Identification of pathogenic microRNAs in *Helicobacter pylori*-associated gastric cancer using a combined approach of animal study and clinical sample analysis

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KEY MESSAGES

1. miR-490-3p is progressively down-regulated in the development of gastric cancer.
2. The tumour suppressive role of miR-490-3p is established both *in vitro* and *in vivo*.
3. DNA methylation is involved in the down-regulation of miR-490-3p in gastric cancer.
4. SMARCD1 is a direct target of miR-490-3p.

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Introduction

Gastric cancer is the fourth most common cancer and the second leading cause of cancer-related death worldwide. *Helicobacter pylori* is estimated to infect at least half of the world's human population. The attributable risk for both intestinal and diffuse types of gastric cancer conferred by *H pylori* is approximately 75%.[1] Only a few studies have addressed the role of miRNAs in inflammatory or pre-neoplastic states of gastric mucosa.[2,3]

In this study conducted from January 2011 to July 2013, we used a multi-level method with a combined approach of high-throughput miRNA array technique, an animal model of *H pylori* infection, clinical sample analysis, and functional assays and identified miR-490-3p as a tumour suppressor in *H pylori*-associated gastric cancer.

Methods

Mouse model of gastric carcinogenesis

To generate a mouse model of gastric carcinogenesis, C57BL/6 mice (6-8 weeks old) were inoculated with *H pylori* (SS1 strain) with or without N-methyl-N-nitrosourea (MNU). 240 p.p.m MNU was given in drinking water starting from about 7 weeks of age for a total of five cycles of a 1-week regimen followed by a 1-week pause. It was freshly prepared twice a week and light shielded. $1x10^9$ CFU/mL *H pylori* in 0.1 mL Brucella broth with 5% foetal bovine serum was inoculated on three alternate days, starting from about 18 weeks of age. Mice were divided into three groups: control, *H pylori*, and *H pylori* plus MNU and kept for 12 months before being sacrificed.

miRNA microarray and data analysis

miRNA microarray was performed as described previously.[4] Agilent mouse whole genome miRNA array (rel15) was used. The miRNA expression values were extracted. Hierarchical clustering and heat map was used to show the non-parametric grouping of the miRNAs.

Cell lines and clinical samples

HFE-145 was obtained from Dr Duane T Smoot of the Howard University. TMK1 was obtained from Dr Eiichi Tahara of the University of Hiroshima. AGS, BGC823, MKN-1, MNK-28, MNK-45, and YCC-10 were purchased from American Type Culture Collection (Manassas [VA], USA). Normal, intestinal metaplasia, gastric cancer tissue and paired gastric cancer and adjacent normal tissue were retrieved from the tissue bank at the Department of Anatomical and Cellular Pathology, Prince of Wales Hospital, Hong Kong.

miRNA quantification

Total RNA was isolated according to the protocol of TRIZOL reagent. Expression of mature miRNAs was determined using TaqMan MicroRNA Assays (Applied Biosystems, USA). U6 served as endogenous control.

Cell viability assay and cell cycle analysis

Cell viability was measured by MTT assay and followed the standard procedure. Cell cycle analysis was determined by propidium iodide staining using the standard procedure.

Cell migration and invasion assay

The migration and invasion assays were performed using sterilised transwell insert chambers according to standard procedures.

Colony formation and anchorage-independent cell growth

For colony formation analysis, 24 hours after transfection, 10^3 viable cells were placed in six-well plates and maintained in complete medium for 2 weeks. Colonies were fixed with methanol and stained with 0.1% crystal violet. Anchorage-independent growth assays were performed in six-well plates by suspending 10^3 transfected cells per well in 0.3% low melting temperature agarose (Sigma) on a 0.5% agarose base layer, both of which contained growth medium. Two weeks later, colonies were visualised by staining with 0.1% crystal violet.

Western blot

Western blot was carried out following standard protocols.

