Significance of Dysregulated Metadherin and MicroRNA-375 in Head and Neck Cancer

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STATEMENT OF TRANSLATIONAL RELEVANCE

This report provides important insight into head and neck cancer (HNC) biology through the use of primary, formalin-fixed paraffin-embedded tumour samples. Through global microRNA (miRNA) profiling, we have identified that under-expression of miR-375 and the over-expression of metadherin as a significantly dysregulated axis in HNC. Importantly, there was a significant correlation between metadherin over-expression with the development of metastases in nasopharyngeal carcinoma (NPC) patients. Given the lack of useful prognostic biomarkers in NPC, metadherin expression may thus hold significant clinical value as a prognostic variable. Furthermore, given that distant disease is a major cause of death for HNC patients, metadherin might also serve as a novel target for therapeutic purposes, which in turn could improve outcome for future HNC patients.
ABSTRACT

Purpose

Despite recent improvements in local control of head and neck cancers (HNCs), distant metastasis remains a major cause of death. Hence, further understanding of HNC biology, and in particular, the genes/pathways driving metastasis is essential in order to improve outcome.

Experimental Design

Quantitative RT-PCR was used to measure the expression of miR-375 and metadherin (MTDH) in HNC patient samples. Targets of miR-375 were confirmed using qRT-PCR, western blot analysis and luciferase assays. Phenotypic effects of miR-375 re-expression and MTDH knock-down were assessed using viability (MTS), clonogenic survival, cell migration/invasion, as well as in vivo tumour formation assays. The prognostic significance of miR-375 or MTDH in nasopharyngeal carcinoma (NPC) was determined by comparing low vs. high expression groups.

Results

MiR-375 expression was significantly reduced (p=0.01), and conversely, MTDH was significantly increased (p=0.0001) in NPC samples. Quantitative RT-PCR, western blots and luciferase assays corroborated MTDH as a target of miR-375. Re-expression of miR-375 and siRNA knock-down of MTDH both decreased cell viability and clonogenic survival, cell migration/invasion, as well as in vivo tumour formation. NPC patients whose tumours expressed high levels of MTDH experienced significantly lower survival, and in particular, higher distant relapse rates (5-year distant relapse rates: 26% vs. 5%; p=0.005).

Conclusions

Dysregulation of miR-375 and MTDH may represent an important oncogenic pathway driving human HNC progression, particularly distant metastases, which is now emerging as a
major cause of death for HNC patients. Hence, targeting this pathway could potentially be a novel therapeutic strategy by which HNC patient outcome could be improved.
INTRODUCTION

Head and neck squamous cell carcinomas (HNSCC) constitute the 5th most common malignancy worldwide (1). Patients with locally advanced HNSCC have 5-year overall survival (OS) rates hovering around 30-45% (2), underscoring a considerable opportunity for improving outcome. Nasopharyngeal carcinoma (NPC) is another malignancy of the head and neck region; however, NPCs are distinct from other head & neck cancers (HNCs) due to their unique etiological, clinical/biological and epidemiological characteristics. Locally advanced NPC patients have a 5-year overall survival rate of ~70% (3), also demonstrating a need for improvement. Hence, it is imperative to acquire a deeper understanding of HNC biology to guide the development and evaluation of novel therapies, in order to improve patient outcome.

MicroRNAs (miRNA) are a novel class of gene regulators which are involved in many biological systems, and recognized to play important roles in human cancers (4). We have recently completed a miRNA profiling study of locally advanced HNSCC, and reported down-regulation of miR-375 as one of the most frequently detected aberrations (5). Preliminary functional analysis demonstrated a potential tumour suppressive role for miR-375 in HNSCC (5). In the current study, we further investigated the role of miR-375 down-regulation in HNC, and determined that metadherin (MTDH) is a target of miR-375 in HNC. MTDH, also known as astrocyte elevated gene 1 (AEG-1) was only recently cloned (6); however, it is rapidly emerging as an important oncogene in many human cancers (7). Furthermore, MTDH over-expression has been observed to significantly correlate with poor prognosis and distant metastases in breast(8), lung(9) and gastric(10) cancers, warranting its further examination in HNC.
MATERIALS & METHODS

Patient information & tissues

With approval from the Institutional Research Ethics Board (REB), 20 diagnostic formalin-fixed paraffin-embedded (FFPE) blocks were collected from HNSCC patients with locally advanced (Stage III or IV) disease, who were previous participants in a Phase III randomized study of hyperfractionated radiotherapy conducted between 1988-1995 (11). Normal epithelial tissues derived from three FFPE blocks of individuals who underwent a tonsillectomy (University Health Network), plus three additional FFPE blocks derived from normal laryngeal squamous epithelial tissues post-laryngectomy (commercially purchased from Asterand, USA) served as controls. In addition, 94 primary FFPE biopsy samples from NPC patients diagnosed from 1993-2000 were evaluated. Eight FFPE blocks of normal nasopharyngeal epithelial tissues derived from patients who underwent a diagnostic quadroscopy served as normal controls. Clinical characteristics of these cancer patients are provided in Table 1.

