

# Comparison of endogenous control genes for normalisation of relative quantitative real-time PCR data in a study characterising microRNA expression in human breast cancer tissues

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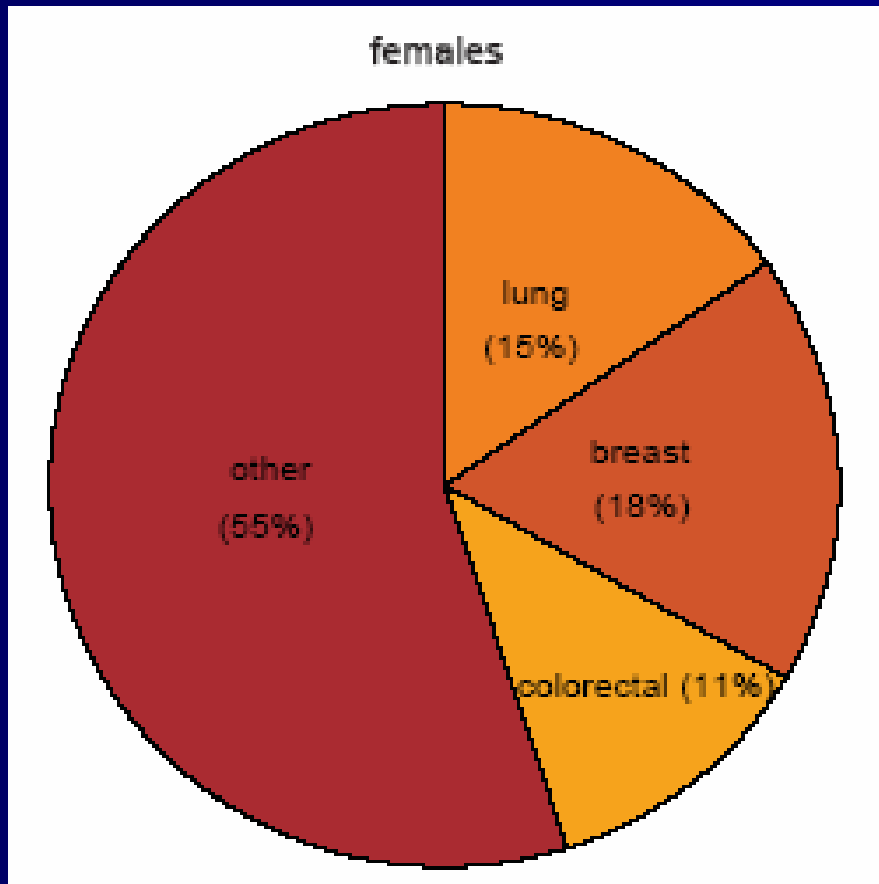
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# Breast Cancer

## Female mortality



- More than 1700 new cases diagnosed in Ireland every year
- 20% of all malignant cancers in Irish women
- Prognostic factors
  - Nodal status
  - Tumour size
  - Histological grade
  - Hormone receptor status

# Gene expression profiling of Breast Cancer

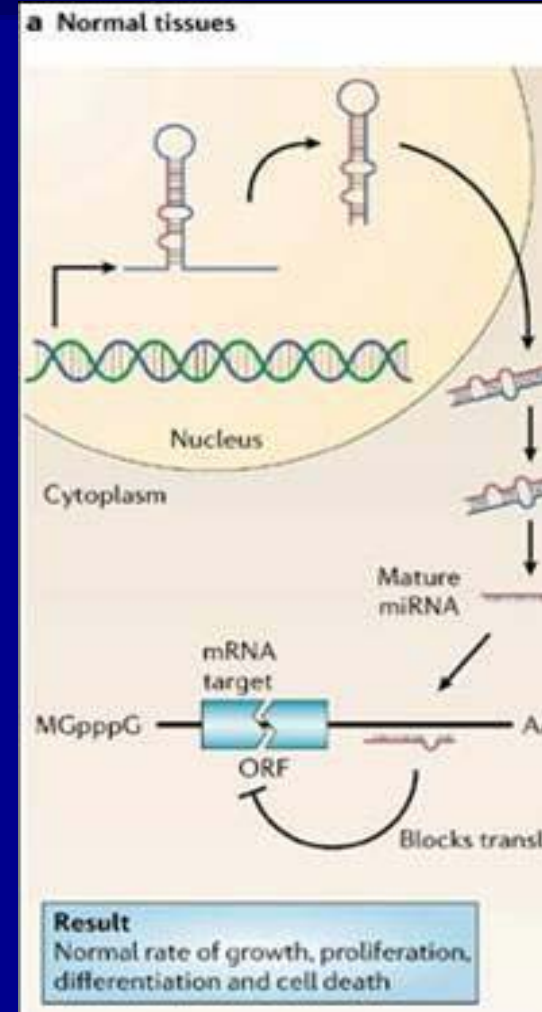
- Patient-tailored treatment
- mRNA expression signatures
  - Oncotype DX™ 21-gene assay (1)
- microRNA expression signatures
  - may be more powerful than mRNA in classifying tumours (2)
  - may aid in prognostication and predicted response to therapy