Construction of lenti-miR-490-3p

miR-490-3p precursor sequences were amplified using the following primers: sense 5'-ccggTCAACCTGGAGGACTCCATGCTGctcgag CAGCATGGAGTCCTCCAGGTTGtttttg-3' and antisense 5'-aattcaaaaaCAACCTGGAGGACTCC-ATGCTGctcgagCAGCATGGAG TCCTCCAGGTTGA-3' and cloned into the pLKO.1-puro vector. pLKO.1-puro vector served as control. Virus packaging was performed in HEK 293T cells co-transfected with pLKO-miR-490-3p or pLKO-ctrl with pCMV-dR8.2 dvpr Vector and pCMV-VSVG Vector using Lipofectamine 2000 (Invitrogen). Viruses were harvested 48 hours after transfection, and viral titre was determined

Animal experiment

TMK-1 cells were infected with lenti-miR-490-3p or control in the presence of 10 μg/mL Polybrene (Millipore). After 48 hours, 2×10^6 cells in 50 μL PBS were injected subcutaneously into the dorsal region of 6-week-old male BALB/c nu/nu mice (n=6 for each group). Tumour volume was measured every 10 days. At the end of the experiment, mice were sacrificed and the tumours excised and weighed.

Luciferase reporter assay

The SMARCD1 3'UTR or the miR-490-3p binding site deleted SMARCD1 3'UTR were subcloned in the pMIR-REPORT™ reporter (Invitrogen). 200 ng of the pMIR reporter together with 1 ng of the pRenilla reporter (Promega) were co-transfected into the cells by lipofectamine 2000 (Invitrogen) according to standard protocols. After 48 hours of incubation, the transfected cells were re-transfected with miR-490-3p mimics or miRNA control and incubated for 3 days. The activity of the firefly luciferase and Renilla luciferase was measured using the Dual-Luciferase Reporter Assay System (Promega)

DNA methylation analysis

Genomic DNA was subject to bisulfite treatment using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA) according to the manufacturer's protocol and stored at -80°C. Bisulfite treated DNA was used as the template in methylation-specific-PCR using primers specific to methylated or unmethylated miR-490-3p promoter.[5]

Statistical analysis

All data were expressed as mean±standard deviation from triplicate experiments. Analysis of variance (ANOVA) was used, followed by the Tukey's t-test or Pearson correlation analysis. A P value of <0.05 was considered statistically significant.

Results

H pylori inoculation induced gastritis, intestinal metaplasia and gastric cancer in mice

The histology of the mouse tissue samples was examined by two pathologists blinded to the treatment group. Four of five mice in the control group had normal histology and the remaining one showed very weak inflammation. For the *H pylori*-inoculated group, three of seven mice exhibited moderate-to-severe inflammation and the tissues were used for miRNA array. In the MNU-plus-*H pylori* group, four mice developed intestinal metaplasia, four developed gastric cancer, and one developed dysplasia.

MicroRNA array analysis and real-time PCR validation

mmu-miR-1, mmu-miR-133a, mmu-miR-133a*, mmu-miR-133b, mmu-miR-203, mmu-miR-205, and mmu-miR-490-3p showed progressive down-regulation in the inflammation–intestinal metaplasia–adenocarcinoma sequence (Fig 1a). Hierarchical clustering (Fig 1b) showed that the expression pattern of miRNAs from the control group and *H pylori* group were the most similar and they were separated first with MNU+Hp (intestinal metaplasia) group and then with MNU+Hp (cancer), suggesting our model correctly depicted gastric carcinogenesis. Real-time PCR validation revealed gradual down-regulation from inflammation to intestinal metaplasia to cancer for miR-133a, miR-133b, miR-203, and miR-490-3p (Fig 1c). This

