MicroRNA signatures in human cancers

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Abstract | MicroRNA (miRNA) alterations are involved in the initiation and progression of human cancer. The causes of the widespread differential expression of miRNA genes in malignant compared with normal cells can be explained by the location of these genes in cancer-associated genomic regions, by epigenetic mechanisms and by alterations in the miRNA processing machinery. MiRNA-expression profiling of human tumours has identified signatures associated with diagnosis, staging, progression, prognosis and response to treatment. In addition, profiling has been exploited to identify miRNA genes that might represent downstream targets of activated oncogenic pathways, or that target protein-coding genes involved in cancer.

Cancer is a complex genetic disease involving structural and expression abnormalities of both coding and non-coding genes. For almost three decades, the alteration of protein-coding oncogenes and/or tumour-suppressor genes (for reviews see REFS 1–3) have been thought to be the causes of tumorigenesis. With the discovery in the past few years of thousands of genes that produce non-coding RNA (ncRNA) transcripts with no significant open reading frame, it has become evident that the genomic complexity of the cancer cell is far greater than expected. Although the identification of long ncRNAs was quite a slow process owing to the difficulties of experimentally cloning non-coding cDNAs of tens or hundreds of thousands of bases[4], the cloning of small members of the ncRNA class was recently extremely successful[5–9].

These microRNAs (miRNAs) are small non-coding RNAs of 20–22 nucleotides, typically excised from 60–110 nucleotide foldback RNA precursor structures (for reviews see REFS 10–12). The biogenesis of miRNAs involves a complex protein system, including members of the Argonaute family, Pol II-dependent transcription and the RNase IIIs Drosha and Dicer[13]. MiRNAs are involved in crucial biological processes, including development, differentiation, apoptosis and proliferation[11,14], through imperfect pairing with target messenger RNAs (mRNAs) of protein-coding genes and the transcriptional or post-transcriptional regulation of their expression[15–17]. The number of human miRNAs reported so far (the July 2006 release of miRBase, at the Sanger Institute[18]) is in excess of 450, twice as many as initial calculations indicated[19], and more than1,000 predicted miRNA genes[20–22] are awaiting experimental confirmation.

Initially identified in B-cell chronic lymphocytic leukaemia (CLL)[23], changes in the expression level of miRNAs have subsequently been detected by different groups in many types of human tumours (several reviews give various details of the link between miRNAs and cancer, such as REFS 24–34). MiRNAs have been proposed to contribute to oncogenesis because they can function either as tumour suppressors (as is the case for *miR-15*a and *miR-16-1*) or oncogenes (as is the case for *miR-155* or members of the *miR-17–92* cluster). The genomic abnormalities found to influence the activity of miRNAs are the same as those previously described for protein-coding genes, such as chromosomal rearrangements, genomic amplifications or deletions and mutations. In a specific tumour, abnormalities both in protein-coding genes and miRNAs can be identified[29]. Homozygous mutations or the combination of deletion plus mutation in miRNA genes are rare events[35], and the functional consequences of heterozygous sequence variations of miRNAs in human cancers have not been identified[36]. Furthermore, the role of polymorphisms in the complementary sites of target mRNAs in cancer patients[37] or individuals with a predisposition to other hereditary diseases[38] have just started to be understood. Therefore, at the present time, the main mechanism that underlies changes in the function of miRNAs in cancer cells seems to be aberrant gene expression, characterized by abnormal levels of expression for mature and/or precursor miRNA sequences compared with the corresponding normal tissues.

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At a glance

- MicroRNAs (miRNAs) located in genomic regions amplified in cancers (such as the *miR-17–92* cluster) function as oncogenes, whereas miRNAs located in portions of chromosomes deleted in cancers (such as the *miR-15a–miR-16-1* cluster) function as tumour suppressors.
- Abnormal expression of miRNAs has been found in both solid and haematopoietic tumours by various genome-wide techniques (including different microarray platforms or bead-based flow cytometry).
- The abnormally expressed miRNAs in human cancers target transcripts of essential protein-coding genes involved in tumorigenesis, such as the Ras oncogenes by *let-7* family members, the BCL2 anti-apoptotic gene by the *miR-15a–miR-16-1* cluster, the E2F1 transcription factor by the *miR-17–92* cluster or the BCL6 anti-apoptotic gene by *miR-127*.
- MiRNA expression fingerprints correlate with clinical and biological characteristics of tumours, including tissue type, differentiation, aggression and response to therapy.
- The fact that consistent abnormal expression of the precursor miRNA, but not of the correspondent active molecule, is found in various types of cancers, raises the possibility that the 'non-active' part of the miRNA molecule could have 'independent' and as yet unknown functions that could be important in tumorigenesis.
- Germline sequence abnormalities were identified in miRNA genes and transcripts, and in targeted sequences in messenger RNAs that are known to be targets of miRNAs. Furthermore, as each miRNA has many targets, inherited minor variations in miRNA expression could have important consequences for the expression of various protein-coding genes involved in malignant transformation. Therefore, it is tempting to propose that both these phenomenons are involved in familial predisposition to cancer.

Bead-based flow cytometry
A high-throughput microRNA (miRNA)-profiling technique in which individual beads are coupled to miRNA complementary probes and marked with fluorescent tags. After hybridization with size-fractioned RNAs and staining, the beads are analysed using a flow cytometer.

Hierarchical clustering
A computational method that groups genes (or samples) into small clusters and then groups these clusters into increasingly higher level clusters. As a result, a dendrogram (that is, a tree) of connectivity emerges.

MiRNA profiling of tumour versus normal tissues

Oligonucleotide miRNA microarray analysis is the most commonly used high-throughput technique for the assessment of cancer-specific expression levels for hundreds of miRNAs in a large number of samples (for reviews see REFS 13,39,40). Another method to determine miRNA expression levels involves the use of a bead-based flow-cytometric technique[41] (FIG. 1). Other developments include quantitative real-time PCR for precursor miRNAs[42] or active miRNAs[43,44], miRAGE — a genome-wide miRNA analysis with serial analysis of gene expression (SAGE)[45] — or the high-throughput array-based Klenow enzyme (RAKE) assay[46]. Each of these techniques has its strengths and weaknesses, but after several years of study using these technologies, and the analysis of more than 1,000 primary tumours, common characteristics of miRNA deregulation in human tumours have emerged.

