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Reciprocal interplay of miR-497 and *MALAT1* promotes tumourigenesis of adrenocortical cancer

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Abstract

Adrenocortical carcinoma (ACC) has high recurrence rates and poor prognosis with limited response to conventional cancer therapy. Recent contributions of high-throughput transcriptomic profiling identified microRNA-497 (miR-497) as significantly underexpressed, while lncRNA *MALAT1* (metastasis-associated lung adenocarcinoma transcript 1) as overexpressed in ACC. miR-497 is located in the chromosomal region 17p13.1, in which there is a high frequency of loss of heterozygosity in ACC. We aim to investigate the interaction of miR-497 and *MALAT1* in ACC and its functional roles in the process of tumourigenesis. In this study, we demonstrated miR-497 post-transcriptionally repressed *MALAT1* while *MALAT1* also competes for miR-497 binding to its molecular target, *EIF4E* (eukaryotic translation initiation factor 4E). We showed that overexpression of miR-497 and silencing of *MALAT1* suppressed cellular proliferation and induced cell cycle arrest through downregulation of *EIF4E* expression. Furthermore, *MALAT1* directly binds to SFPQ (splicing factor proline and glutamine rich) protein, indicating its multifaceted roles in ACC pathophysiology. This is the first study to identify the feedback axis of miR-497-*MALAT1*/*EIF4E* in ACC tumourigenesis, providing novel insights into the molecular functions of noncoding RNAs in ACC.

Key Words

- ▶ miR-497
- ▶ *MALAT1*
- ▶ microRNA
- ▶ long noncoding RNA
- ▶ adrenocortical carcinoma

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Introduction

Adrenocortical carcinoma (ACC) is an ultra-rare and aggressive cancer with an incidence of 0.7–2.0 cases per million populations (Kerkhofs *et al.* 2013). Its 5-year survival rate is lower than 35% (Lughezzani *et al.* 2010). Surgical removal of the tumour is the first-line treatment and the best chance for long-term control of malignancy (Erdogan *et al.* 2013). However, approximately 50% of patients have recurrence 6–24 months after surgical resection (Libe 2015). For patients with advanced or metastatic disease,

mitotane remains the only approved adrenolytic drug that is given in parallel with cytotoxic chemotherapy, but due to its limited response rates and long-term side effects, mitotane is not an effective treatment for most patients with ACC (Scheingart *et al.* 2012).

The traditional view of ncRNAs as merely transcriptional noise is fading and revolutionizing the basic concept of RNA carrying message for protein translation into a broader paradigm of gene regulation

implicated in almost all biological pathways. The ncRNAs form ribonucleoprotein complexes that recognize nucleic acid target sequences through complementarity and consists of ncRNA of different sizes and functions (Kawaji & Hayashizaki 2008). miRNAs are small ncRNAs approximately ~21 nucleotides in length that inhibit canonical target mRNAs translation or degrade mRNA transcript by binding to the 3' untranslated regions (3' UTRs) of mRNA targets.

More than 50% of miRNA genes are located in cancer-associated genomic regions or in fragile sites, suggesting that miRNAs may play an important role in the pathogenesis of cancer (Calin *et al.* 2004). miRNA-497 (miR-497), part of miR-15 family cluster and encoded by the first intron of MIR497HG (Gene ID: 100506755), is located at the chromosomal region 17p13.1, in which there is a high frequency of loss of heterozygosity (LOH) in ACC compared to that of adrenocortical adenomas (Libe *et al.* 2007, Soon *et al.* 2008). Additionally, miR-497 was identified significantly underexpressed in three independent ACC studies (Ozata *et al.* 2011, Chabre *et al.* 2013, Assié *et al.* 2014).

