miR-153 supports colorectal cancer progression via pleiotropic effects that enhance invasion and chemotherapeutic resistance

miR-153 supports colorectal cancer progression

MicroRNAs that facilitate progression and mediate drug resistance in advanced cancers have increased appeal as treatment targets, given the more frequent lack of effective therapies at late stages of disease.

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CRC, colorectal cancer; FOXO3a, Forkhead box O3 transcription factor; MiRNA, microRNA; MMP9, matrix metalloprotease 9.

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**Abstract**

While microRNAs (miRNAs) have been broadly studied in cancer, comparatively less is understood about their role in progression. Here we report that miR-153 has a dual role during progression of colorectal cancer (CRC) by enhancing cellular invasiveness and platinum-based chemotherapy resistance. MiRNA profiling revealed that miR-153 was highly expressed in a cellular model of advanced stage CRC. Its upregulation was also noted in primary human CRC compared to normal colonic epithelium, and in more advanced CRC stages compared to early stage disease. In CRC patients followed for 50 months, 21/30 patients with high levels of miR-153 had disease progression compared to others in this group with low levels of miR-153. Functional studies revealed that miR-153 upregulation increased CRC invasiveness and resistance to Oxaliplatin and Cisplatin both in vitro and in vivo. Mechanistic investigations indicated that miR-153 promoted invasiveness indirectly by inducing MMP9 production, whereas drug resistance was mediated directly by inhibiting the Forkhead transcription factor FOXO3a. In support of the latter finding, we found that levels of miR-153 and FOXO3a were inversely correlated in matched human CRC specimens. Our findings establishes key roles for miR-153 overexpression in CRC progression, rationalizing therapeutic strategies to target expression of this miRNA for CRC treatment.
Introduction

Colorectal cancer (CRC) is the second commonest cause of cancer related death in western societies.\(^1\) Disease progression and metastases are the principal causes of death, and advanced disease occurs in up to 30\% at presentation.\(^2\) Additionally, in patients with localised disease who proceed to apparently curative surgery, 50\% subsequently develop recurrence.\(^2,3\) Despite increasingly sophisticated techniques for therapy to recurrent CRC, the majority of patients remain incurable, highlighting the need for a continued effort to better understand the complexities of disease progression, and identify new directions for treatment strategies.

MicroRNAs (miRNAs) are short non-coding RNA molecules and play a critical role in malignant transformation, underscored by the observation that over 50\% of miRNA genes are located within or close to cancer-associated genomic regions.\(^4,5\) MiRNAs regulate gene expression by binding to the 3\’untranslated region (3\’UTR) of protein-coding mRNAs through sequences in the 5\’-end of the miRNA that are only partially complementary.\(^6\) One consequence of this imperfect complementarity is that each miRNA can regulate multiple target mRNAs and thereby impact functionally diverse programs of gene expression. MiRNA expression is deregulated in CRC with a growing number of oncogenes and tumour suppressor genes under miRNA regulation.\(^7,8\) Importantly, miRNA expression patterns correlate with distinct CRC sub-types and clinical phenotype.\(^8-11\) Emerging data has also implicated miRNAs in disease progression and acquisition of metastatic capabilities in non-colorectal malignancies, through promotion of epithelial-mesenchymal transition and regulation of metastasis associated genes.\(^12,13\) To date however, little is understood about the role of miRNAs in disease progression in CRC and few candidate miRNAs and target genes have been implicated.
In the present study, we hypothesised that deregulated miRNAs contribute to CRC progression. MiR-153 was identified as over-expressed in more advanced CRC using cell models and human tumour samples. Functional and mechanistic studies demonstrate that over-expression of miR-153 can increase CRC invasiveness through upregulation of MMP9, and additionally lead to enhanced platinum-based chemotherapy resistance. This latter effect may be through an inhibitory effect on the Forkhead box O3 (FOXO3) transcription factor which we show is targeted by miR-153, and inversely correlates with miR-153 in human CRC samples. Together, these results point to a significant and novel contribution of upregulated miR-153 in CRC, and suggest that modulation of miR-153 may be a useful strategy in limiting CRC progression.

Materials and Methods

Cell and organotypic cultures, transfections, and cloning

Details of the cell lines, cloning, and transfections are provided in supplementary methods. Organotypic cultures were prepared as previously described. Gels comprised a 50:50 mixture of Matrigel and type I collagen with \(5 \times 10^5\) human fetal fibroblast cells, to which were added \(5 \times 10^5\) SW480 cells stably transfected with 2ug plasmid expression constructs for miR-153 or control scrambled miRNA (CmiR00001-MR04; GeneCopoeia). Media were changed every 2 days, and gels harvested and formalin fixed after 14 days.