MiRNA-expression profiles classify human cancers. To date, every type of tumour analysed by miRNA profiling has shown significantly different miRNA profiles (for mature and/or precursor miRNAs) compared with normal cells from the same tissue (TABLE 1, FIG. 2). Two large profiling studies using various tumours and two distinct technologies to investigate genome-wide miRNA expression have been published[41,47]. Lu *et al.* developed a bead-based flow-cytometric profiling technology that is highly specific. They performed a systematic analysis of 334 leukaemias and solid cancers, and found that miRNA-expression profiles classify human cancers according to the developmental lineage and differentiation state of the tumours. Furthermore, they observed patterns of gene expression for each type of tumour that reflected distinct mechanisms of transformation. For example, the acute leukaemia samples were divided into three clusters that corresponded to the *BCR–ABL*, *TEL-AML1* (acute myeloid leukaemia 1, also known as runt-related transcription factor 1; *RUNX1*) and *MLL* (mixed lineage leukaemia) rearrangements, respectively. An interesting observation was that miRNA expression in tumour samples seems globally lower than in normal tissue, as 129 out of 217 measured miRNAs showed this pattern of expression. One explanation could be that global miRNA levels reflect the state of cellular differentiation. In fact, several profiling studies, including those by Lu *et al.*[41] and by Garzon *et al.*[48], showed that distinct miRNA profiles characterize different haematopoietic differentiation stages.

Using a large-scale microarray analysis of 540 samples, including 363 from six of the most frequent human solid tumour types and 177 normal controls, Volinia *et al.* also found that cancer cells showed distinct miRNA profiles compared with normal cells[47] (FIG. 2). Out of the 228 miRNA genes analysed, 36 were overexpressed and 21 were downregulated in cancer cells versus normal cells. Hierarchical clustering analyses showed that this miRNA signature enabled the tumour samples to be grouped on the basis of their tissue of origin. Several other genome-wide profiling studies have been performed on various cancer types, including CLL[49], breast cancer[50], glioblastoma[51], thyroid papillary carcinoma[37], hepatocellular carcinoma[52], lung cancer[53], colon cancer[45] and endocrine pancreatic tumours[54]. For example, in a study of 104 matched pairs of primary cancerous and non-cancerous lung tissue, Yanaihara *et al.* found a set of 43 differentially expressed miRNAs; 28 were downregulated and 15 were overexpressed in the tumour cells[53]. Murakami *et al.*, using a microarray platform, found that in hepatocellular carcinoma five out of eight differentially expressed miRNAs were downregulated in cancer cells[52]. All these studies support the same view: the alterations seen in cancer cells that express miRNAs consist of both overexpressed and downregulated microRNAs.

One key issue is the level of differential expression that can be considered biologically significant. 10–20-fold differences in miRNA expression levels have been found to be important in papillary thyroid carcinomas[37], however, we share the view that much smaller changes (less than twofold) could also be significant[35,49–51]. This reflects the fact that specific miRNAs target many genes and, as a large number of miRNAs seem to be altered in human tumours, one can postulate that the disruption of two or more cooperating oncogenes and/or tumour suppressors is a likely event[29]. An opposite view is that in some cases the described changes in miRNA expression between normal and tumour cells could be biologically irrelevant: the main interactions with various targets could have antagonistic rather than accumulating biological consequences (for example, the repression of both pro-apoptotic and anti-apoptotic genes).

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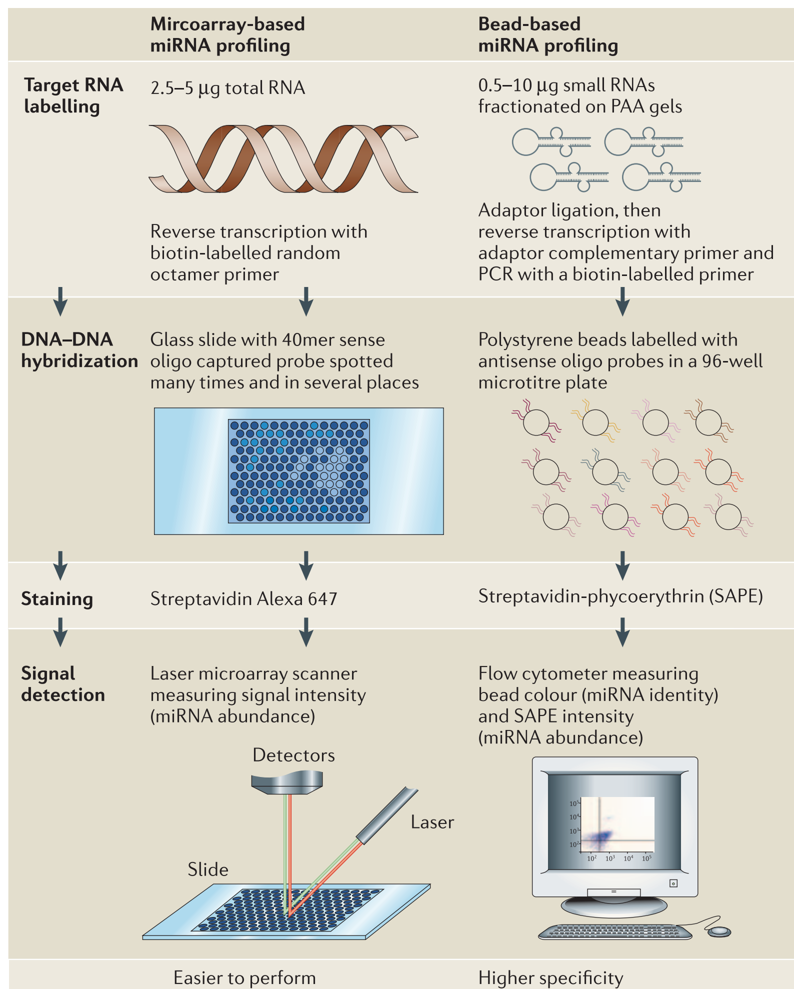

