curve; (PFS): Progression-free survival; (OS): Overall survival; (SEM): Standard error of the mean; (ECOG): Eastern cooperative oncology group.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MVA conceived the study, participated in its design and drafted the manuscript. MBC, MH, VM and IS performed the molecular analyses. MR, LMAA, MQV, ACM and LC made substantial contributions to data acquisition. MVA, VM, MBC, JFT and AF made substantial contributions to data analyses and interpretation. MVA and VM performed the statistical analyses. MJLP and PID reviewed the histological samples. All authors read and approved the final manuscript.

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Supplementary Material

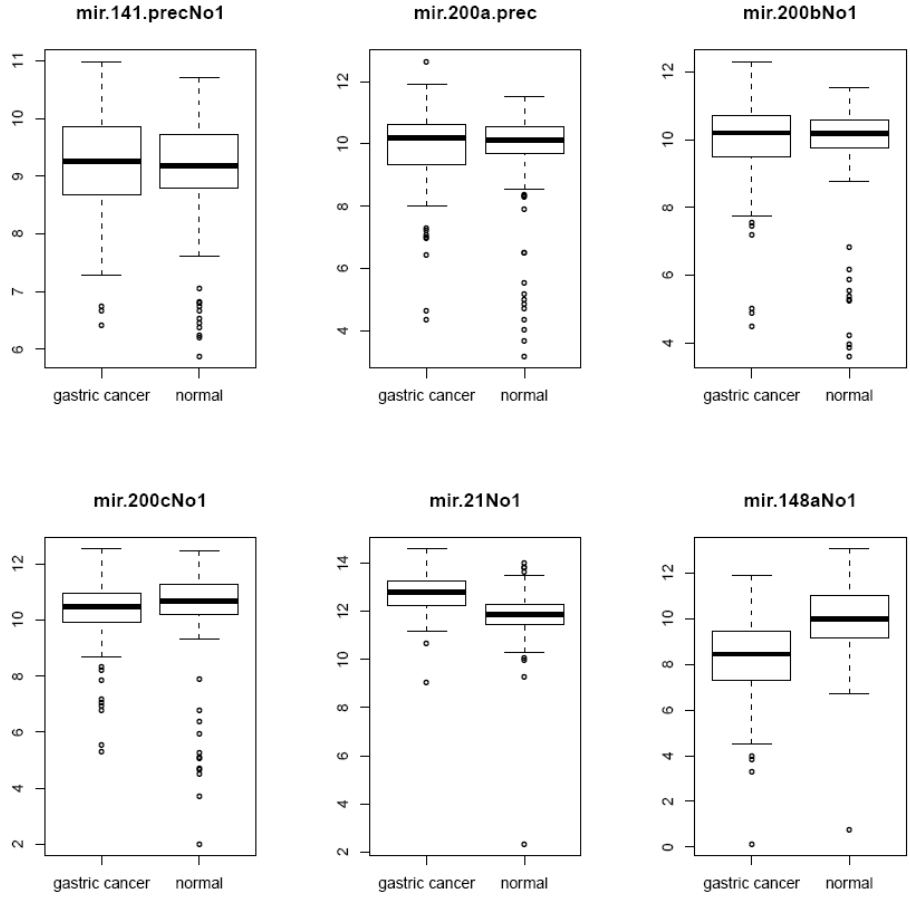

Figure S1. Box plots of the miR-200s family of microRNAs, miR-148a and miR-21 expressions in gastric cancer samples and normal gastric mucosae. Tissue miRNA concentrations were significantly lower for miR-148a ($p<0.0001$) whereas miR-21 was significantly higher ($p<0.0001$) in the gastric cancer samples compared to those in normal gastric mucosae. MiR-200s were not differentially expressed in the paired non-tumour mucosa and cancer samples. MiR-148a and miR-21 were among the differentially expressed microRNAs in gastric cancer signature as defined by Ueda T, et al. The upper and lower limits of the boxes and the lines inside the boxes indicate the 75th and 25th percentiles and the median respectively. The upper and lower horizontal bars denote the 90th and 10th percentiles respectively.

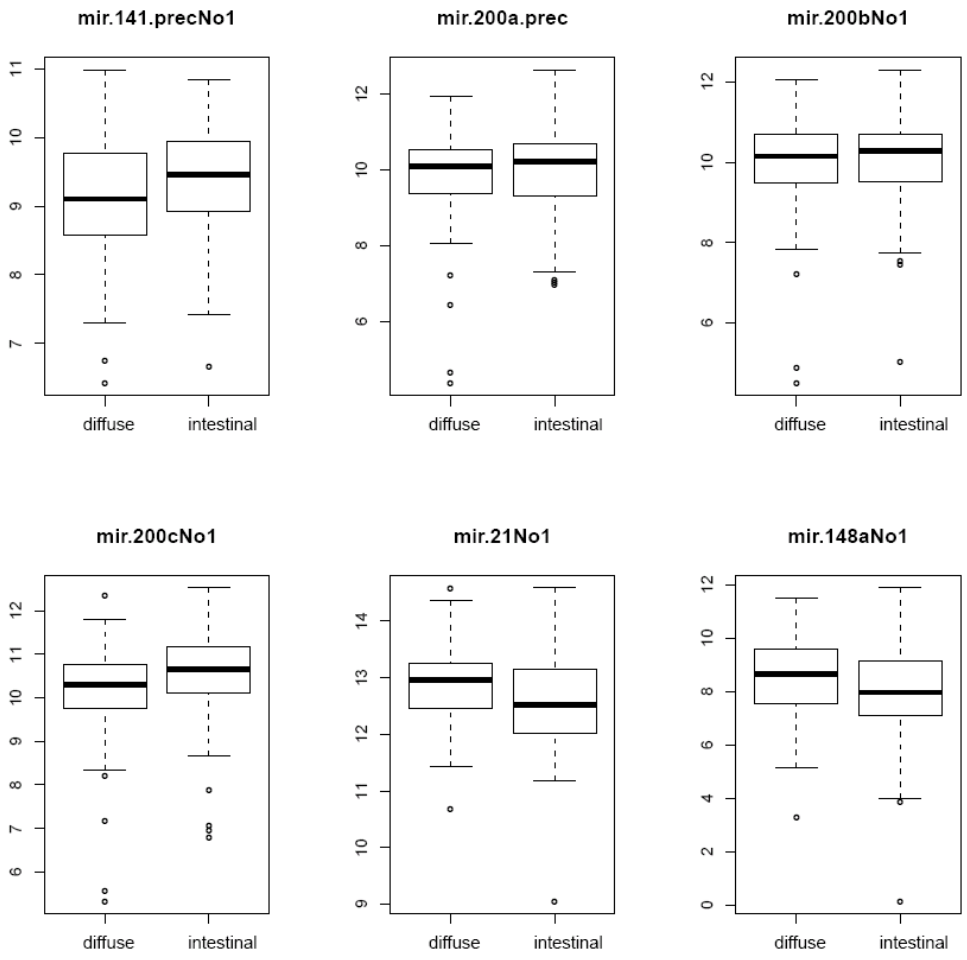

Figure S2. Box plots of the miR-200s family of microRNAs, miR-148a and miR-21 concentrations in gastric cancer samples according to histological type: diffuse or intestinal. Tissue miRNA concentrations were significantly higher for miR-148a (p= 0.004) and miR-21 (p=0.011) in the diffuse type compared to intestinal type. MiR-200s were not differentially expressed according to histological type. MiR-148a and miR-21 were among the differentially expressed microRNAs in gastric cancer signature as defined by Ueda T, et al. The upper and lower limits of the boxes and the lines inside the boxes indicate the 75th and 25th percentiles and the median respectively. The upper and lower horizontal bars denote the 90th and 10th percentiles respectively.

Table S1. MiR-200s family of microRNAs, miR-148a and miR-21 expressions in the gastric cancer samples compared to those in normal gastric mucosae

ID	logFC	AveExpr	t	p.Value	adj.p.Val	B
hsa-mir-148aNo1	1.8297	9.1140	11.5895	0.0000	0.0000	42.0248
hsa-mir-21No1	-0.8297	12.3278	-8.9058	0.0000	0.0000	25.1241
hsa-mir-200a-prec	-0.0834	9.8751	-0.5747	0.5663	0.7652	-7.2715
hsa-mir-200bNo1	-0.0695	9.9517	-0.4916	0.6236	0.7652	-7.3158
hsa-mir-141-precNo1	-0.0501	9.1793	-0.4718	0.6377	0.7652	-7.3254
hsa-mir-200cNo1	0.0436	10.3469	0.2830	0.7775	0.7775	-7.3967

Table S2. MiR-200s family of microRNAs, miR-148a and miR-21 expressions in the gastric cancer samples according to histological type: diffuse or intestinal

ID	logFC	AveExpr	t	p.Value	adj.p.Val	B
hsa-mir-148aNo1	-0.696	8.307	-2.955	0.004	0.021	-1.987
hsa-mir-21No1	-0.306	12.744	-2.566	0.011	0.033	-2.965
hsa-mir-200cNo1	0.285	10.333	1.756	0.081	0.162	-4.581
hsa-mir-141-precNo1	0.189	9.229	1.422	0.157	0.235	-5.076
hsa-mir-200a-prec	0.082	9.940	0.476	0.635	0.762	-5.922
hsa-mir-200bNo1	0.045	10.011	0.262	0.793	0.793	-5.997

List of abbreviations

logFC: a positive results indicated downregulation; a negative results indicated upregulation.

AveExpr: Average expression in all the samples

t: T-test statistic

p.value: Unadjusted p value

adj.p.Val: Adjusted p value, FDR= false discovery rate, according Benjamini and Hochberg.

B: Expression index. A higher B value indicates a higher differential expression.

Table S3. Studies assessing miRNAs expression in blood among gastric cancer patients

Author(s), year	Ref.	Study population			Method qRT-PCR	Source	Timing	Normalization	miRNAs selection
		Country	Cases (stage I/II/III/IV)	Controls					
Zhou H, et al. 2010	40	China	90 (stages NR)	27	SYBR Green	MNC*	Pre- and post-operative (41/49)	U6 snRNA	µParaflo™ microfluidic chip
Tsujiura M, el. 2010	41	Japan	69 (38/13/14/4)	30	TaqMan	Plasma	Pre-operative		Literature and TaqMan qRT-PCR
Liu R, et al. 2011	42	China	142 (29/56/48/23)**	105	TaqMan	Serum	Pre-operative		Solexa, Literature and TaqMan qRT-PCR
Liu H, et al. 2012	43	China	40 (4/12/11/13)	41	SYBR Green	Serum	Pre-operative	U6 snRNA	Microarrays (Agilent) and qRT-PCR
Konishi H, et al. 2012	44	Japan	56 (33 I-II / 23 III-IV)	30	TaqMan	Plasma	Pre-operative***		3D-Gene miRNA microarray
Song M-y, 2012	45	China	68 (31 I-III/ 22 IV/ 29 NR)*	68	TaqMan	Serum	Pre-operative	miR-39	TaqMan low-density array
Wang M, et al. 2012	46	China	65 (33 I-II, 24 III, 8 IV)	NR	SYBR Green	Plasma	Pre-operative	U6 snRNA	Previous work (unpublished data)
Valladares-Ayerbes M, et al. 2012	Present serie	Spain	52 (9 I-II/ 12 III /31 IV)	15	SYBR Green	Whole blood	Pre- and post-operative (15/37)	U6 snRNA and 5S rRNA	Literature, bioinformatics and qRT-PCR in cell lines

NR, not reported. * Blood Mononuclear cells (MNC). **Stage distribution in the all cohort. Validation set included 142 patients. ***Pre- versus post-operative in 29 cases in validation cohort

Table S3. Cont.

Author(s), year	Ref.	Diagnostic performance					PFS and OS Prognostic Value
		Target(s)	AUC	95%CI	Sensitivity	Specificity	
Zhou H, et al. 2010	40	miR-106a and miR-17	0.741	0.620-0.839	62.96	80.49	NR
Tsujiura M, el. 2010	41	miR-106b*	0.721	NR	NR	NR	NR
Liu R, et al. 2011	42	miR-1, miR-20a, miR-27a, miR-34a, miR-423-5p	0.879	0.822-0.936	80	81	NR
Liu H, et al. 2012	43	miR-378	0.861	0.766–0.928	87.5	70.73	NR
Konishi H, et al. 2012	44	miR-451	0.96	NR	96.0	100.0	NR
Song M-y, 2012	45	miR-221	0.700	62-78			
		miR-376c	0.710	62-80	82.4†	58.8†	NR
		miR-744	0.740	65-82			
Wang M, et al. 2012	46	miR-17-5p	NR	NR	NR	NR	Yes, Univariate OS
		miR-20a	NR	NR	NR	NR	Uni and multivariate OS
Valladares-Ayerbes M, et al. 2012	Present serie	miR-200c	0.715	0.597–0.833	65.4	100	Yes, univariate and multivariate OS and PFS

PFS: Progression free survival. OS: Overall survival
† Sensitivity and specificity based on the combination of miR-221, miR-744, and miR-376c

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RESEARCH ARTICLE Open Access

Circulating miR-200c and miR-141 and outcomes in patients with breast cancer

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Abstract

Background: The deregulation of microRNAs in both tumours and blood has led to the search for microRNAs to indicate the presence of cancer and predict prognosis. We hypothesize the deregulation of miR-200c/miR-141 in the whole blood can identify breast cancer (BC), and could be developed into a prognostic signature.

Methods: The expression of miR-200c and miR-141 were examined in bloods (57 stage I-IV BC patients and 20 age-matched controls) by quantitative reverse-transcription PCR. The associations of circulating microRNAs with clinic and pathological characteristics were analysed. Their effects on survival were analysed by the Kaplan-Meier method and Cox regressions.

Results: MiR-200c was down regulated ($P < 0.0001$) in the blood of BC patients, yielded an area under the ROC curve of 0.79 (90% sensitivity, 70.2% specificity) in discriminating BC from controls. Circulating miR-141 was not discriminating. MiR-200c and miR-141 in the blood of BC patients were inversely correlated ($P = 0.019$). The miR-200c levels were numerically higher in stage IV and tumours with lower MIB-1. MiR-141 was significantly higher in the blood of patients with stage I-III, lymph node metastasis, and HER2 negative tumours. High blood expression of miR-200c and/or low expression of miR-141 was associated with unfavourable overall survival (hazard ratio, 3.89; [95% CI: 1.28-11.85]) and progression-free survival (3.79 [1.41–10.16]) independent of age, stage and hormonal receptors.