(1) Paik S, et al. *New England Journal of Medicine*. 2004;351(27).

(2) Lu et al, *Nature*, 2005

# microRNAs

- Negatively regulate gene expression at the transcriptome level
- microRNA → multiple targets
- Mutated or abberantly expressed in cancer
- Novel class of tumour suppressor genes and oncogenes



# microRNA profiling

- Hybridisation-based technologies
  - Northern-blotting
  - Microarray
  - Bead-based hybridisation
- qPCR
  - Increased specificity
  - Low template requirement
  - Large dynamic range
  - Multiplexing capabilities

# Normalization of qPCR data

Raw data



*Correct for*

Technical variation in clinical samples:



*Detect*

**Gene-specific/biological variation**

Commonly normalized using a constitutively-expressed  
**Endogenous Control (EC) gene**

# Considerations

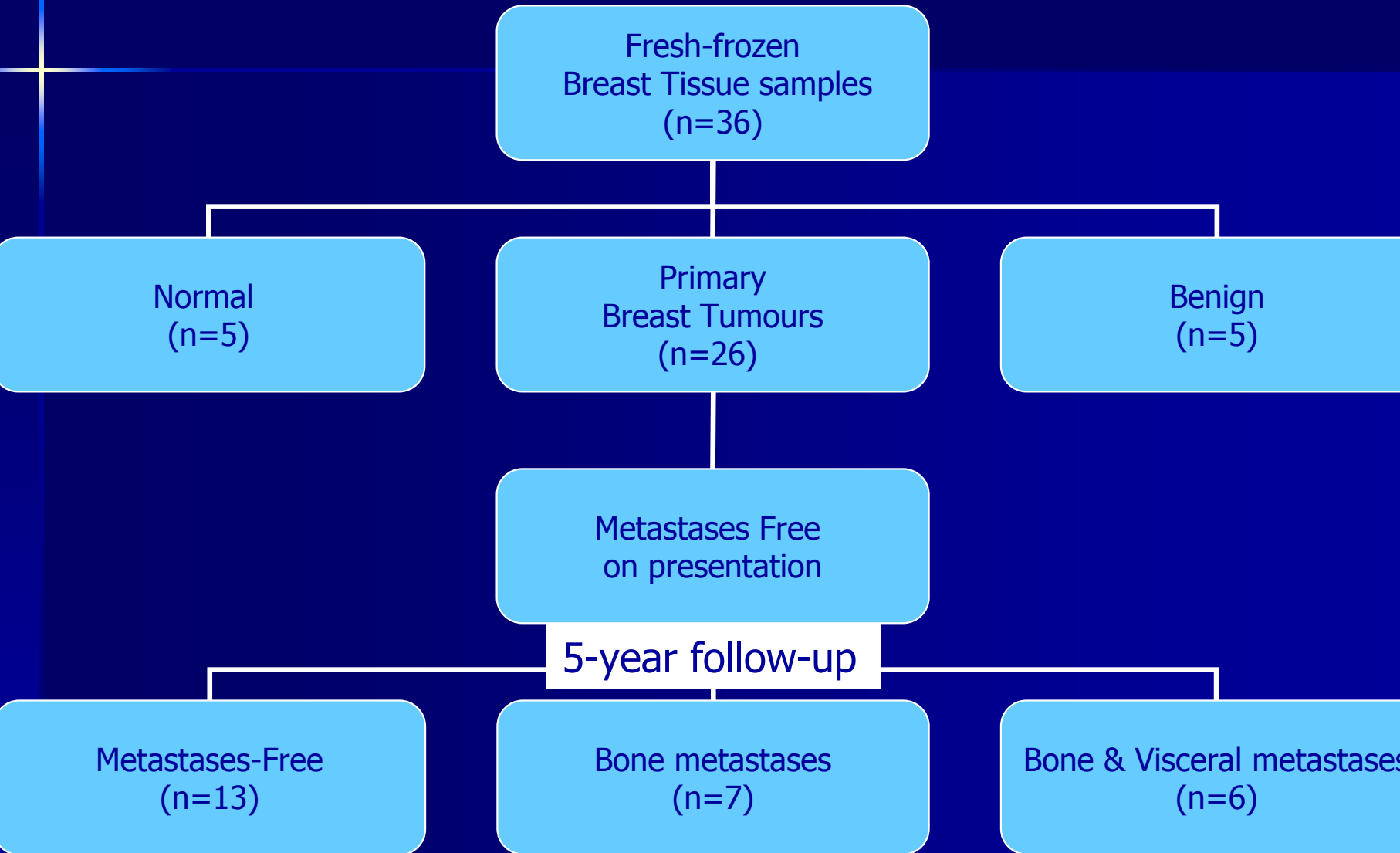
- No established endogenous controls for microRNA profiling studies
  - Let-7a, 18S rRNA, GMN (1)
  - RNU19, RNU66 (2)
- Template= total RNA or small RNA-enriched RNA
- Ideally
  - Same level of expression
  - Same assay chemistry

