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14. ABSTRACT

MicroRNAs are small non-coding RNAs that have an important function in post-translational gene regulation and have been shown to modulate the expression of developmentally important transcription factors. Dysregulation of specific microRNAs has been associated with several cancer types. The study of microRNAs and their potential role in breast cancer is an under-explored area of breast cancer research. This project was designed to explore the possible link between microRNAs and hormone receptors in a breast cancer cell model and how dysregulation of specific microRNAs may be important in the etiology of breast cancer development. In this study we have used microRNA arrays to identify differentially expressed microRNAs between PR positive and PR negative cell lines. We have started to develop novel techniques to validate differential regulation of microRNAs. In addition, we have identified unique microRNA sequences, potentially regulating PR expression. From this study we predict that specific microRNAs have an essential role in the development of hormone dependent breast cancers. Identification of these microRNAs could be used in prognosis of subsets of hormone dependent breast cancer and form therapeutic targets for directed cancer treatment.

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microRNAs, breast cancer, progesterone receptor

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Table of Contents

Introduction

MicroRNAs are small non-coding RNAs that have an important function in post -translational gene regulation and in particular have been shown to modulate the expression of developmentally important transcription factors. Dysregulation of specific microRNAs has been associated with several cancer types. The study of microRNAs and their potential role in hormone-dependent breast cancer is an underexplored area of breast cancer research. At the commencement of this concept grant there were no investigations addressing a role for microRNAs in breast cancer. In the past year there have been two publications investigating the role of specific microRNAs in breast cancer cell models, as well as expression profiling studies examining microRNA dysregulation in breast cancer tumor samples. However, these studies have focused on microRNA overexpression or silencing and have not examined the potential interaction between microRNAs and hormone receptors. This project was designed to explore the possible link between abnormal hormone receptor regulation and microRNAs in a breast cancer cell model and how dysregulation of specific microRNAs may be important in the etiology of breast cancer development.

Body

The overall aim of this concept grant was to investigate the possible interaction between microRNAs and the progesterone receptor.

The first aim of the project was to use microRNA arrays to identify differentially expressed microRNAs between PR positive and PR negative cell lines. At the commencement of this project there were no commercially available microRNA arrays. We adopted two different approaches to establish a methodology for microRNA profiling using microarrays:

1) miRMAX arrays purchased from Rutgers University (*1*). These microRNAs are now commercially available through Invitrogen as NCode arrays. However, one of the major concerns with purchasing

commercial arrays is that new microRNAs are continually being identified, and some of these were not incorporated into the design of these arrays.

2) To overcome this problem we have designed and developed unique microRNA arrays for the analysis of microRNA differentially expressed in breast cancer cell models, in collaboration with Dr Nham Tran (University of Sydney).

The analysis we performed consisted of two steps:

I. In order to verify our model system and microarray technology, we compared the expression of T-47D (PR.ER positive cells) and MCF-7 (PR, ER positive cells) microRNAs with the recent publication of the expression of precursor microRNAs .

II. We compared the differential expression of T-47D C4:2 (PR, ER negative) cells with parent T47D cell lines (example shown in Table 1).

In support of the concept of differential regulation of microRNAs in breast cancer associated with PR expression, two recent papers have reported an association between microRNA expression and breast cancer tumor samples (*2, 3*). Specifically, they have identified an association between hormone receptor status of the tumor and microRNA expression.

Table 1 MicroRNAs differentially expressed between T47D C4:2 and parent cell lines. MicroRNAs were hybridised with an equimolar reference set of endogenous microRNAs (arrays purchased from Rutgers University). Microarray analysis was performed in Genespring. Intensity values were normalised to control channel (reference set) and median centred. Fold change values are the resulting normalised expression of microRNAs in parent cell-lines compared to expression in C4:2 cell-lines. Probes with raw and control channel intensity values of < 100 were excluded in order to exclude probes with low signal above background.

Description	Fold Change
hsa-miR-224	48.56
hsa-miR-210	19.82
hsa-miR-191	6.887
hsa-miR-21	6.77
hsa-miR-301	6.613
hsa-miR-149	6.182
hsa-miR-187	0.182
hsa-miR-125b	0.174
hsa-miR-100	0.129

The second aim was to validate differential regulation of specific microRNAs identified in aim one.

Due to the complexity of this project we have only just begun to validate candidate differentially regulated microRNAs using the established protocols, including Northern blotting. MicroRNA expression profiling

requires adaptation of existing qPCR techniques, due to the short length of the active mature microRNAs and the high degree of homology between related microRNAs. To address this problem, we have started to develop specific qPCR techniques based on primers that form hairpin loops and we now have preliminary data to support the effectiveness of this method. This work has been performed in collaboration with the Preiss Laboratory (Victor Chang, Cardiac Research Institute, Sydney, Australia). To establish the validity of this methodology we are currently examining the expression of let-7, a microRNA associated with cell growth in mammalian cells and development in lower organisms (*4*). Using this specific qPCR methodology we have been able to measure the expression levels of endogenous of let-7 in Hela mammalian cell lines using transient transfection of known quantities of artificial let-7 as controls. This methodology is currently being developed for the expansion to other microRNAs in other cell-lines and will be used to validate the differential expression of microRNAs identified in aim 1.

