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MicroRNA-224 is associated with colorectal cancer progression and response to 5-fluorouracil-based chemotherapy by KRAS-dependent and -independent mechanisms

E B Amankwatia[1], P Chakravarty[2], F A Carey[3], S Weidlich[1], R J C Steele[1], A J Munro[1], C R Wolf[1,4] and G Smith*[,1]

[1]*Division of Cancer Research, Medical Research Institute, University of Dundee, Ninewells Hospital and Medical School, Dundee DD1 9SY, UK;* [2]*Bioinformatics and Biostatistics Service, Cancer Research UK, 44 Lincolns Inn Fields, London WC2A 3PX, UK;* [3]*Department of Pathology, Ninewells Hospital and Medical School, Dundee DD1 9SY, UK and* [4]*CRUK Molecular Pharmacology Unit, Ninewells Hospital and Medical School, Dundee DD1 9SY, UK*

Background: Colorectal cancers arise from benign adenomas, although not all adenomas progress to cancer and there are marked interpatient differences in disease progression. We have previously associated *KRAS* mutations with disease progression and reduced survival in colorectal cancer patients.

Methods: We used TaqMan low-density array (TLDA) qRT–PCR analysis to identify miRNAs differentially expressed in normal colorectal mucosa, adenomas and cancers and in isogeneic *KRAS* WT and mutant HCT116 cells, and used a variety of phenotypic assays to assess the influence of miRNA expression on *KRAS* activity, chemosensitivity, proliferation and invasion.

Results: MicroRNA-224 was differentially expressed in dysplastic colorectal disease and in isogeneic *KRAS* WT and mutant HCT116 cells. Antagomir-mediated miR-224 silencing in HCT116 *KRAS* WT cells phenocopied *KRAS* mutation, increased *KRAS* activity and *ERK* and *AKT* phosphorylation. 5-FU chemosensitivity was significantly increased in miR-224 knockdown cells, and in NIH3T3 cells expressing *KRAS* and *BRAF* mutant proteins. Bioinformatics analysis of predicted miR-224 target genes predicted altered cell proliferation, invasion and epithelial–mesenchymal transition (EMT) phenotypes that were experimentally confirmed in miR-224 knockdown cells.

Conclusions: We describe a novel mechanism of *KRAS* regulation, and highlight the clinical utility of colorectal cancer-specific miRNAs as disease progression or clinical response biomarkers.

Colorectal cancer (ICD-10 C18-C20) is the fourth most common cancer in the United Kingdom and a leading cause of cancer death (Cancer Research UK, 2014). Colorectal cancers initially present as benign polyps (adenomas), the most aggressive of which become malignant, promoting metastasis to lymph node and liver secondary sites. Identification and removal of premalignant adenomas is therefore a clinical priority, and the focus of NHS (National Health Service, 2014) and Scottish Bowel Screening programmes

*Correspondence: Dr G Smith; E-mail: g.smith@dundee.ac.uk

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(Scottish Bowel Screening Programme, 2014). Not all adenomas progress to cancer, however, and we therefore need to better understand the molecular events that influence disease progression, and to develop more specific screening tests for early detection and the identification of polyps at greater risk of progression.

Progression from colorectal adenoma to cancer is driven by the acquisition of loss of function mutations in tumour suppressor genes such as *APC* and *TP53*, and activating mutations in oncogenes including *Kirsten* RAS (*KRAS*). *KRAS* mutations, and phenotypically similar mutations in *BRAF*, promote cell growth by prolonged activation of RAS/MAPK-regulated signalling cascades (Barbacid, 1990). We have described significantly increased *KRAS* mutation burden in advanced Dukes' C cancers and shown that mutation burden is negatively associated with survival (Smith *et al*, 2002; Conlin *et al*, 2005).

Colorectal cancer treatment routinely combines surgical resection with adjuvant or neoadjuvant treatment with 5-fluorouracil (5-FU) or its prodrug capecitabine (Scottish Intercollegiate Guidelines Network, 2011). As with rates of disease progression, response to treatment varies widely, and sensitive, patient-specific biomarkers for treatment selection and assessment of prognosis are urgently required. *KRAS* and *BRAF* mutation status are clinically relevant response biomarkers in patients treated with cetuximab and related drugs, monoclonal antibodies targeted to the epidermal growth factor receptor, a key node in the RAS/MAPK signalling pathway (Lièvre *et al*, 2006; Amado *et al*, 2008; Bokemeyer *et al*, 2008). As biomarker assessment is often limited by the availability of serial tissue samples, recent research has focussed on identification of serum-based biomarkers including microRNAs (miRNAs), small (18–25 nucleotides) noncoding RNAs that function as negative regulators of gene expression, repressing protein translation by binding to target messenger mRNAs (Bartel, 2004). MicroRNAs are frequently differentially expressed in cancer, and have been suggested to influence cancer progression, following negative regulation of both tumour suppressor genes and proto-oncogenes. Previous studies have highlighted significant differences in miRNA expression in colorectal cancer relative to matched normal tissue, where specific miRNAs have consistently increased (e.g., miR-21, miR-135b, miR-183, miR-31, miR-201) or decreased (e.g., miR-145, miR-378) expression (Bandrés *et al*, 2006; Monzo *et al*, 2008; Schetter *et al*, 2008; Chen *et al*, 2009; Motoyama *et al*, 2009; Earle *et al*, 2010; Wang *et al*, 2010; Chang *et al*, 2011; Faltejskova *et al*, 2012; Fu *et al*, 2012; Hamfjord *et al*, 2012; Mosakhani *et al*, 2012; Reid *et al*, 2012). In contrast, miRNA expression in colorectal adenomas is less well described, although recent studies have highlighted differential expression of several miRNAs, including miR-135b (Wu *et al*, 2014) and miR-17 (Kanaan *et al*, 2013).

Individual miRNAs can target multiple mRNAs because of imperfect complementarity in 3′-UTR seed sequences (Lewis *et al*, 2005). Robust bioinformatics approaches are therefore required to predict miRNA target genes, combined with phenotypic validation of predicted miRNA/mRNA interactions. To address these issues in colorectal cancer development, we have performed a quantitative, systematic comparison of miRNA expression in colorectal cancers, matched healthy tissue and colorectal adenomas with low-grade and high-grade dysplasia to identify miRNAs that promote, or that may have utility as biomarkers of colorectal cancer progression, with a particular focus on novel regulators of the RAS/MAPK signalling pathway.

MATERIALS AND METHODS

Ethical approval. All studies involving human tissues were approved by the Tayside Tissue Bank Research Ethics Committee, a devolved sub-committee of the Tayside Committee on Medical Research Ethics.

RNA extraction and quantitation. RNA was extracted from cell lines using Qiagen RNeasy mini kits (Qiagen, Manchester, UK) following the manufacturer's protocol for mammalian cells, with on-column DNase digestion (Qiagen RNase free DNase kit). Tissue samples ($\sim$20 mg) were disrupted by homogenisation using a rotor–stator homogeniser (Polytron, Thermo Fisher Scientific, Waltham, MA, USA) before RNA extraction, following the manufacturer's protocol for animal tissue. RNA was additionally extracted from three 20 μm FFPE tissue sections (combined) using RecoverAll Total Nucleic Acid isolation kits (Life Technologies, Paisley, UK).