Conclusions: Circulating miR-200c and miR-141 were deregulated in BC comparing with controls. Furthermore, miR-200c and miR-141 were independent prognostic factors and associated with distinct outcomes of BC patients.

Keywords: Breast neoplasm, microRNAs, Blood, Biomarkers, Prognostic factors

Background

Breast cancer (BC) is the leading cause of cancer death in women worldwide, accounting for 458,400 deaths in 2008 [1]. Relative survival from BC in women has improved steadily in all developed countries over the past 25 years. By (2012), it was estimated that Spain would have a total of 27,000 new diagnoses of BC among women and currently BC remains the leading cause of death among women in Spain with 6231 deaths and a European age-standardised mortality rate of 18 per 100,000 person-years [2].

Cancer progression and blood-borne metastasis contribute to the great majority of BC deaths. The discovery of specific biomarkers characterizing the metastatic phenotype holds the promises of personalised therapy and improved prognosis prediction in many neoplastic diseases including BC.

Tumour tissue based biomarkers (e.g. size, grade, node status, hormone receptor status, HER2, Ki-67) are widely used in the clinical practice in BC. In addition, gene expression signatures of breast carcinomas have led to new classifications of tumour subgroups and also carry prognostic and predictive information [3]. In contrast, although serum tumour markers, including carcinoembryonic antigen, CA 15.3 and CA 27.59 could provide

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some prognostic information, they are not currently recommended for screening, diagnosis, or routine surveillance after initial treatment [4].

A large amount of data has revealed the correlation between specific tumours and differential microRNA (miRNA) expression profiles, thus providing a new class of disease-specific biomarkers (revised in [5]). MiRNAs are 18- to 25-nt noncoding RNA molecules that regulate protein expression of specific mRNA by either translational inhibition or mRNAs degradation. MiRNAs play different regulatory roles in cancer and have distinct functions in controlling the cell cycle, proliferation, invasion and metastasis. Moreover, miRNA deregulation can induce a pro-inflammatory and pro-metastatic environment and curtail the anti-tumour immunity [6,7].

An increasing number of studies analysing the miRNA expression signatures in BC, their correlates with specific molecular subtypes and their potential clinical relevance have been reported [8-11].

The miR-200 family of miRNAs consists of five members grouped in two independent transcriptional clusters: miR-200a, 200b and 429, located on chromosome 1p36; and miR-200c and 141, located on 12p13. Deregulation of miR-200 family of microRNAs in cancer [12,13] has been related to epithelial to-mesenchymal transition and cell-plasticity, apoptotic response, molecular subtype, oestrogen regulation, control of the growth and function of stem cells and regulation of the downstream transcriptional program that mediate distant metastasis. Also, regulatory functions of miR-200 s in tumour angiogenesis have been recently described [14]. However, in vitro and functional studies have yielded conflicting results regarding the net effect of miR-200 s in suppressing or promoting metastasis in different cellular contexts and cancer types [15-17].

MicroRNAs can be detected in the blood and studies indicate they are particularly stable and abundant [18,19]. Circulating miRNAs could be actively secreted from tumour cells, but also from non-malignant cells, including immune cells, either microvesicle-associated or free, in a selective manner [20]. In addition, passive leakage derived of apoptosis or necrosis of cancer cells tissue or chronic inflammation could be the source of microRNA founded in total blood, serum or plasma.

Our previous study has shown miR-200c in the blood can distinguish with significant specificity and sensitivity, patients with gastric cancer from healthy controls and remarkably, increased expression levels of miR-200c in blood were significantly associated with poor progression-free and overall survivals in gastric cancer patients [21].

Only a few studies have directly examined the role of miRNAs in the prognosis in BC, the vast majority of which were conducted analysing miRNA expression in the primary breast tumour (revised in [22]). However, little is known concerning the relationship between the blood miRNA expression profiles with the prognosis in BC patients. We first performed a Phase I preclinical study by means of computational tools for miRNAs profiling. Selected miRNAs were evaluated by RT-qPCR in BC and hematopoietic cell lines, control bloods, and blood from metastatic BC patients. Based on these results miR-141 and mir-200c were chosen for further analysis in BC patients [23].

Hence, we hypothesised that the quantitative detection of the miR-200 family in the whole blood could be useful as clinical biomarker in BC patients. In that sense, the blood miR-200 cluster expression might correlate with BC diagnosis, staging and prognosis. In the present study, we found that miR-200c and miR-141 expression levels were deregulated in the blood of BC patients. Likewise, the blood levels of miR-200c and miR-141 emerged as compelling and independent prognostic signature for the progression and survival of BC patients.

Methods

Participants

Consecutive female BC outpatients were included from the medical oncology unit at University hospital in La Coruña, Spain. Inclusion criteria were: Confirmed pathologic diagnosis of invasive BC; stage I–III with no prior systemic therapy; stage IV patients with no previous systemic therapy or in confirmed progression after such treatment; written informed consent.

Exclusion criteria were defined as: previous invasive non-breast cancer; coagulopathies or platelets < $20{,}000 \times 10^9$/L; any previous systemic therapy for BC except relapsed or stage IV patients with confirmed progressive disease; prior pelvic radiation; previous bisphosphonate therapy.

The diagnostic work-up included clinical examination, blood sampling with CA 15.3 serum determination, mammography, chest x-ray, abdominal ultrasound and bone scan. Computed tomography scanning of the chest, abdomen and pelvis was performed on stage IV patients.

The patients were followed up clinically every 3 months during the first 2 years, every 6 months for 3 years and in a yearly basis afterwards to monitor disease progression. Mammographic evaluation was performed every year during all the follow up period.

The controls (all females) were recruited from the patients' family and relatives. We only excluded subjects with a previous history of malignant disease. Thus, controls with different chronic but stable diseases (e., hypertension, diabetes mellitus or heart disease) were eligible and consecutively recruited. The control cohort was selected to include an age distribution that was comparable to the patient group.

The peripheral venous blood (PB) for quantitative reverse transcription PCR (RT-qPCR) analysis was collected in EDTA-containing tubes (10 mL). The first 5 mL of collected blood was discarded to avoid contamination with epidermal cells. Then, the PB was further diluted in RNA*later* and frozen at −20°C for storage until RNA extraction

This study was approved by the Ethics Committee of Clinical Investigation of Galicia (Spain) and conducted in compliance with the Helsinki Declaration. Written informed consents were obtained from all the patients and the controls prior to their inclusion in the study.

Pathological analyses

The primary tumour and axillaries lymph nodes collected during surgery were processed on a routine diagnostic basis. Histological type, tumour size and nodal involvement were analysed, and the disease was staged according to the TNM system [24]. Tumour grading was performed according to modified Bloom–Richardson score. Immunohistochemistry (IHC) was performed for oestrogen receptor (ER), progesterone receptor (PgR), Ki-67antigen (MIB-1) and HER2. Immunopositivity was recorded when 10% (ER, PgR) of the nucleus of tumour cells were stained. HER2 required distinct membranous staining for being considered positive (3+). The HER2 status of tumours with an IHC score of 2+ was determined by the fluorescence *in situ* hybridization results.

Residual disease status at the time of blood sampling was classified as R0 when no residual disease was present after surgery, R1 when microscopic residual disease was found and R2 in the presence of macroscopic disease. The patients from whom the blood was obtained before the start of neoadjuvant treatment were categorised as R2. When surgery was not performed, the pathological diagnosis was based on radiological-guided biopsies.

Blood microRNA isolation and reverse-transcription quantitative PCR (RT-qPCR)

MiRNA extraction from blood was performed with the RiboPure-Blood Kit (Ambion Inc, Austin, TX). The procedure was performed using 0.5 mL of whole blood. The mirVana^{TM} RT-qPCR miRNA Detection Kit (Ambion Inc, Austin, TX) was used to detect and quantify miRNA expression. To control input variability and sample normalisation, primer sets specific for the small RNA species U6 snRNA (Ambion, AM30303) and 5S rRNA (Ambion, AM30302) were used. Real-time PCR was performed on the LightCycler® 480 Instrument (Roche, Mannheim, Germany).

The Relative Expression Software Tool (REST) was used to analyse the relative miRNA expression in each sample and to determine the fold difference for every miRNA [25]. The expression levels of the target miRNAs were standardised using an index containing 5S rRNA and U6 snRNA.

All the procedures have been described previously [21]. For details, refer to Additional file 1.

MiRNA analyses were performed with no knowledge of the clinical or follow-up data.

Bioinformatics and microRNAs expression profiling

MiRNA expression data from previously published BC cohorts [9,10] were retrieved. Selected microRNAs, miR-200c and miR-141 were analysed further to assess whether they were associated with clinic and pathologic factors.

The online tool MIRUMIR [26] was used to estimate the power of miR-200c and miR-141 tumour expression to serve as potential biomarkers to predict survival of BC patients. MIRUMIR performs survival analyses across several available data sets. False discovery rate control procedure is implemented to adjust *P*-values for multiple testing. MIRUMIR is freely available at http://www.bioprofiling.de/MIRUMIR.

In addition, the PROGmiR tool [27] available at http://www.compbio.iupui.edu/progmir was also used to study overall survival implications for miR-200c and miR-141 in BC. The BC expression data comes from The Cancer Genome Atlas (TCGA; https://tcga-data.nci.nih.gov/tcga). This dataset include survival data of 727 cases of invasive breast carcinoma. MicroRNA expression data was obtained using the Illumina Genome Analyzer (GA) and HiSeq platforms.

Finally, to more comprehensively profile circulating miR-141 and miR-200c as potential markers of BC, we obtain their expression in serum, plasma or total blood in the genome-wide studies deposited in NCBI's Gene Expression Omnibus (GEO) [28]. The values of the specific miRNAs were retrieved through of the GEO2R web application, available at http://www.ncbi.nlm.nih.gov/geo/geo2r/.

Study design and statistical analyses

The primary aims were to estimate the diagnostic accuracy and usefulness of miRNA as measured by RT-qPCR in the blood of BC patients as a clinical biomarker and to determine its potential prognostic value. The study was performed following the proposed guidelines of the Early Detection Research Network [29]. The design and results are presented in accordance with the REMARK [30] and MIQE guidelines [31].

The potential correlation among blood miRNA levels and the clinical and pathological features of the study subjects were analysed. The normality of the distribution of miRNA expression was analysed using the Kolmogorov-Smirnov test. Thus, parametric or non-parametric statistics

were used, as appropriate. The relationships between miRNAs levels and the quantitative clinical variables were analysed using the Spearman correlation. The Cutoff Finder software [32] was used for receiver operating characteristic (ROC) curves analysis and miRNAs expression cutoffs determinations. The ROC curves were constructed by plotting sensitivity (Y-axis) vs 1-specificity (X-axis) and the areas under the curve (AUC) were calculated. The method used was based on the maximization of Youden's J statistics. In this first step, the cutoff is optimized for discriminating controls and BC patients based on miRNAs expression. In the second step, the Cutoff Finder tool fits Cox proportional hazard models to the dichotomized miRNA expression in the BC cohort and the survival variables (OS and PFS). These prognostic cutoffs are defined as the points with the most significant (log-rank test) split. Hazard ratios (HRs) including 95% confidence intervals are calculated to assess the stability and significance of the dichotomization.

Significances of correlations with overall survival (OS) and progression-free survival (PFS) were determined. PFS was measured as the time between the baseline blood sampling for miRNA analysis and the documentation of first BC progression, based on clinical and radiological findings, second primary tumour or death from any cause (events). OS was measured from the time at which the baseline blood sample was obtained to the date of death from any cause or date of last follow-up. The patients who were alive and progression-free at the time of analysis were censored by using the time between the blood assessment and their most recent follow-up evaluations.

Multivariate survival analyses (PFS and OS) were performed using Cox regression models. All statistical tests were two-sided and *P* values less than 0.05 were considered significant. SPSS Statistics 19.0 for Windows (IBM Corporation, Armonk, NY, USA, 2011) and Graph Pad Prism 5 (GraphPad Software, La Jolla, CA, USA, 2007) were used for data analyses.

The statistical power of the study was estimated *post-hoc*, taking into account a probability of survival at the end of the study of 0.75 in the low-risk miRNA signature group and 0.35 in the poor-prognostic subgroup. The poor-prognostic subgroup was defined by an increased expression of miR-200c and/or down-regulation of miR-141 in the patient's bloods. With the sample size of 57 patients, the study was able to demonstrate by two-sided log-rank test, a significant difference in OS, with an alpha-error of 0.05 and a statistical power higher than 80%.

Results

Patients and clinical data

From November 2006 to May 2008, 57 female patients with histological proven BC were consecutively recruited for this study. The control cohort included 20 cases. The clinical characteristics of the included subjects are shown (Table 1). The mean age was 54.8 years (standard

Table 1 Characteristics of subjects included in the study

Characteristic	Patients	Controls	
	***n* = 57 (%)**	***n* = 20 (%)**	
Age (years, mean ± SD)	55.4 ± 12.8	54.8 ± 14.3	0.853*
<55	28 (49)	12 (60)	0.48**
≥55	29 (51)	8 (40)	
Menopause			
Pre-menopausal	24 (42.1)	N/A	
Post-menopausal	33 (57.9)	N/A	
Histology			
Ductal	50 (87.7)		
Lobular	5 (8.8)		
Other	2 (3.5)		
Histological grade			
1	7 (12.3)		
2	25 (43.9)		
3	23 (40.4)		
TNM Stage			
I	14 (24.6)		
II	13 (22.8)		
III	17 (29.8)		
IV	13 (22.8)		
Lymph nodes involved			
No	20 (35.1)		
Yes	37 (64.9)		
Hormonal Receptors			
Positive	42 (73.7)		
Negative	14 (24.6)		
HER2			
Positive	14 (24.6)		
Negative	42 (73.7)		
MIB1			
<25%	40 (70.2)		
>25%	14 (24.6)		
Type			
Luminal	32 (56.1)		
HER2	14 (24.6)		
Triple negative	10 (17.5)		
R0	28 (49.1)		
R2	29 (50.9)		

Abbreviation: ECOG Eastern Cooperative Oncology Group performance status. Residual Status (R): R0, no residual tumour; R2, macroscopic residual tumour. The number (percentages) of patients with data avalaible is indicated. *Student t-test. **Chi^2 test.

error of the mean [S.E.M.], 3.2; range, 29 to 73 years) in the control group and 55.4 years (S.E.M., 1.7; range, 27 to 83) in the patient group (*t* test, $P = 0.853$).