RNA purification from FFPE samples

To ensure that all tissues analyzed contained >70% tumour cells, a representative section from each patient was stained with H & E, then reviewed by a HNC pathologist (B P-O) to ascertain regions with malignant epithelial cells for macro-dissection. All blocks were processed randomly, with clinical outcome unknown, to avoid experimental bias. Total RNA enriched for small RNA species was isolated using the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Ambion, Inc.), according to the manufacturer’s instructions.

Cell lines and reagents

The human hypopharyngeal HNSCC cell line, FaDu, was obtained from American Type Culture Collection (Manassas, VA), and cultured according to specifications. The Epstein-Barr
virus (EBV) positive NPC cell line C666-1 (12) was maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Wisent, Inc.), and 100 mg/L penicillin/streptomycin. The normal oral epithelial (NOE) cells (Celprogen, Inc.) served as normal controls. All cells were authenticated at the Centre for Applied Genomics (Hospital for Sick Children, Toronto, Canada) using the AmpF/STR Identifiler PCR Amplification Kit (Applied Biosystems), and maintained at 37°C with 5% CO2.

5-aza-2’-deoxycytidine treatment and quantitative CpG methylation analysis

C666-1 and FaDu cells were seeded overnight in 12-well plates and media containing 3µM 5-aza-2’-deoxycytidine (Sigma Aldrich) was added to cells 2 and 4 days after seeding, respectively. On day 5, cells were harvested and RNA was extracted for qRT-PCR analysis of miR-375 expression levels. The CpG islands in the genomic region of miR-375 were identified using the EMBL-EBI CpG Island Finder and Plotting Tool (Supplementary Fig 1A) and 3 amplicons spanning these regions (Supplementary Fig 1B) were analyzed for CpG methylation using the EpiTYPER (Sequenom, Inc.) at the Analytical Genetics Technology Centre (AGTC), University Health Network, Toronto.

Quantification of miRNA and mRNA

MicroRNA expression was assessed by qRT-PCR analysis using TaqMan® microRNA Assays (Applied Biosystems) as previously described (13). Quantitative RT-PCR was also utilized to analyze expression changes of four previously published miR-375 targets: MTPN, USP1, PDK1, ADIPOR2, plus five newly identified miR-375 targets: MTDH, GMFB, RANBP3, SPAG9 and ZNF462. Total RNA was isolated from cells using the Total RNA purification Kit (Norgen, Inc.). One µg of total RNA was reverse-transcribed using SuperScript II Reverse Transcriptase (Invitrogen Canada) as specified by the manufacturer. Quantitative RT-PCR was
performed using SYBR Green PCR Master Mix (Applied Biosystems) and an ABI PRISM 7900 Sequence Detection System (PerkinElmer Biosystems). Primers for PCR amplifications (Supplementary Table 1) were designed using Primer 3 Input (version 0.4.0). Relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method (14).

Construction of plasmids and luciferase assays

Wild-type and/or mutant fragments (150-200 bp) from the 3’UTR of MTDH, MTPN and USP1 containing predicted binding sites for miR-375 were individually amplified by AmpliTaq gold DNA polymerase (Applied Biosystems) using the primers listed in Supplementary Table 2. The PCR products were subsequently purified, digested with SPEI and HINDIII, and then cloned downstream of the firefly luciferase gene in the pMIR-REPORT vector (Ambion, Inc.) to produce the following plasmids: pMIR-MTDH, pMIR-MTDH-MUT, pMIR-MTPN, and pMIR-USP1. Subsequently, FaDu and C666-1 cells were seeded onto 24-well plates one day and three days before transfection, respectively. Cells were then transfected with 100 nM of pre-miR-NEG or pre-miR-375 in the morning; 6 hours later, cells were co-transfected with 100 ng of the reporter plasmid of interest, plus 50 ng of pRL-SV40 (Promega BioSciences) containing the renilla luciferase gene. Luciferase activity was measured 48 hours post-transfection using the Dual-Glo luciferase assay system according to manufacturer’s instructions (Promega BioSciences). Firefly luciferase activity was normalized to that of the renilla luciferase.

