ORIGINAL ARTICLE

Circulating MicroRNAs in blood of patients with prostate cancer

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KEYWORDS
Prostate cancer; MicroRNAs; Micrometastasis; Polymerase chain reaction

Abstract
Introduction: MicroRNAs (miRNAs) are small regulatory RNAs that do not code for proteins. Detection of circulating tumor cells (CTC) would provide diagnostic and prognostic information in prostate tumors (PT). Thus, miRNAs could constitute a promising new class of biomarkers for CTC detection.
Objectives: To analyze circulating microRNAs in whole blood as non-invasive markers in patients with localized prostate cancer and healthy individuals.
Material and methods: A preliminary study including a population of 40 patients with mean age of 71 years and mean PSA of 18.9 ng/ml (range). Regarding the risk group (RG): 33.3% had low risk, 30% intermediate risk and 36.7% high risk. A previous in silico study identified 92 candidates and was followed by another in vivo to verify the findings of the former using array technology by real-time PCR.
Results: Statistical analysis of the results revealed 10 microRNAs candidates with statistically significant differential expression between the different risk groups and healthy controls: hsa-miR-337-3p, hsa-miR-330-3p, hsa-miR-339-3p, hsa-miR-124, hsa-miR-218, hsa-miR-128, hsa-miR-10a, hsa-miR-199b-5p, hsa-miR-200b and hsa-miR-15b.
Conclusions: Our data suggest that circulating microRNAs can act as biomarkers to identify risk groups in CaP.

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PALABRAS CLAVE
Cáncer de próstata; MicroARN; Micrometástasis; Reacción en cadena de la polimerasa

MicroARN circulantes en sangre de pacientes con cáncer de próstata

Resumen

Introducción: Los microARN (miARN) son ARN reguladores de pequeño tamaño que no codifican para proteínas. La detección de células tumorales circulantes (CTC) proporcionaría información diagnóstica y pronóstica en los tumores de próstata (TP). En este sentido los miARN podrían constituir una nueva y prometedora clase de biomarcadores para la detección de CTC.

Objetivos: Analizar miARN circulantes en sangre total como marcardores no invasivos en pacientes con cáncer de próstata localizado e individuos sanos.

Material y métodos: Estudio preliminar con una N poblacional de 40 pacientes con una média de edad de 71 años y un PSA medio de 18, 9 ng/ml (rango). Respecto al grupo de riesgo (GR): un 33,3% bajo riesgo, un 30% riesgo intermedio y un 36,7% alto riesgo. Se realizó un estudio previo in silico que identificó 92 miARN candidatos seguido de otro in vivo para verificar los hallazgos del primero mediante tecnología de arrays de PCR a tiempo real.

Resultados: El análisis estadístico de los resultados reveló 10 miARN candidatos con una expresión diferencial estadísticamente significativa entre los distintos grupos de riesgo y los controles sanos: hsa-miR-337-3p, hsa-miR-330-3p, hsa-miR-339-3p, hsa-miR-124, hsa-miR-218, hsa-miR-128, hsa-miR-10a, hsa-miR-199b-5p, hsa-miR-200b y hsa-miR-15b.

Conclusiones: Nuestros datos sugieren que los miARN circulantes pueden servir como biomarcadores para identificar grupos de riesgo en CaP.

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Introduction

Prostate cancer (PCA) is the most common malignancy in men in western countries and the second most common cause of death from cancer. Rectal examination (RE) and prostate-specific antigen (PSA) are usually applied for the early detection of PCAs. However, the diagnostic value of both methods is limited due to low sensitivity (RE) or low specificity (PSA test). Therefore, a search for new, improved biomarkers is necessary for the diagnosis of PCAs.

MicroRNA (miRNA) has been estimated to modulate the expression of approximately 30% of the protein-encoding genes in humans. An altered expression or the dysfunction of miRNA pathways might affect divergent cellular processes, including the cell cycle, differentiation and proliferation, thereby influencing tumorogenesis and progression.

miRNAs can be exported by cells and circulate in the human blood in a stable form, some circulating miRNAs can help distinguish between patients and healthy individuals. These findings imply their possible use as non-invasive markers to monitor the progression of the disease. In the case of prostate cancer, several previous works have demonstrated that miRNAs derived from epithelial tumors can be detected in blood samples and some are potentially correlated with the risk of disease progression or proved to be predictors of disease aggressiveness. Thus, in this work, we explore the possibility of identifying miRNA genes as signatures of circulating genetic material in PCAs.