The blood was obtained after R0 surgery in 28 patients (49.1%). In 29 (50.9%) patients, the blood samples were obtained before neoadjuvant treatment or in the presence of metastatic disease, both of which were categorised as R2 at the time of blood sampling.

All patients were followed until death or study completion. The last date of follow-up for the survivors was April (2013). Disease progression events occurred in 22 patients (38.6%). The mean PFS was 235.3 weeks (95% CI: 203.6 to 267 weeks). There were 10 progressions among stage I–III patients and 12 progressions of metastatic disease. The mean OS was 264.6 weeks (95% CI: 239.2 to 290 weeks) and 19 patients (33.3%) died. The mean (S.E.M.) follow-up time for the patients still alive at the time of the analysis was 298.2 (2.7) weeks (median, 303.7 weeks; 95% CI: 296.6 to 310.8 weeks).

Expression of miRNA in blood samples

Real-time PCR quantitative assessment of miR-141 and miR-200c were performed using 77 blood samples (57 patients and 20 controls). The Figure 1 depicts relative expression for the blood levels of miR-141 and miR-200c.

The blood expression of miR-141 was not significantly ($P = 0.557$) different in patients compared to healthy controls (Figure 1A). The mean relative miR-141 expression (Figure 1B) was 2.615 (S.E.M., 0.83; 95% CI: 0.89 to 4.34) in controls, 8.81 (S.E.M., 2.29; 95% CI: 4.2 to 13.4) in stage I-III patients and 1.06 (S.E.M., 0.93; 95% CI: 0 to 3.09) in stage IV BC patients ($P = 0.003$ Kruskal-Wallis test. Dunnett's multiple comparison tests: stage I-III *vs* control, $P = 0.099$; stage IV *vs* control, $P = 0.904$). However, the blood levels of miR-141 could not discriminate BC patients from healthy women in ROC analysis.

We compared the expression levels of circulating miR-200c in controls and BC patients. Our data showed miR-200c was downregulated in the blood of BC patients by comparison with its expression in the blood of controls ($P < 0.0001$; Figure 1C). Next, we sought to identify potential differences of the expression levels of miR-200c according to stage. The mean relative miR-200c expression (Figure 1D) was 2.53 (S.E.M., 0.58; 95% CI: 1.31 to 3.74) in controls, 0.41 (S.E.M., 0.13; 95% CI: 0.16 to 0.66) in stage I-III patients and 1.75 (S.E.M., 0.62; 95% CI: 0.41 to 3.09) in stage IV BC patients ($P < 0.0001$; Kruskal-Wallis test. Dunnett's multiple comparison

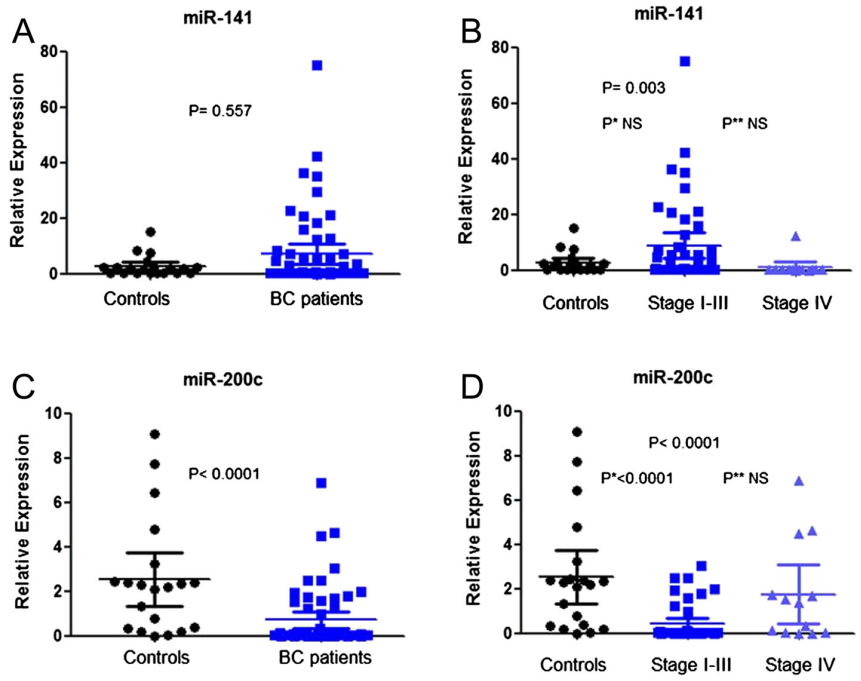

Figure 1 Real time PCR of miR-200c and miR-141 in blood samples. Plots depicting the relative expression for blood levels of miR-141 **(A)** and miR-200c **(C)** between patients and healthy controls, and between stage I-III patients, stage IV patients and healthy controls **(B and D)**. The horizontal bar denotes the mean value for each group. The corresponding *P* values are provided in plots.

tests: stage I-III *vs* control, $P < 0.0001$; stage IV *vs* control, $P = 0.342$).

ROC curve analysis (Figure 2A) showed that the blood levels of miR-200c might serve as negative biomarker for discriminating BC patients from healthy controls, with an AUC (the area under the ROC curve) of 0.79 (95% CI: 0.688 to 0.914; $P < 0.001$). At the cut-off value of 0.165, the sensitivity and specificity were 90.0% and 70.2%, respectively. The odds ratio (OR) according to the cut-off value (Figure 2B) was 0.62 (95% CI: 0.45-0.85; $P < 0.0001$).

The ROC curve analysis using blood miR-200c yielded an AUC of 0.850 (95% CI: 0.738 to 0.957; $P < 0.001$; OR: 0.37) in discriminating stage I-III BC from healthy controls as shown in Figure 2C and D. When comparing the relative miR-200c levels in controls and stage I-II patients (Figure 2E and F), the AUC was 0.82 (95% CI: 0.694 to 0.945; $P < 0.001$; OR: 0.45) with a sensitivity of 90%, and a specificity of 75%.

Clinical and pathological characteristics and miRNA levels in the blood

The clinical and pathological characteristics and the miR-200c and miR-141 expression levels in the blood from BC patients are given (Table 2). The correlations of quantitative clinical and laboratory parameters and miRNAs levels are summarized (Table 3). The miR-200c levels were not related to any of the clinical and pathological characteristics analysed. There was a tendency ($P = 0.054$) to higher levels in the stage IV group compared to stages I-III group. MiR-141 levels were significantly higher in the blood of the patients with lymph node metastasis ($P = 0.014$) and HER2 negative tumours ($P = 0.037$). In stages I to III BC patients, we evaluated the miR-200c and miR-141 levels according to timing of blood sampling (post- vs. pre-operative). The levels of each miRNA in the post- operative vs. pre-operative samples were not significantly different (Table 2). However, the pre-and post-resection samples were not paired from the same patients.

The Spearman order correlation analysis showed that miR-200c expression in the blood of BC patients was inversely correlated with the miR-141 level ($r_s = -0.311$, $P = 0.019$). In the control group however, there was no correlation between miR-141 and miR-200c ($r_s = 0.006$, $P = 0.98$).

Prognostic significance of miR-200c and miR-141 levels in the blood

The HRs for PFS and OS were first estimated considering the actual values of every miRNA as a continuous variable in a Cox regression model. Increasing values for miR-200c were associated with PFS events (HR 1.37; 95% CI: 1.09-1.71; $P = 0.007$) and reduced OS (HR 1.38; 95% CI: 1.11-1.71; $P = 0.003$). In contrast, the miR-141 levels as a continuous variable were not significantly associated with outcomes (HR for PFS, 0.987; 95% CI: 0.95-1.025; $P = 0.498$. HR for OS, 0.986; 95% CI: 0.942-1.032; $P = 0.542$).

To generate survival curves, we converted continuous miRNAs expression values to dichotomous variables, using the Cutoff finder software [32]. This procedure enabled division of samples into classes with high and low expression of microRNA.

Using this approach, miR-141 was down-regulate in the blood of 26.3% (15/57) of the patients. The percentage of patients with miR-141 down-regulation was associated with TNM stage: 18.2% (8/44) in stage I-III patients and 53.8% (7/13) in stage IV patients (Fisher's exact test, $P = 0.027$). In contrast, high expression of miR-200c was found in 24.6% of the patients (14/57). This overexpression was also associated with stage IV (53.8% of the patients; Fisher's exact test, $P = 0.01$).

The Kaplan-Meier analysis and the log-rank test were used to calculate the effect of miR-200c and miR-141 blood expression on patient survival (Figures 3 and 4). Specifically, the mean overall survival and progression-free survival time of patients whose bloods expressed high levels of miR-200c (>1.29 relative expression value) was 201.48 weeks (median, 158.29 weeks) and 162.84 weeks (median, 89.43 weeks) respectively, whereas the mean OS and PFS time of those with low levels of miR-200c expression was 284.7 weeks (log-rank $P = 0.004$) and 258.85 weeks (long-rank $P = 0.022$), respectively (Figure 3B and D). The median was not reached in the low miR-200c subgroup. A significant association between a high miR-200c blood level and poor PFS (HR 3.33; 95% CI: 1.22 to 9.07; $P = 0.019$) and OS (HR 2.79; 95% CI: 1.01 to 7.7); $P = 0.048$) was found, with independence of tumour stage and hormonal receptors status as depicted (Figure 5A).

Moreover, low expression levels of miR-141 (<0.145 relative expression value) in BC patient bloods (Figure 3A and C) were found to be associated with poorer progression-free survival time (mean: 169.37 versus 258.12 weeks; log-rank $P = 0.028$) and overall survival time (mean: 216.01 versus 281.9 weeks; log-rank $P = 0.011$). The median was not reached in the high miR-141 subgroup. A decreased miR-141 level was an indicator of a poor prognosis (HR for death, 2.76; 95% CI: 1.04 to 7.35; $P = 0.042$) independently of stage and hormonal receptors. The association of low miR-141 level with progression events when adjusted for stage and hormonal receptors, however, did not reach the statistical significance (Figure 5A; HR, 2.50; 95% CI: 0.96 to 6.53; $P = 0.061$).

To further evaluate whether blood miR-200 s deregulation can predict BC prognosis, we next performed survival analysis. Kaplan-Meier analysis showed that

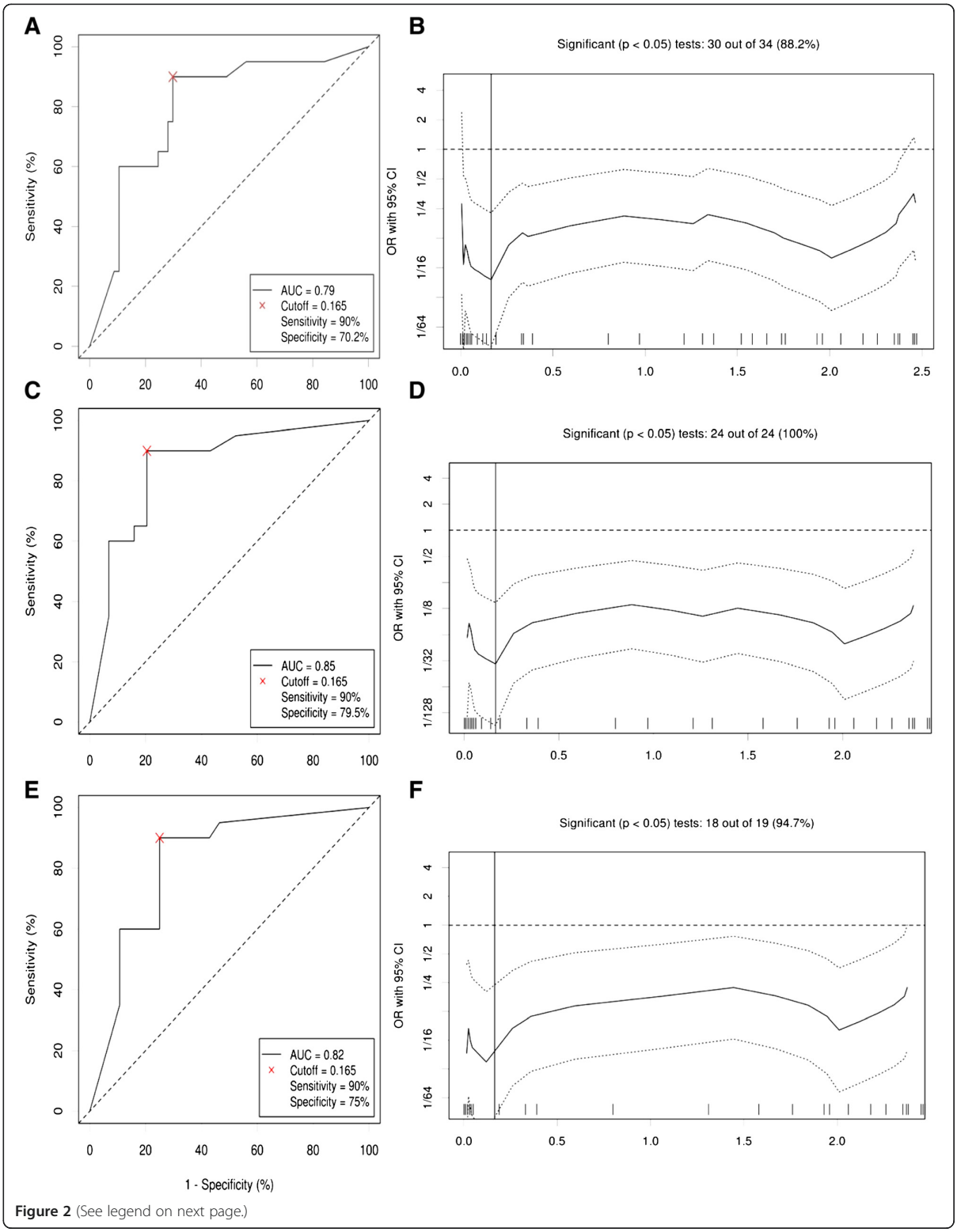

Figure 2 (See legend on next page.)