Figure 1 | **Principles of microarray technology and bead-based flow cytometry used for microRNA profiling.** The process of microarray-based microRNA (miRNA) profiling , which was initially developed by Liu *et al.*[39], is shown as described in most profiling studies on primary tumours. The bead-based miRNA profiling technique was developed by Lu *et al*[41]. Both strategies involve four main steps (shown on the left side): target labelling, DNA–DNA hybridization, staining and signal detection. The different replicates of the spots on the glass slide represent different oligonucleotide sequences corresponding to sequences from the precursor miRNA or active miRNA molecule, whereas the different beads are marked with different fluorescent tags (each colour represents a specific miRNA using up to 100 different colours). The main advantage of the microarray-based miRNA profiling technique is the high standardization of the procedure (which enables tens of samples to be processed in parallel), whereas the main advantage of the bead-based miRNA profiling is that, because hybridization takes place in solution (instead of a glass surface), it offers a higher specificity for closely related miRNAs. The described polyacrylamide gel (PAA) fractionation is an additional processing step in the bead-based system. This figure is modified with permission from REF. 49 © (2004) National Academy of Science, USA.

Significance analysis of microarrays (SAM)
A statistical method used in microarray analyses that calculates a score for each gene, and therefore identifies genes that are significantly associated with an outcome variable, such as the type of analysed tissue (normal or cancerous).

Common miRNA genes are differentially expressed in various cancers, suggesting common altered regulatory pathways. Assembling the statistical analyses of microarray data obtained by two different methods, significance analysis of microarrays (SAM) and prediction analysis of microarrays (PAM) from six solid tumours (lung, breast, colon, gastric and prostate carcinomas and endocrine pancreatic tumours), Volinia *et al.* described a common signature composed of 21 miRNAs differentially expressed in at least three tumour types[47]. At the top of the list were *miR-21*, which was overexpressed in six types of cancer cells, and *miR-17-5p* and *miR-191*, which were overexpressed in five. As the embryological origin of the analysed tumours was different, the significance of such findings could be that these common miRNAs participate in fundamental signalling pathways altered in many types of tumour. Supporting the function of these genes in tumorigenesis, it was found that the predicted targets for the differentially expressed miRNAs are significantly enriched for those that target known tumour suppressors and oncogenes[47]. Furthermore, *miR-21*, the only miRNA overexpressed in all six types of cancer analysed, and which is also overexpressed in glioblastomas[55,51] and cholangiocarcinomas[56], was shown by Meng *et al.* to directly target the tumour suppressor *PTEN* in cholangiocarcinoma cells. *PTEN* encodes a phosphatase that can inhibit growth and/or survival pathways, and its function is altered in advanced tumours of various types, including breast, lung, gastric and prostate[57]. In all these tumours, the frequency of loss-of-heterozygosity (LOH) at the *PTEN* locus is relatively small (less than 10%), leading to the speculation that if *PTEN* is shown to be a target of *miR-21* in the other tumour types then this could be the main mechanism of *PTEN* inactivation. Furthermore, knockdown of *miR-21* in cultured glioblastoma cells triggers apoptosis by a caspase-dependent mechanism[55]. All these data suggest that *miR-21* is an anti-apoptotic and pro-survival factor.

Further examples of miRNAs that are frequently deregulated in cancer and target tumour suppressors and/or oncogenes come from an elegant study performed in the laboratory of F. Slack, which clearly showed that Ras oncogenes are regulated by the *let-7* miRNA family[58]. Another example of fine functional dissection of a miRNA pathway comes from the study of Voorhoeve *et al.*, which shows the oncogenic function of *miR-372* and *miR-373* in testicular germ-cell tumours of adolescents and adults. These miRNAs facilitate the proliferation and transformation of cells that harbour both oncogenic *HRAS* and functional wild-type *TP53* (REF. 59).

Additional support for the function of miRNAs in human cancers has come from transgenic and knockout mouse models, which have been important for understanding the tumorigenic pathways in which deregulated miRNAs are involved. He *et al.* showed that enforced expression of the *miR-17-92* cluster accelerated MYC-induced lymphomagenesis[60], suggesting the oncogenic potential of some of the miRNAs that are overexpressed in B-cell lymphoma samples owing to genomic amplification. Recently, Costinean *et al.* produced the first transgenic mouse that specifically overexpresses a miRNA gene, *miR-155*, in B cells[61]. This gene is overexpressed in various types of B-cell malignancy[62–64]. The transgenic mice developed polyclonal pre-leukaemic pre-B-cell proliferation followed by B-cell malignancy. These findings suggest that *miR-155* deregulation could be an early event in oncogenesis that needs additional genetic alterations for the development of the fully malignant phenotype[61]. The same was also shown for

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Table 1 | **Facts about microRNA-expression profiling in human cancers**

Cancer type*	MiRNA profiling data	Significance	Refs
Chronic lymphocytic leukaemia	A unique signature of 13 genes associated with prognostic factors (ZAP70 and IgVH mutation status) and progression (time from diagnosis to therapy)	MiRNAs as diagnostic markers (the identification of two categories of patients)	49,35
Lung adenocarcinoma	Molecular signatures that differ with tumour histology; miRNA profiles correlated with survival (*miR-155* and *let-7*)	MiRNAs as prognostic and diagnostic markers	53
Breast carcinoma	MiRNA expression correlates with specific pathological features	MiRNAs as prognostic markers	50
Endocrine pancreatic tumours	A signature that distinguishes endocrine from acinar tumours; the overexpression of *miR-21* is strongly associated with both a high Ki67 proliferation index and the presence of liver metastases	MiRNAs as diagnostic and prognostic markers	54
Hepatocellular carcinoma	MiRNA expression correlated with differentiation	MiRNAs as prognostic markers	52
Papillary thyroid carcinoma	MiRNA upregulation (for example, *miR-221* and *miR-222*) in tumoral cells and normal cells adjacent to tumours, but not in normal thyroids without cancers	MiRNAs probably involved in cancer initiation	37 114
Glioblastoma	A specific signature compared with normal tissues	MiRNAs as diagnostic markers	51
Human cancers	MiRNA-expression profiles accurately classify cancers; an miRNA classifier classes poorly differentiated samples better than a messenger RNA classifier	MiRNAs as diagnostic markers	41
Human solid cancers	Common signature for distinct types of solid carcinomas	Specific miRNAs are involved in common molecular pathways	47