Patients and samples

Samples from patients with biopsy proven CRC were obtained fresh at the time of surgery and snap frozen prior to being deposited in a UK Human Tissue Act approved tumour bank. In each case, matched tumour and uninvolved proximal mucosa were
obtained. All patients provided informed consent, and the study was approved by the regional research ethics committee. Pathological verification of diagnosis and staging was in accordance with the Association of Coloproctology of Great Britain and Ireland guidelines. All specimens were reviewed by clinicians with a specialist interest in gastrointestinal pathology using paraffin embedded tissue that was adjacent to or in close proximity to tumour bank tissue. Tumours used in the present study were adenocarcinomas only, and selected at random from the tumour bank before laser capture microdissection (LCM) and RNA extraction. Exclusion criteria included evidence of a hereditary tumour, presence of multiple tumours, and tumours with histologically identified extensive necrosis. A total of 100 human tumours were examined. RNA extraction from tumours is described in supplementary methods.

**MiRNA expression profiling, in silico analyses, and bioinformatics**

MiRNA expression profiling and *in silico* analyses are detailed in supplementary methods. Array data are MIAME compliant and available in the EBI database (http://www.ebi.ac.uk/arrayexpress/experiments/; Accession number E-MEXP-3270).

**Array-based comparative genomic hybridisation**

Array-based comparative genomic hybridisation was performed as previously described and is detailed in supplementary methods.

**Cell lysis, real-time PCR, Western blotting, and functional assays**

Cell lysis, down-stream analyses, and functional assays to determine biological effects of over expression of miR-153 are described in supplementary methods.

**Tissue microarray development and immunohistochemistry**

Tissue microarray development and immunostaining is detailed in supplementary methods.

**In vivo xenograft invasion assay**
Development of a SCID mouse miR-153 knockdown xenograft model is detailed in supplementary methods.

Results

MicroRNA expression profiling in SW480 and SW620 cells

Expression of 328 human miRNAs was determined in the paired CRC lines SW480 and SW620. Figure 1A and 1B demonstrate the top 20 miRNAs up- or down-regulated >4-fold in the metastatic SW620 cells compared with SW480. Some candidates identified have been previously found to be deregulated in CRC by other groups. To further verify microarray data, 9 miRNAs of variable fold change were selected for real-time PCR validation. Consistent with the microarray data, similar fold changes in the selected miRNAs were found with high correlation between microarray and PCR results (supplementary figure 1A and 1B).

Expression of miR-153 is increased in CRC and advanced disease

In order to prioritise differentially expressed miRNA candidates for further study, putative mRNA targets of the top 15 miRNAs were predicted computationally using TargetScan (www.targetscan.org). This gene list was annotated and then subjected to gene-set enrichment analysis using the DAVID bioinformatics resource (see supplementary methods) to identify over-represented biological processes for each candidate miRNA. This data was collated and compared for all 15 overexpressed miRNA candidates, to inspect for enrichment of biological processes associated with disease progression and cancer spread (summarised in Supplementary figure 1C). One miRNA identified in our cell line profiling as upregulated over 12 fold in higher stage CRC was miR-153. Clustered and non-clustered analyses for miR-153 show that based on a p value of 0.001, 55% of the 36 biological processes identified were cancer
associated. In addition, the highest enrichment score obtained for all candidate miRNAs was associated with miR-153 and the biological processes of motility and adhesion with an enrichment score of 12.5, suggesting that deregulation of miR-153 may have important consequences in carcinogenesis and disease progression.

Further assessment with Panther, BioCarta, and KEGG annotation categories for miR-153 in DAVID demonstrated enrichment of genes associated specifically with CRC (p<0.005), and the Wnt, Cadherin, and TGF-beta (p<0.001) signalling pathways, lending additional support for a link between miR-153 deregulation and colorectal tumourigenesis in particular (supplementary file 1).

Prompted by this, we investigated expression of miR-153 in a panel of human CRC cell lines. Five of 10 cell lines examined demonstrated significant upregulation of miR-153 levels compared to SW480 cells (figure 1C). The pattern of miR-153 expression closely correlated with the expression of matching cell lines in the NCI-60 panel as determined through the web application CellMiner. To determine miR-153 expression in vivo we conducted a small scale pilot to examine 10 random tumours of varying stage by laser capture microdissection and miRNA expression analysis. Significantly increased expression of miR-153 was noted in tumour compared to normal tissue (supplementary figure 2A; p<0.0001). As transition between SW480 and SW620 cells principally reflects a change towards a metastatic phenotype, we examined expression of miR-153 in metastatic compared to non-metastatic tumours. Expression of miR-153 in 5 non-metastatic stage 1 tumours was compared with 5 stage 3 and 5 stage 4 tumours with lymphatic or distant organ metastases (supplementary figure 2B). Higher expression of miR-153 was noted in more advanced CRC (p<0.005).
We extended our analysis to an additional unselected group of 83 human samples with known clinical outcome (clinicopathological data presented in supplementary table 1) comprising 23 normal mucosa; 20 stage 1 (T1-2/N0/M0 tumours); 20 stage 3 or 4 (any T/N1-2 or M1); and 20 metastases (liver and lung). Significantly increased miR-153 expression was noted in tumour compared to normal tissue (figure 1D; p<0.005) and with increasing disease stage. Specifically, higher levels were noted in tumours with nodal or distant organ spread (figure 1E; p<0.005). When median expression of miR-153 was used to stratify patients, after a follow up time of 50 months, 21 out of 30 patients in the high miR-153 group developed disease progression compared to 9 out of 30 in the low miR-153 group (figure 1F). Factors associated with improved disease free survival (DFS) by univariate analysis included the absence of Extramural vascular invasion (EMVI) and low miR-153 expression (Log Rank p=0.007; Chi-Square=7.3; Supplementary table 2). Demographic characteristics, histological grading, and the type of surgery did not have a significant impact on survival. Multivariate analysis indicated that both EMVI and miR-153 status were independent prognostic factors (for EMVI p<0.001; Hazard ratio (HR)=9.6; 95% CI=3.63-49.12; for miR-153 status p=0.024; HR=4.7%; CI=1.17-8.74).