(See figure on previous page.)
Figure 2 The role of blood miR-200c in breast cancer diagnosis. Receiver-operating characteristic (ROC) curve analysis **(A)** and odds ratio plot **(B)** using blood miR-200c expression levels for discriminating breast cancer patients (n = 57) and healthy controls (n = 20). ROC curves and odds ratio plots for discrimination of stage I-III BC from healthy controls **(Cand D)** and discrimination of stage I-II patients from healthy controls **(E and F)** are also shown.

patients with higher levels of blood miR-200c and /or low levels of miR-141 had significantly poorer progression-free survival ($P = 0.003$; log-rank test; Figure 4A) and overall survival ($P < 0.0001$; log-rank test; Figure 4B). The results of the Cox proportional hazards model incorporating a "poor prognostic" blood miRNA signature are shown (Figure 5B). Multivariate analyses included age, tumour stage, hormonal receptors and microRNA levels. When paired in an interaction model, high miR-200c and/or low miR-141 levels had a greater association with decreased survival (HR, 3.89; 95% CI: 1.28 to 11.85; P =0.017) and shorter PFS (HR, 3.79; 95% CI: 1.41 to 10.16; P =0.008) than either one alone.

Bioinformatics and microRNAs expression profiling

The miR-200c and miR-141 expression levels by oligonucleotide microarray profiling of a panel of 20 BC samples were retrieved from Mattie et al. [9]. This series included three common phenotypes (9/20, ErbB2-positive/ER-negative; 4/20, ErbB2-positive/ER-positive; 7/20, ErbB2-negative/ER-positive). The tumour miR-141 and miR-200c expressions were not associated with the patient age, hormonal receptors, HER2 overexpression, grade, proliferation index, or p53 mutational status.

The associations between miR-200c and miR-141 tumour expression, molecular subtypes and clinic and pathological factors were assessed using the microRNA expression data (GEO accession number GSE7842) provided by Blenkiron et al. [10], including 93 primary breast tumour samples. For multiple comparisons, P value was adjusted at 0.01. No significant associations between miR-200c and miR-141 with tumour characteristics such as molecular subtype, grade, stage, vascular invasion, ER status, Nottingham Prognostic Index (NPI) as well as TP53 status and HER2 overexpression were found.

Two different datasets, which provide miRNAs expression data and clinical outcomes for BC patients, were identified by MIRUMR online tool [26]. In the first dataset, (accession number GSE37405) low miR-141 tumour expression (P-values corrected by FDR, 0.03308) and low miR-200c tumour expression (P-values corrected by FDR, 0.02324) were associated with a reduced overall survival in high-risk oestrogen receptor positive BC patients (Additional files 2 and 3). By contrast, in the second dataset (GEO accession number GSE22216) that included 189 early primary BC patients, no survival differences were found according to miR-141 ($P = 0.486$) and miR-200c ($P = 0.469$) tumour expression (Additional files 4 and 5).

We also used the PROGmiR tool [26] to create Kaplan-Meier survival plots for miR-200c and miR-141 using the BC TCGA data. Overall survival at 3 and 5 years were not significantly different according tumour levels of miR-200c and miR-141. However, with a longer follow-up, the survival times became significantly better in the high microRNA expressions groups. The hazard ratio and P values for the proportional hazards model are also given (Additional files 6, 7, 8 and 9).

The data about circulating miR-141 and miR-200c expression in three genome-wide studies deposited in NCBI's Gene Expression Omnibus (GEO) were retrieved and analysed [33-35]. These studies included plasma (two studies) or total blood (one study) of control healthy women and early BC patients. The characteristics and results of these studies are depicted (Additional file 10). The levels of miR-141 and miR-200c in the plasma were not significantly different between early BC and controls. However, miR-141 was lower in total blood of the BC cohort in comparison to controls ($P = 0.029$). There was a trend to a negative correlation between circulating miR-141 and miR-200c expression.

Discussion

Blood biomarkers that provide accurate diagnostic and prognostic information for women with BC are urgently required. MicroRNAs are deregulated in BC and histological and molecular subtypes are characterised by specific microRNA profiles. The deregulated expression of miRNAs in both tumour tissues and the blood compartment has led to the search for miRNAs to predict presence of cancer and indicate its overall prognosis [8-11]. To date, most of the studies in BC have focused on the potential role of circulating (plasma or serum) miRNAs as biomarkers for diagnosis and detection of early disease and most of them are based on the testing of multiple miRNAs, using high-throughput technologies [19,33-35]. However, very few studies have explored the capabilities of the blood miRNA expression in predicting the clinical outcome of BC patients.

We hypothesize the deregulated expression of circulating and cellular miRNAs present in the whole blood can identify the presence of BC, and could thus be developed into a prognostic signature. Our study did not pursue the current tendency to examine circulating miRNAs in plasma or serum using high-throughput technologies. In

Table 2 Distribution of clinical and pathological parameters and levels of miR-200c and miR-141 in blood

Parameter	*n*	miR-200c	*P* value	miR-141	*P* value
Age (years)			0.190		0.240
<55	28	0.35 (0.72)		7.71 (12.47)	
≥55	29	1.07 (1.74)		6.4 (15.1)	
Menopause			0.572		0.258
Pre-menopausal	24	0.37 (0.68)		7.8 (12.96)	
Post-menopausal	33	0.97 (1.68)		6.5 (14.5)	
Histology			0.140*		0.712*
Ductal	50	0.69 (1.36)		7.26 (14.22)	
Lobular	5	1.3 (1.95)		10.42 (14.67)	
Other	2	0.01 (0.01)		0.2 (0.02)	
Histological grade			0.106*		0.703*
1	7	1.79 (1.59)		3.84 (4.92)	
2	25	0.36 (0.69)		4.19 (7.61)	
3	23	0.77 (1.74)		11.72 (19.31)	
TNM Stage			0.054		*0.001*
I-III	44	0.41 (0.83)		8.81 (15.16)	
IV	13	1.75 (2.22)		1.06 (3.35)	
Lymph nodes involved			0.216		*0.014*
No	20	1.06 (1.34)		1.53 (3.41)	
Yes	37	0.53 (1.38)		10.02 (16.21)	
Hormonal receptors			0.460		0.887
Positive	42	0.79 (1.52)		5.03 (9.35)	
Negative	14	0.44 (0.86)		13.58 (21.85)	
Oestrogen receptors			0.460		0.887
Positive	42	0.79 (1.52)		5.03 (9.35)	
Negative	14	0.44 (0.86)		13.58 (21.85)	
Progesterone receptors			0.653		0.371
Positive	29	0.59 (1.15)		5.96 (10.53)	
Negative	27	0.82 (1.61)		8.46 (16.84)	
HER2			0.833		*0.037*
Positive	14	0.88 (1.41)		1.24 (2.13)	
Negative	42	0.64 (1.39)		9.14 (15.5)	
MIB1			0.073		0.790
<25%	40	0.90 (1.55)		7.93 (15.24)	
>25%	14	0.19 (0.65)		5.99 (10.26)	
Type			0.809*		0.105*
Luminal	32	0.71 (1.52)		6.21 (10.4)	
HER2	14	0.88 (1.41)		1.24 (2.13)	
Triple negative	10	0.41 (0.86)		18.52 (24.35)	
Residual disease			0.554		0.755
R0	27	0.52 (0.97)		8.69 (17.24)	
R2	30	0.89 (1.66)		5.56 (9.73)	

Table 2 Distribution of clinical and pathological parameters and levels of miR-200c and miR-141 in blood *(Continued)*

Blood sampling^			0.72	0.128
Before surgery	17	0.24 (0.5)	8.69 (17.24)	
After surgery	27	0.52 (0.97)	8.99 (11.59)	

The miRNAs relative expression levels (REL) are shown in arbitrary units. The data represent the mean (standard deviation). *n* indicates the number of patients with data available. ^Timing of blood sampling before or after surgery is indicated for stages I to III patients only. Mann–Whitney test. *Kruskal-Wallis test.

contrast it is focused on the selective testing of two members of the miR-200 family of microRNAs, miR-200c and miR-141, in the whole blood. Although the feasibility of using miRNA expression profile in whole blood as the basis for recognition of several diseases has been demonstrated [18] its potential prognostic value in cancer has not been comprehensively explored.

We found that miR-200c/miR-141 expression in the blood of BC patients is deregulated comparing with controls and, furthermore miR-200c and miR-141 levels were associated with distinct disease-free survival and overall survival of patients. Both of the univariate and multivariate analyses indicated that miR-200c and miR-141 blood levels were independent prognostic factors for BC outcomes.

Our study showed miR-200c in blood was down-regulated in stages I-III BC patients compared to age-matched controls, discriminating these subsets with an AUC-ROC of 0.85, and compared to patients with metastatic disease. In contrast, a tendency to higher levels of miR-141 in the blood of stage I-III BC patients in comparison with controls and stage IV patients was found. MiR-200c and miR-141 were inversely correlated in the blood of BC patients. Since these miRNAs measurements could discriminate metastatic from early stage BC patients and were associated with prognosis, miR-200c/miR-141 blood levels may represent a BC-specific deregulation with potential functional consequences. Indeed, the blood levels of miR-200c and miR-141 seem to mirror the suggested biphasic role of this family of microRNA during metastatic process [15,16].

In our BC cohort, neither miR-200c nor miR-141 circulating levels were significantly associated with age, menopausal status, histological subtype, tumour grade, hormonal receptors or IHC-based subtypes. The miR-200c levels were numerically higher in stage IV and tumours with lower MIB-1 staining. The miR-141 levels were lower in stage IV, lymph node negative patients and HER2 negative tumours. To validate these results, we used previously reported data on miRNAs profiling studies in BC. Similar to our findings, miR-200c and miR-141 were not associated with molecular subtypes or clinic and pathologic factors analysed [9,10].

Table 3 Correlations of clinical and laboratory parameters and miRNA levels in blood of breast cancer patients

	miR-200c		miR-141	
	Spearman's *Rho*	*P* value	Spearman's *Rho*	*P* value
Age	0.217	0.104	−0.208	0.12
Serum Ca 15.3	0.163	0.273	−0.361	0.013
Neutrophils Count	−0.202	0.133	0.094	0.485
MIB1 tumour staining	0.085	0.538	−0.138	0.314
miR-141	−0.311	0.019		

One of the strengths of our study is the capability of the whole-blood miR-200 and miR-141 deregulation to predict PFS and OS was interrogated across a set of BC patients with a comprehensive clinical, pathological and long-term outcome data. Even with a relatively low sample size and few events in our patient population we were able to demonstrate the correlation of these miRNAs to PFS and OS. MiR-200c was the most accurate miRNA individually for predicting PFS and OS, and its prediction accuracy increased by a small margin when used in combination with miR-141. The poor-prognostic profile defined by a high miR-200c and/or low miR-141 in the blood levels had a greater association with decreased survival and shorter PFS than either one alone, and it was independent of age, tumour stage and hormonal receptors status in the multivariate Cox's model.

The sources of miRNAs in the blood are intriguing and whether deregulation in circulating blood miRNAs reflected similar changes in breast tumour tissues is controversial. In that sense, it was surprising to detect reduced concentrations of circulating miR-200c and miR-141 in the whole blood of subsets of our BC patient cohort comparing to age-matched healthy females. Recently Dvinge et al. [17] have demonstrated a global decrease in miRNA expression in breast tumours and described that polycistronic miRNAs can show dependent, independent or even opposite expression patterns in BC. Distinct patterns of miRNAs in circulation and BC tissue had been reported both in murine BC models [36] and clinical series [37].

Furthermore, a recent report suggests that normal and malignant mammary epithelial cells release miRNA into blood and fluids in a specific manner [20]. Microarray and quantitative PCR analyses had indicated the breast tumour cells selectively retain miR-141. In comparison, miR-200c was highly released from cells. The low levels of any particular miRNA in blood could also be caused

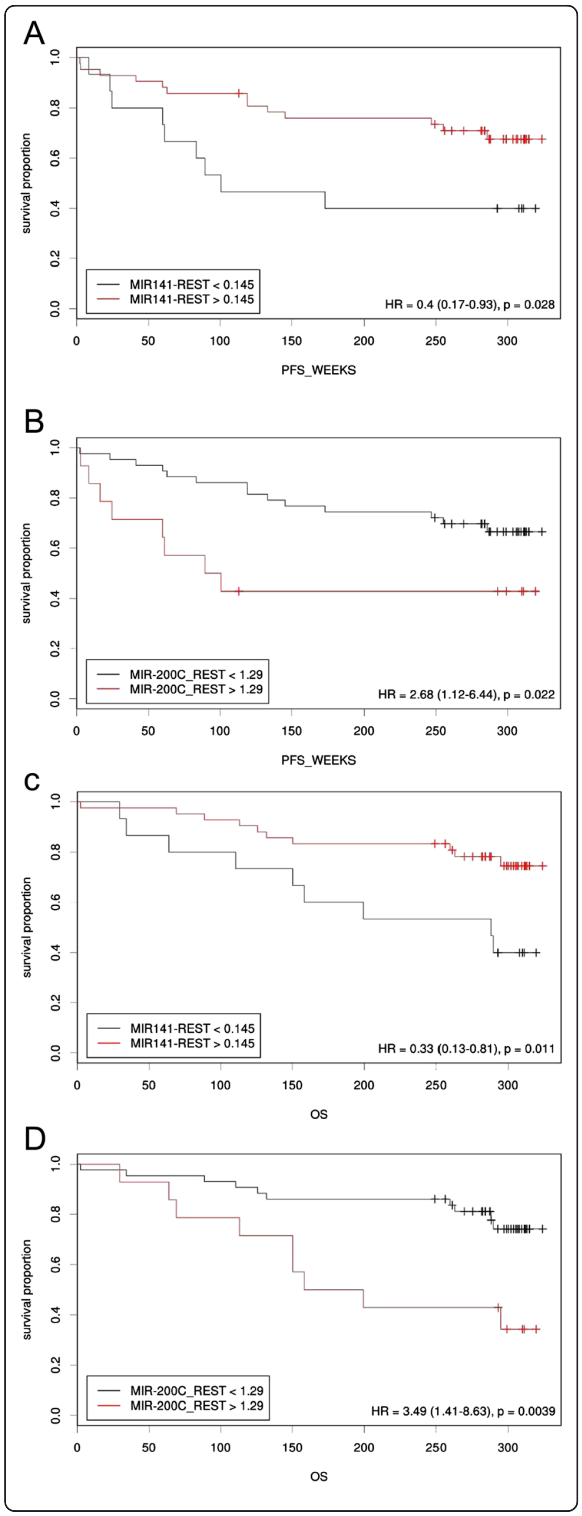

Figure 3 miR-200c and miR-141 expression levels measured in peripheral blood are associated with poor prognosis in breast cancer patients. Kaplan-Meier curves showing **(A and B)** the progression-free survival (PFS) and **(C and D)** the overall survival (OS) of 57 breast cancer patients with high or low blood expression levels of microRNA. Continuous miRNA expression levels measured using RT-qPCR were converted to dichotomous variables using the Cutoff software (see text). The *P* values were computed using the Log-rank test.