Viability and clonogenic assays

Viability of pre-miR-375, or siMTDH-transfected FaDu or C666-1 cells was examined using the CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTS), according to the manufacturer's protocol (Promega BioSciences). The cellular effects of these manipulations were further investigated in FaDu cells using clonogenic assays as previously described (15). Briefly,
cells were re-seeded at low density in 6-well plates in triplicate, and incubated at 37°C under 5% CO2 at 3 days post-transfection. After 10-12 days, plates were washed, fixed in 50% methanol and stained with 0.1% crystal violet, and then the number of colonies was counted. The fraction of clonogenically viable cells was calculated by comparison of pre-miR-375 or siMTDH transfected cells with pre-miR-NEG or scrambled siRNA (siNEG) transfected cells, respectively.

**Cell cycle analysis**

Cell cycle analysis of pre-miR-375 transfected C666-1 cells was performed as previously described (15). Briefly, cells were harvested and washed with fluorescence-activated cell sorting (FACS) buffer (PBS/0.5% BSA), then re-suspended and fixed with ice-cold 70% ethanol. After washing, cells were re-suspended in FACS buffer and incubated with propidium iodide in the dark before analysis in the BD FACScalibur using the FL-2 channel. The flow cytometry data were analyzed using FlowJo software (Tree Star, Inc.).

**Western blot analysis**

Total protein extracts were harvested from cell lines and prepared for immunoblotting as previously described (16). Membranes were probed with anti-Phospho-Akt (Ser473) (D9E) XP® rabbit mAB (Cell Signaling Technology), anti-Akt (pan) (C67E7) rabbit mAB (Cell Signaling Technology), anti-MTDH polyclonal (clone L-19; 1:1000 dilution; Invitrogen, Inc.), anti-β-actin (8H10D10) mouse mAB (1:20,000 dilution; Cell Signaling Technology), or anti-GAPDH monoclonal antibodies (1:15,000 dilution; Abcam, Inc.), followed by secondary antibodies conjugated to horseradish peroxidase (1:2000 dilution; Abcam, Inc.) or IRDye® fluorescent Secondary Antibodies (1:20,000 dilution; LI-COR Biosciences). GAPDH or β-actin protein levels were used as loading controls. Western blots were quantified with the Adobe Photoshop
In vitro migration and invasion assays

Invasion and migration of HNC cells were assayed using the BD BioCoat™ Matrigel™ Invasion Chambers and Control Inserts (BD Bioscience), respectively. Each well of a 24-well plate contained an insert with an 8 µm pore size PET (polyethylene terephthalate) membrane. Inserts coated with a thin layer of matrigel basement membrane matrix were used to measure the ability of the cells to invade through the reconstituted basement membrane. For both migration and invasion assays 1x10^5 cells were seeded inside the insert with medium containing 0.5% serum. High serum (20%) medium was then added to the bottom chamber of 24-well plates to serve as a chemo-attractant. After 48 hrs, the membranes were washed, stained, then separated with a sterile scalpel and mounted on a glass slide. The number of migrating or invading cells was then counted under a light microscope.

Tumor formation assay

All animal experiments utilized 6-8 week-old severe combined immunodeficient (SCID) BALB/c female mice in accordance with the guidelines of the Animal Care Committee, Ontario Cancer Institute, University Health Network (Toronto, Canada). Cells were first transfected with siNEG, siMTDH, pre-miR-NEG, or pre-miR-375 72 hrs before they were harvested and their viability was assessed by the trypan blue exclusion method. Subsequently, 2.5x10^5 viable cells were suspended in 100 µL of growth medium, and injected intramuscularly into the left gastrocnemius muscle of female SCID mice. Tumour growth was monitored by measuring tumour plus leg diameter (TLD) three times a week. Mice were euthanized by CO₂ once TLDs reached 14 mm.
Immunohistochemical (IHC) detection of MTDH expression

Protein expression of MTDH was evaluated in 94 NPC patient samples using immunohistochemistry, which was performed on 5 µm FFPE sections of each patient’s tumour using microwave antigen retrieval, in combination with the Level-2 Ultra Streptavidin system (Signet Laboratories). MTDH expression was detected with the rabbit polyclonal anti-MTDH antibody (1:50 dilution; Invitrogen, Inc.). Expression levels of MTDH were graded according to intensity of immuno-expression.