MiR-153 over-expression is not due to genetic copy number change

Deregulation of miRNAs has been attributed to genomic copy number changes and miRNAs are over-represented in regions of genomic gain in CRC. We therefore sought to determine if miR-153 upregulation in CRC was due to copy number change. SW620 cells were analyzed using a Genome-Wide Human SNP Array 6.0 with the hapmap270.422 data set as reference. MiR-153 is located on chromosome 2q35, however no copy number changes at this locus were identified (supplementary figure 3). To clarify if the miR-153 locus is subject to copy number change in primary CRC,
we examined the Mitelman Database of Chromosome Aberrations. 346 cases of CRC were identified and examined for recurrent and non-recurrent cytogenetic band 2q35 abnormalities. No abnormalities affecting this locus were identified.

**Functional consequences of miR-153 over-expression**

To determine any potential functional roles of over-expressed miR-153 in promoting CRC progression, we investigated the effects of transiently over-expressed miR-153 on a series of cancer-relevant in vitro cell-based assays testing growth and proliferation, invasion and motility, apoptosis and chemosensitivity in SW480 cells. MiR-153 over-expression (supplementary figure 4A) had no effect on proliferation (figure 2A), anchorage-independent growth (figure 2B), or cell migration using scratch and transwell invasion assays without matrigel (data not shown). Over-expression of miR-153 promoted a more invasive phenotype in SW480 cells however, when matrigel-coated transwell invasion assays were examined (figure 2C and D; p<0.05). Apoptosis was assessed in the presence or absence of Cisplatin. Overexpression of miR-153 had little effect on viability in the absence of Cisplatin, however protection against cell death was observed in cells overexpressing miR-153 when treated with Cisplatin (figure 2E and 2F). These findings suggested that miR-153 may have a dual role in CRC progression, promoting enhanced invasiveness and chemosensitivity. Consequently both these processes were assessed further and are described below.

**Over-expression of miR-153 leads to increased invasiveness in CRC via MMP9**

The effect of miR-153 on CRC invasion was tested in additional cell lines with low baseline expression of miR-153. Transient over-expression of miR-153 in DLD-1, Ht29, and AAC1/82 cells (supplementary figure 4E) led to enhanced invasiveness in Matrigel coated transwell assays (figure 3A). Using antimiR-153, inhibition of miR-
153 in CRC cell lines with high baseline levels of miR-153 (supplementary figure 4F) significantly reduced invasion compared to control (figure 3B) without any alteration to proliferation (supplementary figures 4B and 4C). To more closely recreate and model in vivo circumstances, we examined the effect of stably transfected GFP-tagged miR-153 in SW480 cells using a 3-dimensional organotypic co-culture of SW480 cells and human fetal fibroblast cells (figure 3C). Ectopic expression of miR-153 but not control resulted in increased invasion of SW480 cells into the underlying stroma of organotypic cultures (figure 3C (i) and (ii)). Although stable overexpression was achieved in only 50% of cells, immunostaining against GFP illustrated enrichment of miR-153 expressing cells at the invasive front (figure 3C (iii) to (vi)). To determine if miR-153 would stimulate invasion in vivo, we performed subcutaneous implantation of SW620 cells in SCID mice. Cells were transfected with antimiR-153 or control, and injected into opposite flanks of each animal. Inhibition of miR-153 resulted in more spheroid tumours with clean edges compared to control tumours with more locally invasive phenotypes (figure 3D). No difference in size of tumours was noted between control and miR-153 inhibited xenografts (supplementary figure 4D). Analysis of depth of invasion and number of invasive tumour spikes into surrounding stroma and adjacent tissue demonstrated a significant reduction in invasive ability of xenografts transfected with antimiR-153 (figure 3E).