The third aim was to perform bioinformatics analyses to determine microRNA binding sequences within the exon sequences, the 5' and 3' UTRs. As the project has evolved updated algorithms have become available for the prediction of miRNA binding sites, along with databases containing these predictions. Most algorithms are applied only to 3'UTR sequences, however there is no evidence to suggest that microRNAs do not act on exon or 5'UTR sequences and some groups have addressed this possibility by including predicted microRNA binding sites within exon sequences (http://www.targetscan.org).

Initially we did not identify any target sites within the PR 3'UTR, however with newer releases of the algorithms we have identified at least 5 miRNA binding sites at the 3'UTR (see Figure 1). These predictions use the range of available algorithms to predict binding sites from the classes of binding site with the most experimental support.

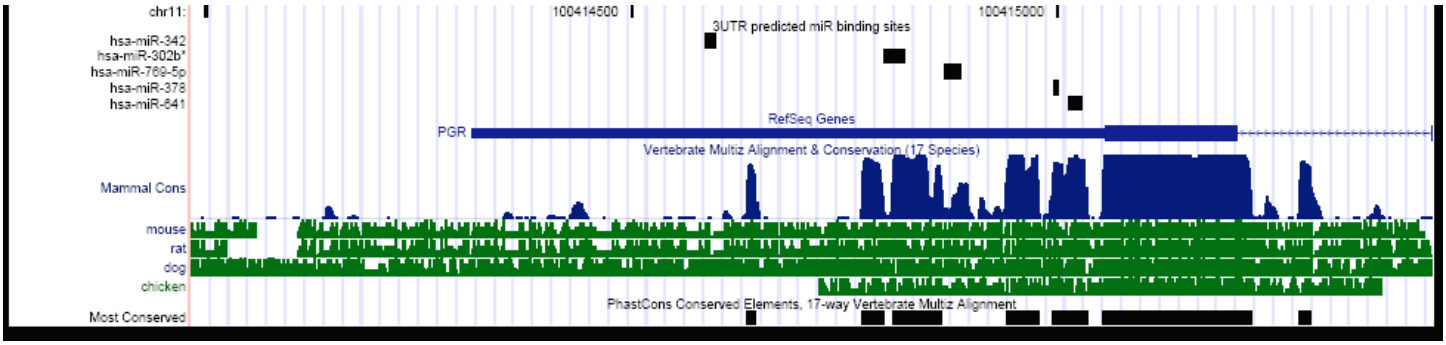

Figure 1 3'UTR predicted microRNA binding sites. Target predictions were downloaded from miRBase (microrna.sanger.ac.uk/), based on the miRanda algorithm and TargetScan 3.0 (http://www.targetscan.org) based on the TargetScanS algorithm. hsa-miR-378 was predicted by TargetScan, and remaining sites were predicted by miRBase. Predictions are shown on a schematic of the PR 3'UTR, generated by UCSC Genome Browser (http://genome.ucsc.edu/).

Although PRA and PRB contain the same 3'UTR, it is possible that differential regulation of these isoforms by miRNAs is dependent on the location of the isoform, i.e. PRA is predominantly expressed in the nucleus whereas PRB is expressed in both the cytoplasm and the nucleus. miRNAs are predicted to be active only in the cytoplasm, and therefore we predict that only cytoplasmic PR will be regulated in this manner, preferentially regulating the PRB isoform.

In addition, we have begun to investigate miR binding to the 5'UTR using a custom implementation of an algorithm to search for 7-nt regions of exact complementarity, which is analogous to the TargetscanS algorithm.