Statistical analyses

All experiments were performed at least three independent times, with the data presented as the mean ± SEM. The statistical differences between treatment groups were determined using a Student’s t test when comparing two treatment groups, or a one-way ANOVA followed by Tukey’s method when comparing more than two treatment groups. Statistical analyses and graphing were performed using Microsoft excel and Graphpad Prism software (Graphpad Software, Inc.).

To determine the potential prognostic significance of miR-375 or MTDH expression, NPC patients were dichotomized into low (<median) or high (≥median) expression groups which were compared based on several clinical parameters: overall survival, disease-free survival, local relapse-free survival, nodal relapse-free survival, or distant relapse-free survival. Median follow-up time for this cohort was 3 years. Using the log-rank test, a p-value < 0.05 was considered significant.

RESULTS

Down-regulation of miR-375 in HNC cell lines and primary NPC patient samples
We have previously reported a high frequency of miR-375 down-regulation in HNSCC samples (5). To further validate the importance of this miRNA in HNC, expression of miR-375 was measured in an additional seven HNC cell lines, and compared to that of the normal oral epithelial (NOE) cell line (Supplementary Fig 2). Down regulation of miR-375 was noted in five of the seven HNC cell lines analyzed (UTSCC-42a, VU147T, HONE-1, HK-1 and C666-1); the C666-1 cells were observed to have the lowest level of miR-375 expression (~16-fold lower than NOEs). We previously reported that pre-miR-375 transfection of FaDu cells inhibited cell growth, along with a modest increase in the sub-G0/G1 population (5). These same analyses demonstrated that miR-375 transfection in C666-1 cells caused a similarly significant reduction in viability to ~60% by day 3, maintained for at least 6 days (Fig 1A). Cell cycle analysis corroborated that transfection of C666-1 cells with pre-miR-375 resulted in a significant increase in the sub-G0/G1 fraction compared to cells transfected with pre-miR-NEG (11.6% vs. 1.5%; p=0.002); no other phases of the cell cycle appeared to be affected (Fig 1B). The expression level of miR-375 was then examined in 92 FFPE NPC compared to 8 normal nasopharyngeal epithelial samples. This analysis demonstrated significantly lower miR-375 expression in NPC vs. normal samples (Fig 1C; p=0.01). Using a cut-off of 2-fold below the mean expression of normal nasopharyngeal epithelial tissues, miR-375 was under-expressed in 69 out of 92 (75%) NPCs; its down-regulation however, was not significantly associated with clinical outcome (Supplementary Fig 3).

**Methylation status of the genomic region containing miR-375**

Methylation has been reported as a potential mechanism of miR-375 silencing in hepatocellular carcinoma (HCC) (17); hence we examined this possible mechanism in both FaDu and C666-1 cells using both bisulfite sequencing and methylation-status related expression
analysis. Bisulfite sequencing conducted on three amplicons spanning the genomic region previously reported to be methylated in HCC (17) demonstrated that ~73% and 65% of the CpG sites in this area were indeed methylated in C666-1 and FaDu cells, respectively (Fig 1D). Furthermore, treatment of both cell lines with the global de-methylating agent 5-aza-2’-deoxycytidine (5-aza) significantly reduced methylation at all CpG sites (Fig 1D), which was associated with the subsequent re-expression of miR-375 (Fig 1E), clearly indicating that one mechanism for miR-375 suppression in HNC cells is hyper-methylation of the promoter or coding regions of miR-375.