Recent studies have also revealed miRNAs may interact with lncRNA to contribute to tumourigenesis (Wang *et al.* 2015, 2016, 2018). lncRNAs are eukaryotic RNA molecules greater than 200 nucleotides in length that have no or limited protein-coding capacity, however, may play a role in transcriptional interference, induce chromatin remodelling and histone modifications, modify alternative exon splicing as well as generate siRNAs (Wilusz *et al.* 2009). lncRNAs are tightly regulated by transcription factors and epigenetic chromatin modifications such as histone methylation, while their expression can be tissue and cell-type specific (Margueron & Reinberg 2010, Mele & Rinn 2016). lncRNAs can act as miRNA sponges by absorbing target miRNAs through their own miRNA reaction elements (MREs) and suppress the targeting mRNAs degradation mediated by miRNAs (Faghihi *et al.* 2010). Studies have also demonstrated that mature miRNAs regulate lncRNAs (Braconi *et al.* 2011, Leucci *et al.* 2013, Liang *et al.* 2013a). Conversely to lncRNAs miRNA sponging mechanism, they may also potentially serve as functional miRNA targets. Noncoding RNAs act in accordance with the current understanding of cancer as an interconnected network that presumably can only be successfully treated when intervening between multiple oncogenic and tumour-suppressive pathways. Thus investigating and understanding this ncRNA crosstalk will give an insight into gene regulatory networks and their implications in ACC.

lncRNA, metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*), was first discovered to be overexpressed in non-small-cell lung tumours (Ji *et al.* 2003). Mounting evidence also shows *MALAT1* is overexpressed in a variety of different cancers such as hepatocellular carcinoma, breast, lung and gallbladder cancers (Lai *et al.* 2012, Ji *et al.* 2014, Feng *et al.* 2016, Sun *et al.* 2016, Zou *et al.* 2016). Moreover, patients with high levels of *MALAT1* are at high risk of metastasis in hepatocellular carcinoma defining it as a prognostic marker (Lai *et al.* 2012). In our previous study of lncRNA microarray analysis in ACC, *MALAT1* was found to be overexpressed in human ACC tissues and the ACC H295R cell line (Glover *et al.* 2015). As bioinformatics analysis of the *MALAT1* sequence shows multiple putative binding sites for miR-497 (DIANA lncBase Predicted v.2, B.S.R.C Alexander Fleming, Athens, Greece), we aimed to investigate the interaction between miR-497 and *MALAT1* as part of a mechanism underlying ACC tumourigenecity. We found *MALAT1* as a bona fide target of miR-497 and exhibited the multifaceted roles of miR-497 and *MALAT1* in ACC malignancy. Our study advances the role of lncRNAs and miRNAs in ACC biology and presents a further potential application of noncoding RNAs in ACC-targeted therapy.

Materials and methods

Clinical tumour samples

Both ACC and normal adrenal cortex (NAC) tissue samples used in this study was obtained from the Neuroendocrine Tumour Bank of the Kolling Institute of Medical Research with ethics approval from the Northern Sydney Area Health Service Human Research Ethics Committee. All patients consented to their samples being used in this study. NAC was harvested from patients by the surgeon whom had performed an adrenalectomy for a benign functional or non-functional adenoma. The normal adrenal was harvested away from the adenoma and adrenal medulla dissected off the cortex. The cortex was snap-frozen in liquid nitrogen and stored at -80°C and histology confirmed by the pathologist prior to use as control tissue. ACC specimens were harvested by the surgeon at the time of surgery from solid areas of the tumour avoiding gross areas of necrosis. Before analysis a section of the frozen sample was analysed by a specialized endocrine pathologist using hematoxylin and eosin staining to exclude any samples with necrosis.

Cell culture and transfections

Human ACC H295R cell line (CRL-2128) was purchased from American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 5% (v/v) fetal bovine serum (FBS) and 1% insulin transferrin selenium (ITS) supplement (Corning, Manassas, VA, USA).

To be able to manipulate the expression of miRNA-497, miRNA mimics (mirVana miRNA mimics, Thermo Fisher Scientific, cat no. 4464066), double-stranded (ds) mature miRNA-497, that function similarly to endogenous miRNA-497, were transfected into H295R cells at a final concentration of 40 nM using Lipofectamine® RNAiMAX (Thermo Fisher Scientific) transfection reagent according to manufacturer's protocol. miRNA scramble sequences were used as negative controls (miR-NC). For miRNA transfections, cells were first transfected with 30–50% confluency, then transfected again 3 days after the first transfection. Cells were collected 3 days after the second completed transfection for the downstream phenotypic studies, including cell apoptosis, cell cycle analysis, cell invasion and migration assays.