by an altered RNA polymerase activity or deregulated processing and exporting factors. Therefore, the extracellular accumulation of mature miRNAs is regulated at levels other than the primary transcript abundance in the tumour cells. Roth et al. [38] had found a very low expression of miR-141 in serum from BC patients and

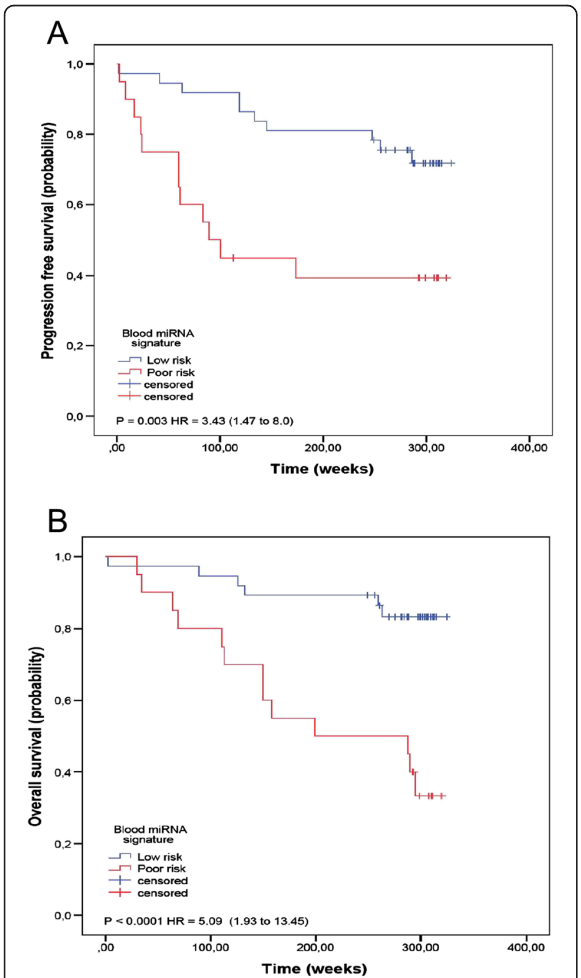

Figure 4 Poor prognostic blood miRNA signature. Kaplan-Meier analysis and log-rank test showed that patients with higher levels of blood miR-200c and/or low levels of miR-141 had significantly poorer progression-free survival **(A)** and overall survival **(B)**.

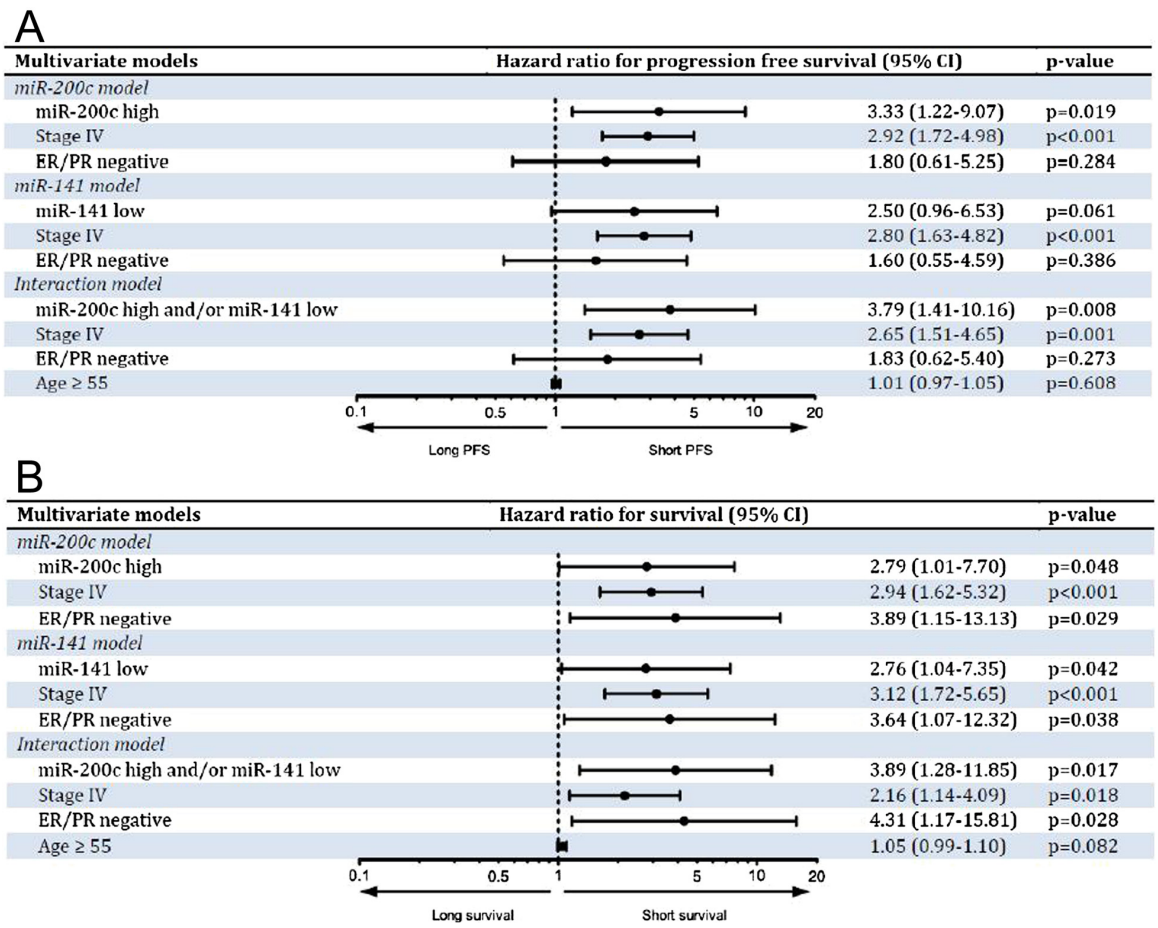

Figure 5 Blood miR-200c and/or miR-141 as prognostic factors in patients with breast cancer. Multivariate models showing the ability of high miR-200c levels, low miR-141 levels, and the combination of both as prognostic factors for predicting progression free survival **(A)** and overall survival **(B)** in breast cancer patients.

healthy females. Moreover the relative yields of miR-141 in serum did not differ significantly between healthy women and women with BC or between M0 and M1 patients. The one previous study that analyse miRNAs in the whole blood in BC patients [34] have included only early stages. They showed a down-regulation of miR-141 in the blood of BC patients while miR-200c was no differentially expressed.

The analysis of miRNA obtained from whole-blood may be advantageous in comparison with serum or plasma determinations, detecting not only those miRNA derived from blood cells comprising circulating tumour cells, but also those secreted in sub-cellular particles such as exosomes or associated with RNA binding proteins and derived from diverse cells and tissues. Compared to serum or plasma, whole blood is easier to collect and has more RNA content, which facilitates reliable and accurate global microRNA expression measurements using less clinical material. Another one of the crucial problems is the efficient and reproducible extraction of small amounts of miRNA from plasma or serum. Therefore, higher yields of miRNAs had been consistently obtained from whole blood samples compared with matched serum or plasma and lower quantification cycles occurred in whole blood compared with matched serum and plasma samples in RT-qPCR experiments [39].

One possibility is that circulating miRNAs are indicative of CTCs and/or metastases. Supporting this concept, Madhavan et al. [40] recently demonstrated that plasmatic levels of miR-200 family members are surrogate markers for CTCs in heavily treated metastatic BC patients and correlate with disease progression and overall survival. However, contradictory results have been described. Roth et al. [38] did not observe any tendency of higher miR-141 levels in serum of CTC-positive BC

patients. Sieuwerts et al. [41] found a significant decreased miR-200c transcript levels in the Ep-CAM$^+$ circulating tumour cells of metastatic BC patients compared with samples from healthy donors. In contrast, mR-141 transcript levels were not differentially expressed. We hypothesised that changes in miR-200c/miR-141 blood transcripts could reflect at least in part, the presence of tumour cells that have undergone or are undergoing epithelial-mesenchymal (EMT) and mesenchymal-epithelial transitions (MET), a dynamic process likely to be important for efficient metastatic colonisation [42]. These previous reports and our results underline the complex relationships between disease and changes in miRNA expression patterns in blood. Furthermore, the contribution of systemic inflammatory, immunomodulatory or proangiogenic processes to the whole blood microRNA profile cannot be ruled out. Maertzdorf et al. [43] found that blood miR-200c and miR-141 expression levels are reduced in chronic inflammatory conditions. In that sense, deregulation of miRNAs in the blood of BC patients could be related, at least in part, to the host immune and inflammatory context in response to BC.

It has been increasingly recognized that miR-200 family of microRNAs plays an important role in the proliferation, invasiveness and migratory properties of BC cells in cell lines [6,11-13] and experimental models; however, a systematic investigation of how miR-200 s deregulation affects the clinical outcome of BC patients has been poorly defined.

In fact, the relative expression of the miR-200 family in BC compared with normal breast tissue and even though profiling data from primary and metastatic BC samples have showed inconsistent results. Some authors [44] have described the upregulation of miR-200c and miR-141 during the transition from normal mammary epithelia to atypical ductal hyperplasia, and maintained their high expression profiles during later stages of invasive ductal carcinoma. The miR-200 family of microRNAs is differentially down-regulated in metastatic lymph node metastasis compared to paired primary tumour in BCs [45]. However, miR-200 expression was found greater in metastases derived from BC than in primary tumours [16,46].

Overexpression of miR-200 s in primary tumour has been associated with an increased risk of metastasis and poor prognosis (in terms of metastasis-free survival) particularly in ER-positive breast cancers [16]. In contrast, the bioinformatics analysis using MIRUMIR and PROGmiR tools indicate an association between lower levels of miR-200c and miR-141 in breast tumours and reduced overall survival.

Although our preliminary results are promising, several limitations in this study are addressed: (i) as the sample size is still small, further validations in large cohorts and in different ethnic groups are recommended; (ii) a remarkable limitation to this and other studies in this field is the lack of standardized procedures. Different pre-analytical and analytical factors affected the quantification of circulating miRNA, including substrate choice (whole blood, antibody-selected cells, plasma or serum), stabilization reagents, centrifugation or filtration to isolate plasma or serum, miRNA extraction procedures, selection of endogenous internal controls, assay choice, individual variation, and the effect of haemolysis. Because miRNAs are present at lower concentrations in plasma and serum than those found in whole blood, all of these variables could increase the assays variability and the stochastic effects when we quantified any microRNA in serum or plasma samples comparing to whole blood. Currently, there are no consistent reference genes suitable for normalizing circulating microRNA expression. Thus, the selection of references to normalize miRNA levels is still rather empirical. A combination of miRNAs for normalization augments the reliability of the data produced, and has been advocated by different studies. In that sense, we used a combination of U6 and 5S as reference genes.

Finally, the clinical utility of any proposed biomarker might be confirmed and validated in independent studies. In that sense our results regarding the prognostic value of circulating miR-200c deregulation in BC are in line with previous results including ours in gastric cancer and the recently reported works in oesophageal and colorectal cancers [21,40,47,48].

In summary, the results of our pilot study indicate that miR-200c and miR-141 levels are deregulated in the blood of BC patients. Based on the differences between cases and healthy controls, the blood miR-200c assay holds promise as a detection marker in BC. Moreover, we were able to verify that miR-200c and miR-141 in whole blood are promising biomarkers of PFS and OS, both independently and in combination. These results will have to be further verified in large study cohorts that include the different stages and molecular subtypes of BC with adequate follow-up. A special attention to technical challenges and standardization must be pursued in the next validation studies. Furthermore, these findings might have relevant implications for other epithelial cancers where the miR-200 s family of microRNA is also deregulated, widening this exciting and growing field.

Conclusions

Breast cancer is the leading cause of cancer death in women worldwide. Blood-borne metastases contribute to the great majority of deaths. The discovery of specific biomarkers characterizing the metastatic phenotype holds the promises of personalised therapy and improved prognosis prediction. MicroRNAs can be detected in the blood

and studies indicate they are particularly stable and abundant.

We hypothesised that the reverse-transcription quantitative PCR detection of miR-200c and miR-141 in the whole blood could be useful as clinical biomarker in breast cancer patients.

Our results indicate that miR-200c and miR-141 levels are deregulated in the blood of breast cancer patients. Based on the differences between cases and controls, the blood miR-200c assay holds promise as a diagnostic marker. Moreover, miR-200c and miR-141 in whole blood are promising biomarkers of progression-free and overall survival, both independently and in combination.

Additional files

Additional file 1: Supplemental methods and results.

Additional file 2: Survival analysis performed with MIRUMIR tool of data from GSE37405 microarray for miR-141 tumour expression. The *P* value is included in the figure.

