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Recent advances in the molecular pathology of micro-RNAs

Compiled and annotated by ***John PC Le Quesne****, Cancer Research UK, Cambridge Research Institute, Li Ka Shing Centre, Robinson Way, Cambridge CB2 0RE, UK and* ***Peter A Hall****, Department of Pathology & Laboratory Medicine, King Faisal Specialist Hospital & Research Centre, PO Box 3354, Riyadh 11211, Saudi Arabia*

Setting the scene: micro-RNAs, their regulation and their deregulation in disease

Micro-RNAs (or miRNAs) are now understood to be one of the major determinants of normal patterns of gene expression in healthy and diseased tissues, although they have only gained this recognition in the last decade. With hindsight, their existence seems almost inevitable. Essentially, they are endogenous antisense oligonucleotides, snippets of RNA without protein-coding potential that bind to and downregulate sets of target mRNAs that contain complementary sites. Thus they exploit the digital nature of the genetic code, as opposed to the more analogue protein-driven processes of classical transcriptional control.

miRNAs function by interfering with the expression of their target mRNAs either by down-regulating mRNA translation or mRNA stability. This is itself a contested point, although current weight of opinion favours the latter. Whichever mechanism is employed, this places miRNAs in the arena of post-transcriptional gene control, alongside other relatively under-studied mechanisms of translational control [1], and alternative splicing [2], both subjects recently reviewed in the 2010 Annual Review Issue of the Journal.

The 1000 or so human miRNAs are themselves part of a much larger, partially discovered group of non-coding RNAs, which have an even broader range of physiological and pathological roles, the subject of another comprehensive review by Taft *et al.* [3] in the 2010 Annual Review Issue .

The physiological roles of miRNAs are manifold, with credible evidence for involvement in metabolism, neuronal plasticity, viral defence and embryonic development. It is this last aspect, particularly their effects upon stem-cell-like behaviour and tissue type determination, which point to their involvement in neoplasia. miRNAs are in many ways analogous to classical transcription factors; they seem to be transcribed and regulated in a similar manner, from conventional promoters, and they can each regulate a range of genes, so long as a suitable sequence element is present. And like transcription factors, many miRNAs have gained notoriety as mediators of the neoplastic phenotype, by either favouring or opposing the various hallmarks of cancer. Farazi *et al.* [4] provide an excellent review of this rapidly expanding field in the 2011 Annual Review Issue.

1. **Dysregulation of protein synthesis and disease**
   John PC Le Quesne, Keith A Spriggs, Martin Bushell, Anne E Willis
   The Journal of Pathology 2010; 220: 140-151. (Invited review)

2. **The pathobiology of splicing**
   Amanda J Ward, Thomas A Cooper
   The Journal of Pathology 2010; 220: 152-163. (Invited review)

3. **Non-coding RNAs: regulators of disease**
   Ryan J Taft, Ken C Pang, Timothy R Mercer, Marcel Dinger and John S Mattick
   The Journal of Pathology 2010; 220: 126-139. (Original paper)

4. **miRNAs in human cancer**
   Thalia A Farazi, Jessica I Spitzer, Pavel Morozov, Thomas Tuschl
   The Journal of Pathology 2011; 223: 102-115. (Original paper)

Technologies and signature generation

miRNAs offer new challenges and opportunities to those wishing to study them in human tissue. Like all RNAs, they are prone to enzymatic degradation and cleavage in dying or damaged tissue, albeit to a lesser extent than mRNA, probably due both to their tiny size (18-22 nucleotides) and to a protein cofactor-shielded microenvironment.

Variations on the standard mRNA detection/quantification technologies are used, including deep sequencing, microarrays, qRT-PCR, Northern blots, and *in situ* hybridisation. Their small size also makes hybridization-dependent methods (i.e. all the above other than deep sequencing) prone to errors related to inaccurate hybridization. One additional consideration is that many assays are unable to distinguish between immature (and therefore inactive) and mature forms of miRNA. These points need to be carefully considered when reading papers on this subject.

Two recent studies of renal cell carcinoma typify the power of these techniques. In the first, Nakada *et al.* [**5**] begin with the observation that E-cadherin is frequently lost in clear cell carcinoma, and this is related to metastasis and poor prognosis. What is the mechanism for this? The authors use microarrays to derive miRNA signatures from frozen clear cell and chromophobe tumour tissue to find that clear cell carcinoma is typified by the loss of miR-200c and miR-141. They go on to show that these miRNAs directly target the transcriptional repressor ZFHX1B, which itself targets E-cadherin, and that the loss of these miRNAs in tumours is related to E-cadherin down-regulation.