*Only data from microarray studies reporting results on human primary tumours were included in this table. IgV_H, immunoglobulin heavy-chain variable-region, MiRNA, microRNA. ZAP70, 70 kDa zeta-associated protein.

the *MYC* oncogene, which is activated by chromosomal translocation in human B-cell neoplasia[65]. In an avian model, oncogenic cooperation in lymphomagenesis and erythroleukaemogenesis between the non-protein-coding *BIC* gene, which was later shown to include the *miR-155* sequence, and MYC has been observed[66], indicating that MYC and *BIC* might cooperate in human tumours. A microarray study of the malignant B cells isolated from *miR-155* transgenic mice compared with non-transgenic controls resulted in a complete list of the deregulated protein-coding genes in these cells, and therefore potentially identified direct and indirect targets of *miR-155*. Of particular note, the expression of 16 different miRNAs was altered in pre-malignant cells from the transgenic mice[61], supporting the view that complex relationships exist between protein-coding genes and miRNAs that affect specific signalling pathways and, probably, direct or indirect interactions exist between *miR-155* and some of the other deregulated miRNAs.

Onco-miRNAs and tumour-suppressor-miRNAs: two different views of the same gene? Genome-wide expression studies also offer intriguing new perspectives on the involvement of miRNAs in human cancer. The first perspective comes from the studies performed by O'Donnell *et al.* on cell models, which showed that in the human B-cell line P493-6, which overexpresses *MYC*, miRNA members of the *miR-17–92* locus have tumour-suppressor activity because their expression decreases the expression of E2F1, and so inhibits MYC-mediated cellular proliferation[67]. However, a different perspective is offered by the results from B-cell lymphomas. The same cluster of miRNAs function as a potential oncogene by cooperating with *MYC* and blocking apoptosis[60]. One possible explanation is that the same miRNA can participate in distinct pathways, having different effects on cell survival, growth and proliferation that are dependent on the cell type and the pattern of gene expression. The combinatorial nature of miRNA–mRNA interactions means that the same miRNA could have different targets and the same mRNA could be targeted by different miRNAs in different cell types. For example, Cimmino *et al.* found that in leukaemic cells with a deletion of the locus on chromosome 13 that contains *miR-15a* and *miR-16-1* miRNA genes, apoptosis is induced as a result of the exogenous re-expression of these miRNAs. *MiR-15a* and *miR-16-1* interact with the anti-apoptotic gene *BCL2* when added back to these cells, however, a similar response is not seen in 293 fetal kidney cells, which normally express *miR-15a*, *miR-16-1* and BCL2 (REF. 68). Therefore, it is probable that the loss of *miR-15a* and *miR-16-1* induces the overexpression of BCL2 in B cells, giving rise to B-cell malignancies. In a different tissue, the overexpression of *miR-15a* and *miR-16-1* might cause the loss of expression of a tumour suppressor that is important for growth control and/or apoptosis. In support of this hypothesis are reports that show that, although *miR-15a* and *miR-16-1* are downregulated in B lymphocytes from patients with CLL[23], the same miRNAs are overexpressed in endocrine pancreatic tumours of neuroectodermal origin[47,54].

The second perspective comes from the finding that the consistent abnormal expression of the precursor miRNAs, but not the active molecules, can be identified in various types of cancer[42,47,49,52,53]. Yanaihara *et al.* found that in their 43 miRNA gene signature, seven precursor miRNAs were differentially expressed in matched lung cancer versus normal lung tissue. In addition, Jiang *et al.*, using a real-time PCR method specifically designed for precursor profiling[42], found significant variations in the

Prediction analysis of microarrays (PAM)
A statistical technique used in microarray analyses that identifies a subgroup of genes that best characterizes a predefined class, and uses this gene set to predict the class of new samples.

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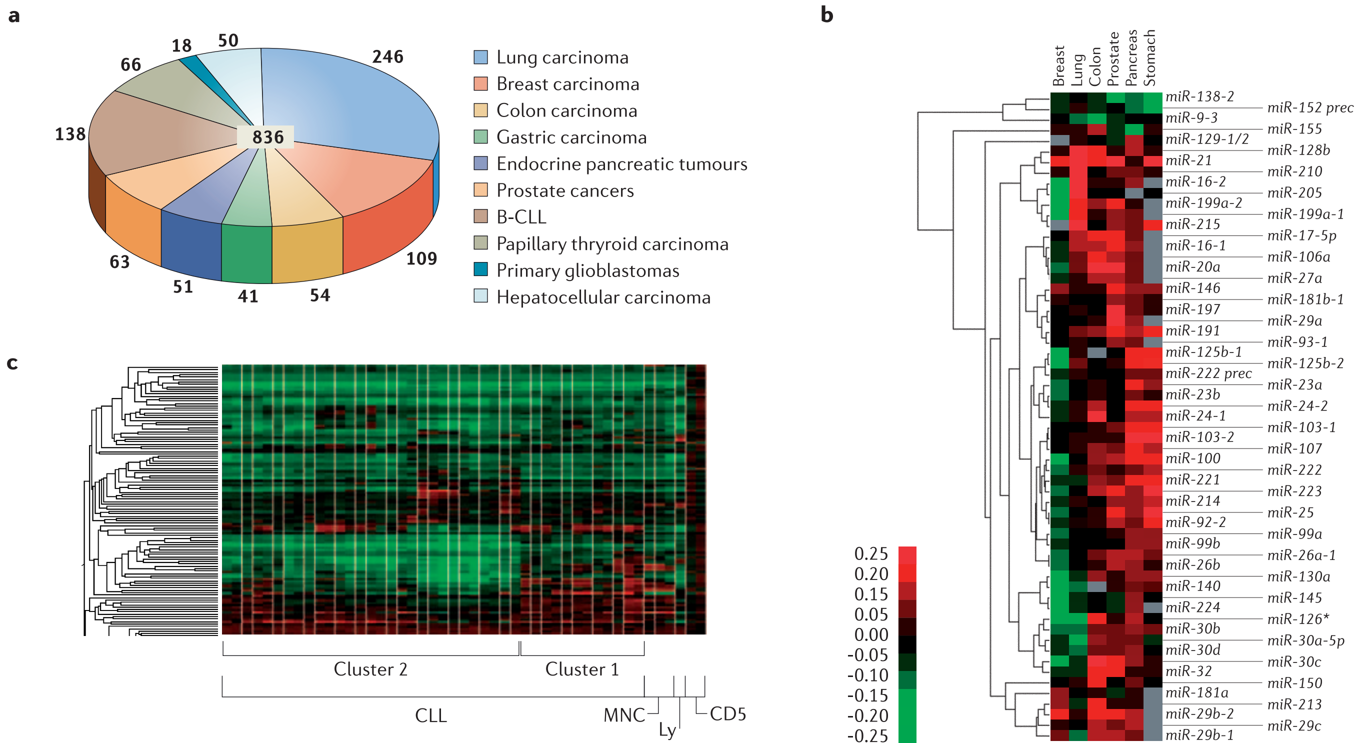