RNA yield and integrity was initially quantitated from A_{260} and A_{280} absorbance readings obtained from a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Loughborough, UK) – all samples selected for further analysis had an A_{260}:A_{280} ratio between 1.7 and 2.0. RNA integrity was further assessed in human tissue samples using an Agilent Bioanalyser 2100 and RNA 6000 Nano LabChip Kit (Agilent Technologies, Wokingham, UK); samples selected for further analysis had RIN (RNA Integrity Number) values >7.

TaqMan quantitative real-time PCR analysis. MiRNA expression was initially investigated using TaqMan low-density miRNA A card arrays (377 miRNAs, 3 endogenous controls and negative control; Life Technologies; http://tools.lifetechnologies.com/content/sfs/manuals/cms_054742.pdf). Reverse transcription (RT) reactions were performed using TaqMan RT reagent kits and Megaplex RT primers (Life Technologies), following the manufacturer's protocol. PCR reactions, combining Megaplex RT products with TaqMan Universal PCR Master Mix II (Life Technologies), were added to each Taqman low-density array (TLDA) card port and PCR reactions run in triplicate on the standard Real-Time PCR program on the 7900 TaqMan real-time system (Applied Biosystems, Warrington, UK). For single probe qRT–PCR miRNA analysis, RT reactions were performed using TaqMan MicroRNA Reverse Transcription Kits (Life Technologies) according to the manufacturer's protocol. Resulting cDNA samples were analysed in triplicate using TaqMan Small RNA Assays, combining miRNA-specific probes (Life Technologies) with TaqMan Universal PCR Master Mix II and run as previously described. Let-7a was used as an endogenous control for all miRNA qRT–PCR analyses.

The mRNA RT reactions were performed, primed with random hexamers, using TaqMan reverse transcription reagent kits (Life Technologies) according to the manufacturer's protocol. PCR reactions were then performed in triplicate using TaqMan Gene Expression Assays (Life Technologies), as described above, using 18S ribosomal RNA as an endogenous control. Gene expression differences in both single probe and TLDA card experiments were identified using SDS 2.3 software (Applied Biosystems) to determine Ct (cycle threshold) values for each target and endogenous control gene for each sample, as previously described (Smith *et al*, 2012).

Cell lines. Isogeneic HCT116 cell lines specifically engineered to express the WT or mutant form of *KRAS* were kindly donated by Dr Bert Vogelstein (John Hopkins University, Baltimore, MD, USA). Isogeneic cell lines were created from the parental HCT116 cell line (heterozygous for *KRAS* WT and G13D *KRAS* mutated alleles) by deleting an allele by targeted homologous recombination to create *KRAS* mutant$^{(KRAS\ G13D/-)}$ and WT$^{(KRASWT/-)}$ HCT116 colorectal cancer cell lines (Shirasawa *et al*, 1993; Torrance *et al*, 2001). Cells were cultured in McCoy's 5A medium with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin and streptomycin) and incubated at 37 °C and 5% carbon dioxide (CO_2). The mouse NIH3T3 embryonic fibroblast

cell line was obtained from Cancer Research UK cell services. NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, at 37 °C in 5% CO_2. 2.5×10^5 cells were transiently transfected in six-well plates with 250 ng of plasmids encoding either WT *KRAS*, WT *BRAF*, *KRAS* G12V, *KRAS* G13D or *BRAF* V6000E using Lipofectamine (Invitrogen, Paisley, UK), and harvested 48 h after transfection as previously described (Smith *et al*, 2010). To create stable cell lines, cells were simultaneously transfected with 250 ng pcDNA3 (Invitrogen), containing a selection marker for geneticin (G418). Geneticin-resistant colonies (formed up to 6 weeks following transfection) were picked using cloning cylinders and expanded in selective media, and each stable cell line tested for *KRAS or BRAF* mRNA and protein expression by qRT–PCR and western blot analysis, respectively.

MiR-224 transfection experiments. HCT116 *KRAS* WT cells (1×10^5 cells per well) were seeded in six-well plates and incubated for 24 h before lipofectamine-based transient transfections with a final concentration of 30 nM miR-224-specific antagomir or miRNA inhibitor negative control (Life Technologies) in serum-free medium. The extent of miR-224 knockdown was assessed by qRT–PCR analysis 24 h following transfection, as previously described.

RAS GTPase ELISA. The amount of GTP-bound *KRAS* in cellular extracts was determined using RAS GTPase Chemi ELISAs (Active Motif, La Hulpe, Belgium) according to the manufacturer's guidelines. Cellular extracts were obtained from cells 24 h following transfection with miR-224 inhibitors or from untransfected cells. Cells were washed with ice-cold PBS and lysed by the addition of complete lysis/binding buffer, and the collection of cell supernatants. Protein concentrations were determined by Bradford assay (Bradford, 1976) and RAS ELISAs performed in triplicate according to the manufacturer's protocol, with endpoint luminescence assessment.

PathScan intracellular signalling array. Protein lysates were obtained as previously described and diluted to 1 mg ml^{-1}. PathScan Intracellular Signalling Array kits (Cell Signaling Technology, Hitchin, UK), slide-based antibody arrays for the simultaneous detection of 18 signalling molecules including *ERK* and *AKT*, were analysed according to the manufacturer's protocol and scanned using Li-Cor Odyssey (Li-Cor Biosciences, Cambridge, UK; excitation 680 nm and detection 700 nm). Relative fluorescent intensities were quantified using Image Studio Software (Li-Cor Biosciences).

MTT cytotoxicity assays. MTT cytotoxicity assays were performed to compare chemosensitivity of HCT116 *KRAS* WT and mutant cells, HCT116 *KRAS* WT cells following miR-224 knockdown and NIH3T3 *KRAS* and *BRAF* WT and mutant cells following treatment with 5-FU, oxaliplatin and irinotecan. Cells (3000 cells per well) were seeded in triplicate in 96-well plates and incubated for 24 h before drug treatment, previously optimised in preliminary experiments (5-FU, 1.25–80 μM; oxaliplatin, 1.25–80 μM; irinotecan, 1–64 μM). Cells were incubated with drug (or vehicle control) for 72 h before addition of 100 μl 5 mg ml^{-1} MTT in phenol red-deficient DMEM. Following a 3-h incubation, the resulting formazan crystals were solubilised in DMSO and absorbance read at 570 nm. Mean absorbance readings for each treatment were calculated as percentages of vehicle controls (designated 100%), plotted against [$\log_{10}$ drug] and IC_{50} values calculated.

