MicroRNAs and prostate cancer

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Abstract

Despite much progress in prostate cancer management, new diagnostic, prognostic and therapeutic tools are needed to predict disease severity, choose among the available treatments and establish more effective therapies for advanced prostate cancer. In the last few years, compelling evidence has documented the role of microRNAs as new broad-spectrum oncogenes or tumour suppressor genes, thus their use as diagnostic, prognostic and therapeutic biomolecules is envisaged. This review extensively and critically summarizes the current knowledge about microRNA deregulation in prostate cancer disease, underlining present limits and future perspectives.

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MicroRNAs: new actors in the cancer scene

In recent years, the discovery of microRNAs has laid a new layer of complexity over the mechanisms regulating gene expression and function (He & Hannon 2004, Chen & Rajewsky 2007). MicroRNAs (miRNAs or miRs) are endogenous non-coding RNAs that can interfere with protein expression either by inducing the cleavage of specific target mRNAs or, in most cases, by inhibiting their translation (Bartel 2004). Thus, miRs offer a fast fine-tuning and energy-saving mechanism for post-transcriptional control of protein expression. Mature miRs are evolutionarily conserved ~22-nt single-stranded RNAs resulting from a multistep processing of longer precursor molecules. miR genes are generally endowed with a Pol II promoter driving their transcription (Lee *et al.* 2004); alternatively, they are located in intronic sequences of host genes with which they are co-transcribed and excised by splicing events (Rodriguez *et al.* 2004). The primary transcripts (pri-miRs) can be thousands of nucleotides long and can contain the precursors of several clustered miRs. The endonucleolytic cleavage by the nuclear enzyme Drosha produces ~70-nt hairpin structures

called pre-miRs (Lee *et al.* 2003). These molecules are then transported to the cytoplasm and are further processed by the ribonuclease Dicer into double-stranded RNAs containing the mature miR and its bulged antisense sequence (miR*; Hutvagner *et al.* 2001, Ketting *et al.* 2001). Finally, the mature miR is selectively incorporated into the RNA-induced silencing complex (RISC) where it elicits the silencing of specific target mRNAs which are recognized by partial complementarity with sequences located in the 3' UTR (Peters & Meister 2007). Given the imperfect nature of these interactions, each miR can bind several different mRNAs and each mRNA can be targeted by multiple miRs, thus implying the existence of an intricate network of gene expression control (Lewis *et al.* 2005).

A rapidly increasing number of physiological processes are now known to be regulated by miRs, which are progressively found to be involved in the regulation of cellular functions ranging from maintenance of stemness to differentiation and tissue development, and from cell cycle to apoptosis and metabolism (Bushati & Cohen 2007, Stefani & Slack 2008, Gangaraju & Lin 2009). Thus, it is intuitive that the aberrant expression of miRs can deeply impact on the multiple features of cell biology, ultimately resulting in complex pathological events such as infective (Baltimore *et al.* 2008, Cullen 2009), cardiovascular (Latronico & Condorelli 2009) and neurodegenerative diseases (Bushati & Cohen 2008), as well as cancer.

This paper is one of 6 papers that form part of a special Focus Section on microRNAs. The Guest Editors for this section were Professor Alfredo Fusco, Naples, Italy, and Professor Carlo M Croce, Columbus, OH, USA.

The hypothesis that miRs can be regarded as new broad-spectrum oncogenes or tumour suppressor genes has opened a revolutionary field of research with exciting diagnostic and therapeutic perspectives. The compelling hint of a widespread miR deregulation in cancer pathogenesis came from the analysis of the genomic distribution of 186 miR genes (Calin *et al.* 2004). In this study, it was demonstrated that more than half of them mapped in cancer-associated genomic regions, namely in chromosomal sites prone to deletions, amplifications or recombinations. These aberrations can result in miR down- or up-regulation, conferring selective advantages to mutated cells. Additional mechanisms of miR deregulation include altered expression of miRs as a consequence of excessive or deficient processing (Karube *et al.* 2005, Sugito *et al.* 2006); aberrant transcription of the precursors by epigenetic silencing of miR promoters (Saito *et al.* 2006, Lujambio *et al.* 2007) or as a result of the activity of oncogenic transcription factors (O'Donnell *et al.* 2005, Chang *et al.* 2008); and more rarely, point mutations in mature miRs or in target sequences that can interfere with normal target recruitment (Iwai & Naraba 2005, Mayr *et al.* 2007).

In order to determine the ultimate effect of these events on miR expression, several platforms have been developed to characterize the full miRNome of cells. Better than complex mRNA profiles, distinct signatures of miRs seem to distinguish different tissues and cells of cancerous or normal origin (Lu *et al.* 2005), thus offering a promising tool to individuate patterns of miR expression that might hopefully gain diagnostic and/or prognostic significance. Besides potential roles in diagnosis and prognosis, these small molecules stand as candidate therapeutic tools: many strategies are being developed to dampen or restore miR levels, thus offering the possibility to interfere at one stroke with multiple molecules or pathways deregulated in tumours.

Prostate cancer: a complex scenario for miR action

In developed western countries, prostate cancer is the most common malignant tumour in men, and is the second highest cause of cancer mortality after lung tumours (Gronberg 2003). Clinically, prostate cancer is diagnosed as local or advanced, and treatments range from surveillance to radiotherapy, radical prostatectomy or androgen-deprivation treatment. Androgen ablation, the mainstay for management of advanced prostate cancer, reduces symptoms in about 70–80% of patients, but most tumours relapse within 2 years to an

incurable hormone-independent state, which is ultimately responsible for prostate cancer mortality (Damber & Aus 2008). The regulatory mechanisms that cause this transition remain largely unknown, and to date, no effective therapy for androgen-independent prostate cancer has been developed.