Additional file 3: Survival analysis performed with MIRUMIR tool of data from GSE37405 microarray for miR-200c tumour expression. The *P* value is included in the figure.

Additional file 4: Survival analysis performed with MIRUMIR tool of data from GSE22216 microarray for miR-141 tumour expression. The *P* value is included in the figure.

Additional file 5: Survival analysis performed with MIRUMIR tool of data from GSE22216 microarray for miR-200c tumour expression. The *P* value is included in the figure.

Additional file 6: Survival analysis performed with PROGMIR tool for miR-141 tumour expression values extracted from TCGA breast cancer dataset (3 and 5 years follow-up). The hazard ratio and *P* value are included in the figure.

Additional file 7: Survival analysis performed with PROGMIR tool for miR-141 tumour expression values extracted from TCGA breast cancer dataset (>5 years follow-up). The hazard ratio and *P* value are included in the figure.

Additional file 8: Survival analysis performed with PROGMIR tool for miR-200c tumour expression values extracted from TCGA breast cancer dataset (3 and 5 years follow-up). The hazard ratio and *P* value are included in the figure.

Additional file 9: Survival analysis performed with PROGMIR tool for miR-200c tumour expression values from TCGA breast cancer dataset (>5 years follow-up). The hazard ratio and *P* value are included in the figure.

Additional file 10: Comparative of miR-141 and miR-200c expression in genome-wide circulating miRNA profiling studies in early breast cancer patients.

Abbreviations

BC: Breast cancer; CTC: Circulating tumour cells; CNA: Circulating nucleic acids; EMT: Epithelial-to-mesenchymal transition; PB: Peripheral venous blood; RT-qPCR: Reverse transcription quantitative PCR; Cq: Quantification cycle; REST: Relative Expression Software Tool; ROC: Eeceiver operating characteristic curve; AUC: Area under the curve; PFS: Progression-free survival; OS: Overall survival; S.E.M.: Standard error of the mean; ECOG: Eastern Cooperative Oncology Group.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MVA conceived the study, participated in data acquisition and analyses and drafted the manuscript. MBC, MH and IS performed the molecular analyses. SA, LC and LMAA made substantial contributions to clinical care of the patients and data acquisition. MBC and AF made substantial contributions to data analyses and interpretation. MJLP and MPS reviewed the histological samples. All authors read and approved the final manuscript.

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Additional file 1.

Supplemental methods and results.

Supplemental methods

Blood microRNA isolation and qRT-PCR

To isolate the miRNA fraction, the RiboPure-Blood Kit was used with the alternate protocol: isolation of small RNAs (Applied Biosystems, Foster City, CA, USA). The procedure was performed using 0.5 mL of whole blood per preparation. The absorbances at 260/280 and 260/230 were assessed using a NanoDropTM 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The purified RNA was further processed using qRT-PCR or stored at -80oC until use.

Reverse-transcription (RT) PCR was performed with 25 ng (up to 6.6 µL) of total RNA using the mirVanaTM qRT-PCR miRNA Detection Kit (Ambion, AM1558) with 2 µL 5X RT Buffer, 1 µL 1X RT Primer (Ambion, miR-200c, AM30096*; miR-141, AM2052*) and 0.4 µL of ArrayScript Enzyme Mix for a total volume of 10 µL.

For the PCR reaction, 10 µL of RT reaction and PCR Master Mix were used. The PCR Master Mix consisted of 5 µL 5X PCR buffer containing SYBR Green I, 0.2 µL SuperTaq 5 U/µL, 0.5 µL PCR primers and 9.3 µL of nuclease-free water for a total volume of 15 µL. Real-time PCR was performed on the LightCycler® 480 Instrument (Roche, Mannheim, Germany). To control input variability and sample normalisation, primer sets specific for the small RNA species U6 snRNA (Ambion, AM30303) and 5S rRNA (Ambion, AM30302) were used. These primer sets were used not only as internal controls but also to verify the integrity of the RNA and the reverse transcription reaction.

Any specimen with inadequate U6 snRNA or 5S rRNA expression would be excluded from the study. For miR-141 and miR-200c, the PCR cycling conditions and analysis were as follows: denaturation at 95oC for 8 seconds; cycling, 40 cycles of 95oC for 5 seconds, 60oC for 5 seconds and 72oC for 2 seconds; melting curve analysis, 1 cycle at 95oC for 5 seconds, 55oC for 1 minute 5 seconds and 95oC continuous; and finally, cooling at 40oC for 10 seconds. The conditions were identical for U6 snRNA and 5S rRNA, except the denaturation step was 1 cycle at 95oC for 6 seconds.

We verified that the amplification of each PCR product was specific using a melting curve analysis. The amplification efficiency was determined for both target and reference genes. Each assay was performed at least in triplicate. The quantification

cycle (Cq) was performed using LightCycler 480 Quantification software (Roche, Mannheim, Germany). For further data analysis, only those miRNAs with a Cq value equal to or below 35, representing detection of one single-molecule template [1] were considered. Positive and negative controls were included in each experiment.

The Relative Expression Software Tool (REST) was used to analyse the relative miRNA expression in each sample and to determine the fold difference for every miRNA. The expression levels of the target miRNAs were standardised using an index containing 5S rRNA and U6 snRNA.

MiRNA analyses were performed with no knowledge of the clinical or follow-up data.

Supplemental results

Online data about tumour expression of miR-141 and miR-200c and prognosis in breast cancer patients

The online tool MIRUMIR was used to perform Kaplan Meier survival analysis. In the first dataset [2], GEO accession number GSE37405, low miR-141 tumour expression (Additional file 2) and low miR-200c tumour expression (Additional file 3) were associated with a reduced overall survival in high-risk oestrogen receptor positive BC patients (P-values corrected by FDR, 0.03308) and 0.02324, respectively]. By contrast, in the second dataset [3], GEO accession number GSE22216, that included 189 early primary BC patients, no survival differences were found according to miR-141 (Additional file 4; P = 0.486) and miR-200c (Additional file 5; P = 0.469) tumour expression. The PROGmiR tool available at http://www.compbio.iupui.edu/progmir was also used to study overall survival implications for miR-141 and miR-200c in BC. The dataset (https://tcga-data.nci.nih.gov/tcga) include survival data of 727 cases of invasive breast carcinoma. The Kaplan-Meier survival plots for miR-141 and miR-200c are given. Overall survival at 3 and 5 years were not significantly different according tumour levels of miR-141 (Additional file 6) and miR-200c (Additional file 8). However, with a longer follow-up, the survival times became significantly better in the high microRNA expressions groups (Additional files 7 and 9). The hazard ratio and P values for the proportional hazards model are also given.

Supplemental references

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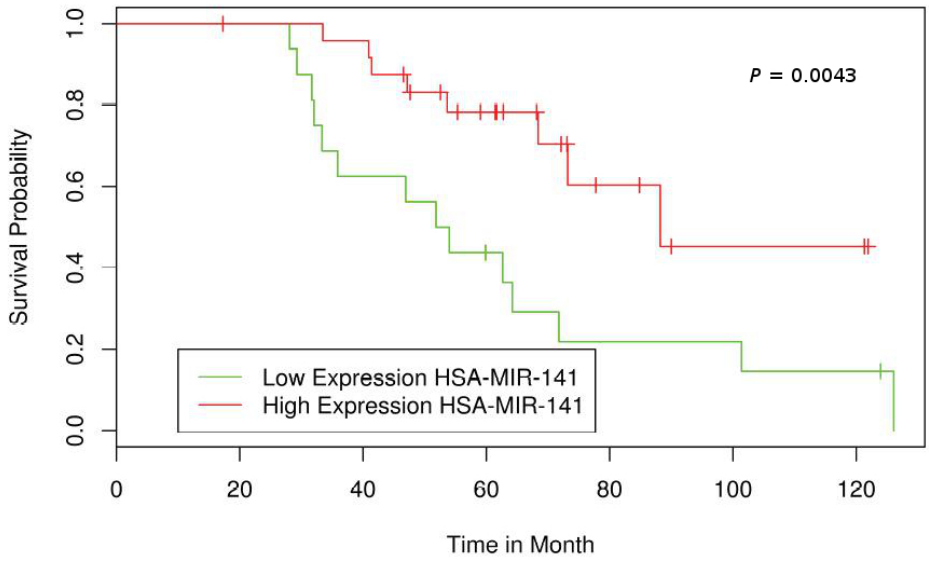
Additional file 2.



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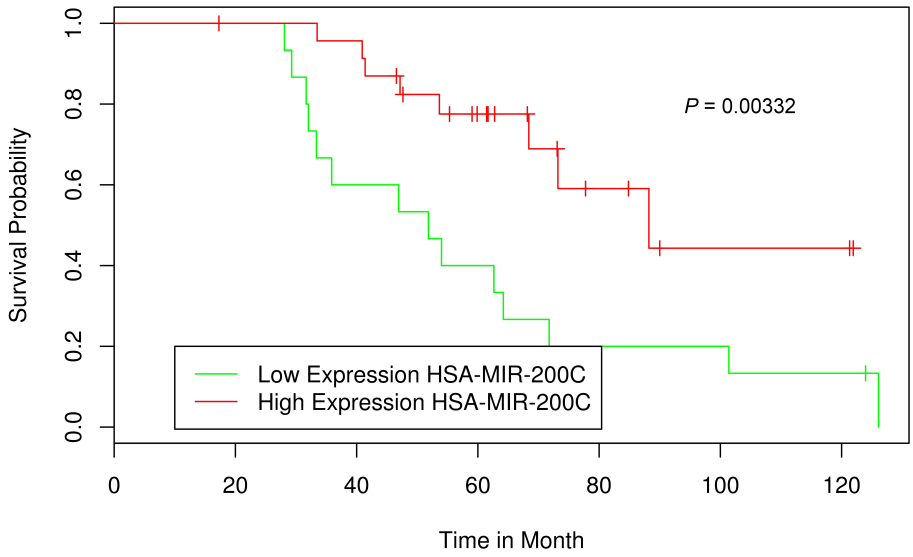

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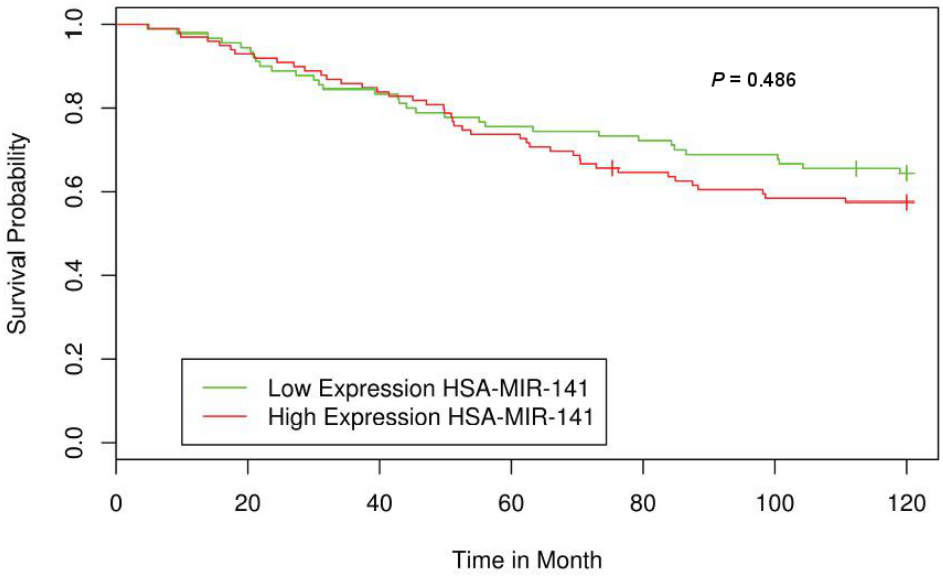

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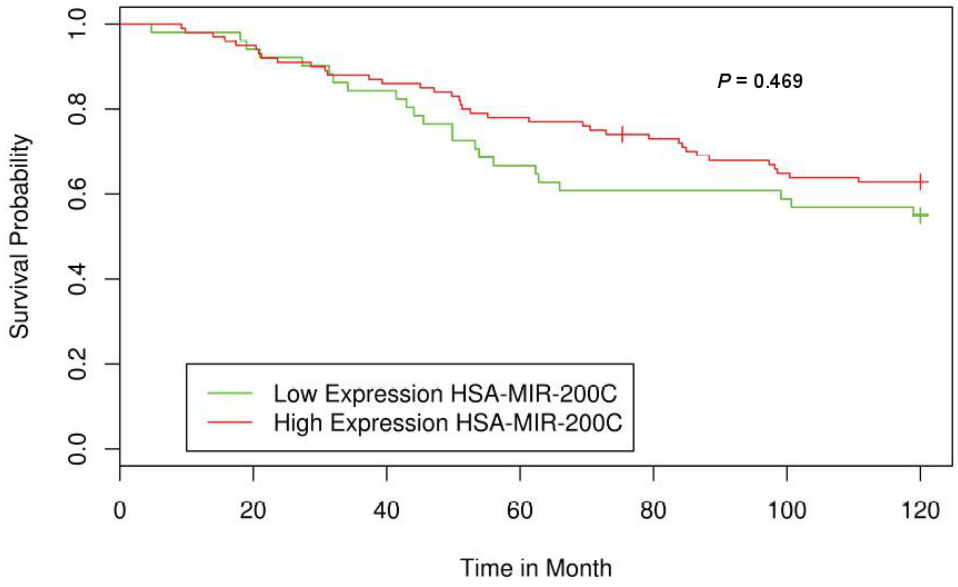