(1) Bandres et al, Molecular Cancer, 2006, 5:29

# Candidate EC genes

Gene Name	Accession number	Function of RNA
let-7a	MI0000060 *	downregulated in lung cancer, targets RAS oncogenes
miR-16	MI0000070 *	Negatively regulates the anti-apoptotic protein BCL2 in CLL patients, recommended by AB
miR-10b	MI0000267 *	expressed in human breast tissue
miR-21	MI0000077 *	Anti-apoptotic factor; upregulated in various cancers
miR-26b	MI0000084 *	expressed in human breast tissue, recommended by AB
RNU48	NR_002745 *	predicted to guide the 2'O-ribose methylation of 28S rRNA
Z30	AJ007733 *	predicted to guide the methylation of the Am47 residue in U6 snRNA

# Samples in study





Sample collection



RNA isolation (small RNA-enriched)



RNA analysis



Reverse Transcription



Real-time PCR



# qPCR

- Amplification efficiency determined for each gene

$$E = (10^{(1/\text{slope})} - 1) * 100$$

- Samples represented in triplicate
- No-template-control for each gene
- Inter-run calibrators for each gene
  - pool of 5 normal breast tissues
  - Standard deviation (0.2-0.3 Ct)

# Relative quantification

- qBase v1.3.5 (Jan Hellemans & Jo Vandesompele)

- Relative quantification model

- Allows use of multiple EC genes
- Adjusts for efficiency of GOI & EC

- Output


- Relative quantification of GOI using user-defined EC genes
- Produces data file of RQ values for further analysis of EC



# geNorm (Vandesompele et al, Genome Biology, 2002)

- **Gene stability measure M**
  - average pairwise variation of a gene with all other genes

- **Pairwise variation V**

	gene A	gene B	
sample 1	a1	b1	$\log_2(a1/b1)$
sample 2	a2	b2	$\log_2(b1/b2)$
sample n	an	bn	$\log_2(an/bn)$  Standard deviation

- **Output**
  - Ranks genes according to their stability
  - Normalisation factor based on geometric average of input EC genes