On the contrary, for early-stage clinically localized disease, radical prostatectomy is generally curative. Nevertheless, treatment is often deferred with active monitoring, particularly in older men. This approach is based on the assumption that many prostate tumours are indolent and progress slowly relative to the patient's life expectancy (Wu *et al.* 2004). At present, the choice of the best treatment for clinically localized prostate cancer is not trivial, because radical surgery and radiotherapy do not offer a clear survival advantage in some patients, and treatment often results in a worse quality of life. During the last 20 years, prostate-specific antigen (PSA) testing has resulted in increased detection of early-stage prostate cancer: while some patients are cured of life-threatening disease, there are concerns about over-treatment and related morbidity (Loeb & Catalona 2007). In fact, serum PSA level, primary tumour stage and Gleason grade do not reliably predict outcome for individual patients. Therefore, the identification of indicators of aggressiveness would be helpful in guiding therapeutic decisions, by distinguishing individuals with potentially life-threatening disease for whom treatment is actually necessary. Diagnostic, prognostic and therapeutic molecules are needed to predict disease severity, choose among available treatments and establish more effective ones for advanced prostate cancer. In this complex scenario, some miRs could find their role.

miR profiling in prostate cancer

miR profiles of different tissues have been reported to be highly informative and more predictive than mRNA characterization to such an extent that poorly differentiated tumours of uncertain origin might be classified on the basis of miR expression (Lu *et al.* 2005). Nevertheless, in prostate cancer the effort of tracing a clear miR profile has proven inconclusive.

At present, much controversy is still present in the literature: conflicting results are often segregating different datasets, possibly because of the study design, underestimated treatments of the patients, methods of sample collection, presence of contaminating cells, and sensitivity and specificity of the platforms used. Table 1 offers an overview of such an inconsistency by summarizing miRs up- or down-regulated in

Table 1 An overview of miR data inconsistency, by summarizing miRs up (↑)- or down (↓)- regulated in prostate cancer disease, as reported in broad-spectrum miR profiles or in studies focused on single miRs. Only the miRs described as aberrantly expressed in at least two different studies are included in the list

	Volinia	Ambrosini	Porkka	Ozen	Tong	Others
<i>let-7a</i>	↓		↓		↑	
<i>let-7b</i>		↓	↓	↓		
<i>let-7c</i>			↓	↓		
<i>let-7d</i>	↑		↓	↓		
<i>let-7f</i>			↓	↓		
<i>let-7g</i>			↓	↓		
<i>let-7i</i>	↑	↑		↓		
<i>miR-100</i>			↓		↓	
<i>miR-125a</i>		↑	↓	↓		
<i>miR-125b</i>			↓	↓	↓	↑
<i>miR-128</i>	↓	↓				
<i>miR-141</i>					↑	↑
<i>miR-143</i>			↓		↓	
<i>miR-145</i>			↓		↓	
<i>miR-146</i>	↑					↓
<i>miR-148</i>	↑		↓			
<i>miR-16</i>	↑		↓	↓		↓
<i>miR-181</i>	↑	↑				
<i>miR-184</i>	↑		↑			
<i>miR-195</i>	↑		↓			
<i>miR-196</i>	↑	↑				
<i>miR-198</i>	↑		↑			
<i>miR-199a</i>	↑		↓			
<i>miR-205</i>		↓	↓		↓	↓
<i>miR-20a</i>	↑				↑	↑
<i>miR-21</i>	↑					↑
<i>miR-218-2</i>	↓	↓				
<i>miR-221</i>		↓	↓		↓	↑
<i>miR-222</i>			↓		↓	↑
<i>miR-23b</i>			↓		↓	↓
<i>miR-25</i>	↑	↑			↑	
<i>miR-26</i>	↑	↑	↓	↓		
<i>miR-27a</i>	↑		↓			
<i>miR-29</i>	↑		↓	↓		
<i>miR-30a</i>			↓	↓		
<i>miR-30b</i>			↓	↓		
<i>miR-30c</i>	↑		↓	↓		
<i>miR-32</i>	↑	↑				
<i>miR-345</i>		↓	↑			
<i>miR-34a</i>	↑			↓		↓
<i>miR-370</i>		↑	↑			
<i>miR-449</i>		↑				↓
<i>miR-92</i>	↑	↑	↓			
<i>miR-93</i>	↑	↑				
<i>miR-99b</i>		↑		↓		

prostate cancer, as reported in broad-spectrum miR profiles or in studies focused on single miRs. miRs described as aberrantly expressed in at least two different studies are included in the list.

The opposite expression of some miRs reported in different studies of prostate tumours mirrors the controversial premises of miRs' overall expression in cancer. In fact, some studies state a widespread down-regulation of miRs in tumours (Lu *et al.* 2005),

consistently with a documented role of miRs in terminal differentiation and a general tendency of tumour cells to a more anaplastic state. Other studies, like the one conducted by Volinia *et al.* (2006) report on a cancer miR signature composed mostly by overexpressed miRs. By comparing total RNA extracted from 363 solid cancers and 177 normal tissues (in regard to prostate, 56 tumours and 7 tissues from non-cancerous individuals), Volinia's study

verified a general up-regulation of miRs in cancer: specifically, 39 miRs were up-regulated and 6 miRs were down-regulated in prostate cancer. These results were in partial agreement with a more focused study conducted with a similar experimental setting by Ambs *et al.* (2008) in which total RNA extracted from 60 macrodissected prostate cancers and 16 surrounding non-tumour tissues was analyzed. Both studies found an up-regulation of *miR-32*, *miR-26a*, *miR-196a*, *miR-181a*, *miR-25*, *miR-93*, *miR-92* and *let-7i* and a down-regulation of *miR-218* and *miR-128*. Moreover, Ambs *et al.* identified some miRs associated with extraprostatic extension of the tumours, including *miR-101*, *miR-30c* and *miR-195*, which were also part of the prostate cancer signature described by Volinia *et al.* The general overexpression of miRs in prostate cancer was also supported by a computational study that verified a reduced abundance of putative miR targets in human prostate tumours by comparing three different gene expression datasets (Sun *et al.* 2009a).