MALAT1 silencing was performed through LNA™ GapmeR (Exiqon, Vedbaek, Denmark), consisting of 14–16 DNA oligonucleotides that has enhanced affinity for the target *MALAT1*. LNA™ GapmeR were also transfected with a similar procedure as the miRNA mimics in H295R cells.

Cell proliferation assay

Cell proliferation was assessed by measuring the absorbance at 490 nm using CellTiter 96 Aqueous One Solution Cell Proliferation Assay according to the manufacturer's instruction (MTS Assay; Promega). ACC H295R cells, at a density of 5.0×10^3 cells per well in a flat-bottomed 96-well plate, were first reverse transfected with miRNA mimics, and then transfected again three days after the first transfection. The day with the completed second transfection was labelled as day 0 and cell proliferation was assessed on the following days 3, 4, 7 and 8.

Cell cycle and apoptosis analysis

For cell cycle analysis, 1.0×10^6 H295R cells were stained with propidium iodide (PI; Sigma Aldrich) at a final concentration of 17.4 µg/mL and the cells were analysed using fluorescence-activated cell sorting (FACS) technique (FACS Calibre, BD Biosciences), flow cytometry histograms were modelled using Modfit LT software (Verity Software

House, ME, USA). For apoptosis analysis, PE Annexin V apoptosis detection kit was used (BD Pharmingen, San Diego, USA) according to manufacturer's instructions on day 4 post transfection. Early-stage apoptosis was determined by Annexin V positive and PI-negative cells on apoptosis graph of FACS assay. Late-stage apoptosis was measured by Annexin V-positive and PI-positive cells.

Invasion and migration

The Cytoselect™ 24-well cell invasion and migration (Cell Biolabs, Inc., San Diego, CA, USA) were used for the cell invasion assay in accordance with the manufacturer's protocol. Briefly, 2.5×10^4 cells in serum-free DMEM were added to the upper chamber of the Matrigel-coated insert, and 500 µL of DMEM containing 5% FBS+1% ITS was added to the lower chamber for culture. The cells were cultured in a humidified atmosphere for 48 h at 37°C and 5% CO₂, and the non-invading cells were removed. The cells were stained using cell stain solution (Cell Biolabs, Inc.) for 10 min at room temperature. The invading cells were extracted using Extraction Solution (Cell Biolabs, Inc.) and measured at OD 560 nm in a plate reader (Synergy™, Winooski, VT, USA). Migration assays were applied similarly and migrating cells were stained, extracted and measured at 24 h.

RNA extraction and RT-qPCR

Total RNA was isolated from tumour tissues, H295R cells using a Qiazol protocol (miRNeasy Mini Kit; Qiagen) and with the RNA quality and concentration tested by the NanoDrop® (ND-1000 spectrophotometer). miR-497 expression profile was validated in a local series of ACC and NAC tissues. The expression level of miRNA-497 day 3 post transfection was measured via quantitative reverse transcription-PCR (RT-qPCR) by using TaqMan® MicroRNA cells-to-C_T™ kit (Applied Biosystems) and commercially available TaqMan® stem-loop RT primers (Applied Biosystems). 10 ng of total RNA were first reverse-transcribed to cDNA using TaqMan miRNA primers. The reverse transcriptase reaction conditions were 30 min at 16°C, 30 min at 42°C, the reactions were then terminated for 5 min at 85°C and chilled at 4°C (Applied Biosystems). The quantitative real-time PCR was performed using the ABI 7900HT Real-time PCR System (Applied Biosystems) under the standard PCR conditions: 2 min at 50°C, then 40 cycles of 15 s at 95°C and 60 s at 60°C. *RNU48* was used as a reference gene for human clinical samples for miRNA profiling.

The same method was also used for gene expression of mRNA targets. Approximately 1 µg of total RNA was used to synthesize cDNA using RNA-to-cDNA Reverse Transcription (Applied Biosystems) and subjected to quantitative real-time PCR reaction conditions: 5 min at 95°C, and 40 cycles of 10 s at 95°C and 20 s at 60°C. PCR was amplified using standard TaqMan gene expression assays (Life Technologies). Target gene expression was normalized to the expression of *GAPDH* within each sample using the relative $\Delta\Delta CT$ method (Livak & Schmittgen 2001). The quantitative real-time PCR was performed using the ABI 7900HT Real-time PCR System (Applied Biosystems) under the standard PCR conditions. Relative expression (RQ) was obtained using the $\Delta\Delta Ct$ method and the differences between groups were assessed using DataAssist version 3.01 (Applied Biosystems).