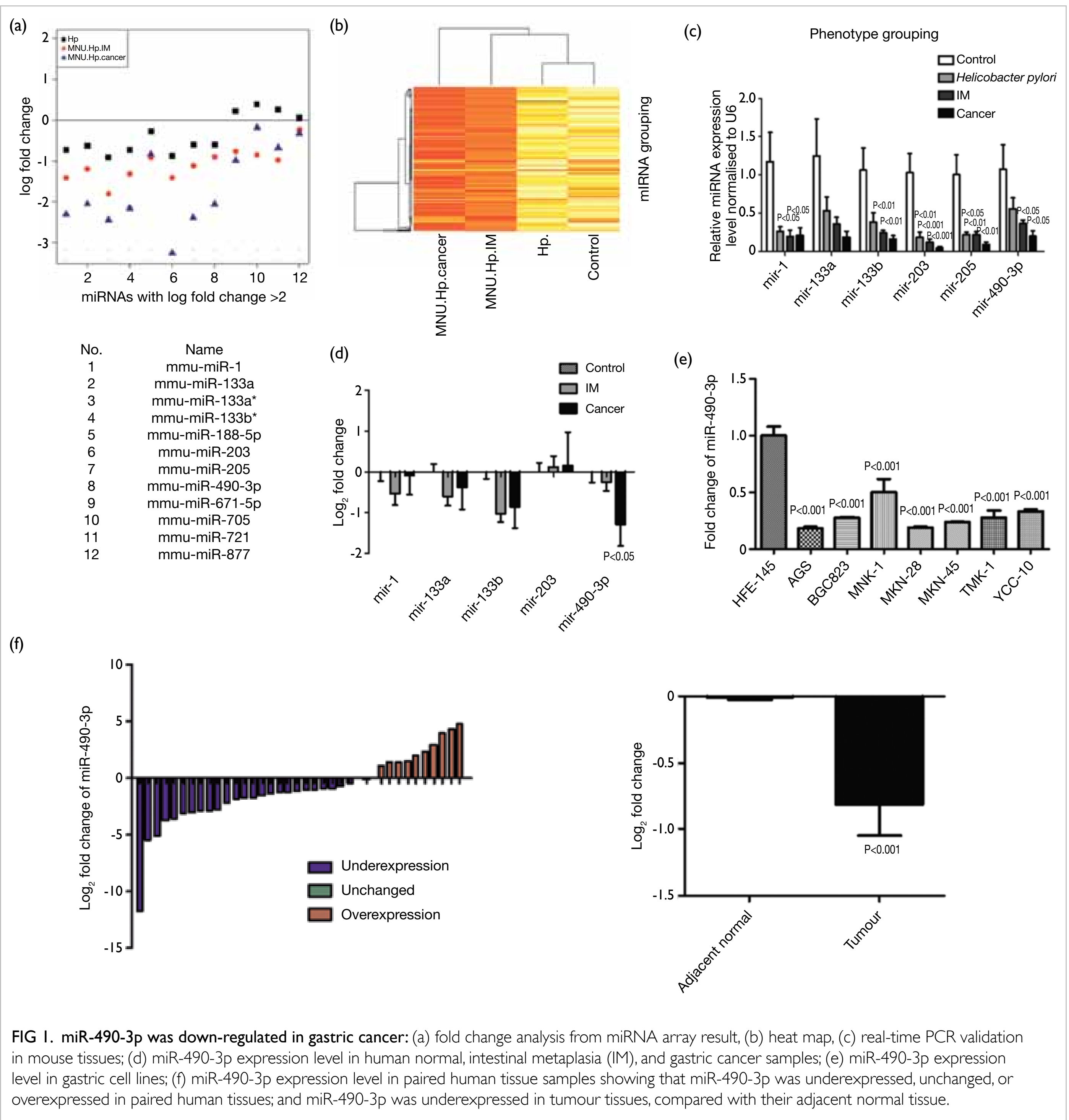

FIG 1. miR-490-3p was down-regulated in gastric cancer: (a) fold change analysis from miRNA array result, (b) heat map, (c) real-time PCR validation in mouse tissues; (d) miR-490-3p expression level in human normal, intestinal metaplasia (IM), and gastric cancer samples; (e) miR-490-3p expression level in gastric cell lines; (f) miR-490-3p expression level in paired human tissue samples showing that miR-490-3p was underexpressed, unchanged, or overexpressed in paired human tissues; and miR-490-3p was underexpressed in tumour tissues, compared with their adjacent normal tissue.

confirmed the validity of the miRNA array data. In human normal, intestinal metaplasia, and cancer tissue samples, down-regulation was found in intestinal metaplasia and cancer groups for miR-1, miR-133a, miR-133b, and miR-490-3p (Fig 1d). A gradual and significant down-regulation was observed only for miR-490-3p, which was then selected for further study.

miR-490-3p was down-regulated in human gastric cancer tissue and human gastric cancer cell lines

To further verify whether miR-490-3p was consistently down-regulated in gastric cancer, the expression level of miR-490-3p was determined in human paired tissue samples. miR-490-3p was significantly down-regulated in all gastric cancer cell

lines, compared with the normal HFE-145 (Fig 1e). miR-490-3p expression level was lower in 25 (69.4%) out of 36 pairs (Fig 1f).