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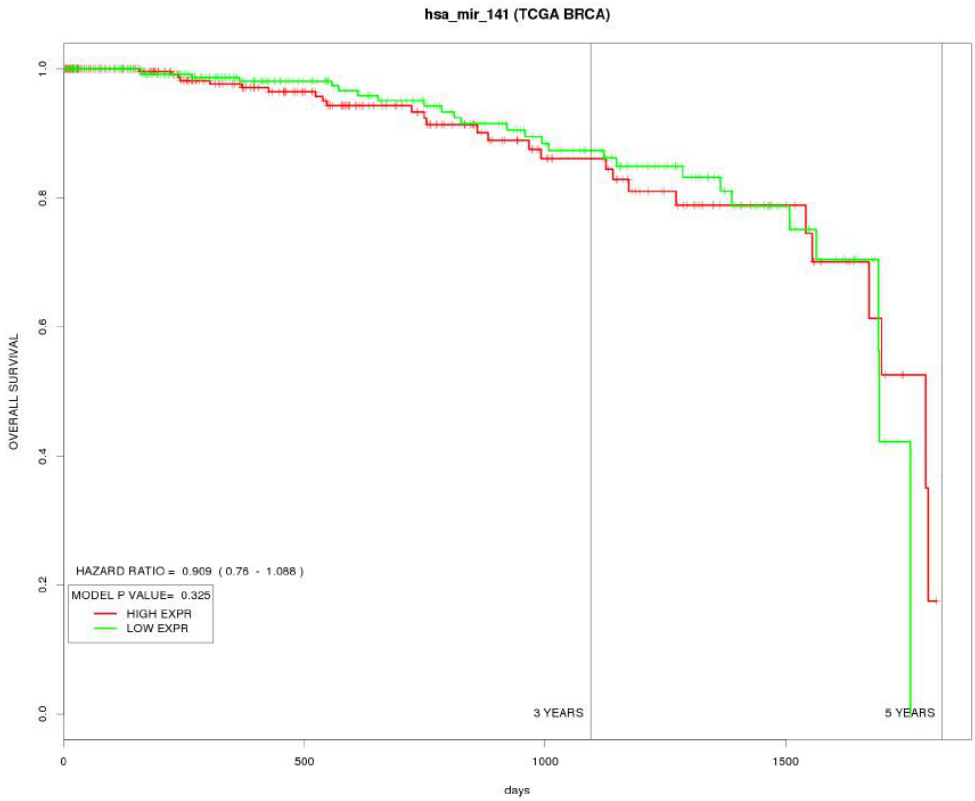

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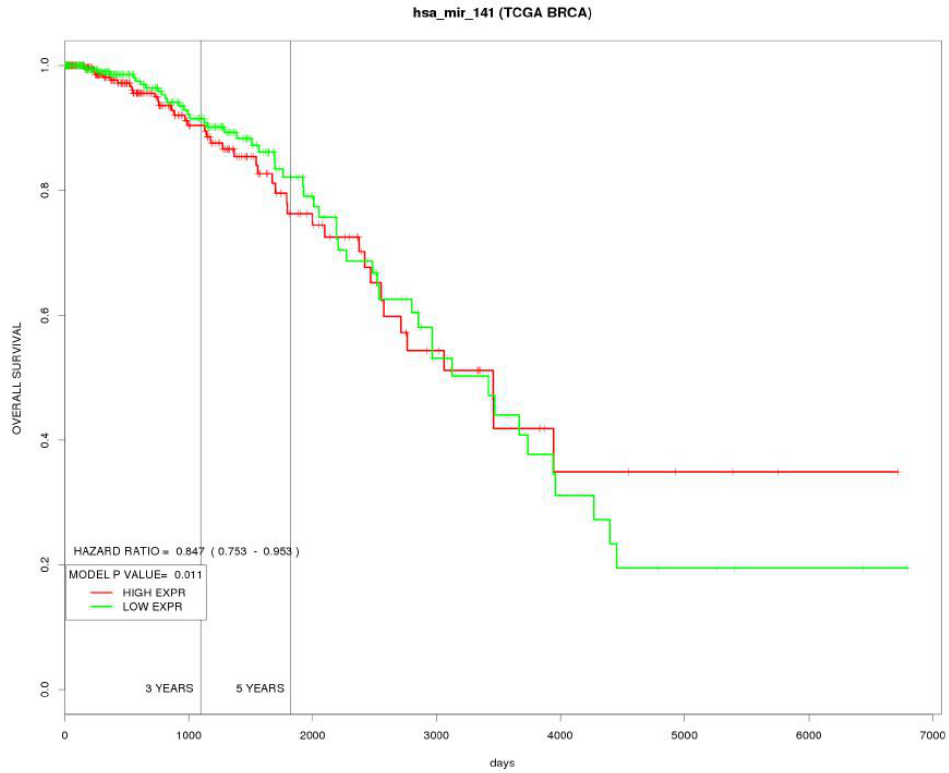

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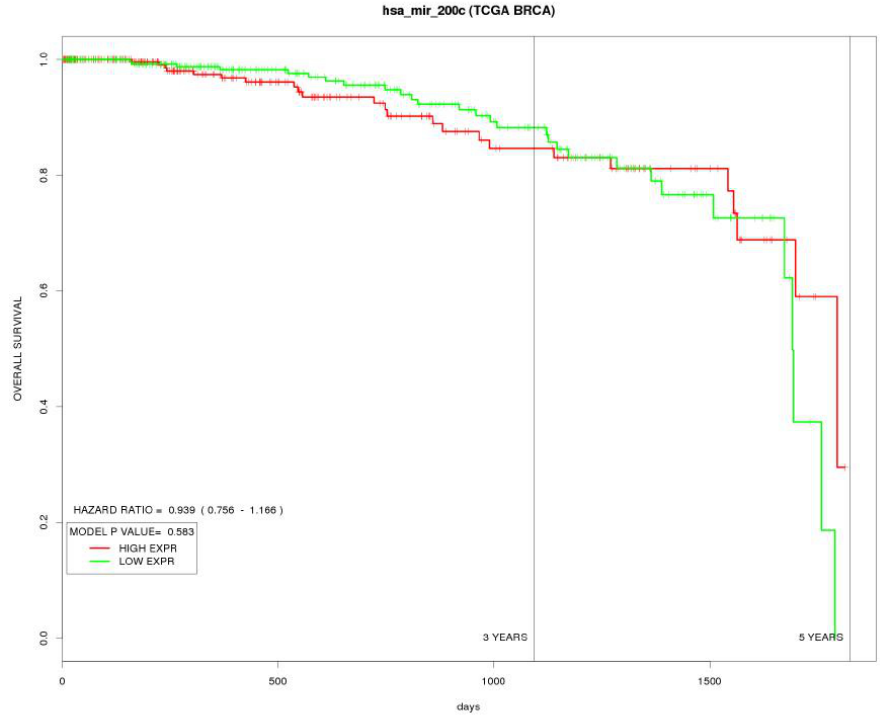

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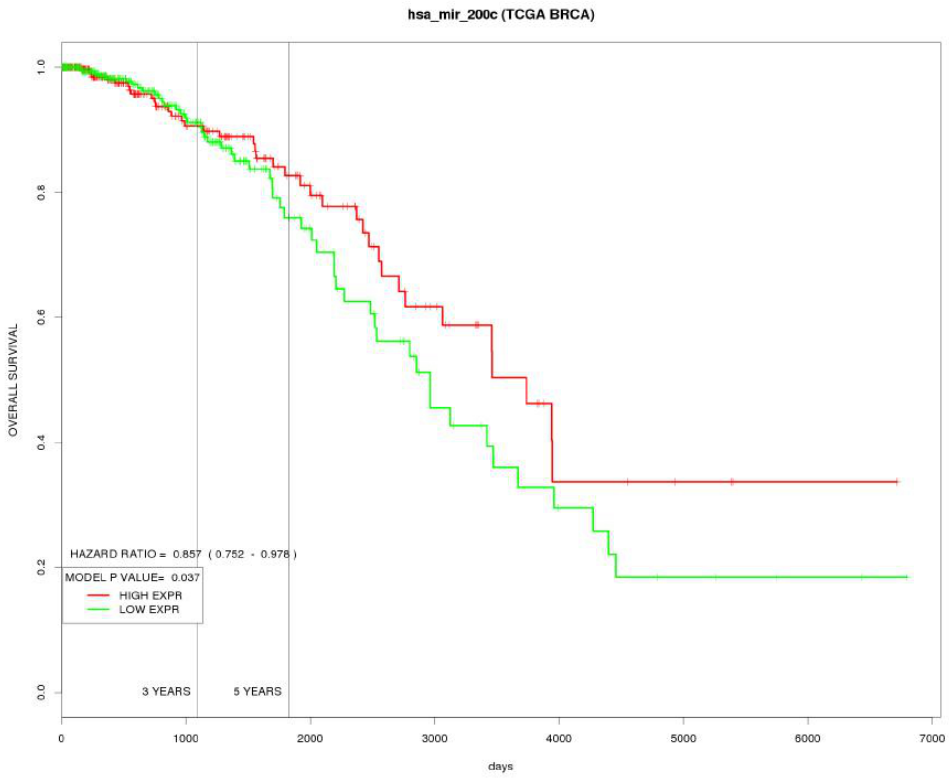

Additional file 10.

Comparative of miR-141 and miR-200c expression in genome-wide circulating miRNA profiling studies in early breast cancer patients.

Study	**Zhao**		**Schrauder**		**Leidner**	
GEO accession number	GSE22981		GSE31309		GSE41526	
Platform	Illumina BeadChip v2		Genion Bioarray		Illumina BeadChip v2	
Substrate	Plasma		Whole Blood		Plasma	
N case / control	20	20	48	57	40	20
Timing of Blood Collection	Presurgery		Presurgery		Pre-op 20 / Post-op 20	
miR-141 (Plattform identification)	ILMN_3168064		188		ILMN_3168064	
Mean ± SD	6.02 ± 1.18	5.82 ± 0.24	5.99 ± 0.53	6.24 ± 0.44	644.02 ± 905.48	509.76 ± 485.99
Range	5.62-10.99	5.66-6.7	4.81-6.87	4.74-7.2	-3.7-4125.7	-0.8 - 1706.2
***P* value**	0,914		***0,029***		0,931	
miR-200c (Plattform identification)	ILMN_3167002		313		ILMN_3168702	
Mean ± SD	9.42 ± 2.41	9.1 ± 2.16	6.25 ± 0.69	6.42 ± 0.49	2407.1 ± 1818.63	2115.77 ± 1474.42
Range	7.07-13.13	6.87-12.89	4.21-7.4	5.09-7.58	93.9-7436.2	416.9- 5231.5
***P* value**	0,525		0,407		0,712	
miRNA Spearman's Correlation	-0,860		0,006		-0,215	
***P* value**	0,599		0,950		0,099	

5. DISCUSIÓN GLOBAL

5. DISCUSIÓN GLOBAL

La diseminación de células tumorales se considera un evento precoz en el desarrollo y la progresión del cáncer. La diseminación hematógena de las células neoplásicas es considerada como la vía fundamental que conducirá a la aparición de metástasis clínicamente relevantes. La presencia de células tumorales en médula ósea actuaría no solo como foco inicial de futuras metástasis óseas, sino como reservorio a partir del cual podrían diseminarse y circular en la sangre secundariamente las células cancerosas [92] y como indicador de la presencia de colonización tumoral visceral a otros niveles. En el desarrollo de los trabajos que presentamos, se aborda el problema de la detección y evaluación del significado clínico de esta enfermedad micrometastásica desde varias vertientes o enfoques.

Primero debemos considerar los dos grandes grupos de pacientes estudiados. Por un lado, pacientes con cánceres gastrointestinales (n= 144), incluyendo las entidades más frecuentes como el cáncer de colon y recto (CCR), el cáncer gástrico y el cáncer de páncreas y por otro lado, el cáncer de mama (n= 57). Indudablemente, el comportamiento clínico y biológico, las alteraciones genéticas y moleculares y las aproximaciones terapéuticas de estas distintas entidades son diferentes. Sin embargo, la presencia de CTD en MO y de CTC, como manifestaciones de enfermedad subclínica micrometastásica, ha sido demostrada en los distintos tumores [17, 22-24, 42-44, 47, 49] y podría considerarse como factor pronóstico. Así, en los distintos artículos presentados en esta tesis, la presencia de EMR se ha asociado con un peor pronóstico. En el primero de los trabajos, la presencia de CTD en MO en cáncer gastrointestinal, detectadas mediante ICQ y criterios estandarizados, se asoció con peor supervivencia global y peor

supervivencia libre de progresión, con diferencias estadísticamente significativas en el análisis multivariante. La presencia de estas CTD en MO no se asoció con otras características clínico-patológicas y se evaluó la potencial relación entre la desregulación de microRNAs en el tumor primario con la presencia de CTD. Aunque no se demostró una correlación con la presencia de CTD, la SG de los pacientes con sobreexpresión de miR-17 (previamente denominado miR-17-5p) y de miR-20a fue significativamente inferior en las curvas de Kaplan-Meier. La sobre-expresión de miR-17 mantuvo su valor pronóstico independiente en el análisis multivariante. Recientemente distintos trabajos han corroborado estos resultados, demostrando el papel de mir-17 en la progresión de la secuencia adenoma-carcinoma [125] y el valor pronóstico de miR-17 en cáncer colorrectal, mediando la resistencia a fármacos, a través de la inactivación de PTEN [126] . En modelos experimentales, miR-17 incrementó la capacidad de invasión de las células tumorales [125] y la proliferación celular [127,128] incluyendo a las células progenitoras.

En los tres siguientes trabajos igualmente la presencia y cuantificación de biomarcadores de RNA (mRNA y miRNAs) en la sangre se asoció con un peor pronóstico en cáncer colorrectal (artículo 2), en cáncer gástrico (artículo 3) y en cáncer de mama (artículo 4).

En segundo término, analizamos de manera global dos compartimentos distintos, la médula ósea (en 38 pacientes) en la primera de las publicaciones y la sangre (globalmente en 163 pacientes) en las tres siguientes. La detección de CT en cada uno de estos compartimentos ha requerido distintas aproximaciones. La ICQ es considerado como el método de referencia para el análisis de CTD en MO. Su valor pronóstico ha sido establecido fundamentalmente en CM y, con menor evidencia, en

otros tumores como el cáncer colorrectal [22-24, 46]. La ICQ se basa en una evaluación morfológica e inmunológica, permite una aproximación semicuantitativa y demostraría de manera directa la presencia de CTD. Sin embargo, la obtención de la MO es más inconveniente para los pacientes y las técnicas de obtención, procesado y evaluación de las preparaciones son laboriosas. El análisis de muestras de sangre, que en sentido amplio podría definirse como una "biopsia líquida" [27-30] es claramente más aceptable y más fácil de implementar en la clínica. Sin embargo debido al menor número de CTC, se requiere de métodos con mayor sensibilidad. Así, se han empleado métodos basados en la PCR para la detección indirecta de las CTC en sangre [60-64]. Estos métodos de análisis de ácidos nucleicos se basan de manera global en la demostración, y de manera óptima en la cuantificación, de secuencias moleculares específicas de las células tumorales, como por ejemplo translocaciones y mutaciones o de transcritos (por ejemplo marcadores de diferenciación epitelial) en compartimentos en donde estas secuencias no deberían encontrarse, al menos con una expresión por encima de un determinado umbral. Quizás esta necesidad de una adecuada sensibilidad y una alta especificidad pueda considerarse uno de los factores limitantes a la hora de aceptar en la clínica el empleo de marcadores moleculares indicativos de la presencia de CTC en sangre.