Luciferase reporter assays

To verify that *EIF4E* is directly regulated by miR-497, a luciferase reporter assay was performed. miR-497 putative-binding sites in the 3'-untranslated region (3'-UTR) regions of *EIF4E* were amplified from human genomic DNA by PCR and cloned into the pMIR-REPORT Luciferase Vector (Part Number AM5795, Life Technologies) between SpeI and SacI restriction sites.

For the *EIF4E*-3' UTR reporter, primer sequences as below were used to amplify a fragment of 830 bp containing two predicted miR-497 seed-binding sequences.

Forward primer: 5'-GACTACTAGTAAATGTGTTCCCTGGCCCACTC-3'

Backward primer: 5'-GACTGAGCTCTGTTCACATGGAAGACACCAC-3'

For the *MALAT1* reporter, primer sequences as below were used to amplify a fragment of 849 bp also containing two seed-binding sequences for miR-497.

Forward primer: 5'-(GCAGCCCGAGACTTCTGTAAAGG)-3'

Backward primer: 5'-(GTGGCAAAATGGCGGACTTTC)-3'

For the reporter assay, the H295R cells were co-transfected along with 50 ng of pMIR Luciferase Reporter Vector, Renilla Luciferase Control Vector (Promega), and 40 nM of miR-497 mimics or double-stranded NC using Lipofectamine 2000 reagent (Thermo Fisher) using manufacturer's protocol. Luciferase activity was measured 2 days after transfection using the Dual-Luciferase Reporter Assay System (Promega) using a luminometer (Veritas Microplate Luminometer, Turner Biosystems, CA, USA). Relative firefly luciferase activity was normalized to the activity of Renilla luciferase activity.

RNA-binding protein immunoprecipitation

RNA immunoprecipitation can be used to identify any RNA species through co-isolation of associated nuclear or cytoplasmic endogenously formed complexes of RNA-binding proteins. Purification of these RNA species also allows interrogation and identification of potential lncRNAs associated with them and can be directly measured downstream using RT-qPCR. RNA-binding protein immunoprecipitation (RIP) was made according to the manufacturer's protocol from the EZ-Magna RIP Kit (EMD Millipore) day 3 post transfection. Approximately, 2×10^7 H295R cells were lysed in RIPA lysis buffer at -80°C overnight. The samples were then centrifuged at 14,000g for 10 min to pellet insoluble protein and the supernatant was collected. Magnetic beads binding antibody of interest for immunoprecipitation were prepared. Mouse SFPQ antibody (sc-101137, Santa Cruz Biotechnology) and nonspecific mouse IgG antibody were used. The RIP lysates and magnetic beads-binding antibody of interest were incubated together rotating overnight at 4°C leading to the immunoprecipitation of RNA-binding protein-RNA complex. Each immunoprecipitate was digested with proteinase K and 10% SDS at 55°C for 30 min after washing with RIP washing buffer. After incubation, centrifuge was used to obtain the supernatant, followed by washing and adding phenol:chloroform:isoamyl alcohol (125:24:1) to separate the phases. Chloroform was added to separate the aqueous phase from the chloroform, and 80% ethanol was used to precipitate the RNA. Each sample was reverse-transcribed using the High Capacity RNA-to-cDNA Kit (Applied Biosystems), followed by qPCR and analysis. All assays were performed in triplicate and independently repeated three times.

Statistics

All numerical results were expressed as the mean $\pm$ s.d. Statistical significance between groups was determined using a paired Student's *t*-test, which was performed using Graph Pad Prism (version 7.0b).

