MicroRNA determination in urine for prostate cancer detection in Mexican patients at the Hospital General “Dr. Manuel Gea González”


ABSTRACT
Multiple molecular markers have been studied in the search for a diagnostic test that will increase the probability of prostate cancer detection and reduce the number of unnecessary biopsies. MicroRNAs are constitutive elements of molecular identification of benign prostatic hyperplasia and prostate cancer and represent a possible tool that could contribute to the development of new opportune and reliable diagnostic strategies for prostate disease.

Objective: To determine the association of microRNA in urine for prostate cancer diagnosis in Mexican patients at Hospital General “Dr. Manuel Gea González”.

Methods: Thirty patients that underwent prostate biopsy from May-July 2010 were included in the study. A urine

RESUMEN
Se han estudiado múltiples marcadores moleculares, en busca de la prueba diagnóstica que aumente la probabilidad de detección de CaP y reduzca el número de biopsias innecesarias. Los microRNA son elementos constitutivos de identificación molecular de HBP y CaP y representan una posible herramienta que contribuya al desarrollo de nuevas estrategias de diagnóstico oportuno y acertado de la enfermedad prostática.

Objetivo: Determinar la asociación de microRNA en orina para el diagnóstico de CaP en pacientes mexicanos en el Hospital General Dr. Manuel Gea González.

Métodos: De mayo a julio de 2010 se incluyó a 30 pacientes sometidos a biopsia de próstata, quienes se les tomó una muestra de orina posterior a masaje prostático previo
sample was taken from each one after prostate massage prior to biopsy, determining microRNA concentration from the sample. Samples with concentrations above 50 ng/mL of microRNA (18/30 samples) were selected; from these samples microRNA amplification and microRNA-373 quantification with real time polymerase chain reaction method were carried out.

**Results:** Of the eighteen patients with microRNA above 50 ng/mL, nine had positive biopsy and nine had negative biopsy. Of the microRNAs-373 quantified, fifty microRNAs showed expression in the eighteen replicas (100%) that were carried out; only twenty-one of the fifty microRNAs were repeated in all samples; nineteen of them were overexpressed (miR-196b, -574-3p, let-7b, -7c, -7d, 7c, -7g, miR-200b, -149, -20b, -17, -184, -20a, -106a, -671-3p, -148a, -429, -31, -100) and only two were underexpressed (miR-150, -328).

**Conclusions:** A group of 21 microRNAs was determined that showed statistically significant expression in the prostate cancer sample group.

**Key words:** Prostate cancer, prostate specific antigen, benign prostatic hyperplasia, miRNA, Mexico.

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**INTRODUCTION**

Prostate cancer (CaP) is the second most frequent malignant neoplasia in men. The most recent figures indicate that 782,600 new cases were diagnosed during 2007 and the number of deaths associated with CaP during that year was estimated 254,000, making CaP the sixth cause of death by cancer in men worldwide.1 Currently, the principal diagnostic tests for CaP are digital rectal examination and prostate specific antigen (PSA) evaluation. However, difficulty in diagnosing early stage CaP still prevails due to the fact that alterations not related to cancer, such as benign prostatic hyperplasia (BPH), present characteristics that are very similar to those of CaP lesions.2,3 Up to now, studies on the Mexican population have not been carried out correlating gene regulation during disease progression as a molecular signature that could be used as a marker for early CaP diagnosis. With the development of genomic technology, efforts have been concentrated on the study of factors at the molecular level that are involved in human cancer onset and progression. Presently the exact causes leading to prostatic adenocarcinoma development have not been explained. Androgens, diet, smoking, sedentary lifestyle, sexual factors, and obesity are known to be among the many putative risk factors. Even though changes in lifestyle and chemical contaminant exposure appear to aid in the reduction of disease incidence, their role in CaP etiology has not been clearly established. An interaction between environmental and genetic factors is involved in cancer pathogenesis. It is believed that genetic factors have a 42% contribution to development and progression risk.4,5 The identification of molecular markers associated with CaP is a scientific contribution that has improved the capacity to detect individuals at risk for developing CaP today. The most widely used marker for CaP diagnosis is prostate specific antigen (PSA). It is a glycoprotein produced exclusively in the prostatic epithelium that is present in small quantities in the serum of healthy males and it accumulates with age. Despite its high sensitivity, serum PSA level test lacks specificity since it does not allow for a reliable difference to be established among BPH, aggressive CaP, and non-aggressive CaP.6,7 PSA3 is a marker in urine that has played an important role in non-invasive CaP diagnosis. It is a prostate-specific microRNA (miRNA), probably not encoded, with an abnormally elevated expression in prostatic neoplasias when compared with adjacent non-neoplastic tissue.8-11
Even though a histological or molecular marker for early CaP prediction does not yet exist, short, non-encoded RNA sequences of 18-22 nucleotides that are expressed and play a role in genetic expression control (miRNAs) are being studied. They can undergo amplification, deletion, or reaccommodation that are linked to cancer.