Figure 2 | **Examples of microRNA profiles in human solid and liquid cancers.** The general consensus is that profiling enabled the signatures that are associated with diagnosis — the process of identifying the nature of a disease — and prognosis — a prediction of the probable course and outcome of a disease — to be defined. **a** | The profiling of 836 solid tissue and haematological primary cancers (and corresponding normal tissues samples) using the microarray technology (as described in REF. 39) and reported to date is shown. The exact number of analysed samples from each tumour type (corresponding normal controls included) is shown. The data were collected from the references cited in the text. **b** | The common expression signature in six solid cancers indicates common altered regulatory pathways involving the same microRNA (miRNA) genes (named on the right side of the dendrogram)[47]. These miRNAs could be considered as 'cancer markers', because variations of their expression could identify the cancerous state. **c** | The distinct clusters identified by miRNA profiles in chronic lymphocytic leukaemia (CLL)[49] were further confirmed to be associated with prognostic factors and disease progression[35]. Note also that various normal haematopoietic samples cluster in a group that is distinct from all CLL samples. MNC, mononuclear cells; Ly, B lymphocytes; CD5, a subset of B lymphocytes largely accepted to represent the equivalent of malignant cells in CLL[93]. Panel b is modified with permission from REF. 47 © (2006) National Academy of Science, USA.

expression of nine precursors in a panel of 32 cancer cell lines[69]. These data raise the possibility that the 'non-active' part of the miRNA molecule could have 'independent' and as yet unknown functions that could be important in tumorigenesis. Putative interaction partners might be the RNA-binding proteins (such as the heterogenous nuclear ribonucleoproteins, hnRNPs), a class of proteins that are abnormally expressed in all types of human cancers[70]. These proteins bind RNA molecules to regulate their stability, and therefore it is possible that in the sequence of several precursor miRNAs such hnRNP-interacting motifs exist. However, there are no data so far that directly attribute regulatory function to a miRNA precursor.

Causes of abnormal miRNA expression

The causes of the widespread disruption of miRNA expression in tumour cells are only partially known and, very probably, various abnormalities in each tumour could contribute to the global miRNA-expression profile. So far, at least three different mechanisms (that could function independently or together) have been described. Such alterations correlate with and could explain the abnormal expression of specific miRNA genes.

The location of miRNAs at cancer-associated genomic regions (CAGRs). Soon after the identification of hundreds of new members of the miRNA family, it was shown that more than half of the known human miRNAs reside in particular genomic regions that are prone to alteration in cancer cells[71]. Such regions include minimal regions of LOH, which are thought to harbour tumour-suppressor genes, minimal regions of amplification, which might contain oncogenes, common breakpoint regions in or near possible oncogenes or tumour-suppressor genes and fragile sites (FRA). FRA are preferential sites of sister-chromatid exchange, translocation, deletion, amplification or integration of plasmid DNA, and tumour-associated viruses such as human papilloma virus (HPV). Of note, miRNA loci were significantly associated with insertion sites of tumorigenic HPV strains linked to endometrial cancers, leading to the possibility that viral insertions in the human genome might disturb small ncRNAs[71].

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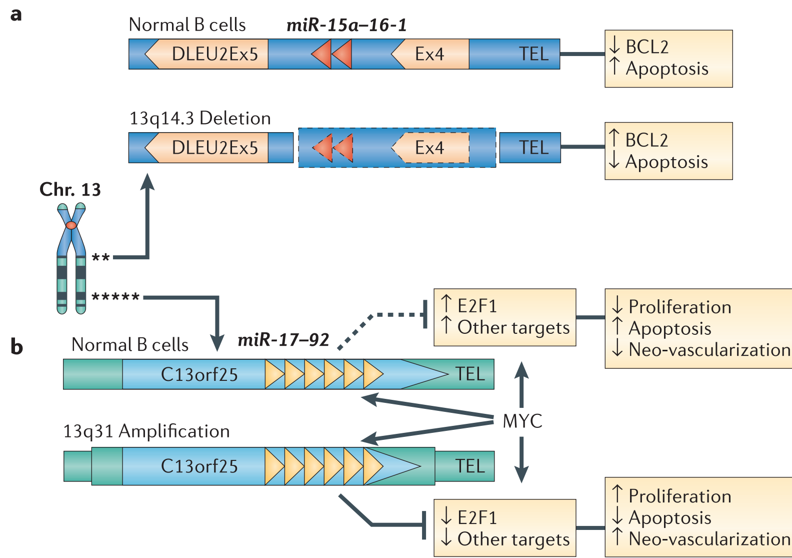

Figure 3 | **Chromosomal alterations at microRNA loci.** The main chromosomal alterations at microRNA (miRNA) loci, loss of heterozygosity and amplification, are identified at two separate regions of chromosome 13. **a** | Shows the 13q14.3 deletion found in chronic lymphocytic leukaemia (CLL) as described in REF. 23. The downregulation of *miR-15a* and *miR-16-1* induces overexpression of the anti-apoptotic BCL2 protein in leukaemia cells[68]. The genomic regions are not drawn to scale, and the cluster on 13q13 is simplified. The functional consequences are shown on the right of the figure. **b** | Shows the 13q13 amplification identified in high-grade lymphomas, as described in REF. 74. The functional consequences are presented on the right side of the figure.The overexpression of *miR-17-5p* and *miR-20a* from the same cluster induces the downregulation of the transcription factor E2F1. It was also shown that the *MYC* oncogene increases the expression of the *miR-17–92* cluster and activates E2F1 (REF. 67), and that the MYC-activated cluster increases tumour angiogenesis[113]. Therefore, the overexpression of the *miR-17–92* cluster is correlated with hyperproliferation, the blocking of apoptosis and neovascularisation. The arrows and the bars represent stimulatory and inhibitory signals, respectively. Orf, open reading frame.