Cell growth assays. Cells were seeded in 6-well plates (1×10^5 cells per well) and incubated for 24 h before trypsinisation at 16, 24, 32 and 48 h time points, counted using a haemocytometer and cell counts plotted against time. Cell doubling time was also compared in HCT116 *KRAS* WT and mutant cells and *KRAS* WT miR-224 knockdown cells using CellTrace Violet Cell Proliferation Kits (Life Technologies) according to the manufacturer's guidelines. Cells were seeded (1×10^6 cells per reaction) in triplicate, labelled with 5 μM CellTrace dye and harvested at 16, 24, 32, 48, 64 and 72 h time points as previously described. Proliferation was quantitated by flow cytometry (Attune Flow cytometer using Attune Cytometric software (Life Technologies)) and ModFit LT software (version 4; Verity Software House, Topsham, ME, USA) used to create a proliferation model and calculate proliferation index (total number of divisions/number of cells that went into division). Proliferation index was plotted against time and doubling time calculated as previously described.

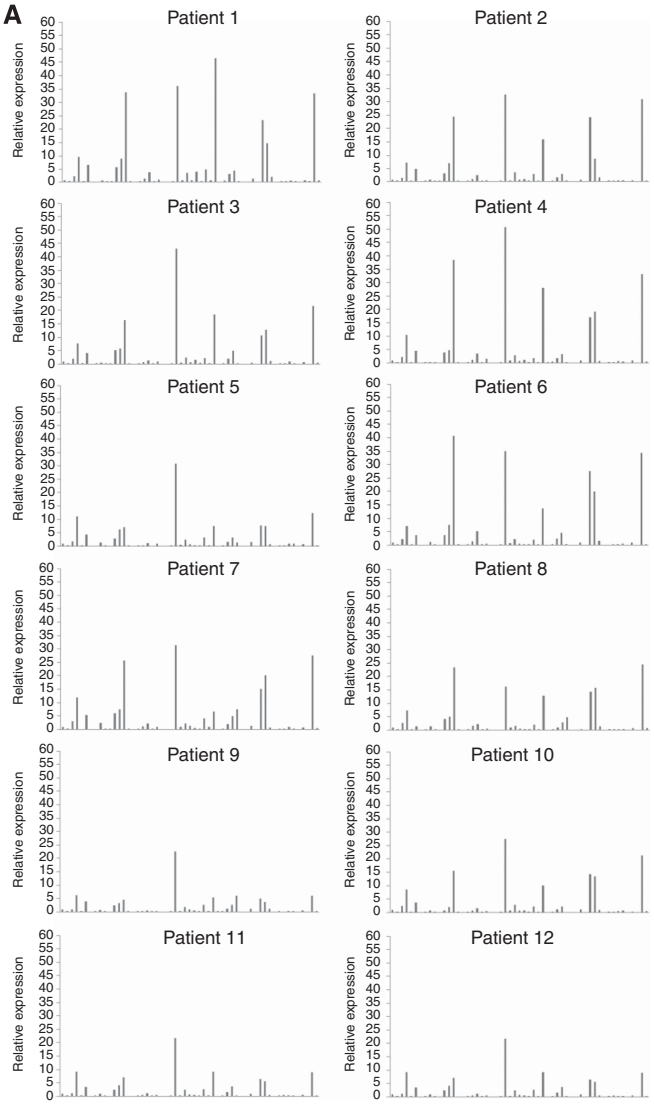
Cell invasion assays. Invasion in HCT116 *KRAS* WT and mutant cells and *KRAS* WT miR-224 knockdown cells was compared using InnoCyte invasion assays (Merck Millipore, Watford, UK) according to the manufacturer's protocol. Cells (1×10^6 per well) were placed in the top chamber in serum-free media, allowed to migrate, fluorescently labelled and relative fluorescence assessed (excitation 485 nm, emission 530 nm).

Bioinformatics and statistics. MicroRNA data from TLDA miRNA cards were analysed using Bioconductor 1.9 (Gentleman *et al*, 2004) running on R 2.6.0 (R Development Core Team, 2008). Initial miRNA screens were performed using individual TLDA cards, with all subsequent experiments performed in triplicate. Cross-sample normalisation of CT values was performed, where the expression of individual miRNAs in each sample was normalised to corresponding let-7a expression. Statistically significantly differentially expressed miRNAs were identified using an empirical Bayes *t*-test (Smyth, 2004) and *P*-values adjusted for multiple testing correction using the Benjamini–Hochberg method (Benjamini and Hochberg, 1995). MicroRNAs with adjusted *P*-values of <0.05 were used to predict target genes identified by at least two of the miRNA target prediction databases: mirDB (www.mirdb.org/miRDB/), miRANDA (www.microrna.org), miRBase (www.mirbase.org), TargetMiner (www.isical.ac.in) and TargetScan (www.targetscan.org). Pathway and biological process enrichment analyses were performed using Metacore (www.thomsonreuters.com/metacore/), employing a hypergeometric distribution to determine enriched gene sets. Independent *t*-tests were used to assess statistically significant ($P \leqslant 0.05$) differences in the means of continuous variables in cell lines, and Mann–Whitney tests were used to compare the medians of continuous variables in patient groups. In all figures, $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.01$, $^{***}P \leqslant 0.0001$ and $^{****}P \leqslant 0.0001$.

RESULTS

Identification of miRNAs differentially expressed in colorectal adenomas and cancers. To perform an initial unbiased analysis of miRNA expression in colorectal cancer, qRT–PCR analysis using TLDA cards was performed as described in Materials and Methods. Colorectal cancer samples ($n = 12$), previously analysed for *KRAS* and *BRAF* mutation status (Weidlich *et al*, 2011), were profiled for miRNA expression and marked interpatient differences in miRNA expression were identified (Figure 1A and Supplementary Figure 1).

Of the 377 miRNAs analysed, 153 (40.6%) were not expressed, 199 (52.7%) expressed in at least 2 cancers and 108 (28.6%) expressed in all 12 cancers, including 18 miRNAs (miR-193b, miR-323-3p, miR-324-3p, miR-331-5p, miR-339-3p, miR-362-5p, miR-365, miR-374, miR-449b, miR-487a, miR-491-5p, miR-494, miR-501, miR-532-5p, miR-574-3p, miR-671-3p, miR-744 and miR-99b) not previously described in colorectal cancer (Supplementary Information and Supplementary Table 1A).