Different results were shown by other groups, who observed a down-regulation of miRs in prostate cancer (Porkka *et al.* 2007). Porkka *et al.* analyzed small RNA obtained from 6 prostate cancer cell lines, 9 prostate cancer xenografts and 13 clinical prostate tissues, specifically 4 benign prostatic hyperplasia (BPH), 5 untreated prostate carcinomas and 4 hormone-refractory prostate carcinomas. A preliminary analysis showed that prostate cancer cell lines and xenografts were clustered by miR expression in nodes correlating with the androgen receptor status. Moreover, a significant association was described between miR levels and the copy number of miR loci reported in previous array comparative genomic hybridization (aCGH) datasets (Saramaki *et al.* 2006). The comparison of the clinical samples identified 51 miRs differentially expressed, specifically 37 down-regulated and 14 up-regulated in carcinoma samples versus the BPH group; 15/37 and 6/14 were altered only in hormone-refractory carcinomas. The miR expression pattern obtained by Porkka *et al.* was different, if not reversed, from Volinia's & Ambs' profiles: only the up-regulation of *miR-184* and *miR-198* and the down-regulation of *let-7a* were in agreement with Volinia's and the down-regulation of *miR-205* and *miR-221* with Ambs'. On the contrary, Porkka's profile discretely overlapped that generated by Ozen *et al.* (2008). In the latter study, the authors compared 16 prostate cancer tissues and 10 normal prostate tissues and observed a widespread down-regulation of miRs in human prostate cancer: among the 85 detectable miRs, 76 were down-regulated,

with a tendency of a more global down-regulation of miRs in the cases with early PSA recurrence. As aforementioned, several miRs were confirmed as down-regulated by Porkka & Ozen, including the *let-7* family (*let-7a*, *let-7b*, *let-7c*, *let-7d* and *let-7g*), *miR-16*, *miR-23a/b*, *miR-99*, *miR-125a/b*, *miR-29 a/b* and *miR-30 a/b/c*.

More recently, Tong *et al.* analyzed 40 formalin-fixed paraffin-embedded prostatectomy specimens with T2a/b stage tumours, including 20 with biochemical relapse within 2 years of prostatectomy and 20 without relapse for 10 years. For each specimen, areas of malignancy were microdissected and compared to uninvolved areas (Tong *et al.* 2009). The main findings of this study were the down-regulation of *miR-23b*, *-100*, *-145*, *-221* and *-222* (in agreement with Porkka's analysis) and the tendency of early relapse tumours to display a distinguished miR signature (particularly, *miR-135b* and *-194* overexpression).

Given these controversial results, it stands that a conclusive miR profile in prostate cancer cannot be drawn. Even though microarray technology applied to miRs offers an appealing tool to set up patient-tailored treatments, the high-throughput nature of these experiments and the relative infancy of miR studies means that results should be interpreted with caution. The successful execution of such studies requires close collaboration between surgeons, pathologists, molecular biologists and bioinformatics' experts because the complexity of biological systems implies various levels at which both interexperimental and intraexperimental variability can occur.

First, mere technical issues can deeply impact on results: the use of total RNA or enriched small RNA can lead to differences in sensitivity and therefore in variability in detectable miRs and in relative percentages of deregulated miRs. Also, the design of probes able to detect both immature and mature miRs can give a distorted view of the active miRs if any alteration of the processing occurs.

Secondly, the quality of starting material is pivotal: tissue handling and choice can drastically affect the interpretation of microarray data. Even though miRs are known to be more stable than mRNA, different methods and timing of tissue storage can result in different levels of miR expression. In this regard, the documented influence of hypoxic stress on miR expression (Kulshreshtha *et al.* 2007) imposes the consideration of a relevant variable, which can bias results for the pre-existence of a hypoxic component of the tumour, which is more likely in cancers of bigger volume. This issue adds further complexity to the problem of tissue heterogeneity. In tumour samples,

heterogeneity derives from high genetic instability and from the contamination with other cell types that can be present in the tissue in varying degrees, such as fibroblasts and myoepithelial, endothelial and inflammatory cells. Moreover, in early-stage focal lesions, tumour cells can be surrounded by a majority of normal cells. On the other hand, a very careful choice of healthy reference tissue is crucial, as this can be contaminated by neoplastic tissue or be the mirror of a hyperplastic stage that could be characterized by a distinct miR signature. In this regard, the analysis of laser-capture microdissected areas of tumour and normal cells offers the most promising solution to these kind of drawbacks.

Thirdly, study design and patient selection can account for considerable variability in results, not only for eventual intrinsic differences in tumour stage and grade but also for probable influences of extrinsic treatments such as radiotherapy (Jossion *et al.* 2008, Shin *et al.* 2009) or androgen deprivation on global and specific miR expression.

Finally, in such a novel and rapidly developing field it cannot be excluded that previously unconsidered mechanisms and variables might need to be elucidated in order to gain better data consistency.

In the following paragraphs, an up-to-date overview of the miRs whose physiological role in prostate cancer development and progression has been specifically investigated is presented. Figure 1 summarizes these miRs, with their targets and their functions, such as those described in prostate cancer studies. The inconsistency of some deregulations with expression profile results has been discussed above and is summarized in Table 1. The following description includes miRs whose alterations have been correlated with genetic alterations and which eventually result in epigenetic reprogramming, miRs acting downstream of transcription factors and miRs whose deregulation has been implicated in prostate cancer progression through the acquisition of invasive features and/or androgen independence.