Identification of mRNA targets of miR-375

To identify the mRNA targets of miR-375, genome wide mRNA expression analysis was performed comparing pre-miR-375 transfected HNC cells with that of pre-miR-NEG transfected cells (unpublished data). Transcripts with >2-fold down-regulation in pre-miR-375 transfected cells were compared to in silico predicted targets from 7 publicly available databases (miRanda, miRDB, RNA22, PITA, miR-Walk, RNAHybrid, TargetScan) generating a list of nine candidate target mRNAs (Supplementary Table 3). As confirmation of the validity of our methodology, two of these genes (MTPN, USP1) had been previously reported as targets of miR-375 (18, 19). The effect of miR-375 transfection on gene expression was analyzed for 6 of the 7 identified genes (optimal primers for AEBP2 could not be generated), as well as two previously reported miR-375 targets (PDK1, ADIPOR2; (19, 20)). Transfection of FaDu and C666-1 cells with pre-miR-375 led to down-regulation of all potential mRNA targets with the exception of ADIPOR2 in FaDu cells (Supplementary Fig 4). Of note, MTDH was consistently down-regulated to the lowest level in both HNC cell lines; moreover, MTDH was the only target predicted by all seven in silico algorithms (Supplementary Table 3).
Down-regulation of MTDH by miR-375 was confirmed at both the transcript and protein level by qRT-PCR and Western blot analysis, respectively (Fig 2A). The direct interaction between miR-375 and MTDH was then verified using luciferase assays. Supplementary Fig 5 depicts the \textit{in silico} predicted binding sites between the miR-375 seed region with the MTDH transcript. The predicted binding site located at nucleotides 3518-3539 in the 3’UTR of the MTDH transcript was supported by all seven \textit{in silico} databases and was therefore selected for plasmid construction. In comparison to control cells transfected with pMIR-REPORT, FaDu and C666-1 cells transfected with pMIR-REPORT-MTDH demonstrated a significant reduction in luciferase activity of 18% and 20% respectively, when co-transfected with pre-miR-375 (Fig 2B). Upon mutating the miR-375 binding site, this inhibitory effect was completely abrogated, corroborating that the observed decrease in luciferase activity was indeed dependent upon an intact miR-375 binding site. Two previously reported mRNA targets, MTPN and USP1, were also demonstrated to be potential targets of miR-375 in both HNC cells (Supplementary Fig 6).

**Effects of MTDH knock-down on HNC cells**

The potential oncogenic role of MTDH was investigated by evaluating the phenotypic consequences of transfecting HNC cells with siRNA directed against MTDH (siMTDH). MTDH knock-down by this siRNA sequence was confirmed at both the transcript and protein level (Fig 2C). Similar to the effects of pre-miR-375 transfection, siMTDH transfection significantly reduced cell viability in both C666-1 (>50%), and FaDu (~20%) cells (Fig 2D). To ensure that the observed cytotoxicity was not due to off-target effects, the MTS experiments were repeated using a second distinct siRNA sequence targeting MTDH, demonstrating a similar effect (Supplementary Fig 7A). As yet another level of corroboration, a rescue plasmid expressing a MTDH transcript refractory to siRNA was co-transfected with siMTDH, which completely
abrogated any siMTDH-mediated cytotoxicity, confirming that this was indeed an MTDH knock-down-specific effect (Supplementary Fig 7B).

Metadherin is frequently reported to promote metastasis in human cancers; hence, the effect of miR-375 and MTDH on HNC cell migration and invasion were evaluated using in vitro trans-well migration assays. Compared to their corresponding negative controls, transfection with pre-miR-375 significantly reduced migration of both C666-1 and FaDu cells by 57% and 80%, respectively (Fig 3A). Similar inhibition of migration was observed in cells transfected with siMTDH, with 75% reduction in C666-1 cells and 86% in FaDu cells (Fig 3A). Moreover, siMTDH and pre-miR-375 transfection also resulted in 86% and 92% reduction in invasion of FaDu cells, respectively (Fig 3B). C666-1 cells are unable to penetrate the matrigel coating used in the invasion assay; hence this property could not be assessed in the NPC model.

**Effects of miR-375 transfection and MTDH knock-down on PI(3)K-Akt signaling**

Western blot analyses were performed to investigate the signaling pathways downstream of miR-375 over-expression or MTDH knockdown in FaDu and C666-1 cells. The PI(3)K-Akt pathway has been previously reported to be activated by MTDH over-expression; specifically increased phosphorylation of Akt (p-Akt), although the mechanism by which this induction occurs remains poorly understood (21-23). Concordantly, a significant reduction in p-Akt was observed in both C666-1 and FaDu cells after siMTDH knockdown (Fig 4A & 4B), phenocopied by miR-375 transfection in FaDu cells (Fig 4B), but not so for C666-1 cells (Fig 4A). Total Akt expression remained unchanged under these conditions.

**MTDH knock-down and miR-375 transfection delayed tumor formation in-vivo**

To investigate the effects of miR-375 over-expression and MTDH knock-down on head & neck tumour forming ability, FaDu and C666-1 cells transfected with siMTDH, pre-miR-375
or corresponding negative controls were injected intra-muscularly into SCID mice. The results demonstrated that both miR-375 over-expression and MTDH knock-down resulted in a significant delay in tumour formation wherein such transfected tumours attained the humane endpoint (TLD = 14mm) ~10-15 days later than tumours transfected with the corresponding negative controls (Fig 4C, 4D). Tumors from both C666-1 and FaDu xenografts were formalin fixed and paraffin embedded for IHC analyses for CD31 and Ki-67, which did not demonstrate any significant difference in their expressions (data not shown).