B

	Normal *vs* LGA	Normal *vs* HGA	Normal *vs* cancer
	Fold change	Fold change	Fold change
miR-15b	3.32	2.83	1.86
miR-27a	5.45	8.35	6.83
miR-29c	9.17	15.29	5.87
miR-92a	3.72	4.66	4.01
miR-93	3.15	3.46	4.25
miR-224	7.41	5.86	5.15
miR-500	2.22	7.29	3.13

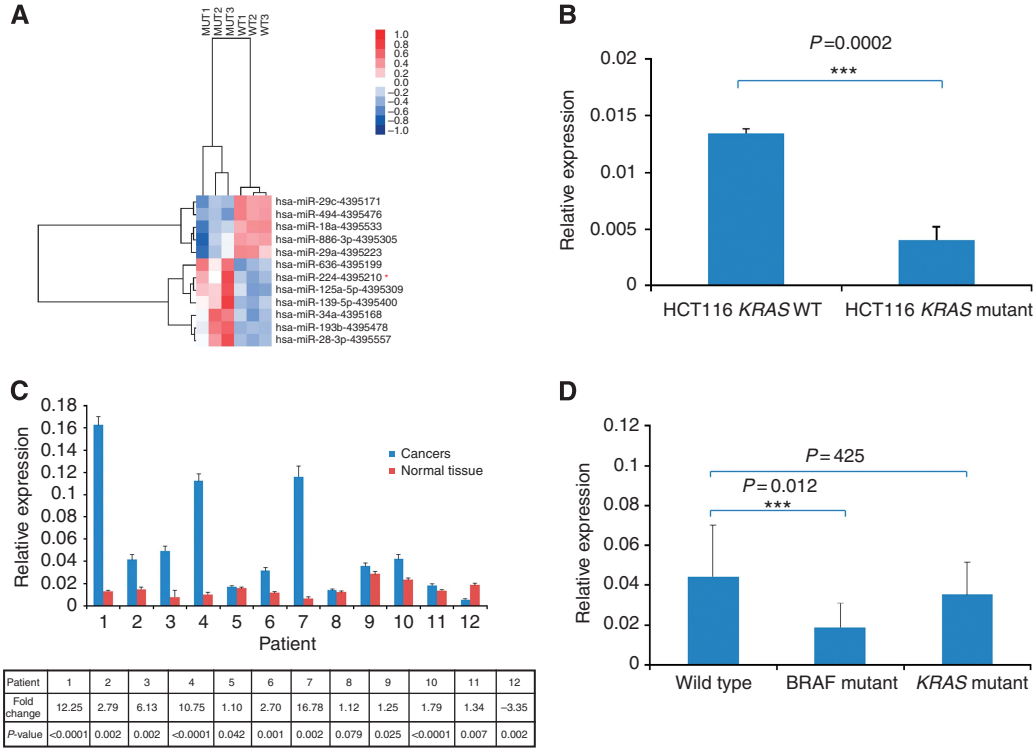
C

	Normal *vs* LGA	Normal *vs* HGA	Normal *vs* cancer
	Fold change	Fold change	Fold change
miR-125b	–7.28	–7.04	–2.12
miR-127-3p	–3.38	–2.85	–1.76
miR-133a	–3.75	–4.29	–27.26
miR-139-5p	–3.01	–3.19	–13.35
miR-145	–3.32	–3.47	–9.42
miR-149	–3.49	–2.16	–3.32

Figure 1. Identification of miRNAs differentially expressed in normal colorectal mucosae, colorectal adenomas and colorectal cancers. qRT–PCR analysis (TaqMan low-density array A cards, $n = 377$ unique miRNA targets) was used, as described in Materials and Methods, in an initial screen to identify miRNAs expressed in normal mucosae ($n = 3$), colorectal adenomas ($n = 6$) and colorectal cancers ($n = 3$), and to assess interindividual differences in miRNA expression. (**A**) Representative data summarising the expression of the first 55 miRNAs on the TLDA array are illustrated, with additional data summarised in Supplementary Figure 1. MicroRNA expression is illustrated relative to the expression of the invariant miRNA let-7a. MicroRNAs with consistently increased ($n = 7$; **B**) or decreased ($n = 6$; **C**) expression, comparing normal mucosae with colorectal adenomas and cancers, are highlighted.

To identify differentially expressed miRNAs, normal patient-matched colorectal mucosa samples ($n = 3$) and colorectal adenomas (high-grade dysplasia ($n = 3$), low-grade dysplasia ($n = 3$)) were analysed. In all colorectal tissues, 111 miRNAs (29.4%) were expressed (Supplementary Information and Supplementary Table 1B). The expression of seven miRNAs was significantly increased (Supplementary Table 1B) and six miRNAs decreased (Supplementary Table 1C) in low- and high-grade adenomas and cancers compared with normal colorectal mucosa.

Prioritisation of miRNAs associated with *KRAS* mutation status. To investigate the influence of *KRAS* mutation status on miRNA expression, we used TLDA qRT–PCR analysis to compare miRNA expression in isogeneic HCT116 colorectal cancer cell lines, expressing either WT or mutant *KRAS* (Shirasawa *et al*, 1993; Torrance *et al*, 2001). We identified 12 differentially expressed miRNAs (Figure 2A), 3 of which (miR-224, miR-29c and miR-139-5p) were also differentially expressed in colorectal cancer. Unlike miR-29c and miR-139-5p, miR-224 expression was increased in both colorectal adenomas and cancers, and was prioritised for more detailed analysis, following replication of our TLDA data using a small TaqMan miR-224 RNA assay to confirm differential miR-224 expression in HCT116 *KRAS* WT and mutant cells (3.3-fold; $P = 0.0002$; Figure 2B), and in colorectal cancers relative to normal colorectal mucosa ($n = 12$; $P = 0.005$; Figure 2C). To further investigate whether miR-224 expression was influenced by *KRAS* or *BRAF* mutation status, we compared miR-224 expression in an extended cancer panel, selected by previously determined mutation status ($n = 41$; 18 *KRAS* mutant, 9 $BRAF^{V600E}$ mutant and 14 *KRAS* and *BRAF* WT cancers; Supplementary Information and Supplementary Table 2). MiR-224 expression was significantly decreased ($P = 0.012$) in *BRAF* mutant cancers (Figure 2D), although not in *KRAS* mutant cancers, possibly as a consequence of mixed *KRAS* genotype; ideally, we would have restricted our analysis to *KRAS* G13D mutant cancers to facilitate direct comparison with our cell line



Patient	1	2	3	4	5	6	7	8	9	10	11	12
Fold change	12.25	2.79	6.13	10.75	1.10	2.70	16.78	1.12	1.25	1.79	1.34	−3.35
P-value	<0.0001	0.002	0.002	<0.0001	0.042	0.001	0.002	0.079	0.025	<0.0001	0.007	0.002

Figure 2. Identification of miRNAs differentially expressed in isogeneic *KRAS* WT and mutant HCT116 colorectal cell lines. qRT–PCR analysis (TaqMan low-density array A cards, $n = 377$ unique miRNA targets) was used, as described in Materials and Methods, to identify miRNAs differentially expressed in isogeneic *KRAS* WT and mutant HCT116 colorectal cell lines. (**A**) Heatmap summarising differentially expressed miRNAs, highlighting miR-224 (*), the expression of which was also increased in both colorectal adenomas and colorectal cancers (Figure 1B). qRT–PCR analysis was also used to confirm increased miR-224 expression in (**B**) *KRAS* WT HCT116 colorectal cancer cells, (**C**) colorectal cancers relative to normal mucosae ($n = 12$) and (**D**) an extended colorectal cancer series ($n = 41$) subdivided according to *KRAS* and *BRAF* genotype, using an independent TaqMan small RNA assay, as described in Materials and Methods. MicroRNA-224 expression is illustrated relative to the expression of the invariant miRNA let-7a; all samples were analysed in triplicate, with experimental errors calculated as previously described (Smith *et al*, 2012).

data, but insufficient cancers with this rare genotype were available for analysis.