Results

miR-497 is underexpressed in ACC

To confirm the underexpression of miR-497 in ACC, RT-qPCR was performed on an independent cohort of 19 ACC and 10 NAC tissue samples. This analysis showed that miR-497 was significantly underexpressed in ACC

Figure 1

miR-497 exerts tumour suppressive roles in ACC. (A) miR-497 is underexpressed in ACC tumour samples compared to NAC. There is also a reduction of miR-497 expression in ACC H295R cell line ($n = 3$). Data are presented as Tukey box plot; median expression is represented by the solid line within the box and the outliers are represented by the dots outside the boxes. (B) miR-497 replacement had growth inhibition in H295R cells. (C) Extra 5% of cellular events underwent early apoptosis post transfection of miR-497 when compared to miR-NC. (D) miR-497 increased cell percentage in the G1 phase by 6% cells. miR-497 decreased cell percentage in the S phase by 15%. (E) Significant decrease in cell invasion by 44% following miR-497 replacement. (F) miR-497 reduced migration by 31% compared to miR-NC. Error bars show s.d., $n = 3$. * $P < 0.05$, **** $P < 0.0001$.

with a 23-fold reduction compared to NAC (Fig. 1A). miR-497 was also underexpressed in ACC H295R cells compared to NAC tissues (Fig. 1A).

miR-497 promotes tumour-suppressive functions in ACC

The functional roles of endogenous miR-497 in the pathogenesis of ACC were investigated upon the replacement of miR-497. The restoration of miR-497 expression in H295R cells following transfection of miR-497 mimics was confirmed using RT-qPCR (Supplementary Fig. 1, see section on [supplementary data](#) given at the end of this article). Restoration of miR-497 resulted in significant inhibition of cell proliferation in H295R cells when compared to miR-NC ($P < 0.05$) (Fig. 1B). The greatest reduction in the inhibition of cell growth was identified on days 7 and 8 with a decrease in cellular proliferation by 73 and 83%, respectively.

The effect of miR-497 on cell apoptosis and cell cycle was further investigated. The Annexin-7AAD staining method demonstrated that the early-stage apoptosis was increased by 5% following transfection with the miR-497 mimics ($P < 0.05$) (Fig. 1C). There was no difference in late-stage apoptosis when miR-497 treated was compared to miR-NC (data not shown). Cell cycle analysis demonstrated cells transfected with miR-497 had significantly increased cell percentage in G1 phase and reduced in S phase when compared to miR-NC (Fig. 1D).

Subsequently, we analysed the effects of miR-497 on the migration and invasion ability of ACC cells. Using transwell assays, we observed that both the invasive (Fig. 1E) and migratory (Fig. 1F) activities of ACC cells were suppressed with miR-497 by 44 and 31%, respectively ($P < 0.05$).

miR-497 targets lncRNA MALAT1 expression through direct binding

To further confirm the direct interaction of miR-497 and MALAT1, we cloned fragments of MALAT1 encompassing two putative-binding sites into the multiple cloning sites of the pMIR-REPORT miRNA Expression Reporter Vector (Fig. 2A). The H295R cells were co-transfected with miR-497 mimics along with the pMIR-REPORT reporter vector, and miR-497 overexpression resulted in a significant decrease in luciferase activity when compared to miR-NC (Fig. 2B).

Furthermore, miR-497 overexpression reduced transcripts of MALAT1 in H295R cells (Fig. 2C). We were also interested to observe if MALAT1 acted as a miR-497 sponge, by knocking down MALAT1 and subsequently performing RT-qPCR to measure the expression of miR-497. There was an increase in miR-497 expression after silencing MALAT1; however, it did not reach statistical significance in miR-497 expression after MALAT1 knockdown compared with miR-NC (Fig. 2D).

Figure 4

Functional effects of miR-497 and *MALAT1* on cancer-related genes. (A) *SFPQ* is underexpressed in ACC tissues compared to NAC. There is also a reduction of *SFPQ* expression in ACC H295R cells ($n = 3$). The data are presented as Tukey box plot; median expression is represented by the solid line within the box. (B) The expression of *SFPQ* mRNA transcript was increased by ~1.8-folds in H295R cells following *MALAT1* knockdown. (C) RNA immunoprecipitation with *SFPQ* antibody followed by RT-qPCR to examine *MALAT1* expression. Data were normalized to mouse IgG as negative control (*MALAT1* expression equals to 1). (D) miR-497 overexpression reduced the levels of *EIF4E* by ~78%. (E) Co-transfection of the luciferase reporter vector containing 3' UTR of *EIF4E*, respectively along with miR-497 mimics suppressed luciferase activity. (F) *MALAT1* knockdown reduced levels of *EIF4E* mRNA expression by ~63%. Bioinformatics tools including Miranda, TargetScan and Diana Tools were utilized to determine putative miRNA targets. Total RNA was collected from H295R cells following day 3 post transfection. mRNA expression of the targets was measured by RT-qPCR. Error bars show s.d., $n = 3$. * $P < 0.05$, *** $P < 0.001$.