Cell growth during animal development depends on molecular signals that carefully coordinate cellular proliferation and death processes and the speed with which they are carried out. However, cancer is characterized by cellular growth in which cell identity has been lost and there is extensive cell proliferation and alteration in the process of cellular death.

Perhaps the most direct link between MiRNA function and human cancer was demonstrated in the study by Calin et al. in which they observed that encoded miR-15a and miR-16-1 are found in the 13q14 chromosomal region, a chromosomal deletion that is commonly found in patients with B-cell chronic lymphocytic leukemia (B-CLL). This means that miR-15a and miR-16-1 are absent or subexpressed in tissue of patients with B-CLL. It was later reported that the action target for both miRNAs was the 3’UTR region of the BCL2 gene transcript, which is a potent apoptosis inhibitor. This strongly suggests suppressive tumor activity on the part of these two miRNAs.

More than half of the miRNAs are located in human genomic regions that are susceptible to undergoing amplifications, deletions, or rearrangements in malignant tumors, which reinforces the hypotheses about the important role of miRNAs in cancer pathogenesis. Based on this information, it could be said that the history of tumor development is reflected in its particular miRNA expression pattern.

Prostate cancer progression is a process that involves multiple molecular alterations, many of which are reflected in changes in cell genetic expression. Due to the regulatory role of miRNAs in the expression of different genes and to their tissue-specificity, it becomes evident that the identification of CaP-related miRNA could provide valuable information on molecular alterations associated with CaP pathogenesis. Recently, different studies have been carried out on miRNA expression in clinical CaP samples. One study on the expression profile of 228 miRNAs in 56 CaP samples and in 7 normal prostate tissue samples was able to identify significant differential expression of 42 miRNAs; 39 (87%) were overexpressed and 6 (13%) were subexpressed in CaP samples. This shows that miRNA expression in tumor tissue can provide valuable information enabling tumor classification and can be used in the development of new diagnostic technologies for types of cancer that currently lack exact molecular markers, as is the case with CaP.

**OBJECTIVE**

To determine the association of miRNA in urine with CaP diagnosis in Mexican patients at the Hospital General Dr. Manuel Gea González.

**METHODS**

A prospective cross-sectional study was carried out. Thirty patients having undergone prostate biopsy from May-July 2010 at the urology service of the Hospital General Dr. Manuel Gea González were included. All patients presented with PSA above 4 ng/dL or digital rectal examination (DRE) that was clinically suspicious of prostate cancer (CaP). Prior to biopsy, prostate massage was carried out, after which 30 mL of urine were collected. Urine samples were mixed with 5 mL buffer solution (RNalater® RNA Stabilization Reagent, AMBION, Austin TX, USA) to stabilize RNA and they were kept in refrigeration at -70°C until processed.

Total RNA and miRNA extraction from different urine samples: Each sample was centrifuged at 3000 rpm for 15 minutes at 15°C and supernatant was decanted to obtain a cell pellet. Pellet was washed, adding 10 mL phosphate buffered saline (PBS) at pH of 7.4. It was centrifuged at 3000 rpm for 10 minutes at 15°C and supernatant was decanted, and miRNA concentration was determined in the sample. Samples with miRNA concentrations above 50 ng/mL (18/30 samples) were selected. MiRNAs were amplified and 373 miRNAs were quantified with real time polymerase chain reaction (PCR) using ABI 7900HT Real Time PCR System (Applied Biosystems Llc, Foster City, CA USA) equipment and DataAssist™ (Applied Biosystems Llc, Foster City, CA USA) software. This process was carried out under the following thermocycler conditions: 50°C for 2 minutes and 95°C for 15 seconds (activation), followed by 40 cycles of 95°C for one minute (denaturalization) and 60°C for two minutes (alignment and extension).