As pre-mir-224 is transcribed from the q arm of the X chromosome, we hypothesised that expression may vary in male ($n = 18$) and female ($n = 23$) patients, although no significant expression differences were identified ($P = 0.331$). Similarly, miR-224 expression did not vary with cancer stage.

Phenotypic consequences of miR-224 knockdown. To investigate the functional role of miR-224, a miR-224-specific antagomir was transiently transfected into HCT116 *KRAS* WT cells. MicroRNA-224 and negative control antagomir concentrations were optimised, with maximum knockdown occurring 24 h following transfection with 30 nM miR-224 antagomir ($P = 1.1 \times 10^{-6}$; Supplementary Figure 2A). Knockdown (>85% relative to basal expression) was maintained for 96 h following transfection (Supplementary Figure 2A), highlighting an experimental window in which to investigate the phenotypic consequences of reduced miR-224 expression.

As miR-224 expression is significantly higher in *KRAS* WT cells, we hypothesised that miR-224 knockdown may phenocopy *KRAS* mutation by increasing *KRAS* activity. The effect of miR-224 knockdown on the amount of GTP-bound *KRAS* was therefore investigated using a RAS ELISA. *KRAS* activity was higher (2.5-fold, $P = 0.002$) in *KRAS* mutant than in WT HCT116 cells (Figure 3A) and, consistent with our hypothesis, miR-224 knockdown in *KRAS* WT cells significantly increased RAS activity (1.9-fold, $P = 0.001$). To investigate *KRAS* specificity, *NRAS* and *HRAS* expression was additionally compared in HCT116 *KRAS* WT and miR-224 knockdown cells, but no significant differences identified (Supplementary Figure 3), although *KRAS* expression was itself significantly increased following miR-224 knockdown (1.7-fold, $P = 0.007$).

PathScan intracellular signalling array targets were then compared in WT HCT116 and miR-224 knockdown cells. Consistent with increased *KRAS* activity, *ERK* phosphorylation was increased in miR-224 knockdown cells (1.6-fold; $P = 0.006$; Figure 3B). Similarly, *AKT* phosphorylation was significantly higher (tyrosine 308 1.4-fold, $P = 0.01$; serine 473 1.3-fold, $P = 0.04$) in miR-224 knockdown cells, suggesting that miR-224 directly influences the pro-survival RAS/AKT/PI3K pathway. Our analysis identified several additional signalling molecules (*STAT1*, *STAT3*, *AMPK*, *HSP27*, *PRAS40* and p38 (*MAPK11*)) the expression of which was also significantly increased following miR-224 knockdown, highlighting novel candidate miR-224 target genes (Supplementary Figure 4).

MTT cytotoxicity assays were used to investigate the effect of miR-224 knockdown on sensitivity to 5-FU, oxaliplatin and irinotecan, the drugs used to treat colorectal cancer. MicroRNA-224 knockdown increased the sensitivity of HCT116 *KRAS* WT cells to 5-FU, with a 1.9-fold decrease in IC_{50} ($P = 0.04$, Figure 3C) but did not influence sensitivity to oxaliplatin or irinotecan (data not shown). To investigate whether 5-FU chemosensitivity was *KRAS* dependent, we additionally compared 5-FU sensitivity in novel NIH3T3-derived cell lines, stably transfected with WT *KRAS*, WT *BRAF*, various *KRAS* mutants and $BRAF^{V600E}$ (Figure 3D).

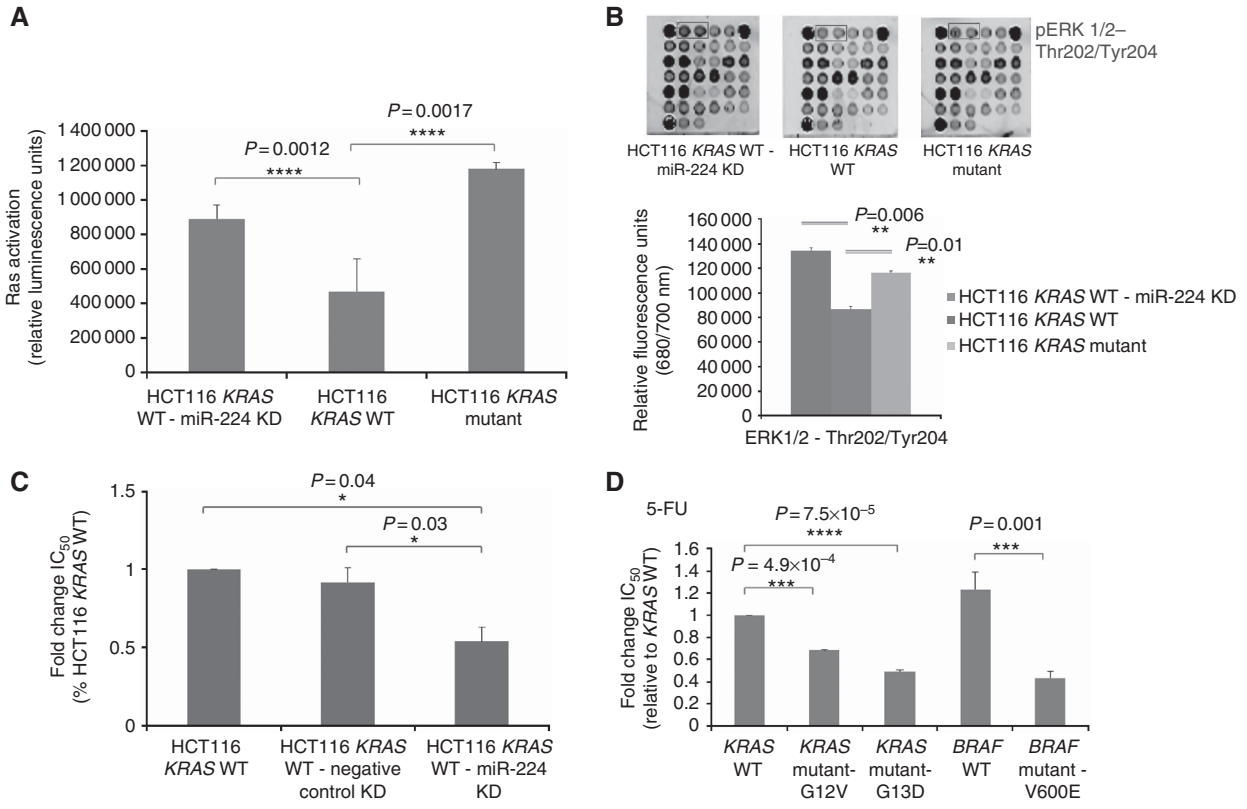


Figure 3. Characterisation of miR-224 knockdown phenotypes. (**A**) A quantitative RAF-binding RAS ELISA was used, as described in Materials and Methods, to assess the influence of miR-224 knockdown on the amount of GTP-bound RAS in cell extracts from *KRAS* WT, *KRAS* WT-miR-224 knockdown and *KRAS* mutant HCT116 cells. Each sample was assessed in triplicate and data represent mean ± s.e.m. of relative luminescence units. (**B**) PathScan intracellular signalling arrays were used, as described in Materials and Methods, to assess the influence of miR-224 knockdown on the phosphorylation of *ERK1/2* and additional substrates summarised in Supplementary Figure 2. Each sample was analysed in duplicate, with relative fluorescence at 680/700 nm quantified using a Li-Cor Odyssey fluorescence imaging system. MTT chemosensitivity assays were used, as described in Materials and Methods, to compare sensitivity to 5-FU in (**C**) *KRAS* WT HCT116 cells, cells transfected with a nonspecific negative control antagomir and miR-224 knockdown cells and (**D**) NIH3T3 mouse fibroblast cells stably expressing equivalent levels of WT *KRAS* and *BRAF* or *KRAS* (G12V and G13D) or *BRAF* (V600) mutant proteins. All experiments were performed in triplicate and IC_{50} values for each cell line calculated using GraphPad Prism (La Jolla, CA, USA).