Our initial findings demonstrated that miR-153 over-expression promoted a more invasive phenotype only in Matrigel-coated transwell invasion assays but not in non-Matrigel coated assays. A principal constituent of Matrigel is type IV collagen, which is also one of the main substrates for Matrix Metalloprotease enzyme 9 (MMP9). MMP9 is a key effector in extracellular matrix degradation and strongly implicated in CRC associated invasion, with levels progressively increasing from early node-
negative to metastatic CRC, making it an optimal candidate to examine.\textsuperscript{22,23} To test if MMP9 may play a role in miR-153 induced invasion, SW480 cells were transfected with miR-153 or control miRNA and medium supernatants sampled for MMP9 activity. Figure 4A and 4B demonstrate that raised levels of MMP9 activity were detected after transfection with miR-153, but not control miRNA. We also assessed levels of the other member of the Gelatinase sub-family, MMP2, however no effect was identified (data not shown). We next tested invasiveness of SW480 cells transduced with miR-153 in the presence of a selective MMP9 inhibitor. Inhibition of MMP9 using an MMP9 inhibitor or siRNA to MMP9 abrogated the enhanced invasiveness mediated by miR-153 (figure 4C and 4D). Finally, to determine if increased activity of MMP9 was due to raised levels of MMP9 transcript, we examined for MMP9 mRNA after forced expression of miR-153 (figure 4E). No change in MMP9 mRNA was identified, indicating that increased gelatinolytic activity is not consequent to a change in MMP9 transcript production or stability. Collectively, these studies suggest that miR-153 may enhance invasiveness through an increase in MMP9 activity.

**MiR-153 over-expression enhances platinum based chemoresistance in CRC via a direct effect on FOXO3a**

Our initial functional screen identified a possible role for miR-153 in mediating chemoresistance to Cisplatin (figures 2E and 2F). Although Cisplatin is an effective platinum-based anti-neoplastic agent with activity against a variety of gastrointestinal and other solid tumours\textsuperscript{27,28}, it is infrequently used in the current management of CRC. Conversely, Oxaliplatin, a newer generation platinum-based compound, now represents a standard of care for patients with stage III and IV CRC when combined with Fluoropyrimidine based agents\textsuperscript{18,28}. We therefore evaluated effect of miR-153
overexpression on both Cisplatin and Oxalplatin mediated apoptosis. Figure 5A demonstrates that overexpressed miR-153 had a similar effect in protecting against Oxalplatin mediated apoptosis. We also examined sensitivity of SW480 and SW620 cells to Oxalplatin. As might be predicted from the higher levels of miR-153 in SW620 cells, SW620 cells were more resistant to Oxalplatin mediated apoptosis, even with high doses of Oxalplatin (figure 5B). Upregulation of miR-153 but not scrambled miRNA reduced abundance of activated Caspase 3 when SW480 cells were treated with Cisplatin, indicating that miR-153 is functioning as an anti-apoptotic factor in this context (figure 5C and 5D). To explore gene targets of miR-153 which may mediate this effect, we examined for putative targets using four established miRNA target prediction programs, miRanda (www.microrna.org), PicTar (www.pictar.org), TargetScan (www.targetscan.org), and Diana-microTv3.0 (http://diana.cslab.ece.ntua.gr/microT/). To reduce false positives, candidates were only considered if they were predicted by at least three methods. One candidate identified by this approach was the Forkhead/winged helix box class O3a (FOXO3a) tumour suppressor protein. FOXO proteins are a conserved subfamily of transcription factors and orchestrate programs of gene expression involved in differentiation, apoptosis, and DNA damage responses. Deletion of FOXO alleles confers a tumorigenic phenotype in mice models while inactivation or silencing leads to a well described chemoresistance to Cisplatin in colon, ovarian, bladder, and oral squamous cell carcinomas, making it an optimal target to investigate. FOXO3a protein levels were examined in the SW480/620 cell model, and found to inversely correlate with miR-153 expression levels (figure 6A). To experimentally verify FOXO3a as a target of miR-153, we examined the effect of miR-153 over-expression on endogenous FOXO3a protein levels. Figure 6B and 6C demonstrate a significant
reduction in FOXO3a protein levels in different cell lines after miR-153 overexpression. We also evaluated if miR-153 could impact the transcript levels of FOXO3a, and noted a significant reduction in FOXO3a mRNA in the presence of miR-153 (Figure 6D).