Materials and methods

Clinical blood samples

In the period between September 2010 and October 2012, 78 patients diagnosed with non-metastatic PCAs who had not received any neoadjuvant hormonal or radiotherapeutical treatment were selected for our study. They were diagnosed by an echo-directed transrectal biopsy along with the taking of 10 cylinders and following the performance of PSA analysis. Out of this population, 30 patients were selected for initial analysis; the other 10 cases in the study are represented by the healthy population (N=10). The mean age was 71 years (range 56–83), the mean total PSA 25.73 ng/ml (range 2.2–425), free PSA 1.09 ng/ml and the mean free/total PSA 17.46%, with a mean of 41% of positive cylinders and a median of 7 for Gleason grade. Perineural involvement was present in 20% of the samples in the pathological anatomy study.

Those patients were classified into subgroups, obtaining 10 (33.3%), 9 (30.0%) and 11 (36.7%) patients with low-, intermediate- and high-risk PCAs, respectively. Ten patients with low-risk prostate cancer showed a mean age of 69 years, mean total PSA of 5.82 ng/ml and mean PSA l/t of 19%, with a mean of 17% of positive cylinders at biopsy, with a Gleason score of 6 and with no perineural involvement in any sample.

Nine patients classified as intermediate risk showed a mean age of 71 years, mean total PSA of 4.31 ng/ml and mean PSA l/t of 14%. The mean of positive cylinders was of 31% with a median of 7 for Gleason grade.

Eleven patients constituted the high-risk group with a mean age of 74 years, mean total PSA of 61.36 ng/ml, mean PSA l/t of 21%, a mean of 77% of positive cylinders and a median of 8 for the Gleason grading system. We observed data of perineural infiltration in 45% of cases.

10 control patients were also recruited and matched by mean age with the individuals affected by PCAs. All patients gave their consent to donate biological material, in compliance with the requirements of the Clinical Research Ethics Committee of Galicia.

The clinical features of the patients are shown in Table 1.
Table 1  Clinical characteristics of the patients.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Prostate cancer</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± DT</td>
<td>71 ± 6</td>
<td>65 ± 7.5</td>
</tr>
<tr>
<td>Median (range)</td>
<td>70 (56–85)</td>
<td>69 (53–74)</td>
</tr>
<tr>
<td>Clinical stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cT1c</td>
<td>18 (60%)</td>
<td></td>
</tr>
<tr>
<td>cT2a</td>
<td>10 (33.3%)</td>
<td></td>
</tr>
<tr>
<td>cT2c</td>
<td>1 (3.3%)</td>
<td></td>
</tr>
<tr>
<td>cT3</td>
<td>1 (3.3%)</td>
<td></td>
</tr>
<tr>
<td>PSA group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2.5</td>
<td>1 (3.3%)</td>
<td></td>
</tr>
<tr>
<td>2.5–10</td>
<td>21 (70%)</td>
<td></td>
</tr>
<tr>
<td>10–20</td>
<td>2 (6.67%)</td>
<td></td>
</tr>
<tr>
<td>&gt;20</td>
<td>6 (20%)</td>
<td></td>
</tr>
<tr>
<td>Risk group according to biopsy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>11 (36.7%)</td>
<td></td>
</tr>
<tr>
<td>Intermediate</td>
<td>9 (30%)</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>10 (33.3%)</td>
<td></td>
</tr>
<tr>
<td>Perineural involvement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>6 (15.8%)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>23 (76.67%)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Gleason</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>10 (26.3%)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>11 (36.67%)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>5 (13.2%)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>4 (10.5%)</td>
<td></td>
</tr>
</tbody>
</table>

MicroRNA extraction and real-time polymerase chain reaction with quantitative reverse transcriptase

Peripheral blood samples were taken from patients before therapy and from EDTA control tubes. In order to isolate the miRNA fraction, we used the Ribopure kit following the isolation protocol by preserving small RNAs (Applied Biosystems, Foster City, CA, USA). The procedure was performed using 0.5 ml of whole blood. These differential expression studies of risk groups with PCa were conducted on oligonucleotide membranes provided by the Hexion company on 96-well plates that included 92 human miRNAs collected in the miRBASE 16.0 database (http://www.mirbase.org/). Real-time PCR was performed using the LightCycler® 480 instrument (Roche, Mannheim, Germany).

The relative expression for each miRNA under study was calculated using the Relative Expression Software Tool (REST). 8

Design of the microRNA matrix

The genes studied in the oligonucleotide membrane are listed in Annex 1 (Supplementary material).

The RNA sequences to be analyzed were downloaded from the Sanger mirbase 16th databases (www.mirbase.org/).