Consistent with our miR-224 knockdown data, all mutant *KRAS* cells (G12V and G13D mutants illustrated as examples) and *BRAF* mutant (V600E) cells were more sensitive to 5-FU (Figure 3D).

Bioinformatics prediction of miR-224-regulated pathways. Individual miRNAs regulate multiple target genes because of imperfect complementarity with 3′UTR seed sequences. MiRNA target prediction databases use unique algorithms to predict miRNA target genes *in silico*, ranked by predicted affinity. We used five miRNA target prediction databases (mirDB, miRANDA, miRBase, TargetMiner and TargetScan) to predict targets of miRNAs differentially expressed in HCT116 *KRAS* WT and mutant cells (Figure 2A), prioritising targets common to at least two databases for further analysis. Enriched pathways and processes significantly associated with each predicted target were identified using the bioinformatics tool Metacore, and several common cancer-related processes and pathways identified, including the epithelial–mesenchymal transition (EMT), cell adhesion, cytoskeleton remodelling and cell proliferation (Supplementary Information and Supplementary Table 3). A similar approach was used to compare HCT116 WT cells before and after miR-224 knockdown (Supplementary Information and Supplementary Table 4).

Experimental validation of bioinformatics predictions. The influence of miR-224 knockdown on cell proliferation was investigated by quantitative cell growth assays and flow cytometry analysis. Consistent with our bioinformatics predictions, miR-224 knockdown significantly increased cell doubling time (23.5 to 26.1 h; $P<0.001$ and 19.8 to 27.5 h; $P=0.01$, respectively). The effect of miR-224 knockdown on cell cycle kinetics was investigated by flow cytometry analysis, but no significant differences in cell cycle progression were identified, either in untreated cells or following acute treatment with 5-FU (data not shown).

To explore the predicted association between miR-224 and EMT, qRT–PCR analysis was used to compare the expression of candidate EMT genes *CDH1* (E-cadherin), *CDH2* (N-cadherin) and *VIM* (Vimentin) in *KRAS* WT and miR-224 knockdown cells. *CDH1* (1.12-fold; $P=0.005$, Figure 4A) expression was significantly increased in miR-224 knockdown cells, although such subtle variation in gene expression is unlikely to significantly influence the EMT phenotype. In contrast, Vimentin expression was decreased (1.7-fold; $P=0.04$, Figure 4B), whereas *CDH2* was not expressed in HCT116 cells (data not shown).

InnoCyte invasion assays were then used to show that miR-224 knockdown significantly reduced invasion in HCT116 *KRAS* WT cells (1.75-fold; $P=0.009$, Figure 4C). HCT116 *KRAS* mutant cells were significantly less invasive (1.2-fold; $P=0.04$) than HCT116 *KRAS* WT cells, further confirming our hypothesis that miR-224 knockdown in *KRAS* WT cells phenocopies *KRAS* mutation. In NIH3T3 cells stably expressing WT or mutant *KRAS*, however, G13D *KRAS* mutant cells were significantly more invasive than NIH3T3 *KRAS* WT cells (4.6-fold; $P=0.002$).

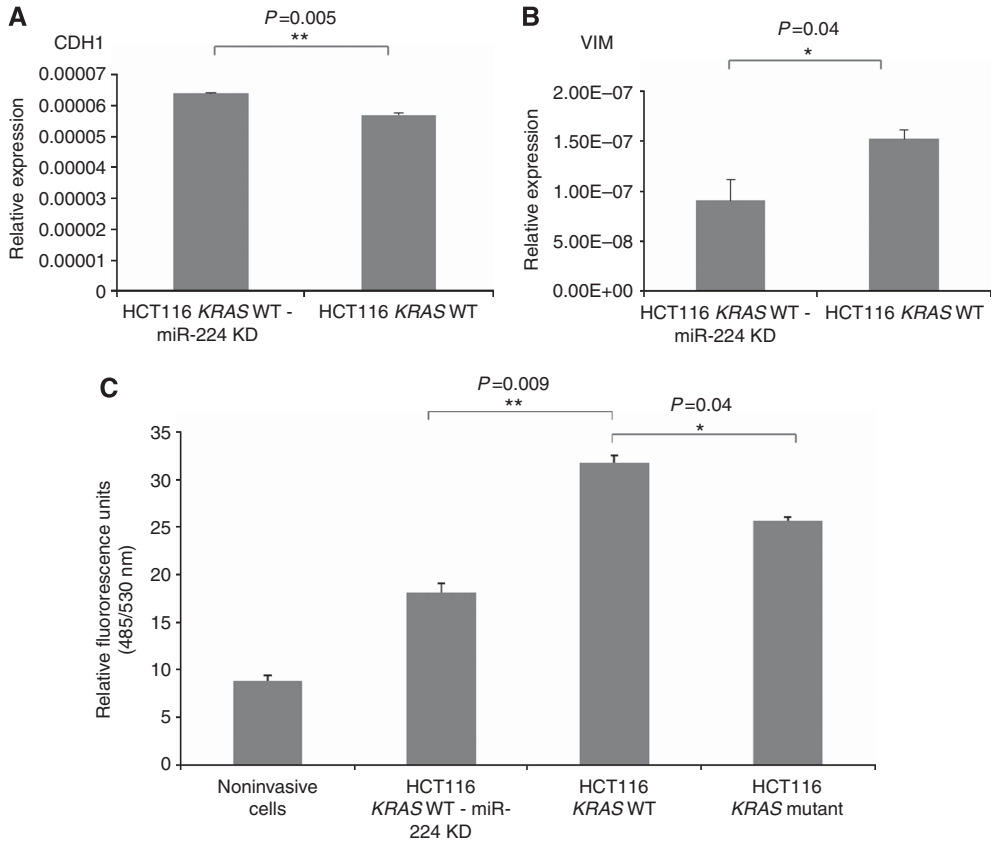


Figure 4. Confirmation of predicted EMT and altered invasion phenotypes in miR-224 knockdown cells. qRT–PCR analysis was used, as described in Materials and Methods, to compare the expression of (**A**) E-cadherin (*CDH1*) and (**B**) Vimentin in WT *KRAS* HCT116 and WT *KRAS* HCT116-miR-224 knockdown cells, with gene expression differences represented relative to the expression of 18S ribosomal RNA. All samples were analysed in triplicate, with experimental errors calculated as previously described (Smith *et al*, 2012). (**C**) Innocyte invasion assays were used to model and quantify cell invasion *in vitro* following Calcein AM labelling, as described in Materials and Methods. Each sample was analysed in triplicate and invasion in WT *KRAS*, mutant *KRAS* and WT *KRAS* HCT116-miR-224 knockdown cells compared with noninvasive cells (no serum control).