**Over-expression of MTDH in primary HNC samples**

The relevance of MTDH in HNC was further investigated in primary human HNC samples in the previously evaluated 20 HNSCC (5), as well as the same 92 NPC samples for which miR-375 expression has already been examined (Fig 1C). Significantly higher MTDH transcript levels were detected in both HNSCC (p=0.048) and NPC patient samples (p<0.0001) compared to corresponding normal controls as measured by qRT-PCR (Fig 5A & B). Using a 2-fold cut-off, MTDH mRNA over-expression was detected in 65% and 93% of HNSCC and NPC samples, respectively. Over-expression of MTDH at the protein level was corroborated in 94 NPC patient samples (including the 92 previously investigated cases) using immunohistochemistry (IHC), illustrating that MTDH expression was predominantly localized to the cell membrane or cytoplasm, with little to no immuno-staining observed in the surrounding stroma (Fig 5C). The vast majority of NPC samples had cytoplasmic MTDH immuno-expression (91 out of 94 cases, or 97%), with only one sample showing nuclear staining (Fig 5D). Strikingly, when dichotomized based on MTDH transcript expression level, NPC patients with high expression (≥median) had significantly worse overall (HR=2.34; p=0.04), disease-free (HR=2.08; p=0.04), and distant relapse-free (HR=6.38; p=0.005) survival compared to patients...
with low MTDH (<median) expression (Fig 5E). The association of MTDH expression with two additional factors that are potentially predictive of distant relapse in NPC patients was also explored; namely treatment, and stage. Firstly, MTDH expression was not different as a function of treatment (chemoradiation vs. radiation alone; Supplementary Fig 8A). Additionally, when the radiation only group of patients was analyzed, MTDH remained a powerful predictor of distant relapse (Supplementary Fig 8B). A similar trend was observed for the chemoradiation patients, but the difference was not statistically significant (p=0.17), likely due to the small sample size (Supplementary Fig 8B). Secondly, MTDH expression was interestingly inversely related to stage, being lower in Stage III/IV vs. Stage I/II patients (p=0.04; Supplementary Fig 8C). Importantly, however, when patients were further categorized according to both stage (I/II vs. III/IV), as well as MTDH expression (<median vs. ≥ median), this was a highly significant association (p=0.006; Supplementary Fig 8D). Specifically, Stage I/II patients with low MTDH expression had 0% distant relapse; conversely, Stage III/IV patients plus increased MTDH expression had the highest risk of distant relapse (5-year rate of 37%), suggesting that using both variables could be quite informative in terms of prognosticating for metastatic disease in NPC.

Due to the small sample size and mal-distribution of outcome of the HNSCC patients (the majority of the 20 samples had relapsed disease), survival analysis was not performed on this cohort.

DISCUSSION

Our recent study profiling miRNA expression in locally advanced HNSCC reported a high frequency of miR-375 under-expression in these tumours, and suggested its potential tumor suppressor function (5). The current report demonstrates that miR-375 is also down-regulated in
75% of NPCs and confirms a tumour suppressor function for this miRNA in HNC cell lines. Furthermore, MTDH has been verified as *bona fide* target of miR-375 in HNC. During the preparation of this report, MTDH was also identified independently by another group as a target of miR-375 in HNSCC (24). However, to the best of our knowledge, this study is the first to support a role for the dysregulation of miR-375 and MTDH in promoting metastases in HNCs; particularly in NPC.

MiR-375 was first described in the development and function of pancreatic islet cells (25, 26), and more recently in human embryonic stem cell development (27). Mounting data however, suggest that miR-375 might also be playing an equally important role in human cancers. Several groups have recently demonstrated a tumour suppressor role for miR-375 (20, 28-31), congruent with our own data in this study. Reduced miR-375 expression has in fact been reported to be associated with worse outcome for patients with esophageal carcinoma (32, 33); high miR-221 to low miR-375 ratio has also been suggested as a potential diagnostic marker for HNSCC (30). Conversely, several groups have also described a potential oncogenic role for miR-375 (34-36), underscoring that similar to many other miRNAs, miR-375 can function as both a tumour suppressor and an oncogene, probably dependent upon context and cancer type.