***MALAT1* competes for miR-497 binding to its molecular targets**

To further investigate whether molecular targets of miR-497 may interact with *MALAT1* in ACC, we first sought to identify mRNA targets of miR-497. We cross referenced putative targets of miR-497 using several prediction algorithm tools; DIANA-microT-CDS version 5.0 (B.S.R.C Alexander Fleming, Athens, Greece), DIANA-miRPath version 6.0 (B.S.R.C. Alexander Fleming), miRanda (Memorial Sloan-Kettering Cancer Centre, NY, USA) and TargetScan version 7.1 (Whitehead Institute for Biomedical Research, MA, USA). While *SFPQ* is not a putative target of miR-497, we identified a number of potential miR-497 targets for further validation. Selected putative targets derived from major cancer-related signalling pathways are listed in Supplementary Table 1.

Restoration of miR-497 significantly reduced mRNA transcripts of eukaryotic initiation factor 4E (*EIF4E*) by 4.5-fold in H295R cells (Fig. 4D). Furthermore, knockdown of *MALAT1* also significantly reduced mRNA transcripts of *EIF4E* by 2.7-fold change when compared to NC ($P < 0.05$) (Fig. 4F).

The 3'-UTR sequence of *EIF4E* containing the potential binding sequences for miR-497, were further cloned into the multiple cloning sites of the pMIR-REPORT miRNA

Expression Reporter Vector (Supplementary Fig. 2). The H295R cells were co-transfected with miR-497 mimics or miR-NC along with the pMIR-REPORT reporter vector and the luciferase activity was measured. The experimental results showed that the miR-497 mimics downregulated the luciferase activity of the luciferase reporter (Fig. 4E) when compared to miR-NC. This confirms the direct binding of miR-497 with *EIF4E*. These results further support the potential role of *MALAT1* acting as miR-497 sponge whereby knockdown of *MALAT1*, relieving the competition for miR-497 binding to *EIF4E*, led to the reduction of *EIF4E*.

Discussion

We report for the first time the reciprocal interplay of miRNA and lncRNA in ACC and its critical roles during the tumourigenic process. This study demonstrates miR-497 functions as a tumour suppressor through post-transcriptional repression of lncRNA *MALAT1* while *MALAT1* also competes for miR-497 binding to its molecular targets. We have shown that overexpression of miR-497 and silencing of *MALAT1* indeed suppressed cell proliferation by inducing cell cycle arrest by regulating

EIF4E expression. Furthermore, we have identified the feedback axis of miR-497-*MALAT1*/*EIF4E* promotes the tumourigenesis in ACC.

miR-497 is a highly conserved miRNA, located on the human chromosome 17p13.1, the genomic locus for p53. miR-497 belongs to the miR-15 family which includes miR-15a, miR-15b, miR-16-1/2, miR-195 and miR-424 and shares the same 3' UTR-binding seed sequence (AGCAGCA). High frequencies of LOH in the region of chromosome 17p13.1 and its association with malignancy have been described in ACC (Libe *et al.* 2007, Soon *et al.* 2008). However, there was no significant driver gene that has been identified in the region for ACC tumourigenesis (Libe *et al.* 2007). It was previously shown that LOH in this region implicated *TP53*; however, both the latest comprehensive whole genome studies of ACC revealed only 16 and 17.8%, respectively of ACC were mutated in the *TP53* gene (Assié 2014, Zheng *et al.* 2016).