Several reports show that this computationally identified association is significant for miRNA expression. Zhang *et al.* showed, by using high-resolution array comparative genomic hybridization (aCGH), that the miRNA loci have a high frequency of genomic alteration in human cancers[72]. Furthermore, about 75% of the miRNAs analysed showed concordance between mature miRNA levels and DNA copy number, and, importantly, their genomic data were in agreement (81% concordance for amplified and/or overexpressed miRNAs, and 60% concordance for deleted and/or downregulated miRNAs, respectively) with the expression data published with an independent set of breast cancers by Iorio *et al*[50]. Other examples of concordance by two independent reports, involve two miRNA clusters located at chromosome 13 (FIG. 3). The cluster *miR-15a–miR-16-1*, located at 13q14.3 in a region deleted in B-cell chronic lymphocytic leukaemia and pituitary adenomas was shown by northern blots to be downregulated in most patients with these tumours[23,73]. The cluster *miR-17–92*, located at chromosome 13q31, in a region amplified in B-cell lymphomas and lung cancers, was found to be overexpressed using microarray studies by He *et al.*[59], and by Hayashita and colleagues and Tagawa and Seto using northern blot analyses in lung cancers and malignant lymphomas, respectively[74,75].

Epigenetic regulation of miRNA expression. DNA hypomethylation, CpG island hypermethylation and histone-modification losses represent epigenetic markers of malignant transformation[76]. Therefore, several groups have investigated whether such events also affect miRNA expression. Scott *et al.* showed that in breast cancer cells histone deacetylase inhibition is followed by the extensive and rapid alteration of miRNA levels[77]. Furthermore, Saito *et al.* found that the combined treatment of human bladder cancer cells with 5-aza-2′-deoxycytidine (5-Aza-CdR) and the histone deacetylase (HDAC) inhibitor 4-phenylbutyric acid (PBA) has a significant effect on the expression of miRNAs[78]. Seventeen miRNAs (out of 313 screened by a microarray assay) were upregulated more than threefold, and *miR-127* was the most differentially expressed. This miRNA is located in a CpG island at chromosome 14q32, a region that is involved in several types of translocations identified in haematological cancers and deleted by LOH in solid tumours[71]. Furthermore, the combined treatment was accompanied by a decrease in DNA methylation and an increase in active histone markers around the transcription start site of *miR-127*. The investigators showed that *miR-127* can translationally repress the transcription of the zinc-finger repressor *BCL6* (REF. 78). Therefore, the induction of *miR-127* by 5-Aza-CdR and PBA treatment in cancer cells might have an anticancer effect by downregulating anti-apoptotic factors, such as *BCL6*, and inducing apoptosis[78].

The effects of DNA demethylation and histone deacetylation on miRNA expression could be tissue-specific, as the treatment of non-small cell lung cancer (NSCLC) cells with demethylating agents and/or HDAC inhibitors does not have a significant influence on miRNA expression[36,53]. Supporting this assumption is the fact that *miR-127* was not found to be expressed in normal lung cells by microarray analysis[39], and was not differentially expressed between cancerous and non-cancerous lung tissue[53].

Abnormalities in miRNA-processing genes and proteins. The protein machinery that is involved in the biogenesis of miRNAs is complex. Theoretically, alterations of these proteins (for a comprehensive description see REF. 34) should have dramatic effects on miRNA expression. In fact, it was shown that a failure of the Drosha processing step could explain the downregulation of miRNAs observed in primary tumours[79]. Furthermore, in a large study of patients with NSCLC, Karube *et al.* found that the expression levels of Dicer, but not Drosha, were reduced in a fraction of lung cancers. This correlated with reduced post-operative survival and poor tumour differentiation status, both of which are associated with a poor prognosis[80,81]. A similar correlation with reduced survival in patients with NSCLC was found for the expression of *miR-155* and *let-7* (REFS 53,82). These results suggest that patient survival could be a consequence of both aberrant miRNA processing and aberrant expression. The conditional knockout Dicer mouse has developmental defects, including lung epithelium abnormalities[81,83], but no reports on tumour formation are available,

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Box 1 | **Putative mechanisms of cancer predisposition by microRNA alterations**

In most familial cases of cancer, culprit genes have not been identified despite several decades of investigation. So it is possible that members of the new category of non-coding RNAs (ncRNAs) are involved in cancer predisposition. Germline sequence abnormalities were identified in microRNA (miRNA) genes or transcripts[35], and in targeted sequences in messenger RNAs (mRNAs) that interact with miRNAs[37].

Predisposing mutations in miRNAs, although rare events, can affect miRNA activity in at least three distinct ways: first, variation in the expression levels of miRNAs, by abnormal processing, if located in the miRNA themselves (as shown in REF. 112) or very close to the gene (as shown in REF. 35); second, an alteration of the spectrum of interactor mRNAs when present in the active molecule; and third, an alteration of putative interactions with proteins (such as heterogenous nuclear ribonucleoproteins), if located in the precursor molecule. A destabilizing effect of the miRNA–mRNA interaction was described for germline single nucleotide polymorphisms found in the two 3′UTR recognition sequences in the *KIT* oncogene that determine the interaction with *miR-221*, *miR-222* and *miR-146*, as reported by He *et al*[37]. As each miRNA has many targets, and, as a large number of miRNAs seem to be altered in human tumours, one can postulate that the disruption of two or more cooperating oncogenes and/or tumour suppressors is a likely event[29]. The accumulation of additional somatic events that occur in genes or ncRNAs, including miRNAs, is necessary for the development of the malignant phenotype (see also FIG. 4).

and Dicer-deficient embryonic stem cells injected into nude mice do not form tumours[84].