miR-490-3p inhibited cell viability, migration, invasion, colony formation, and anchorage-independent cell growth

MTT assay showed that the growth of AGS and TMK-1 was significantly inhibited but the growth of normal HFE-145 was not influenced (Fig 2a). Consistently, the proliferation of AGS and TMK-1 was also significantly reduced by miR-490-3p as determined by Brdu incorporation assay (Fig 2b). MiR-490-3p inhibited cell growth by inducing cell cycle arrest (Fig 2c), which was further confirmed by western blot (Fig 2c). Overexpression of miR-490-3p also significantly inhibited migration, invasion, colony formation, and anchorage-independent cell growth in both cells (Figs 2d-g).

miR-490-3p inhibited in vivo tumour growth

We constructed lenti- miR-490-3p for *in vivo* delivery of miR-490-3p and determined its anti-tumour effect in nude mice. Compared with the control group, lenti-miR-490-3p significantly inhibited tumour size and tumour weight (Fig 2h).

DNA methylation is involved in the down-regulation of miR-490-3p in gastric cancer

In both cells' cell lines, 5AZA treatment restored the expression of miR-490-3p significantly (Fig 3a). As miR-490-3p was located in the intronic region of a host gene CHRM2 (cholinergic receptor, muscarinic 2), we hypothesised that they may share the same promoter. Correlation of miR-490-3p with CHRM2 confirmed this hypothesis (Fig 3b). Using methylation-specific PCR, DNA methylation in the promoter of CHRM2 was higher in human tumours compared with adjacent normal tissue and also higher in five of six human gastric cancer cell lines, compared with the normal gastric epithelial cell HFE-145 (Fig 3c). These findings suggest that promoter DNA methylation is at least in part responsible for the down-regulation of miR-490-3p in human gastric cancer.