We next determined if the effect of miR-153 in promoting CRC chemoresistance to Oxaliplatin could be altered by introduction of exogenous FOXO3a. As shown by other groups, FOXO3a was found to mediate chemosensitivity to Oxaliplatin (supplementary figure 5B)\textsuperscript{30-36}. In addition, figure 6E demonstrates that overexpression of FOXO3a can reverse the observed effect of miR-153 on Oxaliplatin chemosensitivity. To determine if the miR-153-FOXO3a interaction may also be contributing to the enhanced invasiveness observed with miR-153 over-expression, we first examined the effect of FOXO3a overexpression in mediating CRC invasiveness. Figure 6F shows that while miR-153 can successfully mediate increased invasiveness in transwell invasion assays, overexpression or siRNA-mediated inhibition of FOXO3a is unable to replicate this effect. Additionally, when FOXO3a was overexpressed in SW480 cells, medium supernatants sampled for MMP9 showed no increase in MMP9 activity compared to controls, unlike miR-153 overexpression (figure 6G). Similarly, other groups have noted no changes in invasiveness or MMP9 activity from over-expressed wildtype FOXO3a, further supporting the hypothesis that the observed effects of miR-153 on invasion and MMP9 are unlikely to be mediated through FOXO3a.\textsuperscript{30,37}

The 3’UTR of FOXO3a contains one phylogenetically conserved miR-153 binding site (figure 6H) with a 7mer-A1 seed-matched site in the 5’ region of miR-153, ranking as one of the top scoring probabilities of conserved targeting (P\textsubscript{CT}) as described by Friedman and colleagues.\textsuperscript{38} To determine if the effect on FOXO3a is
mediated via this predicted miR-153 binding site, we cloned a 542bp region of the 3’UTR of FOXO3a containing the single putative miR-153 binding site downstream of the Renilla luciferase open reading frame. Wildtype 3’UTR and a mutant form in which the putative seed-binding site was mutated were evaluated. As shown in figure 6I, when miR-153 precursor was co-transfected with wildtype FOXO3a 3’UTR reporter construct, significant repression in activity was noted. Mutation of the miR-153 binding site eliminated the observed repression, supporting a direct association between miR-153 and FOXO3a. Similar results were obtained in HCT116 CRC cells, indicating this is not unique to SW480 cells.

We next sought to determine if expression of FOXO3a was altered in human CRC. Using a custom-designed tissue microarray, we examined and scored the intensity and proportion of cells staining for FOXO3a in 10 normal colorectal mucosa samples, 10 adenomatous polyps, 20 stage 1 CRC, and 20 stage 3/4 tumours. Representative sections are presented in figure 7A and analysis of the scoring presented in 7B. FOXO3a expression was significantly lower in advanced cancer stages (p<0.0005). Analysis of matched tumours for expression of miR-153 and FOXO3a showed a significant and inverse correlation between miR-153 and FOXO3a (figure 7C; R=-0.75; p<0.0001; 95% CI = -0.86 to -0.52). Taken together, these results suggest that miR-153 mediated resistance to platinum based chemotherapy could be mediated through reduced levels of FOXO3a.

**Discussion**

Disease progression in CRC is a complex multi-step process. During progression, cancer cells acquire the ability to invade beyond normal cellular boundaries, intravasate into blood and lymphatics, journey to distant organs, extravasate and proliferate in a different microenvironment, concomitantly eluding anti-tumour host
immunity and defying chemotherapeutic agents. Although to date miRNAs have been principally identified as mediators of tumorigenesis, emerging data is also uncovering their role as important modulators of different steps in metastasis and disease progression, serving as promoters\textsuperscript{7,12}, or inhibitors\textsuperscript{13}, of these processes.

Here we report on the pleiotropic actions of a poorly understood miRNA, miR-153 in promoting CRC progression. We used the SW480/SW620 cell model for CRC progression and conducted miRNA profiling to identify differentially expressed miRNAs between the two cell lines. One candidate identified by our screen was miR-153, which was upregulated by over 12 fold in the more advanced SW620 cells. MiR-153 is a conserved miRNA first detected at high levels in brain tissue, and implicated in development of neurodegenerative conditions.\textsuperscript{39,40} Intriguingly, miR-153 levels are reduced in human Glioblastoma Multiforme,\textsuperscript{41} and increased in endometrial adenocarcinomas.\textsuperscript{42} To our knowledge, miR-153 has not been implicated in CRC before. To determine the relevance of this miRNA, we examined miR-153 expression in a panel of CRC cell lines, in human CRC tumour samples compared to normal mucosa, and subsequently across a panel of pre-defined CRCs of different clinical stage. MiR-153 expression in the CRC cell lines examined in our study showed similar expression patterns to matching CRC lines in the NCI-60 panel, further verifying our findings\textsuperscript{43}. MiR-153 was over-expressed in tumours compared to normal tissue, and consistent with our cellular model, expressed at higher levels in metastatic compared to non-metastatic tumours. As the main mechanism for development of CRC is chromosomal instability,\textsuperscript{44} and miRNAs are over-represented in regions of genomic copy number change in CRC, we examined the miR-153 locus for copy number change. Little is understood about the regulation of miR-153 and systematic evaluation of the literature did not identify any studies describing genetic
or epigenetic regulation of miR-153 specifically. MiR-153 resides in the gene locus for the PTPRN gene, which encodes a member of the protein tyrosine phosphatase family (supplementary figure 3). Analysis of our cell line model and the Mitelman database suggest that copy number change in this region is a rare event however. Intriguingly, recent data from DNA methylation profiling in ovarian cancer has shown that promoter hypermethylation of the PTPRN gene is significantly associated with longer patient survival, supporting a model in which miR-153 silencing may be protective against disease progression\textsuperscript{46}. At present however, no data showing coregulation of PTPRN and miR-153 has been described.