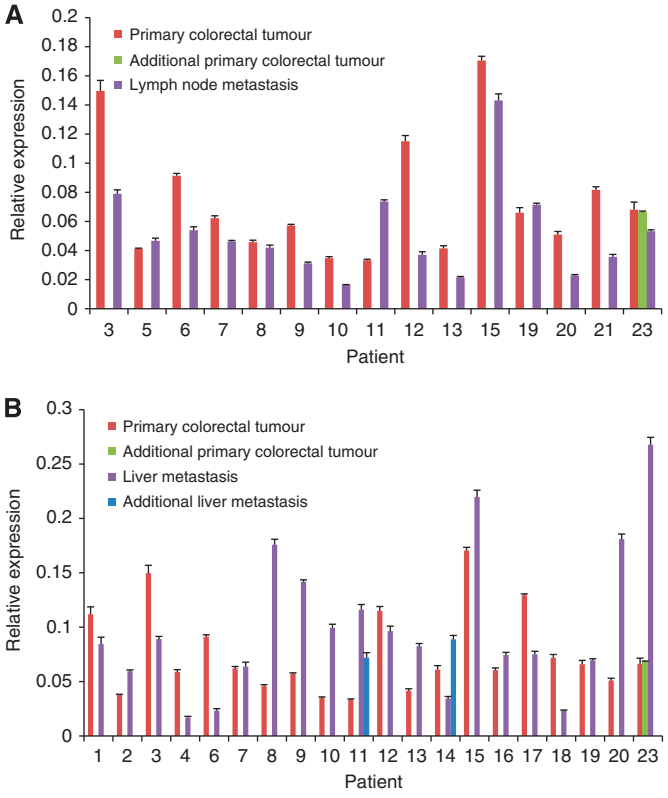
MiR-224 expression in metastatic colorectal disease. To investigate whether miR-224-dependent invasion differences influenced disease progression, a matched series of primary colorectal cancers and lymph node ($n=15$) and liver ($n=20$) metastases from 24 patients were selected for analysis and miR-224 expression compared within each matched pair by qRT–PCR analysis as previously described. MicroRNA-224 expression was significantly decreased in the majority (11 of 15) of lymph node metastasis (Figure 5A) but significantly and more abundantly increased in 13 of 20 liver metastases (Figure 5B). We attempted to further correlate miR-224 expression with patient gender, differentiation, cancer site and *KRAS* genotype, but found no significant associations, although our patient cohort was too small for meaningful assessment.

DISCUSSION

We have previously described the importance of dysregulated *KRAS* signalling in the development and progression of colorectal cancer (Smith *et al*, 2002; Conlin *et al*, 2005), and now aimed to identify miRNAs associated with disease progression and/or regulation of the RAS/MAPK signalling pathway. We identified several candidate miRNAs, one of which, miR-224, was differentially expressed in colorectal adenomas and cancers, and also in *KRAS* WT and mutant HCT116-derived colorectal cancer cell lines. MicroRNA-224 is one of the several miRNAs that have previously shown to be differentially expressed in colorectal cancer (Monzo *et al*, 2008; Arndt *et al*, 2009; Sarver *et al*, 2009; Wang *et al*, 2010; Fu *et al*, 2012; Liao *et al*, 2013; Yuan *et al*, 2013), and increased miR-224 expression in colorectal adenomas has been described by Bartley *et al* (2011) and Oberg *et al* (2011), suggesting that it is an early event in colorectal cancer development.

A miR-224/*KRAS* regulatory interaction has not previously been described, although miR-224 has been shown to repress the tumour suppressor gene *RKIP* (*RAF kinase inhibitory protein*) that inhibits metastasis in various cancers by dissociating the interaction between *RAF1* and *MEK* (Huang *et al*, 2012). Inhibition of miR-224 would therefore be predicted to increase RAS/MAPK signalling, either through activation of *KRAS* or promotion of RAF1/MEK binding following *RKIP* activation. Consistent with this hypothesis, we described increased *KRAS* activity and increased *ERK* and *AKT* phosphorylation following miR-224 knockdown in HCT116 colorectal cancer cells, whereas in colorectal cancer patients, *RKIP* expression has previously been shown to inversely correlate with patient survival and predict metastatic spread (Al-Mulla *et al*, 2006). Increased RAS activity following miR-224 knockdown is of particular interest, as our bioinformatics analysis identified the guanine nucleotide exchange factor *SOS2* (*sons of sevenless 2*) gene as a candidate miR-224 binding partner, promoting dissociation of *KRAS* and GDP, and favouring GTP binding (Pierre *et al*, 2011).

Several additional miRNAs (miR-146b-3p, miR-486-5p, miR-92a, miR-127-3p and miR-378) have previously been shown to be differentially expressed in *KRAS* WT and mutant colorectal cancers



Patient	Fold change	*P*-value
3	−1.89	0.005
6	−1.69	0.0004
7	−1.35	0.004
9	−1.83	3.1E−05
10	−2.10	0.003
11	2.21	2.9E−05
12	−3.11	0.0004
13	−1.92	0.005
15	−1.19	0.01
20	−2.23	0.003
21	−2.29	0.0001
23*	−1.28	0.0004

Patient	Fold change	*P*-value
1	−1.32	0.04
2	1.57	0.0007
3	−1.68	0.008
4	−3.48	0.0003
6	−3.92	1E−05
8	3.84	0.0008
9	2.48	3.7E−05
10	2.86	0.001
11 (i)	3.48	0.003
11 (ii)	2.16	0.01
12	−1.19	0.04
13	1.99	0.0003
14 (i)	−1.77	0.009
14 (ii)	1.46	0.006
15	1.29	0.007
16	1.23	0.01
17	−1.72	0.0007
18	−3.07	0.003
20	3.55	0.0002
23 (i)	4.04	3.4E−05
23 (ii)	3.93	0.001

Figure 5. Comparison of miR-224 expression in paired primary and metastatic colorectal cancers. qRT–PCR analysis was used, as described in Materials and Methods, to compare miR-224 expression in primary colorectal cancers ($n = 20$) and matched (**A**) liver and (**B**) lymph node metastases. MicroRNA-224 expression is illustrated relative to the expression of the invariant miRNA let-7a; all samples were analysed in triplicate, with experimental errors calculated as previously described (Smith *et al*, 2012). Fold changes in miR-224 expression and associated *P*-values are summarised for sample pairs where significant differences in gene expression were identified.