We have previously demonstrated in a significant body of work that there is a minimum 10.4Mb LOH in ACC compared to adrenal adenoma (Soon *et al.* 2008). In that study the region of 17p13.1-13.3 were fine-mapped utilising 12 microsatellite markers in 23 ACCs and 14 adenomas demonstrating LOH in 74% of cancers and only 14% of adenomas. This region of LOH contains the *TP53* gene and miR-497 and we surmise that 497 is a key tumour suppressor gene lost in this region in three quarters of adrenal cancers. This finding by Soon *et al.* is consistent with the current study demonstrating a significant reduction in the expression of miR-497 in the current cohort of ACCs compared to normal adrenal and is analogous to the initial discovery of miR-15a/16 as tumour suppressor genes as they were underexpressed in chronic lymphatic leukaemia (CLL) and located at 13q14 – a region that was frequently deleted in CLL (Calin *et al.* 2002).

miR-497 has been identified to act as a potential tumour suppressor in various cancers (Li *et al.* 2011, Shen *et al.* 2012, Furuta *et al.* 2013, Luo *et al.* 2013, Wang *et al.* 2013, Ma *et al.* 2017). Further studies to investigate the high frequencies of LOH and its association with the underexpression of miR-497 in ACC may help to characterize the tumour-suppressive roles of miR-497 as a driver in ACC.

The interaction network of miRNA-mRNA-lncRNA has been revealed to play critical regulatory roles in cancer biology (Xiao *et al.* 2015, Lu *et al.* 2016, Wang *et al.* 2017). *MALAT1* has been reported that it modulates splicing factors and regulates alternative splicing of a subset of pre-mRNAs, however, upstream control of

MALAT1 has not been elucidated (Tripathi *et al.* 2010). Accumulating studies have shown some lncRNAs are under the regulation of miRNAs to reduce their stability. For example, oncogenic lncRNA *HOTTIP* is frequently upregulated in hepatocellular carcinoma and post-transcriptionally targeted by tumour suppressive miR-125b (Tsang *et al.* 2015). *MALAT1* has also been found to be silenced by miR-101 and miR-217 in oesophageal squamous cell carcinoma, which leads to significant reduction of cell proliferation by the G2/M cell cycle arrest (Wang *et al.* 2015). More studies have shown that lncRNAs interact with miRNA by competing for miRNAs binding to their downstream mRNA targets (Cesana *et al.* 2011, Tay *et al.* 2011, Feng *et al.* 2016, Wu *et al.* 2017). An *in vitro* study in gallbladder cancer, demonstrated *MALAT1* could directly bind to miR-363-3p and effectively function as a miRNA sponge to modulate the expression of its endogenous target myeloid cell leukaemia-1 (*MCL-1*) (Wang *et al.* 2016). In hepatocellular carcinoma, *MALAT1* also functions as a competing endogenous RNA for miR-30a-5P to regulate vimentin and promotes migration and invasion (Pan *et al.* 2018).

In our published study (Glover *et al.* 2015), *MALAT1* was previously assessed to be overexpressed in ACC in a discovery cohort of ten ACCs compared to six NACs and consequently validated in an expanded cohort of 20 ACCs and six NACs. In this study, we confirmed the underexpression of miR-497 and identified not only miR-497 negatively regulates the expression of *MALAT1*, and *MALAT1* also reversely competes for miR-497 binding to *EIF4E*, alleviating the negative effect of miR-497 on *EIF4E*.

The expression and activation of *EIF4E* has been reported to be associated with transformation and tumourigenesis while acting as a proto-oncogene in cancers (Wendel *et al.* 2004, Graff *et al.* 2008). In gastric and breast cancer, downregulation of *EIF4E* resulted in the reduction in proliferation and cell cycle arrest *in vitro* (Liang *et al.* 2013b, Nasr *et al.* 2013). We also found that restoration of miR-497 significantly slowed proliferation and arrested cell cycle partially by regulating the expression of *EIF4E* in H295R cells. In our next phase of study, it would be interesting to explore the direct effect of *EIF4E* regulation on the development of ACC by examining the loss-of-function studies in H295R cells using gene editing to silence the *EIF4E* gene transcript. Overall, our findings in this study suggest that the feedback axis of miR-497-*MALAT1*/*EIF4E*, as demonstrated in Fig. 5, may act as a key mediator in the tumourigenesis of ACC, providing novel insights into the molecular functions of noncoding RNAs in ACC.