To better understand how deregulation of miR-153 impacts CRC, we conducted functional studies to examine effects of ectopic miR-153 and inhibition of miR-153. We observed two broad effects of miR-153 upregulation, leading to increased cellular invasiveness, and enhanced platinum-based chemotherapy resistance. To dissect the mechanisms behind miR-153 mediated increase in invasiveness, we examined production of MMPs after miR-153 overexpression. The gelatinase MMP9 is strongly implicated in CRC progression, and secretion is higher in metastatic murine CRC compared to non-metastatic tumours,\textsuperscript{45} while in the SW480/620 human CRC model, MMP9 expression is higher in metastatic SW620 cells.\textsuperscript{14} SW620 cells are more invasive than SW480 cells in a variety of in vitro assays, and overexpression of MMP9 in SW480 cells leads to a more invasive phenotype.\textsuperscript{14,47} Additionally, multiple in vivo studies have demonstrated increased MMP9 transcript,\textsuperscript{23} protein,\textsuperscript{48} and bioactivity\textsuperscript{49} in more advanced human CRC compared to earlier stages, and increased MMP9 is an independent predictor of overall, cancer specific, and disease-free survival.\textsuperscript{23,49} In the present study, we noted increased MMP9 activity after transfection of cells with miR-153. Mir-153 transfected cells demonstrated enhanced invasion
through matrigel, a basement membrane-like matrix rich in collagen type IV (the main substrate of MMP9), and inhibition of MMP9 was sufficient to abrogate this effect. The mechanism by which miR-153 upregulates MMP9 activity remains unclear at present, however the results of our gene set enrichment analysis for putative miR-153 targets demonstrated over-representation of a number of transcription factors which may provide insights into this process (supplementary files 1 and supplementary figure 1C).

To better understand how miR-153 increases resistance to platinum based agents, in silico analysis was performed with miRNA target predicting algorithms. One candidate identified was the FOXO3a transcription factor. Functionally, FOXO3a has striking similarities to p53, and the two transcription factors share many downstream targets. Although inactivating mutations in FOXO3a in human cancer have not been described, loss-of-function of FOXO3a frequently occurs by post-translational modifications. FOXO3a is a critical molecule in initiating apoptotic programs, and upregulates pro-apoptotic genes such as Bim and PUMA, while downregulating anti-apoptotic genes. FOXO3a is a well described regulator of the response to Cisplatin in several malignancies including CRC, and silencing endogenous FOXO3a impairs cytotoxicity of this chemotherapeutic agent. Accordingly, we tested if miR-153 could be mediating its effect through FOXO3a. Our results show a consistent and inverse relationship between miR-153 and FOXO3a levels in CRC cell lines and tumours, and support a model in which tumour over-expression of miR-153 reduces FOXO3a transcript and protein levels, impairing the apoptotic response to Cisplatin, and mimicking the role of oncogenic kinases such as Akt in inactivating FOXO3a. Our results verify that the miR-153/FOXO3a interaction is a direct one, as miR-153 interacts with the 3’UTR of FOXO3a through a specific seed binding site to bring...
about repression of a FOXO3a-3’UTR luciferase fusion gene. Expression of FOXO3a is clearly a tightly regulated process, and our findings demonstrate that miR-153 represents a further layer of regulation and providing further fine-tuning of FOXO3a function.

Collectively our findings suggest that overexpression of miR-153 has an important and dual role in promoting disease progression in CRC through enhanced cellular invasion and reduced chemosensitivity. The ability of one miRNA to have a dual function in disease promotion is not unprecedented\textsuperscript{14} and indeed the predicted promiscuity of miRNAs by target prediction has long hinted at this. Such pleiotropy also exposes opportunities for exploiting the miRNA system for therapeutic manipulation with potential to correct more than one corrupted pathway by targeting one molecule only, giving added value in the design and development of any novel targeted therapy. Our present findings also add to the weight of evidence incriminating miRNAs in the mechanisms behind cancer progression and metastasis, and identify miR-153 as a further molecule in the new class of “metastamirs”.\textsuperscript{51}

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FIGURE LEGENDS

Main document figure legends

Figure 1. MiR-153 is upregulated in advanced stage CRC cell lines and human tumours. MiRNA expression profiles were determined in SW480 and SW620 cells (A, B). Upregulated (A) and downregulated (B) miRNAs in the metastatic SW620 cell line compared with SW480. miRNAs previously linked to CRC are indicated with "**". (C) Endogenous miR-153 expression by quantitative RT-PCR in a panel of CRC cell lines (*,p<0.05; **,p<0.01). (D and E) Expression levels of miR-153 were examined by qPCR in 83 human samples comprising 23 normal mucosa; 20 stage 1 (T1-2/N0/M0 tumours); 20 stage 3 or 4 (any T/N1-2 or M1); and 20 metastases (liver and lung). Significantly increased miR-153 expression was noted in tumour compared to normal tissue and with increasing disease stage (p<0.005). (F) Disease free survival for patients according to low or high expression of miR-153 (Kaplan-Meier).