Focusing on single miRs: starting from genetics

It is widely accepted that tumour initiation and progression occur through the accumulation of genetic aberrations leading to deregulated expression of genes located in amplified or deleted regions, namely classical oncogenes or tumour suppressor genes. It is now increasingly clear that the same concept can be

Figure 1 miRs whose physiological role in prostate cancer has been thoroughly investigated. miR targets and functions, such as those described in prostate cancer studies, are shown.

applied to miR genes, which have already been found to be enriched in known cancer-associated genomic regions at the onset of their discovered function in cancer pathogenesis (Calin et al. 2004).

Chromosomal CGH and loss of heterozygosity (LOH) studies have verified that in early-stage tumours genomic DNA losses prevail over gains, with 6q, 8p, 10q, 13q, 16q and 17p being implicated most often in these alterations. In hormone-refractory prostate cancer, LOH frequency raises three- to fourfold and chromosomal gains also become prevalent: amplifications at 7p, 7q, 8q and Xq are common late events associated with an aggressive phenotype (Visakorpi et al. 1995, Nupponen et al. 1998a,b, Porkka & Visakorpi 2004, Saramaki et al. 2006). In particular, gain of the 8q region is the most frequent (80–90% of cases) and huge chromosomal alteration in advanced prostate tumours, involving nearly the whole 8q arm. Even though more than one oncogene can be included in such an aberration (Reiter et al. 2000), *c-MYC* amplification at 8q24 is recognized as the main event related to prostate tumour progression. Few genes have been described as the main targets of losses of heterozygosity, including *NKX3-1* at 8p21, *PTEN* at 10q23.3 and *KLF5* at 13q21 (Dong 2001). Despite the presence of two important tumour suppressor genes at 13q, namely *BRCA2* and *RBI*, no correlation between LOH and down-regulation of these genes has been observed (Latil et al. 1999, 2002). A detailed analysis of microsatellite markers on 13q identified a minimal region of loss of 800 kb at 13q14.2–q14.3 (Ueda et al. 1999, Yin et al. 1999, Chen et al. 2001). This allelic imbalance is observed in about 65% of prostate tumours and has been found to be associated with early biochemical relapse (Brookman-Amissah et al. 2007).

miR-15a/miR-16

Interestingly, the above-mentioned region encompasses two miR genes, *miR-15a* and *miR-16*, frequently deleted or down-regulated in chronic lymphocytic leukaemia (Calin et al. 2002) and shown to induce apoptosis by post-transcriptional repression of the antiapoptotic protooncogene *BCL2* (Cimmino et al. 2005).

Based on the hypothesis that these miRs might be the previously unidentified tumour suppressor genes in prostate cancer, our group investigated their expression and function in prostate (Bonci et al. 2008). By qPCR analysis of 20 prostate tumour-derived primary cultures compared to their normal counterpart and by *in situ* hybridization analysis of 15 prostate tumour biopsies, *miR-15a/miR-16* levels were found to be down-regulated in the vast majority of the cases (up to

85% of the samples). By using the model of two prostate cell lines, the normal immortalized RWPE-1 cells and the prostate cancer LNCaP cells with reduced *miR-15a/miR-16* levels, the functions and targets of these miRs were investigated. A stable lentivirus-mediated sequestering of *miR-15a/miR-16* in RWPE-1 cells induced an increase in proliferation and migration *in vitro* and allowed these cells to form small tumour masses in nonobese diabetes/severe combined immunodeficiency (NOD/SCID) mice. On the contrary, lentivirus-mediated *miR-15a/miR-16* reconstitution in LNCaP cells resulted in a dramatic apoptotic effect *in vitro* and in considerable regression of tumour xenografts *in vivo*. Moreover, antagomiR-15a/antagomiR-16 injection in normal prostates of BALB/C mice resulted in hyperplastic morphology of the gland, with alterations of normal acini architecture. These observations were, at least in part, explained by the evidence that *miR-15a/miR-16* are able to post-transcriptionally repress the expression of *BCL2*, *CCND1* (i.e. cyclin D1) and *WNT3A*, thus interfering with multiple oncogenic activities. *BCL2* up-regulation has been described as a common event, especially in hormone-refractory prostate cancer, where it can support survival of cells in the absence of androgens (McDonnell et al. 1992, Colombel et al. 1993). *CCND1* is a well-known promoter of cell proliferation and its regulation by *miR-15a/miR-16* is in line with the evidence of a more widespread role of these miRs in the control of cell cycle progression (Linsley et al. 2007, Liu et al. 2008). *WNT3A* has been implicated in prostate cancer pathogenesis by enhancing β -catenin stabilization and its transcriptional activity, as well as by activating pivotal oncogenic pathways such as v-akt murine thymoma viral oncogene homolog (AKT) and mitogen-activated protein kinase (MAPK) (Almeida et al. 2005, Yun et al. 2005). β -catenin stabilization leads to the transcription of several oncogenic genes (such as *CCND1* itself, *MYC*, *MMPs* and *endothelin*) and, by acting as a co-activator of androgen receptor, it promotes its activity in the presence of low androgen levels (Schweizer et al. 2008). AKT and MAPK activation can also provide prostate cancer cells with survival and proliferation signals in a hormone-deprived environment (Yeh et al. 1999, Graff et al. 2000). Moreover, a recent report showed that *miR-15a/miR-16* regulate vascular endothelial growth factor (VEGF) levels (Karaa et al. 2009), thus implying a role of these two miRs in the control of angiogenesis. In conclusion, several lines of evidence support the concept that *miR-15a/miR-16* loss might contribute by different means to prostate cancer progression.