In the second study Weng *et al.* address the use of formalin-fixed paraffin-embedded tissue for the analysis of miRNA expression. They demonstrate that miRNAs purified from cores of formalin-fixed paraffin-embedded tumour tissue can successfully be profiled by microarray, deep sequencing and qRT-PCR, and that this data is of

a quality comparable to that obtained from fresh frozen tissue [6]. This important paper has great implications, as it opens up the possibility of using diagnostic tissue archives for miRNA studies.

5. **Genome-wide microRNA expression profiling in renal cell carcinoma: significant down-regulation of miR-141 and miR-200c**
   C Nakada, K Matsuura, Y Tsukamoto, M Tanigawa, T Yoshimoto, T Narimatsu, LT Nguyen, N Hijiya, T Uchida, F Sato, H Mimata, M Seto and M Moriyama
   The Journal of Pathology 2008; 216: 418-427. (Original paper)

6. **MicroRNA profiling of clear cell renal cell carcinoma by whole-genome small RNA deep sequencing of paired frozen and formalin-fixed, paraffin-embedded tissue specimens**
   Lihong Weng, Xiwei Wu, Hanlin Gao, Bing Mu, Xuejun Li, Jin-Hui Wang, Chao Guo, Jennifer M Jin, Zhuo Chen, Maricela Covarrubias, Yate-Ching Yuan, Lawrence M Weiss and Huiqing Wu
   The Journal of Pathology 2010; 222: 41-51. (Original paper)

Dysregulation of specific miRNAs in cancer: players in regulatory networks

Numerous individual miRNAs have been identified as having tumour-suppressing or oncogenic properties. Indeed it is becoming clear that they are players in complex regulatory networks that encompass well-known genes involved in cancer such as p53, myc and HIF1α.

Once such example is miR-34, which is an important mediator of p53 function. Subramanian *et al.* find using microarray profiling that miR-34a loss is associated with progression from neurofibromas to malignant peripheral nerve sheath tumours, and that this is accompanied by a mRNA signature suggesting p53 inactivation [7].

In Burkitt's lymphoma, Leucci *et al.* identify a minority of cases that do not have an activating translocation involving c-myc. C-*myc* mRNA has target sites for let-7 and miR-34 [8]; they go on to show that miR-34 is often down-regulated in translocation-negative cases, and that knockdown of miR-34 in cell lines will de-repress myc expression, providing an intriguing model to help explain these anomalous cases.

Building on their earlier work in clear cell renal carcinoma, Nakada *et al.* investigate the role played by miR-210 [9], which is seen to be upregulated in these tumours. They show that miR-210 is upregulated by the hypoxia inducible factor HIF1α, and that in cell lines, exogenous miR-210 disrupts normal spindle formation leading to centrosome amplification and aneuploidy, in a mechanism that is partly mediated by the miR-210 target E2F3.

7. **Genome-wide transcriptome analyses reveal p53 inactivation mediated loss of miR-34a expression in malignant peripheral nerve sheath tumours**
   Subbaya Subramanian, Venugopal Thayanithy, Robert B West, Cheng-Han Lee, Andrew H Beck, Shirley Zhu, Erinn Downs-Kelly, Kelli Montgomery, John R Goldblum, Pancras CW Hogendoorn, Christopher L Corless, Andre M Oliveira, Sarah M Dry, Torsten O Nielsen, Brian P Rubin, Jonathan A Fletcher, Christopher DM Fletcher and Matt van de Rijn
   The Journal of Pathology 2010; 220: 58-70. (Original paper)

8. ***MYC* translocation-negative classical Burkitt lymphoma cases: an alternative pathogenetic mechanism involving miRNA deregulation**
   E Leucci, M Cocco, A Onnis, G De Falco, P van Cleef, C Bellan, A van Rijk, J Nyagol, B Byakika, S Lazzi, P Tosi, H van Krieken and L Leoncini
   The Journal of Pathology 2008; 216: 440-450. (Original paper)