The influence of genetic abnormalities in small-RNA-processing proteins could have a more significant influence than is recognized at present. Recently, a new class of germline-specific small ncRNAs (26–31 nucleotides long), named PIWI-interacting RNAs (piRNAs), was discovered and found to bind the testis-specific murine PIWI protein orthologues[85–89]. Intriguingly, the human homologue of this subgroup of the Argonaute proteins, named HIWI, is located in the genomic region 12q24.33 that is genetically linked with the development of testicular germ-cell tumours in adolescents and adults. In addition, *HIWI* is overexpressed in most testicular seminomas analysed so far[90]. The expression of *HIWI* in human gastric cancer is associated with proliferating cells[91]. These correlations raise the possibility that piRNAs could represent another example of cancer-altered small ncRNAs, specifically in relation to PIWI overexpression.

MiRNA profiling as a new clinical tool

As active players in important human oncogenic signalling pathways, miRNAs should affect cancer diagnosis and prognosis (TABLE 1). As discussed above, several reports have already shown this for several tumour types[49,50,62]. But other reports indicate that miRNA profiles can be used to diagnose tumour types that cannot be determined on the basis of tumour biopsy samples.

MiRNA profiling as a diagnostic tool. Metastatic cancer of unknown primary site (CUP) is one of the 10 most frequent cancer diagnoses worldwide, and constitutes 3–5% of all human malignancies[92]. Patients with CUP present with metastases (late-stage disease) without an established primary tumour (that is, a site at which the tumour has initially developed and from which it has metastasized). The study of Lu *et al.* produces an important advance in the diagnosis of this peculiar type of cancer. Analysing 17 poorly differentiated tumours with non-diagnostic histological appearance, they showed that the miRNA-based classifier was much better at establishing the correct diagnosis of the samples than the mRNA classifier[41]. This result is exciting — profiling a few hundred miRNAs has a much better predictive power for CUP diagnosis than profiling several tens of thousands of mRNAs. As miRNA expression changes with differentiation, the poorly differentiated tumours have lower global expression levels of miRNAs compared with well-differentiated tumours from control groups[41]. The reduced expression levels of miRNAs in poorly differentiated tumours is probably why miRNA profiling is effective in the diagnosis of CUP.

CLL is the most common adult leukaemia in the Western world[93,94]. During its natural history, this indolent disease can evolve into an aggressive type of lymphoma and/or leukaemia, including Richter syndrome[95]. Furthermore, as the prognosis of most cases of CLL is relatively good, treatment after diagnosis is started only if poor prognostic markers are evident[96]. Few such markers have been found, but those that have include high expression levels of the 70 kDa zeta-associated protein (ZAP70) and an absence of mutations in the immunoglobulin heavy-chain variable-region gene (IgV_H). By performing a miRNA-profiling screen on 144 patients with CLL, a unique signature of 13 miRNAs (out of the 190 analysed) was shown to differentiate cases on the basis of a good or bad prognosis, or on the presence or absence of disease progression[35]. Among them, *miR-16-1* and *miR-15a* were expressed at lower levels in patients with a good prognosis, in agreement with early reports that 13q14.3 genomic deletions at the locus that harbours these genes correlate with a favourable course of the disease[97]. Among the genes found to be downregulated in the group of patients with a bad prognosis were members of the *miR-29* family. This could be explained by the recent findings that *TCL1*, an oncogene that is overexpressed in CLL cells from patients with aggressive disease[98], is a target of *miR-29* genes (Y. Pekarsky, unpublished observations). Therefore, these findings support a model in which two different molecular types of CLL with opposite clinical behaviours exist: one with a good prognosis and slow disease progression characterized by deletions of chromosome 13q14.3 and low levels of *miR-15a* and *miR-16-1*, and a second that is aggressive and prone to develop Richter syndrome, characterized by low levels of expression of *miR-29* genes and high expression levels of TCL1 protein. As *BCL2* is consistently overexpressed in CLL samples[99], *miR-15a* and *miR-16-1* might have additional targets important for progression; furthermore, other mechanisms of BCL2 deregulation (such as the minority of cases that have a BCL2 translocation[100]), and other interacting miRNAs might be involved.

MiRNA profiling as a prognostic tool. Lung cancer is the leading cause of death from cancer in males worldwide[101]. Therefore, the identification of new prognostic markers (markers that correlate with disease evolution) could be a significant advance for the identification of patients that would benefit from more aggressive therapy. In univariate analyses, the expression of both *miR-155* (high levels) and *let-7a-2* (low levels)has been shown to correlate with poor

Richter transformation
Denotes the development of high-grade non-Hodgkin lymphoma, prolymphocytic leukaemia, Hodgkin disease or acute leukaemia in patients with chronic lymphocytic leukaemia. Current treatments are aggressive, but prognosis is poor.

Univariate versus multivariate analyses
Univariate analysis explores each variable in a data set separately and describes the pattern of response to the variable. It describes each variable on its own. Multivariate statistical analysis describes a collection of procedures that involve the observation and analysis of more than one statistical variable at a time. It describes all variables together.

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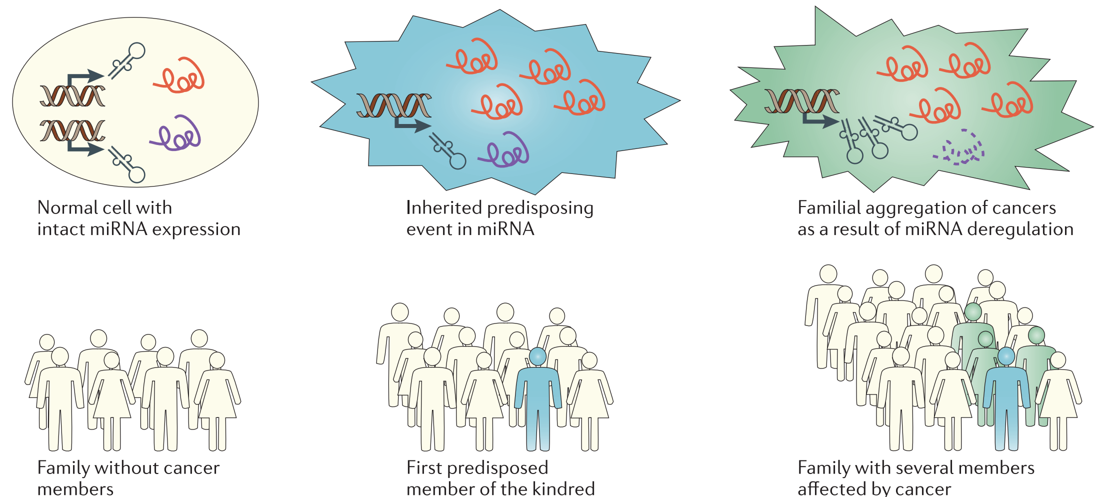