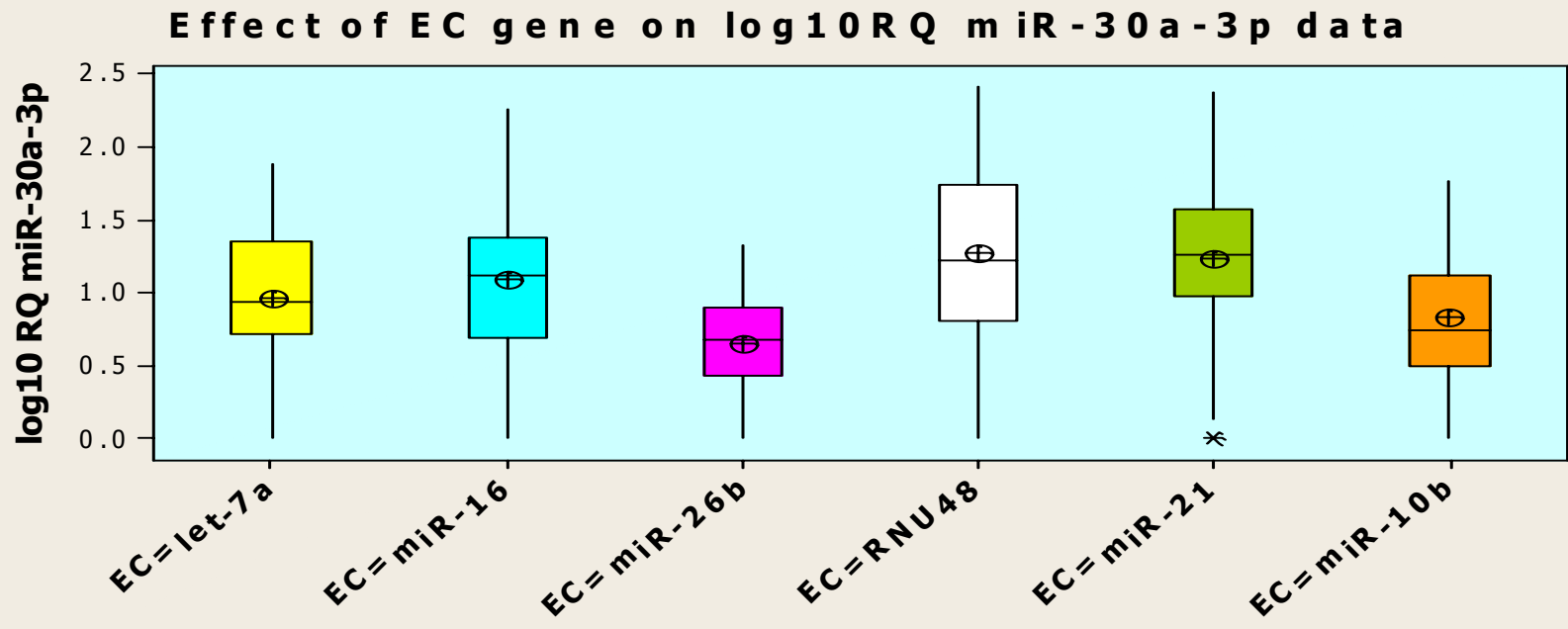
# NormFinder

(Andersen et al, Cancer Research, 2004)

- estimation of both the intra- and the inter-group expression variation
- Combines these to give a stability value for each gene.
- **Requirement**
  - No prior expectation of expression difference between groups
- **Output**
  - Ranks genes according to their stability
  - Recommends best combination of two genes

# Further investigation

- No significant variability in RQ of target gene when additional gene is used for normalisation
- Significant variability in RQ of target gene depending on single EC gene used



# Conclusions

- EC genes need to be validated in context of study
- let-7a is most stably expressed in this cohort
  - Minimum of three genes recommended for reliable normalisation
- sn(o)RNAs may not be suitable for every application
- Microarray → additional candidates

- Work conducted at:

National Breast Cancer Research Institute,  
University College Hospital,  
Galway, Ireland



- Wed. 28<sup>th</sup> March, 9:50am, Lecture Hall 14  
Evaluation of endogenous control genes for real-time quantitative PCR in breast cancer tissues.  
McNeill R.E., Miller, N. and Kerin, M.J.