(Ragusa *et al*, 2010; Mosakhani *et al*, 2012). Of these, miR-92a, a member of the miR-17-92 cluster, has been most extensively characterised, shown to directly target the anti-apoptotic protein *BIM* (Tsuchida *et al*, 2011), and to predict both metastatic spread and survival (Zhou *et al*, 2013). It will therefore be of particular interest to investigate in future experiments whether the expression of miR-92a, like miR-224, correlates with sensitivity to 5-FU-based chemotherapy in colorectal cancer patients.

Similar to our findings in colorectal cancer, previous studies have described increased miR-224 expression (and increased expression of other genes located on chromosome Xq28) in hepatocellular carcinoma (HCC), as a result of alternative epigenetic regulation of miRNA expression by the histone deacetylases *HDAC1* and *HDAC3*, and the histone acetylase protein *EP300* (Wang *et al*, 2012). Like colorectal cancer, progression of HCC has been associated with increased miR-224 expression, as a consequence of both increased *EP300* expression and NF-κB-mediated response to inflammation (Scisciani *et al*, 2012). We have previously described amplification of the q arm of the X chromosome in both colorectal adenomas (Leslie *et al*, 2006) and colorectal cancers (Leslie *et al*, 2003); it will therefore be of particular interest in future studies to investigate whether similar epigenetic and inflammation-mediated mechanisms of miR-224 regulation are important in colorectal cancer.

MicroRNA-224 knockdown in HCT116 *KRAS* WT cells increased cell doubling time, suggesting that miR-224 influences disease progression by increasing cell proliferation or by inhibiting apoptosis. MicroRNA-224 has recently been shown to promote proliferation through repression of the tumour suppressor gene *SMAD4* (Wang *et al*, 2013; Zhang *et al*, 2013b), and to influence apoptosis in hepatocellular carcinoma through negative regulation of apoptosis inhibitor protein-5 (API-5; (Wang *et al*, 2008). Consistent with these findings, we confirmed increased pro-proliferative *ERK* and *STAT3* phosphorylation following miR-224 knockdown, and increased *STAT1* phosphorylation that influences tumour suppressor and anti-angiogenesis pathways (Battle *et al*, 2006). A recent report describing miR-224 targeting of the cyclin-dependent kinase inhibitor *CDKN1A* ($p21^{cip1}$; Olaru *et al*, 2013) is of particular interest, as our own bioinformatics analysis identified the related gene CDKN1B ($p27^{kip1}$) as a miR-224 target. Consistent with our findings, Liao *et al* (2013) have recently suggested that miR-224 regulates the G1/S cell cycle transition by downregulating $p21^{cip1}$ and $p27^{kip1}$ in colorectal cancers, and may therefore have clinical utility as both a prognostic marker and therapeutic target.

Intriguingly, we have shown that miR-224 knockdown increased 5-FU chemosensitivity, and have suggested that this may result from the creation of a *KRAS* 'mutant-like' phenotype. In support of this hypothesis, we further demonstrated that introduction of mutant *KRAS* or *BRAF* to NIH3T3 cells also significantly increased 5-FU chemosensitivity, whereas Klampfer *et al* (2005) reported protection from 5-FU-induced apoptosis after targeted deletion of a mutant *KRAS* allele in HCT116 cells, and promotion of cell death following 5-FU challenge in cells transfected with inducible mutant *KRAS* (V12). However, as *KRAS* mutation status independently influences disease progression (Smith *et al*, 2002), it is challenging to assess the unique influence of mutation status on chemotherapy response in colorectal cancer patients.

Our bioinformatics analysis suggested that miR-224 may play a role in regulation of the EMT, and may therefore influence cell invasion and migration. Consistent with this hypothesis, miR-224 knockdown resulted in increased E-cadherin and decreased Vimentin expression. Our bioinformatics data predict miR-224

targeting of additional EMT-associated genes including claudins (*CLDN11* and *CLDN14*) and desmogleins (*DGS2*), groups of proteins involved in cell adhesion (Lamouille *et al*, 2014). Our own data in HCT116 colorectal cancer cells and recent similar studies in HepG2 hepatocellular cancer cells suggest that miR-224 promotes cell migration and invasion by activation of AKT signalling and direct targeting of the tumour suppressor gene *PPP2R1B* (Ma *et al*, 2012) and rho GTPase-activating proteins *ARHGAP9* and *ARHGAP21* that deactivate *CDC42* (Scisciani *et al*, 2012). MicroRNA-224 may also influence invasion and metastasis by direct or indirect regulation of matrix metalloproteinases, enzymes secreted by cancer cells that degrade the extracellular matrix and aid dissemination. For example, miR-224 has been shown to regulate both PAK4, a *CDC42* effector that plays a role in cytoskeleton remodelling, and the matrix metalloproteinase *MMP9* (Li *et al*, 2010) through direct targeting of the transcription factor *HOXD10* (Li *et al*, 2014). Consistent with our own data, *MMP9*, like miR-224, was previously shown to be differentially expressed in primary colorectal cancers and matched liver and lymph node metastases (Illemann *et al*, 2006). Similar phenotypic associations have been proposed for additional miRNAs, including miR-29c and miR-139-5p that, like miR-224, are differentially expressed in colorectal cancer and in KRAS WT and mutant HCT116 cells. MicroRNA-29c, for example, has recently been shown to promote EMT by regulation of β-catenin signalling in colorectal tumours (Zhang *et al*, 2014a), whereas decreased miR-29c expression has been associated with relapse in colorectal cancer patients (Kuo *et al*, 2012). In previous TLDA analysis, miR-139-5p was shown to inhibit colorectal cancer cell growth (Schepeler *et al*, 2012), and has recently been described as a Notch1-dependent tumour suppressor in colorectal cancer cell lines and xenograft models (Zhang *et al*, 2014b), emphasising the need to extend our miR-224 analysis to additional candidate miRNAs in future studies.

Our data highlight significant differences in miR-224 expression in alternative metastatic sites, where expression is decreased in the majority of lymph node metastases, but increased in liver metastases. These differences may help to rationalise apparent discrepancies in the conclusions of recent studies by Zhang *et al* (2013a) who confirm increased miR-224 expression in colorectal cancer and suggest that miR-224 may have utility as a relapse biomarker, whereas Yuan *et al* (2013) alternatively suggest that miR-224 suppresses metastasis in SW480 colorectal cancer cell line-derived xenografts. Although the regulatory role of miR-224 (and additional microRNAs) will almost certainly be influenced by the genetic background of individual colorectal cancers, our data suggest that miRNA expression is influenced by anatomical site, and highlight the potential utility, particularly of stably expressed serum microRNAs, as clinical response biomarkers, although additional quantitative analyses of much larger clinical series will be required to support this conclusion. Although serum levels of miR-224 have not yet been studied as clinical response biomarkers in colorectal cancer, serum miR-224 expression has recently been associated with the development of monogenic diabetes (HNF1A-MODY; (Bonner *et al*, 2013), shown to be increased in Crohn's disease (Fujioka *et al*, 2014) and renal cell carcinoma patients (Cheng *et al*, 2013) and to predict response to combination (R-CHOP) chemotherapy in diffuse large B-cell lymphoma patients (Song *et al*, 2014).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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