Figure 4 | **The involvement of microRNAs in cancer predisposition.** MicroRNA (miRNA) alterations could predispose to cancer development. Here we propose a model in which structural and/or expression abnormalities of miRNAs in the germline could represent an inherited predisposing event (blue person). For simplicity, we present only the situation of gene deletion, but all other types of loss-of-function or gain-of-function mutations described for protein-coding genes can be involved in the disruption of miRNA expression. To develop a malignant tumour, a second genetic event has to occur in a somatic cell (green people): this can be an alteration in a protein-coding gene, or a 'hit' in another miRNA (shown here as amplification). The consequences are on the levels of expression of various target mRNAs: the overexpression of target oncogenes (red protein) in the case of miRNA deletion, and the downregulation of target tumour-suppressor genes in the case of miRNA amplification (purple protein).

survival in 104 United States patients with lung cancer; by multivariate analyses the expression of *miR-155* also correlated with a poor prognosis when all clinical variables were considered together[53]. In an independent study of 143 Japanese patients with lung cancer, reduced *let-7* expression was found to significantly correlate with a shorter survival time after potentially curative surgery[82]. In both studies, about two thirds of the cases were adenocarcinomas (a type of NSCLC). By multivariate analyses, a low expression level of *let-7* was an independent prognostic factor for the stage of the disease, and correlated with significantly shorter survival[82]. In a meta-analysis of 29 studies that investigated the role of Ras oncogenes in the survival of patients with NSCLC cancers, Ras overexpression was associated with a reduced survival time[102]. As *let-7* negatively regulates Ras[58], these results show the existence of a link between *let-7*, Ras expression and the life expectancy of patients with lung cancer.

A miRNA perspective in cancer therapy

The potential for using miRNAs in cancer is now being explored. The theoretical rationale is based on the fact that miRNAs are natural antisense interactors that regulate many genes involved in eukaryotic survival and proliferation. Also, miRNA-expression profiles have been shown to change during treatment with gemcitabine, and the modulation of some miRNAs (including the overexpression of *miR-21*) increases the sensitivity of cholangiocarcinoma tumour cells to this chemotherapeutic agent *in vitro*[56]. Such results offer the experimental bases for the use of miRNAs as therapeutic targets. The development of modified miRNA molecules with longer *in vivo* half lives and efficiency, such as the locked nucleic acid (LNA)-modified oligonucleotides[103], the anti-miRNA oligonucleotides (AMOs)[104] and the 'antagomirs'[105] is the first step towards bringing these fundamental research advances into medical practice. Future *in vivo* experiments of miRNA transgenics and knockouts will offer valuable information about safety and efficacy.

Antagomirs
A new class of chemically engineered oligonucleotides that efficiently and specifically silence endogenous miRNAs in mice.

A new form of cancer predisposition?

The expression levels of many genes show abundant natural variation in species that range from yeasts to humans. This trait, termed 'gene expression phenotype' has an inherited component, which was shown to be present through familial aggregation studies in humans and simple segregation studies in yeast[106,107]. Cheung *et al.*, using microarray studies of human lymphoblastoid cells, identified a set of genes with a high degree of variation in expression levels between individuals[106]. The variation in expression among unrelated individuals was 3–11 times greater than between monozygotic twins, and the variance among siblings was 2–5 times greater than that between twins. These data suggest that genetic differences among individuals contribute to the variation in expression phenotype for some genes[106]. Because of the particular way in which miRNAs function — by targeting a number of functionally important protein-coding genes — we propose that variations in the expression and/or sequence of miRNAs, if inherited in the germline, could be important in cancer predisposition by affecting the levels of expression of tumour-suppressor genes or oncogenes (BOX 1, FIG. 4).

A significant proportion of patients with cancer are members of kindreds that develop the same type of cancer without a specific Mendelian inheritance, or that develop different types of cancers. This type of familial predisposition represents an important medical problem, and the

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identification of the predisposing gene(s) could offer early diagnosis and potential cancer prevention. Despite various efforts, the basis of this type of predisposition is largely unknown. A clear example comes from cases of familial CLL: although rare mutations in the ataxia telangiectasia mutated (*ATM*)[108,109] or *ARLTS1* genes[110], as well as in the *miR-15a* and *miR-16-1* transcripts[35], have been identified in families with two or more members with CLL, the culprit genes are still unknown for most cases.

In the United States, prostate cancer affects African-American males at a rate that is disproportionate with the number of White males diagnosed with the disease, and genetics could have an important role, but no target genes have been identified after decades of research[111]. We analysed miRNA expression levels in a pilot set of 18 samples of normal prostate tissue derived from two distinct human populations, 10 Caucasians from the United States and 8 African-Americans collected by the US National Cancer Institute Cooperative Prostate Cancer Tissue Resources. Using two different methods of microarray analyses, PAM and SAM, we identified both significantly overexpressed and downregulated miRNAs in the African-American individuals compared with the Caucasian individuals (G.A.C., C.M.C., S. Volinia and S. Ambs, unpublished data). The list of miRNAs that are at least three times differentially expressed includes *miR-301*, *miR-219*, *miR-26a*, *miR-1b-1* and *miR-30c-1*. Therefore, different human populations show variations in the global expression pattern of miRNAs expressed in a specific tissue. Whether such variations represent an inherited family trait remains an open question that should be solved by future large studies aimed at assessing the global miRNA expression profiles in cancers with high heritability, such as familial breast cancers or familial CLLs.

In conclusion

The question of whether miRNAs represent the 'dark side' of cancer predisposition remains to be answered, but there is no doubt that these 'strangers' in the genomic galaxy are involved in the regulation of pathways that are involved in the development and progression of many types of tumour.

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Competing interests statement
The authors declare no competing financial interests.

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