Así, como nuevo enfoque reseñable, tres de los trabajos encuadrados en esta tesis se ocupan esencialmente de la búsqueda sistemática de nuevos biomarcadores basados en RNA en sangre y de su análisis de manera prospectiva en la clínica.

La selección inicial de los diferentes marcadores moleculares de mRNA de CTC se ha efectuado con un diseño de estudio preclínico de fase I [129] y mediante una combinación de análisis bioinformático, mediante la herramienta "Digital Differential

Display" [130], análisis de la literatura y del estudio bioestadístico de los datos de expresión publicados en bases de datos de disposición pública. Adicionalmente hemos evaluado la expresión de los diferentes mRNA y miRNAs en distintas líneas celulares tumorales. Los resultados de nuestros trabajos demuestran la validez de estas aproximaciones para la búsqueda y selección de nuevos biomarcadores [99,131]. Así, en el primero de los trabajos publicado en 2012 (artículo 2), se analizan dos nuevos biomarcadores de mRNA, AGR2 y LGR5, en sangre en pacientes con carcinomas de colon y recto. En los dos trabajos siguientes (artículos 3 y 4), se evalúa el potencial de los microRNAs como indicadores moleculares indirectos de la presencia de CTC en sangre en pacientes con cánceres gástricos y de mama.

Distintas publicaciones han demostrado como existe una heterogeneidad en las características de las CTC, con diferentes capacidades tumorogénicas [73, 74] y con células en procesos de plasticidad epitelio-mesenquimal [106], con pérdida de la expresión de marcadores epiteliales clásicos incluyendo Ep-CAM, CKs y E-cadherina. Resulta por tanto de especial relevancia contar con marcadores moleculares de CTC que se expresen durante estos procesos de plasticidad epitelio-mesenquimal que experimentan las células neoplásicas durante su migración en el torrente circulatorio, pero también que reflejen de alguna forma el potencial de las células de actuar como "iniciadoras" de metástasis [132].

El gen AGR2 codifica una proteína secretada de 17 kDa, que pertenece a la familia de las proteínas disulfuro isomeras [133]. Inicialmente se seleccionó como potencial mRNA biomarcador de CTC en base a los resultados de expresión obtenidos mediante DDD y en líneas celulares y especímenes de cáncer gastrointestinal [131].

Sus potenciales funciones en cáncer y su papel oncogénico han sido recientemente revisados [134]. En un trabajo reciente y con resultados concordantes con nuestros hallazgos previos, la expresión en sangre del mRNA de AGR2, junto con CK19 y CK20, permitió identificar la presencia de CTC en pacientes con CCR, aún en ausencia de células circulantes mediante el sistema Cell-Search [135].

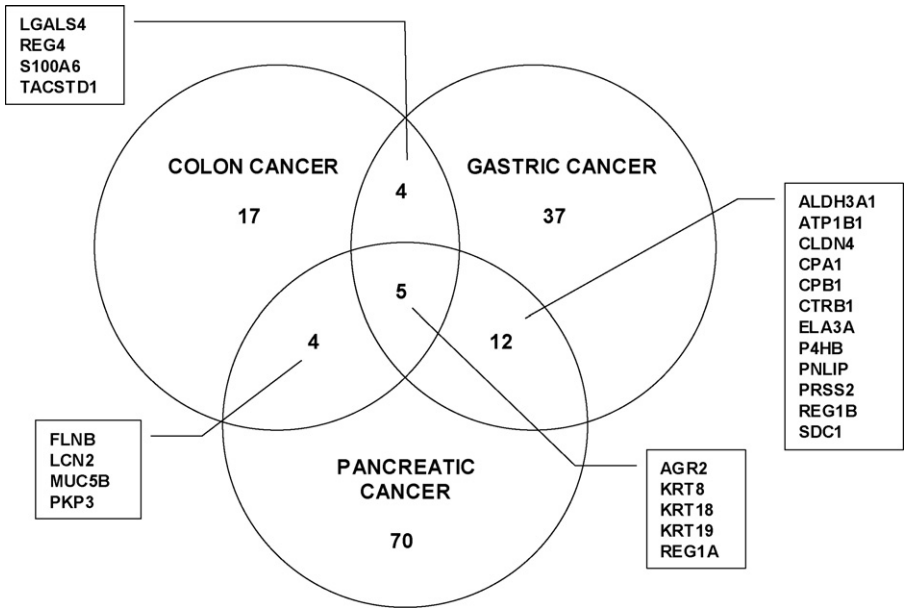

Figura 5. Diagrama de Venn que muestra los genes sobre expresados en bibliotecas de cDNA de cánceres colónicos, gástricos y pancreáticos, mediante DDD. Obtenido de Valladares-Ayerbes M, et al. [131].

El gen LGR5 (receptor 5 acoplado a proteína G rico en residuos con leucina) ha sido identificado recientemente como marcador de células progenitoras en distintos tejidos incluyendo el epitelio colónico [136] y en cáncer colorrectal [137], favoreciendo la proliferación celular a través de la activación de la vía Wnt/β-catenina [138].

En nuestro estudio, presentado en el artículo 2, evaluamos la expresión de LGR5 en sangre como potencial marcador de células tumorales circulantes con fenotipo de células progenitoras. Recientemente, Kantara C, et al. [139] han demostrado la presencia, en sangre en ratones con xenoinjertos de tumores de colon y en pacientes con CCR, de células LGR5 positivas y con capacidad de formar esferoides multicelulares, característica funcional asociada con las células progenitoras. Resulta interesante reseñar como estas células tumorales, aunque expresan marcadores epiteliales como CK19, no presentan expresión de Ep-CAM, quizás debido a procesos de EMT. Estos hallazgos pueden explicar de alguna forma, resultados falsos negativos que se obtendría con métodos de identificación o aislamiento de CTC basados en la expresión de este marcador [100, 140, 141].

Como se ha referido previamente, el papel de los microRNAs en la regulación de diferentes procesos relacionados con cáncer [120] es cada vez más reconocido. Adicionalmente, distintos trabajos han puesto de manifiesto la presencia de miRNAs circulantes en sangre en los pacientes con cáncer, postulándose su potencial valor para el diagnóstico y la estratificación del pronóstico. En los artículos 3 y 4 que presentamos, la cuantificación de miR-200c en sangre permitió identificar subgrupos de pacientes con cáncer gástrico y con cáncer de mama con un pronóstico diferente. Los microRNAs de la familia miR-200 participan como reguladores clave en el mantenimiento del fenotipo epitelial y de la plasticidad epitelio mesenquimal. Se ha sugerido un papel dual en el proceso de las metástasis: su regulación a la baja favorecería la invasión y la circulación de células tumorales y su re expresión en los células diseminadas favorecería el establecimiento de las colonias metastásicas. El potencial valor pronóstico de la cuantificación de miR-200s dependería por tanto del contexto en que se determinara, del tipo de tumor y del momento evolutivo de la

enfermedad. Así, en tres recientes meta-análisis se ha demostrado cómo la determinación de niveles aumentados de miR-200c en sangre (incluyendo plasma o suero) se asocia significativamente con una peor supervivencia en pacientes con cáncer [142-144].

En los tres trabajos que presentamos respecto a la detección de CTC en sangre se emplea una metodología basada en RT-qPCR. La expresión de los diferentes biomarcadores se cuantificó mediante SYBR-green, de manera relativa. Se estimaron los puntos de corte mediante análisis de las curvas ROC, incluyendo un número significativo de controles sin cáncer (en total, n= 58). El aislamiento de los biomarcadores moleculares (mRNA o miRNA) se efectuó a partir de sangre completa. Distintos autores han propuesto el empleo de métodos de "enriquecimiento" de las posibles CTC en sangre, bien mediante sistemas inmunomagnéticos, métodos de filtración en función del tamaño celular o por gradientes de densidad. Nuestros trabajos analizan los biomarcadores de RNA en la sangre completa, ofreciendo en teoría la posibilidad de detección del RNA celular y del presente en plasma, suero, unido a proteínas o en partículas subcelulares [145].

De manera global, la presencia de enfermedad micrometastásica, tanto en MO como en sangre y con las distintas metodologías, se ha asociado en cada uno de los grupos de pacientes analizados en los trabajos presentados, con un peor pronóstico. Distintos estudios de cohortes, análisis sistemáticos y meta-análisis han confirmado este valor pronóstico. Sin embargo, cada uno de los métodos podría tener ventajas en situaciones clínicas concretas. Por ejemplo, a pesar de la aprobación del sistema CellSearch para la detección de CTC por las autoridades sanitarias, su introducción

sistemática en la práctica clica resulta aún controvertido, no siendo recomendado, por ejemplo en cáncer de mama [146].

En el CCR metastásico la detección de CTC en sangre mediante el sistema CellSearch se correlaciona con un peor pronóstico [84, 87, 147]. Se está evaluando en ensayos clínicos prospectivos [148-149] si seleccionar la intensidad del tratamiento en función del número de CTC en sangre, entre otros parámetros, puede ser de utilidad para los pacientes con CCRm. Sin embargo, la sensibilidad del sistema CellSearch puede no ser adecuada para detectar la presencia de EMR en pacientes con CCR en estadios más precoces [150]. En estos pacientes, el estudio de MO mediante ICQ o métodos moleculares en SP serían más adecuados para identificar en estudios prospectivos, por ejemplo, que pacientes podrían beneficiarse de tratamiento complementario o para monitorizar la eficacia de este tratamiento.

Los distintos biomarcadores de mRNA (AGR2 y LGR5) y de microRNA (miR-200s, especialmente miR-200c) analizados en los trabajos que presentamos, podrían formar parte de paneles de múltiples biomarcadores potencialmente útiles para la estratificación pronostica en pacientes con cáncer avanzado y para la identificación de subgrupos de pacientes con enfermedad tumoral precoz y mayor riesgo de recaída. Esta hipótesis debería ser evaluada en el contexto de estudios clínicos prospectivos.

6. CONCLUSIONES

6. CONCLUSIONES

6.1. En cáncer colorrectal, gástrico y pancreático, el estudio inmunocitoquímico con anticuerpos anti-CK demostró la presencia de CTD en un 42,1% de los pacientes.

6.2. La presencia de CTD no se asoció con el estadio, el grado histológico, la invasón vascular o perineural o la extensión de la resección quirúrgica. El perfil de expresión de los microRNAs miR-17, miR-20a y miR-21 no se asoció con la presencia de CTD.

6.3. La detección de CTD se asoció con un peor pronóstico, de manera independiente. El aumento en la expresión de microRNAs de la familia miR-17-92 en los tumores se asoció con peor SLP y peor SG, en el análisis multivariante.

6.4. Los niveles del mRNA de AGR2 en sangre en pacientes con cáncer de colon y recto se encuentran elevados respecto a los controles. El nivel de LGR5 se encontró elevado en los pacientes con estadio IV frente a los estadios más precoces y los controles. No existió correlación entre los niveles de ambos mRNAs.

6.5. En los pacientes con CCR la expresión en sangre de AGR2 se correlacionó con estadios pT3–pT4 y con el grado histológico. LGR5 se correlacionó con el grado histológico y la cirugía R2. La cuantificación de AGR2 y LGR5 en sangre se asoció de manera independiente en el estudio multivariante, con una peor supervivencia libre de progresión, pero no con la SG.

6.6. Mediante RT-qPCR es posible detectar y cuantificar en sangre la presencia de microRNAs epiteliales específicos, como los microRNAs de la familia de miR-200s.

6.7. Los niveles de mir-200c en sangre pudieron diferenciar con un área bajo la curva ROC de 0,715, una sensibilidad del 65,4% y una especificidad del 100%, a los pacientes con cáncer gástrico de los

controles. En pacientes con cáncer de mama, los niveles de mir-200c y miR-141 en sangre se encontraron desregulados, respecto a los controles. Las pacientes con cáncer de mama globalmente presentaron niveles significativamente reducidos de miR-200c, con un AUC de 0.79 (sensibilidad de 90% y especificiad de 70.2%).

6.8. En pacientes con cáncer gástrico, el nivel de mir-200c en sangre se asoció con el estadio y con el número de gánglios linfáticos afectos. En cáncer de mama los niveles de miR-200c no se asociaron significativamente con parámetros clínicos y patológicos analizados. La expresion en sangre de miR-141 en cáncer de mama fue superior en los estadios I-III, en pacientes con gánglios positivos y en tumores HER2-negativos

6.9. En la cohorte de pacientes con cáncer gástrico, en el estudio multivariante, el nivel de miR-200c en sangre se asoció significativamente con la SLP y la SG. En las pacientes con cáncer de mama, niveles en sangre más elevados de miR-200c, la reducción de miR-141, o la combinación de ambos factores, se asoció de manera significativa con la SG y con la SLP, de manera independiente.

En base a estas conclusiones experimentales, podemos afirmar:

I). La presencia de enfermedad mínima residual, detectada por inmunocitoquímica y RT-PCR, en pacientes con tumores digestivos y cáncer de mama supone un factor pronóstico desfavorable.

II). El análisis del perfil de expresión de microRNAs de la familia miR-17-92 en el tumor, puede añadir información pronostica independienete de la detección de CTD.

III). Biomarcadores de RNA en sangre, como AGR2 y LGR5 en cáncer colorrectal, miR-200c en cáncer gástrico y miR-200c y miR-141 en cáncer de mama tienen un valor pronóstico y son de utilidad para la estadificación molecular.

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