Figure 2. Transient overexpression of miR-153 in SW480 cells had no effect on proliferation in MTS assay (A) or soft agar colony forming ability (B). (C and D) Matrigel coated transwell invasion assays demonstrate increased invasion of SW480 cells transiently transfected with Pre-miR-153 (relative levels of miR-153 shown in (E)) compared to control miRNA. (D) Representative phase contrast micrograph of invading SW480 cells. (F) Representative flow cytometry data of SW480 cells transfected with control or miR-153 miRNAs. Relative expression level of miR-153 is provided in adjacent bar chart. (G) Analysis of apoptosis in SW480 cells in the presence or absence of Cisplatin at 50ug/ml. Data presented represents results from at least three independent experiments expressed as means ± SD. * p<0.05.
Figure 3. (A) Increased invasion after upregulation of miR-153 was noted in CRC cell lines with low baseline expression of miR-153 (relative expression of miR-153 is shown in supplementary figure 4E). (B) MiR-153 inhibition using antimiR-153 in CRC cell lines with high baseline levels of miR-153 significantly reduced invasion compared to control (relative expression of miR-153 shown in supplementary figures 4E and 4F). (C) 3-dimensional organotypic co-cultures of SW480 cells and human fetal fibroblast cells show increased invasion of SW480 into stroma after upregulation of miR-153. Adjacent bar chart illustrates comparative expression of miR-153. (Ci and Cii) Representative sections of organotypic gels immunostained with an anti-cytokeratin antibody demonstrate enhanced invasiveness of SW480 cells in which stable, ectopic expression of GFP-tagged mi-153 has been induced. (Ci,ii-Cvi) Consecutive sections were immunostained with an anti-GFP antibody and demonstrate enrichment of miR-153 expressing SW480 cells at the invasive front compared with control miRNA. (Transfected cells contain plasmid constructs which co-express GFP). (D) Representative images of mouse tumour xenografts with inhibition of miR-153 demonstrate a clean edge of tumour spheroid and fewer invasive fronts into the surrounding stroma (Dii and Diii) in contrast to controls with a more locally invasive tumour phenotype (Di and Div). Arrows in Di and Diii show invasive tumour fronts extending and involving surrounding muscle. Arrow heads in Dii and Div indicate the sharp and clean edges of the expanding SW620-antimiR-153 tumour spheroid. Adjacent bar chart illustrates comparative expression of miR-153. (E) Quantitation of the vertical depth of tumour invasion and the number of invasive tumour spikes. Twenty random high power fields of the tumour stroma interface were analysed per slide and mean values obtained. Results are expressed relative to control
anti-miR findings. Vertical depth of invasion was measured between the deepest point of invasion and base of the main tumour mass.

**Figure 4.** Upregulation of miR-153 enhances invasiveness through increased MMP9 activity. (A) Representative experiment showing zymographic activity in medium supernatants of SW480 cells after transfection with Pre-miR-153 or control. Adjacent bar chart illustrates comparative expression of miR-153. (B) Assessment of MMP9 activity by determination of band intensity. Results are expressed as relative changes in MMP activity against control miR. (C) Transwell invasion assays in the presence of MMP9 inhibitor (100nM; 4C) with representative expression of miR-153 shown in (4D). (4E) Transwell invasion assays in the presence of siRNA to MMP9 with representative expression of miR-153 shown in (4F), and levels of MMP9 inhibition depicted in supplementary figure 5A. (G) Effect of miR-153 or control Pre-miR on MMP9 transcript levels. Results show data from at least three independent experiments.*, p<0.05.

**Figure 5.** (A) Overexpression of miR-153 in SW480 cells provides resistance to apoptosis from both Cisplatin (50ug/ml) and Oxaliplatin (30ug/ml), compared to no chemotherapy. Data presented represents results from at least three independent experiments expressed as means ± SD. * p<0.05. Relative expression of miR-153 is shown in supplementary figure 4G. (B) Effect of Oxaliplatin treatment at various doses on cell viability in SW620 and SW480 cells. SW620 cells are more resistant to Oxaliplatin mediated cell death when compared to SW480 cells. (C) Western blot analysis for levels of pro- and cleaved caspase 3 after SW480 cells were transfected with miR-153 or control for 48 hours after treatment with Cisplatin. (D) Relative
signal intensities for pro- and cleaved caspase 3 were determined by densitometry and normalized to Actin. Results are presented relative to control-miR expression which was set at 1, and relative expression levels of miR-153 are provided in supplementary figure 4H.