miR-101

miR-101 is another miR for which genomic loss has been described as a causative mechanism of deregulation (Varambally *et al.* 2008). *miR-101* loci on chromosome 1 (*miR-101-1*) and chromosome 9 (*miR-101-2*) were found to be somatically lost in 37.5% of clinically localized prostate cancer and 66.7% of metastatic disease. Moreover, according to public domain aCGH datasets, this event seems to be common to a broad range of cancer types. *miR-101* overexpression in DU145 prostate cancer cells attenuated their proliferation *in vitro* and *in vivo* and markedly impaired their invasive potential. *miR-101* decrease during prostate cancer progression was found to parallel an increase in enhancer of zeste homologue 2 (EZH2), which was demonstrated as a direct target of *miR-101* (Varambally *et al.* 2008). The primary activity of EZH2, a master regulator of important cellular processes, is to trimethylate histone H3 lysine 27, thus leading to widespread gene silencing (Yu *et al.* 2007, Cao *et al.* 2008). EZH2 has been described as being overexpressed in many solid tumours, promoting cell proliferation, anchorage-independent growth and invasion. Nevertheless, the mechanism of its elevation is unclear so far. The deletion of *miR-101* may be the missing molecular link by which a genetic event can deeply impact on cell epigenetics.

miR-449

Similarly, *miR-449* has been reported to influence cell transcriptome by modulating histone deacetylase 1 (HDAC1) levels (Noonan *et al.* 2009). HDACs prevent transcription through the removal of acetyl groups from histones, thereby promoting chromatin condensation. The relevance of its overexpression in cancers is supported by the existence and the efficacy of HDAC inhibitors in clinical practice. HDAC1 has been described as overexpressed in up to 70% of prostate tumours (Weichert *et al.* 2008), and HDAC inhibitors proved effective in animal models of prostate cancer (Qian *et al.* 2007, Wedel *et al.* 2008). The levels of *miR-449* were found to be lower in prostate tumours than in matched controls. Its reintroduction in PC3 prostate cancer cells resulted in growth arrest, apoptosis and a senescence-like phenotype (Weichert *et al.* 2008, Noonan *et al.* 2009). Reduction of *miR-449*, putatively by loss of the prostate cancer susceptibility locus 5q12, may therefore contribute to HDAC1 increase and downstream epigenetic silencing.

miR-34

Apart from acting upstream of widespread transcriptional changes through the modulation of epigenetic processes, miRs can work downstream of master transcription factors and thereby mediate their functions. Several recent studies have implicated the *miR-34* family of miRs in the p53 tumour suppressor network (Bommer *et al.* 2007, He *et al.* 2007). The expression of *miR-34* is markedly induced by DNA damage and oncogenic stress in a p53-dependent manner. *miR-34* activation can recapitulate the elements of p53 activity, inducing cell cycle arrest and apoptosis by the down-modulation of proteins such as CDK4, CDK6, cyclin D1, cyclin E2, E2F3 and BCL2 (Chang *et al.* 2007, Raver-Shapira *et al.* 2007, Tazawa *et al.* 2007). On the contrary, loss of *miR-34* can impair p53-mediated cell death. These effects have also been observed in prostate cancer, where *miR-34* was found to be absent in androgen-refractory, p53-defective PC3 and DU145 cell lines. *miR-34* reconstitution in PC3 cells impaired cell growth and camptothecin resistance (Fujita *et al.* 2008). Interestingly, *miR-34* also inhibits *SIRT1*, a gene that hinders p53-dependent apoptosis, promoting survival under genotoxic and oxidative stress (Yamakuchi *et al.* 2008). *SIRT1* is elevated in a variety of cancer cells, including PC3 and DU145, where it contributes to chemoresistance. Therefore, *miR-34* participates in a positive feedback loop that enhances p53 activity by suppressing *SIRT1*. Its loss recapitulates, at least in part, the loss of p53.

miR-23

Likewise, by targeting glutaminase, *miR-23* has been found to participate in the pro-tumourigenic network resulting from MYC overexpression, which is thought to be the most common alteration in prostate cancer. One of the effects of this up-regulation is the increase in oxygen consumption and the induction of mitochondrial mass and function (Li *et al.* 2005). Cancer cells depend on continued mitochondrial function for metabolism, including for glutaminolysis. Glutamine is a major source of energy and its catabolism provides nitrogen and carbon substrates for anabolic processes; on the other hand, glutamine catabolism produces the substrate for the synthesis of glutathione, which protects cells from reactive oxygen species. High MYC expression results in strong induction of mitochondrial glutaminase, the enzyme converting glutamine to glutamate. This effect was found to be mediated by MYC-induced *miR-23* repression (Gao *et al.* 2009). According to Porkka's analysis, *miR-23*

levels are reduced in prostate cancer, while elevated levels of MYC parallel glutaminase levels. Thus, *miR-23* loss may connect a genetic alteration to metabolic anomalies in cancer cells.

miR-146

Also belonging to the group of reduced miRs, *miR-146* was identified as more consistently down-regulated in androgen-independent prostate cancer cell lines (including LNCaP-C81, LNCaP C4-2B and PC3) than in androgen-dependent cell lines (LNCaP and PC3-AR9; Lin et al. 2007). This finding was confirmed by fluorescent *in situ* hybridization analysis in human prostate cancer tissue arrays. *miR-146* contribution to prostate cancer progression has been identified in its capacity to repress ROCK1 expression (Lin et al. 2008), a downstream effector of hyaluronan-mediated signalling on CD168 receptor. Hyaluronan is synthesized by stromal fibroblasts in response to paracrine factors produced by tumour cells, and this crosstalk is crucial for the development and progression of hormone-refractory prostate cancer. In particular, adhesion of tumour cells to host cell layers and subsequent transcellular migration are pivotal steps in cancer invasion and metastasis. ROCK1-mediated oncogenic mechanisms include the direct phosphorylation of myosin light chain, which leads to increased cell migration and invasion (Itoh et al. 1999), activation of AKT/TOR/eIF4E signalling and increase in macrophage colony-stimulating factor (M-CSF) cytokine production to facilitate osteolytic metastasis (Bourguignon et al. 2003).