SMARCD1 is a direct target of miR-490-3p

To identify the direct target of miR-490-3p, we

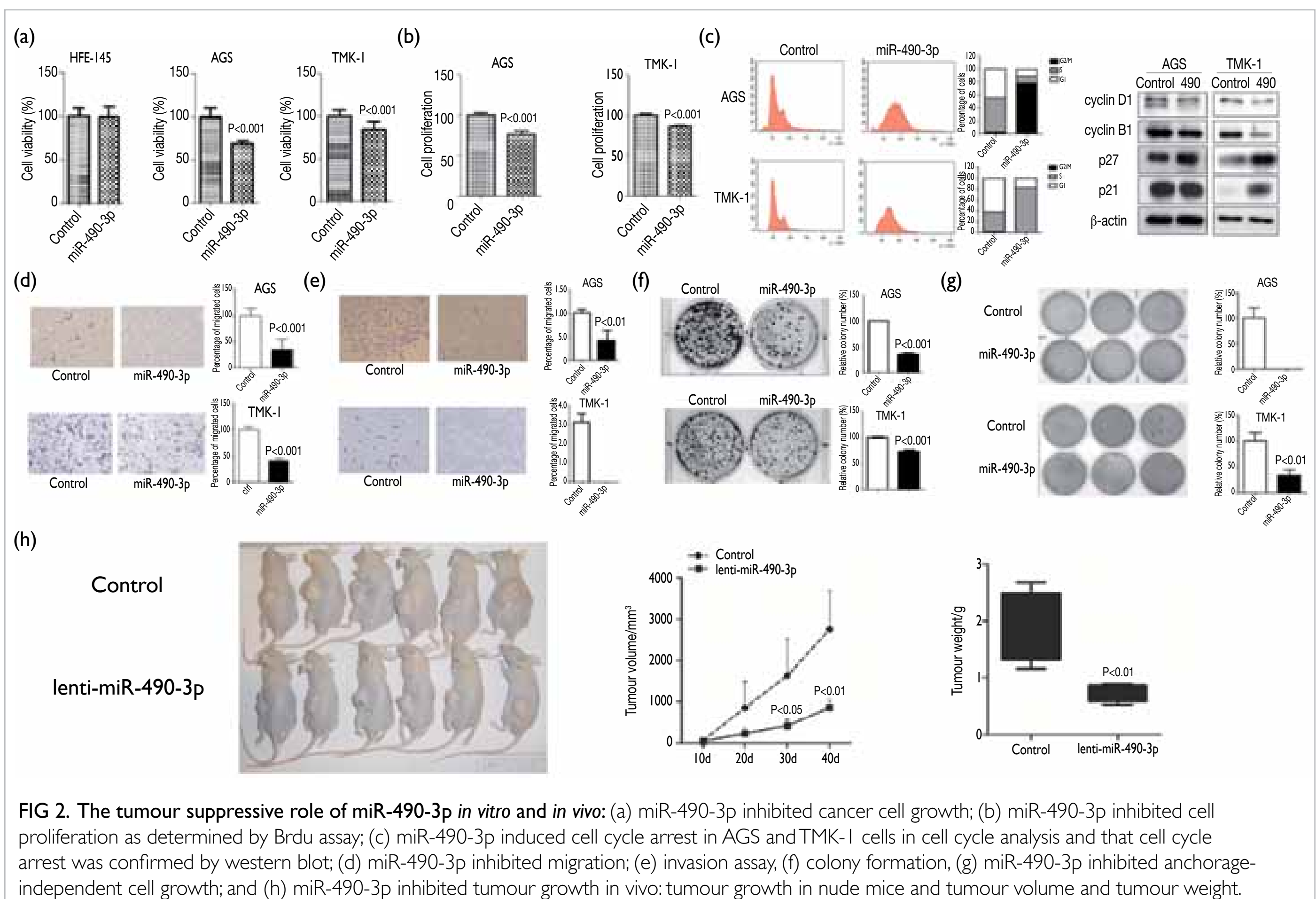

FIG 2. The tumour suppressive role of miR-490-3p *in vitro* and *in vivo*: (a) miR-490-3p inhibited cancer cell growth; (b) miR-490-3p inhibited cell proliferation as determined by Brdu assay; (c) miR-490-3p induced cell cycle arrest in AGS and TMK-1 cells in cell cycle analysis and that cell cycle arrest was confirmed by western blot; (d) miR-490-3p inhibited migration; (e) invasion assay, (f) colony formation, (g) miR-490-3p inhibited anchorage-independent cell growth; and (h) miR-490-3p inhibited tumour growth in vivo: tumour growth in nude mice and tumour volume and tumour weight.