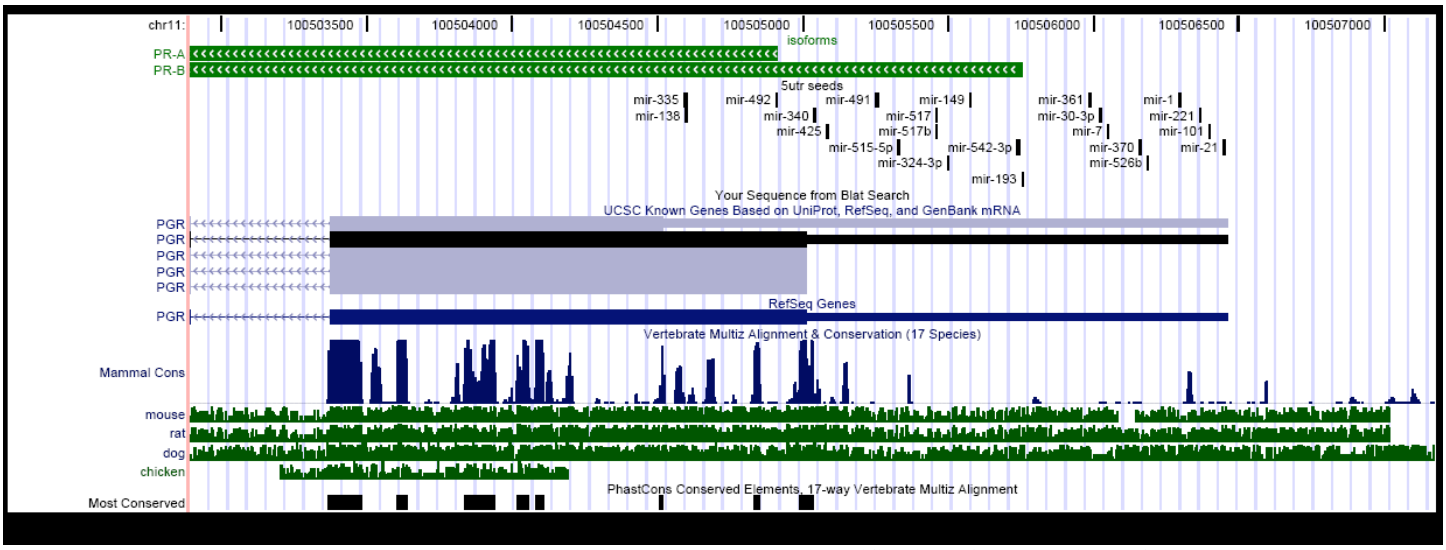

Figure 2 5'UTR predicted binding sites. Target predictions were based on identification of short regions of exact complementarity to the 5' end of the microRNA, analogous to the TargetScanS algorithm. Predictions are shown as a schematic of the PR 5'UTR generated by Genome Browser (http://genome.ucsc.edu/). The extent of PR A and PR B generating transcripts is also shown.

Key research accomplishments

The key research accomplishments of this project to date are as follows:

- Identification of a potentially new regulatory mechanism: progesterone receptor mediation by microRNAs
- Identification of microRNAs differentially regulated between PR+ and PR- breast cancer cells
- Development of new techniques and technology, both laboratory based and bioinformatics for investigation of this regulatory mechanism
- Bioinfomatics analysis of potential microRNA binding sites in progesterone receptor sequence based on the range of existing algorithms, which survey 3'UTR sequences
- Generation of potential microRNA binding sites using a novel speculative approach based on existing algorithms, in which 5'UTR sequences were surveyed
- These techniques can be used to further explore microRNAs and their association with PR and ER action
- An ongoing part of this project has been to develop novel cell lines expressing variant PR and ER isoforms to study interaction between microRNAs and steroid hormone receptors.

Reportable outcomes

- Cohort of novel breast cancer cell lines with various steroid hormone receptor isoforms.
- Development of bioinformatics for analysis of the association between microRNA targeting and mRNA expression levels, specifically relating to steroid hormone action.

Conclusions

We have used custom-made microRNA arrays to investigate differential expression of microRNAs in breast cancer cell lines with different hormone receptor status. A number of microRNAs were identified that appear to be differentially regulated, and these will be confirmed using further microarray analysis and verification using other experimental techniques. microRNA expression profiling is still in its infancy, and the limited range of

available techniques necessitates the development of novel methods. As part of this project we have developed novel techniques for the investigation of microRNA expression. These techniques are currently being used to identify and validate differential regulation of specific microRNAs that are potentially regulate steroid hormone receptors. In addition we have developed novel approaches to assess potential microRNA binding of hormone receptors using bioinformatics. Analysis of progesterone receptor sequence using these approaches has identified unique predicted microRNA binding sites, that could play a part in the post-transcriptional regulation of PR by microRNAs. The techniques that have been developed as part of this project can be used in further studies of microRNA involvement in hormone-dependent breast cancers.

MicroRNA regulation of hormone receptors has not been explored. From this study we predict that specific microRNAs have an essential role in the regulation of hormone receptors. We further predict that microRNAs are involved in dysregulation of hormone receptor expression, and have an important role in the development of hormone dependent breast cancers. Identification of these specific microRNAs essential for normal hormone action, and demonstrating the loss or gain of these specific microRNAs in breast cancer cell models, could be used in prognosis of subsets of hormone dependent breast cancer. Additionally, these microRNAs could form therapeutic targets for directed treatment of these cancer types.

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