Interestingly, it has been demonstrated that *miR-146* inhibits the translation of the SDF-1 receptor CXCR4, and that megakaryopoiesis is controlled by a cascade pathway, in which the promyelocytic leukemia zinc finger (PLZF) factor suppresses *miR-146* transcription and thereby activates CXCR4 translation (Labbaye et al. 2008). Although reported in a completely different system, this targeting may have pathogenic relevance in prostate cancer metastasis. As a matter of fact, haematopoietic cells home to bone during embryonic development; the chemokine SDF-1 (expressed by osteoblasts and endothelial cells) and its receptor CXCR4 play a key role in this process (Kucia et al. 2005). Increasing evidence is supporting the idea that the SDF-1/CXCR4 axis influences the formation of the premetastatic niche and the recruitment of endothelial progenitor cells at the primary tumour site with subsequent angiogenesis (Wang et al. 2006). Several different tumour types, including the

osteotropic prostate and breast cancers, have been shown to overexpress CXCR4 (Mochizuki et al. 2004, Luker & Luker 2006), possibly at the post-transcriptional level (Sun et al. 2003). In prostate cancer, CXCR4 protein expression is significantly elevated and its levels are associated with poor prognosis (Akashi et al. 2008). Thus, it is conceivable that *miR-146* loss might contribute to enhanced prostate cancer aggressiveness by increasing multiple pro-metastatic proteins, including ROCK1 and CXCR4.

miR-205

Also described as implicated in migration control, *miR-205* was reported to be down-regulated in prostate cancer cell lines and in 23 of 31 prostate carcinomas compared to the untransformed RWPE-1 cell line and normal prostate tissues respectively (Gandellini et al. 2009). *miR-205* restoration in DU145 prostate cancer cells resulted in marked morphological changes resembling a reverse epithelial–mesenchymal transition. Specifically, *miR-205*-transfected cells tended to shift from a fibroblast-like elongated phenotype to a large, flattened, polygonal shape and to aggregate in tightly packed colonies. The enhanced cellular adhesion of these cells was confirmed by a strong increase in E-cadherin and β -catenin expression at cell–cell contacts. Consistently with a role in the negative control of epithelial–mesenchymal transition, an event preceding metastasis, *miR-205* reintroduction significantly reduced migratory and invasive capabilities of DU145 and PC3 cells. These effects were partially explained by *miR-205* targeting of PRKCE (PKC ϵ) and ZEB2 mRNAs. The role of these molecules in prostate cancer progression is sustained by ZEB2-mediated repression of E-cadherin transcription (Peinado et al. 2007) and by PKC ϵ -induced increase in proliferation, apoptosis resistance and androgen independence (Wu & Terrian 2002). Unexpectedly, PKC ϵ interference rather than ZEB2 interference produced a phenotype more reminiscent of the one observed in *miR-205*-expressing cells. Interestingly, gene expression profile of DU145 transfected with *miR-205* precursor revealed a general induction of genes involved in the assembly of cell junctions and the down-regulation of several pro-tumourigenic factors, some of which have already been associated with prostate cancer progression, such as IL-6 (Culig et al. 2005), caveolin-1 (Williams et al. 2005), EZH2 (Varambally et al. 2002, Bracken et al. 2003), ERBB3 (Li et al. 2006), E2F1 and E2F5.

miR-21

Although not extensively investigated in prostate cancer, *miR-21* is noteworthy as in the large-scale study conducted by Volinia *et al.* it was the only miR up-regulated in all the analyzed tumours, including breast, colon, lung, pancreas, prostate and stomach cancers. Additional studies demonstrated elevated *miR-21* expression in hepatocellular carcinomas (Kutay *et al.* 2006), ovarian cancer (Iorio *et al.* 2007, Nam & Kim 2008), cervical carcinoma (Lui *et al.* 2007), multiple head and neck cancer cell lines (Tran *et al.* 2007), papillary thyroid carcinoma (Tetzlaff *et al.* 2007) and some other solid tumours. More recent studies indicate that *miR-21* is also up-regulated in leukaemic cancers (Fulci *et al.* 2007, Lawrie 2007, Jongen-Lavrencic *et al.* 2008, Navarro *et al.* 2008). Therefore, abundant *miR-21* may be a general feature of tumour cells.

In prostate cancer, *miR-21* was found to be elevated in PC3 and DU145 androgen-independent cell lines (Li *et al.* 2009). Blocking *miR-21* by antisense oligonucleotides did not affect proliferation, but it sensitized cells to staurosporine-induced apoptosis and impaired cell motility and invasion. In this study, myristoylated alanine-rich protein kinase C substrate (MARCKS) was identified as a new *miR-21* target in prostate cancer cells. Given the involvement of MARCKS in cellular processes such as cell adhesion, spreading, membrane trafficking and cell motility (Arbuzova *et al.* 2002), it is conceivable that the observed effects were due, at least in part, to modified MARCKS levels. However, lessons from other cancers suggest that a wider spectrum of genes might be altered in *miR-21*-overexpressing cells. Validated targets of *miR-21* enclose several genes mainly implicated in suppressing cell migration and invasion, including PDCD4 (Asangani *et al.* 2008, Frankel *et al.* 2008), PTEN (Meng *et al.* 2007), TPM1 (Zhu *et al.* 2007), SPRY2 (Sayed *et al.* 2008) and the metalloprotease inhibitors TIMP3 and RECK (Gabriely *et al.* 2008).

miR-221/miR-222

Along with *miR-21*, the two closely related *miR-221* and *miR-222* are among the most frequently over-expressed miRs in cancer. Several reports have consistently described the up-regulation of either one miR or both miRs in a wide spectrum of human tumours, including glioblastoma (Ciafre *et al.* 2005), melanoma (Felicetti *et al.* 2008), and hepatocellular carcinoma (Fornari *et al.* 2008), as well as in thyroid (Pallante *et al.* 2006), kidney and bladder (Gottardo *et al.* 2007), gastric (Kim *et al.* 2009), pancreatic