Chromosomal loss and promoter hypermethylation are two common mechanisms for inactivation of tumour suppressor genes. Deletion of chromosome 2q35 has indeed been reported in HNSCC (37). In our current work, bisulfite sequencing demonstrated that CpG islands proximal to the miR-375 locus are highly methylated (Fig 1D), and exposure to 5'-aza-2-deoxycytidine resulted in a significant re-expression of miR-375 (Fig 1E), corroborating that hypermethylation is indeed one important mechanism for miR-375 under-expression in HNC,
similar to the previous report in HCC (17). Hence, chromosomal loss and hypermethylation-mediated silencing are two likely mechanisms leading to miR-375 down-regulation in HNC.

In terms of MTDH over-expression in HNC, miR-375 down-regulation is clearly one documented mechanism, but the down regulation of other miRNAs could also play a role in this process. As an example, miR-26a was recently reported to be under-expressed in breast cancer, targeting both MTDH and enhancer of zeste homolog 2 (EZH2) (38). Our own group has previously reported significant miR-26a down-regulation in primary HNSCC (5), as well as NPC (39); hence, under-expression of miR-26a might indeed be another mechanism for MTDH over-expression in HNC. A third potential mechanism for MTDH over-expression could be chromosomal amplification; given that 8q22 (genomic location for MTDH) is also frequently amplified in both NPC and HNSCC (40, 41). Indeed, this mechanism for MTDH over-expression has already been reported for both breast (8) and hepatocellular carcinomas (42). Taken together, these conglomerate reports illustrate that MTDH over-expression in HNC is likely mediated by a complex interplay between several factors including chromosomal amplification and targeting by multiple miRNAs.

The human \textit{MTDH} gene was first cloned in astrocytes, where it was shown to be up-regulated in response to treatment with TNF-\textit{\alpha}, gp120, or HIV infection; therefore initially denoted as “Astrocyte elevated gene 1” (AEG-1)(6). The mouse homologue was cloned shortly thereafter in a murine model for metastatic breast cancer; discovered due to its role in selectively homing metastatic breast cancer cells to the lung (43). Since these seminal observations, MTDH has rapidly emerged as an important mediator in the development and progression for several human cancers (7). In fact, MTDH over-expression has already been associated with poor outcome for breast (8), liver (42), esophageal (44), renal cell (45), colorectal (46), and non-small
cell lung cancers (9), frequently associated with metastases and chemoresistance. However, the molecular mechanisms by which MTDH mediates metastases and/or chemoresistance remain to be clearly elucidated. Some of the suggested downstream signalling events have included activation of PI3K-Akt (21), NF-κB (47), MAPK and Wnt (42), up-regulation of MMP-9 (48) and FOXO3a (22, 44), suppression of FOXO1 (23), as well as induction of EMT (47).

Herein, we report the over-expression of MTDH in both HNSCC and NPC. Due to small sample size (n=20), the significance of MTDH over-expression observed in the HNSCC samples was marginal (p=0.048), therefore we sought out additional evidence to support these findings. Using a publicly available online (http://www.ebi.ac.uk/gxa/array/U133A) data set mined by Lukk et al. (49), we identified that MTDH was also significantly over-expressed in their HNSCC samples by 2.1-4.2 fold (p<0.08). Furthermore, another group has also recently reported the significant over-expression of MTDH in HNSCC using 20 paired HNSCC/Normal samples (24); hence we are confident regarding MTDH over-expression in primary HNSCC. We also report, for the first time, a role for MTDH in promoting a metastatic phenotype in HNC, particularly in NPC. The oncogenic function of MTDH was demonstrated both in vitro and in vivo wherein MTDH affected cell migration and invasion, potentially mediated in part via phosphorylation of Akt. The clinical impact of this observation is extremely important; due to the advent of intensity-modulated radiation therapy (IMRT), the 5-year local control rates for NPC are excellent, at ~90% (3). However, the 5-year OS for NPC remains modest at ~70% due to distant metastases (3). Hence, therapies that effectively reduce metastases would be of extreme importance in improving survival for NPC patients. As MTDH is primarily expressed at the plasma membrane of breast cancer cells (43), a DNA vaccine therapy targeting MTDH has been developed, demonstrating some preliminary success in reducing tumour burden and lung
metastases in a mouse model of breast cancer (50). However, such approaches might not be applicable to HNC, given that MTDH expression appears to be predominantly cytoplasmic in this disease (Fig 5C).

In conclusion, a novel pathway of miR-375 down-regulation, leading to MTDH over-expression has been documented in human HNC, which has significant clinical implications as a mechanism by which HNC metastases could develop. Further unraveling of the MTDH protein structure and function, as well as the molecular mechanisms by which it activates a metastatic cascade could lead to the development of small molecule inhibitors, which can be examined as a potential therapeutic strategy by which outcome can be improved for future patients with HNC.