The Bioconductor code of the programming environment allows the detected fluorescence to normalize during the amplification process in regard to an endogenous control and to report the quantity of miRNAs in the problem sample in relation to a standard.

The Urology Department of the Hospital Gea González provided the human resources and the Instituto Nacional de Medicina Genómica (INMEGEN) provided the transport tubes and material for carrying out microRNA amplification. All procedures were performed in accordance with the General Health Law in Matters of Health Research Regulations; Second Title,
Chapter 1, Article 17, Section II, minimum risk research, with informed consent form.

Student's t test was applied to determine statistically significant changes.

RESULTS

Initially RNA was isolated from the 30 urine samples of patients diagnosed with CaP and 30 diagnosed with BPH. Concentration of the recovered RNA was quite variable. Only 18 samples with miRNA concentration above 50 ng/ul. were selected from the sample total; 9 from the CaP group (positive biopsies) and 9 from the BPH group (negative biopsies). Isolated RNA quality was evaluated by measuring absorption at 260 nm and 280 nm. The 260nm/280nm relation in the selected samples was under 1.5 and over 0.7. The quantity of RNA used for the reverse transcriptase (RT) reaction was adjusted to 75 ng. Selective preamplification reaction for miRNAs was triplicated in order to increase the number of copies of each miRNA and to improve their detection during real time PCR reaction. Preamplification was especially useful in the present study since nucleic acid concentration in the urine is lower than that in tissue.

The difference of miRNA expression between CaP and BPH was obtained by means of comparing the expression profiles of both conditions. A change in miRNA expression level in CaP that was twice as high as that in the BPH samples was considered positive at a statistically significant level of P <0.05.

A set of 21 miRNAs was determined that presented statistically significant differences between both groups in all the samples; overexpression was identified in 19 miRNAs (miR-196b, -574-3p, let-7b, -7c, -7d, 7e, -7g, miR -200b, -149, -20b, -17, -184, -20a, -106a, -671-3p, -148a, -429, -31, -100) and subexpression in 2 (miR -150, -328).

DISCUSSION

Previous reports by different groups have shown that it is feasible to use mRNA isolated in urine as a CaP marker. PCA3 non-coding mRNA determination has shown a sensitivity of 0.69% and a specificity of 0.86%. The PCA3 non-coding mRNA determination has shown a sensitivity of 0.69% and a specificity of 0.86%.

The ability to discriminate between CaP and other types of non-malignant neoplasias such as BPH is considerably increased with the combined use of various markers. MicroRNA expression signatures are potentially useful in the diagnosis, prognosis, and prediction of therapeutic response in different types of human disease, such as cancer, as well as in the differentiation of malignant and non-malignant neoplasm. Other studies have shown the presence of placental miRNAs in the mother's plasma, as well as detectable quantities of miRNAs in fluids such as blood, saliva, and even urine; and these molecules have been found in relatively stable forms, protected from degradation. Advances have been made in the development of methods for the study of miRNAs present in organ tissue and in cells.

Current estimations predict that close to 30% of human genes are regulated by a miRNA and that each miRNA has approximately 200 target transcripts in vertebrates. Variability in the quantity of recovered RNA can be explained by the interindividual difference in the quantity of prostate cells that are sloughed and poured into the urine. The number of patients and samples used in the present study was very small, making it difficult to ensure that the expressed miRNAs found were directly associated with CaP. However, studies carried out at other institutions have found miRNA expression results similar to those of the present study.

The detection and quantification of miRNAs in urine as CaP markers is potentially useful; the challenge that lies ahead is to identify and validate that set of miRNA markers for the purpose of opportunistically establishing prognostic groups that can provide orientation with respect to therapeutic decisions in the treatment of patients with prostatic disease and to enable adequate progression prediction to be carried out.

CONCLUSIONS

A group of 21 miRNAs was determined that showed significant expression difference in the CaP sample group.

The detection and quantification of miRNAs in urine as CaP markers is potentially useful.

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