(Lee *et al.* 2007), ovarian (Dahiya *et al.* 2008) and prostate cancers (Galardi *et al.* 2007). In prostate cancer cell lines and primary tumour cells, an inverse correlation between the expression of *miR-221/miR-222* and the cell cycle inhibitor p27^{Kip1} (Galardi *et al.* 2007, Mercatelli *et al.* 2008) has been described. Consistently, ectopic introduction of *miR-221/miR-222* in low expresser LNCaP cells strongly increased their growth potential by inducing a G₁–S shift in the cell cycle, and it was able to enhance their clonogenicity in soft agar and their tumorigenicity in SCID mice. Conversely, anti-*miR-221/222* treatment of high expresser PC3 cells impaired their colony-forming potential *in vitro* and reduced tumour growth *in vivo* (Mercatelli *et al.* 2008). The documented influence on the levels of CDK inhibitors p27^{Kip1} and p57^{Kip2} by *miR-221/miR-222* can easily explain the observed effects on cancer cell proliferation. In particular, p27^{Kip1} is a well-established marker of poor prognosis in several human tumours, including prostate cancers. However, further studies are needed to unravel the role of *miR-221/miR-222* in the progression to androgen independence. A recent study comparing androgen-dependent and androgen-independent LNCaP cells has described *miR-221/miR-222* as massively increased in castration-resistant cells (Sun *et al.* 2009b). Overexpression of *miR-221* or *miR-222* in androgen-dependent LNCaP cells significantly reduced the dihydrotestosterone (DHT)-induced up-regulation of PSA expression and increased androgen-independent growth. Conversely, knocking down *miR-221* and *miR-222* in the hormone-resistant clone impaired the growth rate of these cells and restored the sensitivity to DHT in terms of PSA transcription and of hormone-stimulated proliferation. Thus, increased *miR-221/miR-222* levels seem to contribute to androgen-independent growth and to be necessary for maintaining the castration-resistant phenotype. Speculatively, this notion may fit together with the reported observation that androgens down-regulate *miR-221/miR-222* levels, thereby helping to explain the controversial low levels of these two miRs documented in microarray studies (Ambs *et al.* 2008). In the scenario of an early, hormone-dependent disease, androgens may play a protective role by keeping *miR-221/miR-222* levels low. Loss of this restraint might accompany, as a cause or as an effect, prostate cancer progression.

miR-125b

In a similar effort to identify miRs associated with prostate cancer progression towards androgen independence, *miR-125b* was described as up-regulated by

androgens and overexpressed in androgen-independent LNCaP sublines compared to parental cells (Shi *et al.* 2007). These controversial findings suggest that androgen-independent regulation of *miR-125b* may also exist. Similar to *miR-221/miR-222*, transfection of synthetic *miR-125b* stimulated the growth of LNCaP cells in the absence of androgens, whereas anti-*miR-125b* inhibited the growth of androgen-independent cells. In this study, the pro-apoptotic Bak1 was identified as a target of *miR-125b*. Bak1 expression has also been reported to be associated with the progression of prostate cancer, with it being reduced in hormone-refractory tumours (Yoshino *et al.* 2006). However, as Bak1 interference did not significantly increase proliferation of prostate cancer cells, further studies are needed to elucidate the role of this miR. Overall, the oncogenic role of *miR-125b* in prostate cancer progression needs further investigation as three miR expression profiles report it as down-regulated in prostate cancer (Porkka *et al.* 2007, Ozen *et al.* 2008, Tong *et al.* 2009). Moreover, *miR-125b* has been described as a tumour suppressor gene in human breast cancer, where it can negatively regulate the expression of both *ERBB2* (*HER2*) and *ERBB3* (*HER3*) (Scott *et al.* 2007). Although miRs can act as oncogenes or tumour suppressor genes in different cellular contexts, it is puzzling that increased expression of *HER2* has been reported in prostate cancer and considered to be a mechanism related to androgen independence (Craft *et al.* 1999).

miR-32, miR-106b-25-93 and miR-126*

miR-32 gene and the cluster *miR-106b/miR-25/miR-93* are located in the intronic regions of *C9orf5* and *MCM7* respectively. These miRs have been described as up-regulated in prostate cancer as a consequence of the overexpression of their host genes (Ambs *et al.* 2008). Whereas the role of *C9orf5* in cancer is unknown, *MCM7* amplifications had already been associated with prostate cancer (Ren *et al.* 2006). However, little is known about the functional relevance of these co-regulations, and further studies are needed to address whether the oncogenic effects of these amplifications are due to either the host genes or the miRs. *miR-32* was found to inhibit the expression of BIM, a proapoptotic member of the BCL2 family, whose down-regulation can contribute to the resistance of tumour cells to death stimuli (Ambs *et al.* 2008). Likewise, the *miR-106b* cluster was found to play an antiapoptotic, growth inhibitory activity by suppressing E2F1 and p21/WAF1 (Ambs *et al.* 2008). E2F1 is also a target of *miR-17-5p* and *miR-20a*, two miRs

belonging to the *miR-17-92* cluster, which were found to be up-regulated in many tumours, including prostate (Volinia *et al.* 2006, Sylvestre *et al.* 2007).

Conversely, it has been demonstrated that *miR-126** deficiency in prostate cancer cells is due to poor expression of its host gene, EGF-like domain 7. The absence of *miR-126** results in high levels of its natural target prostein, a prostate antigen whose reduction impairs LNCaP cell migration and invasion (Musiyenko *et al.* 2008).

MYC and miR: an attempt to weave a net

In the last few years, both large-scale and focused studies have shed light on the identity of miRs whose deregulation might have a pathogenic significance in prostate cancer disease. In some cases, miR deregulations have been proposed or demonstrated as consequences of single genetic or epigenetic events occurring during tumour progression. However, it may be intriguing to wonder if a common upstream event might also contribute to widespread miR deregulation. In this regard, oncogenic transcription factors may amplify their effects by deregulating the activity of sets of miRs, and thereby indirectly influence a huge spectrum of targets. In particular, it is remarkable that several reports have demonstrated the existence of a tight link between miRs and MYC, albeit in experimental systems different from prostate. The c-MYC oncogenic transcription factor is pathologically activated in many human malignancies (Adhikary & Eilers 2005, Meyer & Penn 2008) including prostate cancer, where amplification of the 8q region is one of the most frequent somatic lesions (Jenkins *et al.* 1997) and c-MYC activity is known to induce androgen-independent prostate cancer growth (Bernard *et al.* 2003).