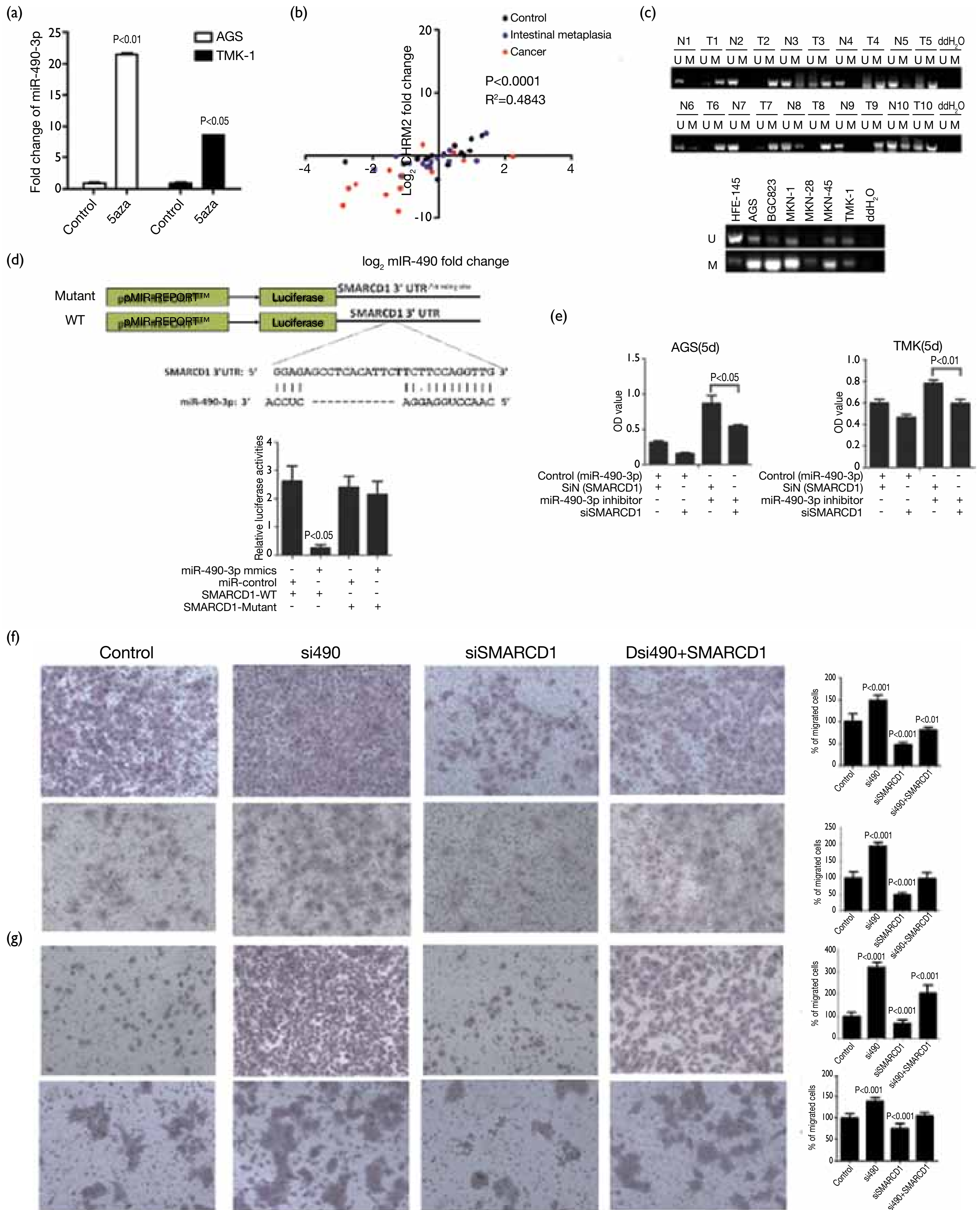

FIG 3. Upstream regulation and downstream target of miR-490-3p: (a) 5aza treatment restored miR-490-3p expression in cell lines, (b) negative correlation of miR-490-3p and CHRM2 in human tissue samples, (c) methylation-specific-PCR in tissue and cell lines, (d) dual luciferase reporter assay, and rescue experiments of (e) MTT assay, (f) cell migration, (g) cell invasion.

used a combined method of in silico prediction, whole genome mRNA array and luciferase assay. SMARCD1 was predicted to be a direct target of miR-490-3p by miRwalk and DIANA. Whole genome mRNA array using cell lines after overexpressing miR-490-3p showed a consistent down-regulation of SMARCD1 in both AGS and TMK-1 (fold change -2.056, P<0.01, data not shown). The array result was successfully validated by real-time PCR. Moreover, miR-490-3p reduced the SMARCD1 3'-UTR luciferase activity (Fig 3d) suggesting that miR-490-3p can target the SMARCD1 gene directly. Rescue experiments showed that the tumourigenic effect of miR-490-3p inhibitors was significantly attenuated by co-transfection of siSMARCD1 (Figs 3e-g). Taken together, these results demonstrat that SMARCD1 is a direct target of miR-490-3p.

Discussion

Despite advances in techniques to inhibit protein-coding genes using small molecules or biological agents, many cancers are unresponsive and resistant to the drugs currently used. Their potential intolerable side effects are a major concern for patients. New and more creative approaches are needed for more effective treatment of cancer. With the discovery of miRNA in 1993, miRNA became one of the important agents in cancer research and enabled development of new cancer therapeutics. In this study using a mouse model of gastric carcinogenesis, miR-490-3p was dysregulated in the development of gastric cancer; it was down-regulated in gastric, colon, bladder, and prostate cancer.

As miRNAs coordinate in cancer pathogenesis, and the phenotypical changes result from multiple interactions between miRNAs and the transcriptome, it is vital to clarify their regulating mechanisms, biological functions, and downstream targets of miR-490-3p before evaluating their usefulness in the treatment of cancer. miRNAs have clinical advantages in their use as biomarkers for diagnosis, prognosis, and treatment outcome for cancer. miR-490-3p is not only a tumour suppressor but also showed progressive down-regulation in the process of gastric carcinogenesis. It may thus be useful for early diagnosis before development of adenocarcinoma. Clinical data are still lacking to determine whether miR-490-3p is related to prognosis and treatment outcome. The experimental findings in the present study will lead to a better understanding of the cancer network regulated by miRNAs and provide new insights and opportunities in the area of miRNA research.

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