9. **Overexpression of miR-210, a downstream target of HIF1α, causes centrosome amplification in renal carcinoma cells**
   Chisato Nakada, Yoshiyuki Tsukamoto, Keiko Matsuura, Tung Lam Nguyen, Naoki Hijiya, Tomohisa Uchida, Fuminori Sato, Hiromitsu Mimata, Seto Masao and Masatsugu Moriyama
   The Journal of Pathology 2011: Accepted Manuscript; DOI: 10.1002/path.2860. (Original paper)

Global dysregulation of miRNA synthesis

While some studies suggest deregulation of specific miRNAs, in fact most reports to date indicate a tendency toward global reduction in miRNA functionality in tumours and transformed cell lines. Key stages in miRNA biosynthesis and function include the maturation of primary micro-RNA transcripts in the nucleus (by the Drosha/DGCR8 microprocessor complex), nuclear export (mediated by exportin-5), the maturation of pre-miRNAs in the cytoplasm (by the ribonuclease Dicer) and the packaging of the mature miRNA into a functional complex (including the proteins AGO2 and TNRC6A). Defects in all of these stages have been implicated in oncogenesis and cancer progression.

Dicer deletion is known to be incompatible with embryonic development, and tissue-specific knockouts often reveal that Dicer is essential for normal organogenesis. The liver is no exception; when Dicer was knocked out in the mouse liver by Sekine *et al.* [**10**], morphology was unaffected, but zonation was severely perturbed, particularly the establishment of periportal gradients of gene expression.

The loss of Dicer expression is related to poor outcome in several tumours. Faggad *et al.* [**11**] show that in ovarian serous carcinoma, the loss of Dicer predicts poor outcome, and that in cell lines Dicer knockdown is related to global down-regulation of miRNAs and ER expression.

In a study of gastric and colorectal cancers, Kim *et al.* demonstrate that microsatellite instability is related to frameshift mutations in several genes involved in miRNA processing and activity. In particular, *AGO2* and *TNRC6A* are frequently mutated and down-regulated in both these tumours [**12**].

In another gastric cancer study [**13**], Tchernitsa *et al.* identify loss of Dicer and Drosha expression by immunohistochemistry in gastric tumours as compared to matched normal tissue, and show that Drosha expression predicts survival. Using array technology, they also identify miRNA signatures that differentiate between node-positive and node-negative cancers.

10. **Dicer is required for proper liver zonation**
    Shigeki Sekine, Reiko Ogawa, Michael T Mcmanus, Yae Kanai and Matthias Hebrok
    The Journal of Pathology 2009; 219: 365-372. (Original paper)

11. **Prognostic significance of Dicer expression in ovarian cancer – link to global microRNA changes and oestrogen receptor expression**
    Areeg Faggad, Jan Budczies, Oleg Tchernitsa, Silvia Darb-Esfahani, Jalid Sehouli, Berit M Müller, Ralph Wirtz, Radoslav Chekerov, Wilko Weichert, Bruno Sinn, Christin Mucha, Nasr E Elwali, Reinhold Schäfer, Manfred Dietel and Carsten Denkert
    The Journal of Pathology 2010; 220: 382-391. (Original paper)

12. **Somatic mutations and losses of expression of microRNA regulation-related genes *AGO2* and *TNRC6A* in gastric and colorectal cancers**
    Min S Kim, Ji E Oh, Yoo R Kim, Sang W Park, Mi R Kang, Sung S Kim, Chang H Ahn, Nam J Yoo and Sug H Lee
    The Journal of Pathology 2010; 221: 139-146. (Original paper)

13. **Systematic evaluation of the miRNA-ome and its downstream effects on mRNA expression identifies gastric cancer progression**
    Oleg Tchernitsa, Atsuko Kasajima, Reinhold Schäfer, Ralf-Jürgen Kuban, Ute Ungethüm, Balazs Györffy, Ulf Neumann, Eva Simon, Wilko Weichert, Matthias PA Ebert and Christoph Röcken
    The Journal of Pathology 2010; 222: 310-319. (Original paper)

miRNAs and tumour progression and metastasis

The preceding discussion supports the idea that miRNA dysregulation can contribute to all the classical hallmarks of cancer. However, it is probably most often linked to metastasis and tumour progression, often by inducing epithelial-to-mesenchymal transitions. In particular, the miR-200 family (miR-200a-c, miR-141, miR-429) is often implicated. These miRNAs indirectly up-regulate several determinants of the epithelial phenotype, including E-cadherin, by down-regulating transcriptional repressors such as ZEB1 [see above and 5].

