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MicroRNAs IN BREAST CANCER —OUR INITIAL RESULTS

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ABSTRACT

MicroRNAs (miRNAs) are small [~21 nucleotide (nt)] non coding RNAs (ncRNAs) that regulate gene expression posttranscriptionally. About 3.0% of human genes encode for miRNAs, and up to 30.0% of human protein coding genes may be regulated by miRNAs. Currently, more than 2000 unique human mature microRNAs are known. MicroRNAs play a key role in diverse biological processes including development, cell proliferation, differentiation and apoptosis. These processes are commonly dysregulated in cancer, implicating miRNAs in carcinogenesis, where they act as tumor supressors or oncogenes. Several miRNAs are associated with breast cancer. Here we present our initial results of miRNA analyses of breast cancer tissues using quantitative real time-polymerase chain reaction (ReTi-PCR) (qPCR) involving stem-loop reverse transcriptase (RT) primers combined with TaqMan® PCR and miRNA microarray analysis.

Keywords: Breast cancer, microRNA (miRNA), Microarray, Real time-polymerase chain reaction (ReTi-PCR), Stem-loop reverse transcriptase (RT) primers.

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INTRODUCTION

MicroRNAs (miRNAs) are small [~21-nucleotide (nt)] non coding RNAs (ncRNAs) that mediate posttranscriptional gene regulation by pairing with the 3' untranslated region (3'UTR) of messenger RNAs (mRNAs), acting as translational repressors, and regulating gene expression posttranscriptionally. After the discovery of the first mi-RNA in the roundworm *Caenorhabditis elegans*, these short regulatory RNAs have been found to be an abundant class of RNAs in plants, animals and DNA viruses. About 3.0% of human genes encode for miRNAs, and up to 30.0% of human protein coding genes may be regulated by miRNAs [1]. Currently more than 2000 unique human mature microRNAs are known [2]. MicroRNAs play a key role in diverse biological processes including development, cell proliferation, differentiation and apoptosis [1]. Thus, potentially all cellular pathways may be governed by miRNAs, which may contribute to the fine tuning of gene expression on a global level. The importance of miRNAs in gene regulation will be better appreciated when their function or deregulation, or that of the cellular machinery mediating their biosynthesis and function, will be identified among the underlying causes of several genetic disorders. Indeed, it is easy to conceive that protein over expression resulting from defective miRNA-based mRNA regulation may compromise normal cell function and cause

genetic diseases [3]. Accordingly, altered miRNA expression is likely to contribute to human disease, including cancer [1]. In cancer, miRNAs function as regulatory molecules, acting as oncogenes or tumor suppressors [4]. Amplification or over expression of miRNAs can down regulate tumor suppressors or other genes involved in cell differentiation, thereby contributing to tumor formation by stimulating proliferation, angiogenesis and invasion, *i.e.,* they act as oncogenes. Similarly, miRNAs can down regulate different proteins with oncogenic activity, *i.e.,* they act as tumor suppressors [5,6].

Several miRNA are associated with breast cancer. It has been shown that there are differences not just between normal and breast cancer tissue, but also between different breast cancer subtypes [7-12].

Several methods for global miRNA profiling are currently in common use. These include quantitative real time-polymerase chain reaction (ReTi-PCR) (qPCR) involving stem-loop reverse transcriptase (RT) primers combined with TaqMan® PCR (Life Technologies, Carlsbad, CA, USA) analysis, qPCR with locked nucleic acid primers (Exiqon, Vedback, Denmark), qPCR using poly(A) tailing (Qiagen, Hilden, Germany; Stratagene, La Jolla, CA, USA), high-throughput sequencing of small RNA libraries and microarray analysis. We have recently initiated a study of microRNAs in breast cancer tissues with a main aim to search for breast cancer diagnostic and prognostic markers.

MATERIALS AND METHODS

Fresh frozen tissues (normal and malignant) from patients with breast cancer were obtained from the Institute of Pathology, Medical Faculty, Skopje, Republic of Macedonia. Histopathological data were obtained from all patients. The protocol used for miRNA quantification in tissue samples included three separate procedures: extraction of total RNA, RT- and ReTi-PCR assays. RNA extraction was performed using an RNeasy Kit (Qiagen). Quality and quantity of total RNAs were checked on 1.0% agarose gels and a nanodrop spectrophotometer. RNA samples were dissolved in RNase-free water and stored at –80°C. MicroRNA quantitation was performed by stem-loop RT-PCR followed by Taq-Man® PCR analysis [13] using TaqMan® MicroRNA Reverse Transcription Kit, TaqMan® Universal PCR Master Mix and five TaqMan® MicroRNA Assays (miR-155, miR21, miR-125b and miR-145 and RNU6b as a control gene) (Life Technologies). The RT-PCR mix was made on ice in a final volume of 15 μL, following the manufacturer's protocol. Thermal cycling conditions were: 30 min. at 16°C, 30 min. at 42°C and 5 min. at 85°C. The ReTi-PCR assay was performed in duplicates in a total volume of 20 μL consisting of 7.7 μL ddH_2O, 1.0 μL 20 × TaqMan® Small RNA Assay, 10.0 μL 2 × TaqMan® Universal PCR Master Mix and 1.3 μL RT reaction product (Life Technologies). Thermal cycling conditions were: enzyme activation and initial denaturation at 95°C for 10 min., followed by 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. The expression of each miRNA relative to RNU6b was determined using the ΔΔCt method. MicroRNA microarray analysis was done using a complete labeling and hybridization kit (Agilent Technologies, Santa Clara, CA, USA) following the manufacturer's protocol [14].

RESULTS AND DISCUSSION

Here we present our initial results of miRNA analysis of breast cancer and normal tissues that included quantitative ReTi-PCR (qPCR) involving stem-loop RT primers combined with TaqMan® PCR (Life Technologies) and miRNA microarray analyses. A total of 35 patients with breast cancer were analyzed using ReTi-PCR of four miRNAs (miR-155, miR-21, miR-125b and miR-145). Our initial results showed that miR-155 and miR-21 are up regulated and miR-125b and miR-145 are down regulated in breast cancer. Our results are in agreement with other published studies for miRNA expression profiling in breast cancer [7,8,11,12,15,16]. MicroRNA microarray analysis was performed in several patients and the data were analyzed by R and MeV statistical programs. Nine miRNAs were differentialy expressed, of which seven were up regulated (miR-155, miR-146a, miR-150, miR-210, miR-21, miR-106b and miR-142-3p) and two were down regulated (miR-139-5p and miR-320c). These up regulated miRNAs were also shown to be differentially expressed in breast cancer by other authors [7-9,15]. Nevertheless, additional testing and analyses are needed to establish accurate and precise miRNA markers that will contribute to the improvement of diagnosis and prognosis in breast cancer patients.

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