In lymphoma cells, c-MYC was found to directly activate the expression of the *miR-17-92* cluster, a group of six miRs, including the above-mentioned *miR-17-5p* and *miR-20*, which is known to contribute to lymphomagenesis and to be involved in many human cancers by promoting cell proliferation, suppressing apoptosis and inducing angiogenesis (Mendell 2008). Interestingly, a later report documented a MYC-induced widespread down-regulation of miRs by direct binding of the transcription factor to promoters or conserved regions upstream of the regulated miRs (Chang *et al.* 2008). The functional significance of this down-regulation was demonstrated by the evidence that enforced expression of repressed miRs diminished the tumourigenic potential of lymphoma cells *in vivo*. The list of down-regulated

miRs included members of the *let-7* family, *miR-15a*, *miR-16*, *miR-22*, *miR-26*, *miR-29*, *miR-30*, *miR-34*, *miR-146*, *miR-150* and *miR-195*. Moreover, c-MYC was reported to activate the expression of *miR-106b* (Sampath *et al.* 2009) and to induce EZH2 over-expression by repressing *miR-26* (Sander *et al.* 2008), which may act in concert with *miR-101* (Varambally *et al.* 2008). Finally and interestingly, c-MYC is known to drive tumorigenesis by increasing genomic instability by means of single nucleotide substitutions and double-stranded breaks (Prochownik 2008). Thus, c-MYC can alter miR expression either directly by modulating miR promoters or indirectly by inducing genetic alterations that can result in aberrant miR levels. Speculatively, the strong enrichment of prostate cancer-associated miRs among MYC-regulated miRs might be more than a mere coincidence.

miRs towards clinical biochemistry and therapeutics

Despite much progress, our understanding of miR alterations in prostate cancer is still in its infancy. Once a clearer overview of the pathological significance of each alteration is reached and the statistical value of any deregulation is confirmed in a vast cohort of patients, selected miRs might be appealing candidates for clinical biochemistry and therapeutics. First, miRs have an unusually high stability in formalin-fixed tissues (Li *et al.* 2007, Xi *et al.* 2007), and this feature makes their detection by *in situ* hybridization or quantitative PCR analysis possible. Secondly and more interestingly, miRs have proven to be present in a markedly stable form in the plasma and serum fluids of humans and other animals. The levels of miRs in serum are reproducible and consistent among individuals of the same species (Gilad *et al.* 2008), while specific expression patterns of serum miRs have already been identified for pregnancy, diabetes and different cancers, thus providing evidence that serum miRs contain fingerprints distinctive of given conditions (Chen *et al.* 2008, Lawrie *et al.* 2008, Taylor & Gercel-Taylor 2008). The comparison of a case–control cohort of serum samples collected from 25 individuals with metastatic prostate cancer and 25 age-matched male control individuals showed increased expression of *miR-100*, *miR-125b*, *miR-141*, *miR-143* and *miR-296* in the prostate cancer serum pool (Mitchell *et al.* 2008). In particular, *miR-141* was proven to be the best prostate cancer biomarker, with it being 46-fold up-regulated and expressed in an epithelial cell type-specific manner. Thus, serum miRs hold special promise for

the discovery of powerful and sensitive biomarkers, which are easily detectable in non-invasive assays that might overtake the antibody-related drawbacks of proteomics approaches (Rifai *et al.* 2006). As stated previously, diagnostic, prognostic, predictive and therapeutic markers are needed to help in forecasting disease severity, choosing therapies and monitoring responses. Moreover, unpredictable advances may come from the investigation of a putative functional role of these circulating miRs. The most compelling hypothesis envisages that these small RNAs, packed in microvesicles called exosomes, work as extracellular messengers mediating a new form of cell–cell communication (Valadi *et al.* 2007). Speculatively, miRs may play a role in the crosstalk between the primary tumour site and the premetastatic microenvironment.

Finally, discovering miRs and their role in the pathogenesis of cancer and other diseases has opened a new biomedical scenario that could use miR agonists or antagonists in order to restore or block the function of a given miR. The multitargeting nature of miRs makes them both powerful and difficult to handle, given the risk of unintended off-target effects that need to be carefully evaluated. The development of antagomiRs, specific and effective miR antagonists that are suitable *in vivo*, paved the way to miR-based therapeutic strategies (Krutzfeldt *et al.* 2005, 2007). The first therapeutic application of antagomiRs has proven promising: in mice and non-human primates, the effective and non-toxic antisense-mediated blocking of liver-specific *miR-122* resulted in reduced cholesterol synthesis and improved fatty acid metabolism (Elmen *et al.* 2008). Nonetheless, the undeniable appeal of these results cannot ignore that the pharmacokinetics of any non-tissue-specific miR in organs less receptive than liver, such as prostate, may prove more challenging (Petri *et al.* 2009). Similarly, the *in vivo* reintroduction of deficient miRs is a major therapeutic hurdle. Several strategies have been developed to increase small RNA half-life in the blood, facilitate transduction across biological membranes and mediate cell-specific delivery of siRNA (Muratovska & Eccles 2004). miR-based therapies may take advantage of such strategies.

In conclusion, our knowledge of miRs in cancer is still limited, and much inconsistency of data has to be overcome before a diagnostic and/or therapeutic application of miRs can be envisaged. However, the rapid expansion of this field and the extent of the perspectives offered let us foresee exciting future advances.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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