In an elegant study [**14**], Castilla *et al*. used multiplex qRT-PCR to determine the difference in miRNA profiles between the epithelial and mesenchymal components of a set of endometrial carcinosarcomas. They identify several miRNAs, including the miR-200 family, that are down-regulated in the mesenchymal component, with accompanying over-expression of E-cadherin repressor proteins.

Baffa *et al.* combined microarray expression data from a number of different primary tumours (colon, bladder, breast and lung) with matched lymph node metastases [**15**]. They derived a signature that is associated with secondary lymph node deposits across tumour types; again, this includes losses in the miR-200 family, as well as changes in many other miRNAs. They also demonstrate by immunohistochemistry that this dysregulation is often accompanied by changes in the expression of predicted target proteins, providing a model for the effects of miRNA dysregulation in lymph node metastasis.

Finally, in a study of primary human cell lines derived from squamous cell carcinomas of the head and neck [**16**], Lo *et al.* show that miR-200c is relatively down-regulated in flow-sorted stem-cell-like populations, and that its restoration reduces this capacity, in a manner that is partially mediated by the miR-200c target *BMI1*. They also show miR-200c loss also favours the mesenchymal phenotype, and that in xenograft models miR-200c inhibits tumour metastasis.

14. **Micro-RNA signature of the epithelial–mesenchymal transition in endometrial carcinosarcoma**
María Ángeles Castilla, Gema Moreno-Bueno, Laura Romero-Pérez, Koen Van De Vijver, Michele Biscuola, María Ángeles López-García, Jaime Prat, Xavier Matías-Guiu, Amparo Cano, Esther Oliva and José Palacios
The Journal of Pathology 2011; 223: 72-80. (Original paper)

15. **MicroRNA expression profiling of human metastatic cancers identifies cancer gene targets**
Raffaele Baffa, Matteo Fassan, Stefano Volinia, Brian O'Hara, Chang-Gong Liu, Juan P Palazzo, Marina Gardiman, Massimo Rugge, Leonard G Gomella, Carlo M Croce and Anne Rosenberg
The Journal of Pathology 2009; 219: 214-221. (Original paper)

16. **MicroRNA-200c attenuates tumour growth and metastasis of presumptive head and neck squamous cell carcinoma stem cells**
Wen-Liang Lo, Cheng-Chia Yu, Guang-Yuh Chiou, Yi-Wei Chen, Pin-I Huang, Chian-Shiu Chien, Ling-Ming Tseng, Pen-Yuan Chu, Kai-Hsi Lu, Kuo-Wei Chang, Shou-Yen Kao and Shih-Hwa Chiou
The Journal of Pathology 2011; 223: 482-495. (Original paper)

Conclusion

So what is the future for miRNAs and their impact in pathology? The field is growing at an extraordinary pace and it is clear that understanding miRNA function and dysfunction will contribute to our understanding of disease mechanisms. Over and above this it may be that monitoring miRNA expression in pathological material will provide new diagnostic and predictive insights. Finally therapeutic strategies based on the manipulation of miRNAs may be possible.

Questions

The following questions can be answered by reading and reflecting upon the above annotation and the papers that are cited within it. Within the Royal College of Pathologists **Continuing Professional Development (CPD) scheme**, CPD points may be earned by writing reflective notes on the papers in this Virtual Issue and the questions are designed to act as a focus for this activity. To do this, you may wish to use the Royal College of Pathologists' **reflective notes form**.

Question 1 Outline the stages of miRNA synthesis and activity.

Question 2 What are the technical issues that influence the ability to detect miRNAs in tissue samples?

Question 3 List the major mechanisms of post-transcriptional gene regulation.

Question 4 Briefly describe some well-characterised physiological functions of miRNAs.

Question 5 Compare and contrast the ways in which oncogenic and tumour-suppressing transcription factors become activated or silenced.

Question 6 What are the relative merits of microarrays, *in situ* hybridisation and deep sequencing in the analysis of miRNA expression in tumour tissue?

Question 7 How do miRNAs become dysregulated in cancer?

Question 8 Which miRNAs are known to be involved in tumour metasasis, and how do they exert this effect?

Question 9 What influence does Dicer depletion have on the malignant phenotype, and why might it have these effects?

Question 10 How does miR-34 dysregulation influence the malignant phenotype?