The sequences listed in Annex 1 were studied using microRNA.org (http://www.mirnadb.org/), dbDEMC (http://159.226.118.44/dbDEMC/index.html), smiRNAdb (http://www.mirz.unibas.ch/cloningprofiles/), GeneHUB-GEPIS (http://research-public.gene.com/Research/genentech/genehub-gepis/index.html) and miRex (http://miracle.igib.res.in/mirex/) programs in order to simulate situations by using expression data from previous experiments. The in silico study of miRNA expression profiles was carried out by using the databases mentioned above to obtain information on those miRNA markers with a minimal expression in normal blood and a maximum expression in different sample types such as prostate adenocarcinoma tissue, LNCaP and PC3 cell lines and prostate tumor biopsies taken with a core needle, simulating in each case our subsequent in vivo study conducted on peripheral blood from patients affected by low-, intermediate- and high-risk PCa versus those healthy cases used as controls.

Statistical methods

The statistical analysis was performed using the statistical package SPSS 19.0 for Windows (SPSS, Chicago, IL) and a level of 0.05 was considered statistically significant.

Results

Circulating microRNAs in prostate cancer risk groups and controls

In order to investigate the differential expression levels of circulating microRNAs by using the real-time PCR technique, we compared miRNA expression profiles using the REST program in the different PCa risk groups and the control group of healthy subjects. This way, we obtained a list of upregulated and downregulated miRNAs for the high-, intermediate- and low-risk groups with respect to the prostate biopsy. Within the high-risk group, we found 68 upregulated and three downregulated circulating miRNAs, within the intermediate-risk group 12 upregulated and 32 downregulated ones and, finally, the low-risk group only provided 37 downregulated miRNAs. It is worth emphasizing in descending order of significance in terms of relative expressions hsa-miR-337-3p, hsa-miR-330-3p, hsa-miR-339-3p, hsa-miR-124, hsa-miR-218, hsa-miR-128, hsa-miR-10a, hsa-miR-199b-5p, hsa-miR-200b and hsa-miR-15b. Fig. 1 and Annex 1 (Supplementary material) show the graphics regarding the expression of these markers in the three risk groups.

By using the Multi Experiment Viewer 4.6.2 (MeV)-genomic analysis software, we built a heatmap (Fig. 2) with 92 differentially expressed miRNAs among the three risk groups under study and the healthy population. We applied a technique of hierarchical data classification to conduct a study on how many miRNA expression patterns were obtained and it resulted in four expression types. The first typology would include those miRNAs that were less expressed in the study population, and thereby in increasing order until reaching typology 4 which includes the most expressed miRNAs in the study population.
Figure 1  (A) Means of microRNA expression candidates by risk groups. The figure shows the distribution of means for -3p, miR330-3p, miR339-3p and miR214 markers in relation to the three risk groups studied in the patient population. (B) Means of microRNA expression candidates by risk groups. The figure shows the distribution of means for miR218, miR103, miR128, and miR199b-5p markers in relation to the three risk groups studied in the patient population. (C) Means of microRNA expression candidates by risk groups. The figure shows the distribution of means for miR200b and miR15b markers in relation to the three risk groups studied in the patient population.
Circulating MicroRNAs in blood of patients with prostate cancer

Diagnostic information

The hypothesis of our study was to forecast the values in a series of nominal variables, including the risk group in relation to the prostate biopsy, Gleason score and perineural involvement, thereby predicting which patients will be included in one category or the other of the dependant variables under study. In order to contrast the hypothesis stated, we took into account the different miRNAs studied that will act as indicators. In order to make predictions about the risk group and Gleason score variables, we used the technique of multivariate linear regression analysis (corrected $R^2 = 1$, $p < 0.01$), where the 92 miRNAs under study were combined to make a correct diagnosis of the dependant variable “risk group”, including the hsa-miR-188-5p, hsa-miR-187 and hsa-miR-196b markers.

For the dependant variable “Gleason score”, the regression equation (corrected $R^2 = 0.866$, $p < 0.01$) included the hsa-miR-135a marker.

Finally, the dichotomous variable “perineural involvement” was studied using binary regression techniques. Only the hsa-miR-339-3p marker was a part of the binary logistic regression equation ($p = 0.022$). The classification on the part of this marker in relation to the dependant variable is correct in 76.2% of the cases with no perineural involvement and in 83.3% of the cases that did show perineural involvement. The hsa-miR-339-3p marker appeared as a poor prognosis marker, since the relative risk $\text{Exp}(B) = 1.146$.

Discussion

Among solid tumors, PCa is one of the oncological diseases with a higher incidence in western countries. PCa is currently recognized as a multifactorial disease. Although an environmental contribution is recognized in the development of prostate cancer, it is believed that genetic predisposition plays an important role in the development of the disease. Lately, genomic research has provided material that, in the near future, will allow us to perform a classification at the molecular level, which will allow us to distinguish the different subtypes and to establish a better stratification of PCa.