Figure 5

Schematic of miR-497-MALAT1/EIF4E interplay feedback axis. LOH at 17p13.1 as shown in Libe *et al.* (2007) leads to the underexpression of miR-497. This affects the feedback loop between miR-497 and MALAT1 and their downstream targets, EIF4E and SFPQ, which regulates cellular proliferation and cell cycle arrest. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-19-0036>.

This study is the first to show the reciprocal relationship between miR-497 and *MALAT1*. Such effect has not been previously studied in a non-adrenal cell line. However, bioinformatics platform, Diana Tools LncBase Predicted v.2 (B.S.R.C Alexander Fleming), identifies that *MALAT1* sequences contain putative binding sites for miR-497, demonstrating its potential interaction in other cell types. In a study of hepatocellular carcinoma (HCC) (Yan *et al.* 2018), expression of miR-497 and *MALAT1* was identified to be inversely correlated; yet, this study did not show the direct interaction between miR-497 and *MALAT1*.

Alternative putative targets of miR-497 have been studied in other cancers. For example, the WEE1 G2 checkpoint kinase (WEE1) gene has been experimentally validated in the study of neuroblastoma (Creevey *et al.* 2013). However, we did not detect any change in the mRNA expression of WEE1 following restoration of miR-497 in the ACC cells. Some other molecular targets, such as Inhibitor of nuclear factor kappa-B kinase subunit beta (*IKK β*) gene and Wnt family member 7A (*WNT7A*) gene have also been validated in prostate cancer and bladder cancer, respectively (Iteasaki *et al.* 2014, Kong *et al.* 2015). These molecular targets are critical to tumour progression and their examination in H295R cells may further reinforce the tumour-suppressive roles of miR-497 in ACC. For a more complete analysis, whole transcriptome profiling using RNAseq will likely identify further molecular targets of miR-497 to enhance the understanding of the roles of miR-497 in ACC development.

lncRNA *MALAT1* has been reported to show extensive regulatory functions in cell proliferation, migration and invasion in various cancers (Hirata *et al.* 2015, Feng *et al.* 2016, Gu *et al.* 2017). Although the oncogenic roles of overexpression of *MALAT1* is not yet fully understood, it has been identified that *MALAT1* is associated with SFPQ, promoting the process of tumourigenesis. SFPQ

is a DNA- and RNA-binding protein, involved in transcriptional regulation. In colorectal cancer (CRC), *MALAT1* competitively binds to SFPQ and releasing PTBP2 (polypyrimidine-tract-binding protein) from the SFPQ/PTBP2 complex to promote cell growth and migration (Ji *et al.* 2014). In a similar pattern in lung adenocarcinoma, hypoxia exposure upregulated the expression of *MALAT1*, which binds to SFPQ and releases it from the GAGE6 promoter, leading to the promotion of cell proliferation, migration and invasion (Li *et al.* 2009). Our data demonstrated *MALAT1* is overexpressed in ACC and can directly bind to SFPQ, although the detail of the binding location still needs further investigation in ACC cells.

In addition to SFPQ, some other molecular targets of *MALAT1* have been studied in ovarian and cervical cancers. For example, apoptotic markers such as Bcl-2-associated X protein (BAX) and B-cell lymphoma (BCL2) were downregulated by *MALAT1* in ovarian cancer (Bai *et al.* 2018). *MALAT1* particularly promoted proliferation and invasion by stimulating the expression of caspase-3 and caspase-8 and inhibiting expression of BCL2 in cervical cancer (Guo *et al.* 2010). This warrants further investigation of these additional molecular targets in H295R cells to assess the other roles of *MALAT1* in ACC.

In summary, this study demonstrates miR-497-mediated regulation of lncRNA *MALAT1*, and inversely *MALAT1* may potentially act as a miR-497 sponge-releasing downstream oncogenic targets. The feedback loop between miR-497 and *MALAT1* could functionally reduce cell proliferative effects and induce cell cycle arrest by mediating *EIF4E* expression. Our data provide new insights into the molecular mechanisms of ACC tumourigenicity in the context of noncoding RNAs.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-19-0036>.

Declaration of interest

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

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