**Figure 6.** FOXO3a levels are reduced in SW620 cells and FOXO3a is a target of miR-153. (A) Western blotting for FOXO3a in SW480 and SW620 cells with corresponding analysis of signal intensities using polyclonal anti-FOXO3a. Results are presented relative to SW480 cells which were set at 1.0. (B) Western blot analysis for endogenous FOXO3a in SW480, DLD1, and Ht29 cells after transfection with control or miR-153 precursor miRNA. Representative figure for miR-153 relative levels is presented in supplementary figure 4I. (C) Relative signal intensities for FOXO3a in Western blots (n=3) were determined by densitometry. (D) Effect of miR-153 or control miRNAs on FOXO3a transcript levels. Total RNA was extracted 48 hours after transfection of SW480 cells and qRT-PCR performed. Results illustrate data from 3 independent experiments and are expressed as means ± SD. (E) Overexpression of miR-153 in SW480 cells provides resistance to apoptosis from Oxaliplatin (30µg/ml), which can be reversed by exogenous FOXO3a. Relative expression of miR-153 is shown in supplementary figure 4J. (F) Knock-in or knock-down of FOXO3a does not influence invasion compared with miR-153. (G) Zymographic activity in medium supernatants of SW480 cells after transfection with Pre-miR-153, FOXO3a expression construct, or control. Comparative expression of miR-153 is shown in supplementary figure 4K. (H) The 3’UTR of mammalian FOXO3a mRNA contains a phylogenetically preserved miR-153 binding site. (F) Dual luciferase reporter assays were performed with FOXO3A-3’UTRwt (containing
wildtype 3’UTR of FOXO3a) and FOXO3A-3’UTRmut (containing a mutation of 4 of the amino acids in the predicted miR-153 binding site) vectors. *, p<0.05.

**Figure 7.** FOXO3a expression in CRC. (A) Representative images from immunostaining on a tissue microarray, showing FOXO3a expression in normal, stage 1, and stage 3/4 CRC. (B) FOXO3a expression is lower in more advanced disease stages (p<0.0005). (C) Correlation of FOXO3a expression and miR-153 levels in human tumour samples.
Figure 1

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Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

**Table A**

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**Graph C**

miR-153 expression (R.U.)

**Graph D**

miR-153 expression (R.U.)

**Graph E**

miR-153 expression (R.U.)

**Graph F**

Cumulative proportion of Disease free survival

No. at risk

Low miR-153

High miR-153

Time to recurrence

Low miR-153

High miR-153

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Figure 3

A) Ratio of invading cells

B) Ratio of invading cells

C) Control miR vs. miR-153

D) Control antimiR vs. AntimiR-153

E) Relative level of miR-153 expression

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**Figure 5**

**A**

![Graph showing the effect of no treatment, Cisplatin, and Oxaliplatin on cell viability with and without miR-153](image)

**B**

![Graph showing the effect of Oxaliplatin concentration on cell viability for SW480 and SW620 cell lines](image)

**C**

![Western blot images for Pro-caspase 3, Cleaved Caspase 3, and Actin](image)

**D**

![Bar graph showing relative levels of Pro-Caspase 3 and Cleaved Caspase 3 with and without miR-153](image)
Figure 6

A

SW660

FOXO3a
Actin

B

Control miR
miR-153

FOXO3a
Actin

SW660
DLD1
HT29

C

Relative level (% of control)

D

Relative level (to control)

E

+ Oxaliplatin

F

Invasion (relative level)

G

175 KDa

H

MMP 9

Hsa-FOXO3a 5'--UAAGGCUAGAAACUUGU-3'
Ptr-FOXO3a 5'--UAAGGCUAGAAACUUGU-3'
Mmu-FOXO3a 5'--UAAGGCUAGAAACUUGU-3'
Rno-FOXO3a 5'--UAAGGCUAGAAACUUGU-3'
Cfo-FOXO3a 5'--UAAGGCUAGAAACUUGU-3'
Gga-FOXO3a 5'--UAAGGCUAGAAACUUGU-3'

Hsa-miR-153 3'--CUAGGAAACGCUAG-5'

I

Relative level (to control)

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Figure 7

A

Normal colon

Stage 1

Stage 3/4

B

C

FOXO3a expression

FOXO3a expression

miR-153 expression / R.U.

R = -0.75; p < 0.0001
(95% CI = -0.89 to -0.52)
miR-153 supports colorectal cancer progression via pleiotropic effects that enhance invasion and chemotherapeutic resistance

Lei Zhang, Karen Pickard, Veronika Jenei, et al.

Cancer Res Published OnlineFirst August 15, 2013.

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