Recently, several miRNA expression profiles have been reported in prostate tumors and they all share an extensive overall deregulation of miRNA. Besides, it was demonstrated that miRNA differential expression profiles in prostate cancer can be firmly correlated with clinical expression, thus being used as diagnostic and prognostic indicators. There is a large number of miRNAs which have been found to be abnormally expressed in prostate cancer, being directly associated with its development. Numerous miRNAs (oncomirs) are overexpressed in PCs, negatively regulating many tumor suppressor genes and leading to tumor growth and metastasis. For instance, hsa-miR-221 and hsa-miR-222 markers have been reported to be overexpressed in PCs, being directly related to metastasis and tumor growth through repression of the p27kip1 white gene. Another miRNA which also has oncomir function is the hsa-miR-21 gene, which is being overexpressed in PCs, playing a part in tumor growth, as well as in the invasion and metastasis processes. On the other hand, the tumor-suppressing role of miRNAs in PCs is associated with the ability to interfere with cell migration and invasion, as well as to mediate and promote cellular apoptosis. In fact, the loss of tumor suppressor miRNAs is a quite common mechanism associated with PCa. For example, the loss of hsa-miR-101 locus associated with the overexpression of gene EZH2 was found in 37.5% of the clinical samples of localized prostate cancer and in 66.7% of the samples of metastatic prostate cancer. Likewise, the overexpression of hsa-miR-101 is associated with low levels of EZH2, as well as with growth and invasiveness suppression in prostate cancer cells. In our work, we studied the expression profiles of 92 circulating miRNAs from patients with PCa and 10 healthy controls in order to determine the expression profiles of these miRNAs depending on the risk group in relation to the prostate biopsy and to associate the differential expression of these markers with different clinicopathological variables. To the best of our knowledge, this is the first study analyzing miRNA expression in whole blood from patients with localized PCa and controls. The statistical study of the results revealed 10 candidates as the best markers with differential expression among PCa risk groups and healthy controls: hsa-miR-337-3p, hsa-miR-330-3p, hsa-miR-339-3p, hsa-miR-124, hsa-miR-218, hsa-miR-128, hsa-miR-10a, hsa-miR-199b-5p, hsa-miR-200b and hsa-miR-15b.

MiRNAs have the ideal properties of a biomarker. Firstly, their expression is frequently deregulated in most human diseases. Secondly, numerous studies show that miRNAs can be used for cancer stratification, monitoring, prognosis and even therapy. Thirdly, a few years ago it became apparent that miRNAs circulate in plasma, serum and urine. This would allow for monitoring of blood levels, in a minimally invasive way for the patient and with low costs for the health system. Fourthly, their blood levels remain stable for considerable periods of time. Fifthly, miRNA quantification is a standardized and easy-to-implement method in a molecular biology laboratory. Finally, very recently, several studies began to be published demonstrating firm correlations between blood miRNA levels with the clinical of the patient (stratification, prognosis, therapeutic response) in several diseases, thus emphasizing the significant clinical value of circulating miRNAs.

In our study, the analysis of the association with the clinical of the patient showed that, on the whole, hsa-miR-188-5p, hsa-miR-187 and hsa-miR-196b would be good predictors to determine through a blood check the risk group of a patient affected by prostate cancer. In order to predict the Gleason score, the statistical analysis of our data showed that just determining hsa-miR-135a in blood and subjecting it to a linear regression equation is sufficient to predict it. Finally, in the case of the clinical variable “perineural involvement”, we found that knowledge of the relative expression of the hsa-miR-339-3p marker in blood would be sufficient to determine whether a patient shows this perineural invasion or not, along with the knowledge that it is also a risk marker, that is to say, the risk of perineural involvement increases for each unit increase in this marker.

The limitations of this study go hand in hand with a small sample size, a fact which will be complemented in the medium term by a broader panel of recruitment cases, assuming that a massive measurement of the candidates for
this study will determine its indications and diagnostic and
diagnostic profitability in a more accurate manner.

It should be emphasized that when we considered
publications related to the diagnostic and clinical potential of
the study of miRNA in fluids, we found a great heterogeneity
of studies and results in this regard. Differences in miRNA
evaluation, quantification and detection methods, the type
(pre-miRNA or mature forms) and number of the miRNAs
evaluated, their origin and collection time (serum, plasma
or blood cells obtained before or after surgery), as well as
the clinicopathological characteristics associated with each
of the patients in the study are variables which should be
taken into account when trying to explain these causes of
heterogeneity.

Our results confirm previous publications on (a) the possi-
bility of detecting miRNA in whole blood; (b) the levels of
miRNA expression given outline risk groups in PCa; and (c)
certain miRNAs included in the study stand out as promising
markers with a diagnostic potential.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data Associated with this article can be
found in the online version, at http://dx.doi.org/

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