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REFERENCES


Table and Figure Legends

Table 1. Clinical characteristics of HNC patients studied. Clinical descriptors of 94 NPC and 20 HNSCC patients whose tumour samples were analyzed during this work.

Figure 1. Down-regulation of miR-375 in HNC cells (A) Viability of C666-1 cells as assessed by the MTS assay 3 and 6 days post-pre-miR-375 or pre-miR-NEG transfection. (B) Cell cycle analysis as performed by flow cytometry on C666-1 72 hours post-pre-miR-375 or pre-miR-NEG transfection. (C) MiR-375 expression in 92 primary NPC biopsy samples, compared to 8 normal nasopharyngeal epithelial control tissues as measured by qRT-PCR. (D) Bisulfite sequencing analysis of the CpG sites within a ~1200 bp region around the genomic locus encoding miR-375. Methylation status of three amplicons was analyzed on C666-1 and FaDu cells, treated with either 5-aza-2’-deoxycytidine (FaDu-AZA/C666-1-AZA), or DMSO (FaDu-V/C666-1-V). (E) qRT-PCR analysis of miR-375 expression in FaDu and C666-1 cells after treatment with 5-aza-2’-deoxycytidine relative to vehicle-control (DMSO) treated cells. Each experiment was performed at least three independent times, with the data presented as the mean ± SEM. *indicates p<0.05

Figure 2. Identification of mRNA targets of miR-375 (A) MTDH expression at both the mRNA (left) and protein (right) level after pre-miR-375 or pre-miR-NEG transfection in C666-1 and FaDu cells. (B) Relative luciferase activity in C666-1 or FaDu cells after co-transfection with pMIR-REPORT, pMIR-MTDH or pMIR-MTDH-MUT vectors and pre-miR-375 or pre-miR-NEG. (C) MTDH expression at both the mRNA (left) and protein (right) level after siMTDH or siNEG transfection in C666-1 and FaDu cells. OD, optical density; *indicates p<0.05
Figure 3. Migration and invasion of miR-375 or siMTDH in HNC cells. (A) Representative images (left) and bar graphs (right) depicting the migratory ability of C666-1 and FaDu cells after pre-miR-375 or siMTDH transfection compared to corresponding negative controls. (B) Representative images (left) and bar graphs (right) depicting the invasive ability of FaDu cells after pre-miR-375 or siMTDH transfection compared to corresponding negative controls. *indicates p<0.05; **indicates p<0.01; ***indicates p<0.001.

Figure 4. Down-stream signalling, and in vivo effects of miR-375 and siMTDH in HNC models. Representative western blot images and relative quantification of p-Akt protein levels in (A) C666-1 and (B) FaDu cells transfected with pre-miR-375 or siMTDH, compared to corresponding negative controls. (C) & (D) Tumour-plus-leg diameter measurements of (C) C666-1 and (D) FaDu cell xenografts transfected with siMTDH, pre-miR-375 or corresponding negative controls. OD, optical density; *indicates p<0.05; ** indicates p<0.01.

Figure 5. MTDH Expression in HNC patient samples. MTDH expression measured by qRT-PCR in (A) 20 HNSCC and (B) 92 NPC patient biopsy samples, in comparison to 6 normal laryngeal epithelial tissues, and 7 normal nasopharyngeal epithelial tissues, respectively. (C) Representative images of immunohistochemical analysis of MTDH expression in primary NPC biopsy samples. Arrows indicate tumour cells exhibiting (C) cytoplasmic membrane or (D) nuclear expression of MTDH. T, tumor cells; E, endothelial cells. (E) Kaplan-Meier plots of overall survival, disease-free survival, and distant relapse-free survival for NPC patients dichotomized based on high (≥median) vs. low (<median) MTDH transcript expression level.
### Clinical Descriptors for the 94 NPC Patients

#### Age
- Median: 46
- Range: 16-79

#### Stage
<table>
<thead>
<tr>
<th>Frequency</th>
<th>Percent</th>
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<tbody>
<tr>
<td>I</td>
<td>8</td>
</tr>
<tr>
<td>II</td>
<td>32</td>
</tr>
<tr>
<td>III</td>
<td>28</td>
</tr>
<tr>
<td>IV</td>
<td>26</td>
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</tbody>
</table>

#### Relapse
- No relapse: 64 (68%)
- Local relapse only: 7 (7%)
- Local + Distant relapse: 13 (14%)
- Distant relapse only: 10 (11%)

#### Treatment
- Radiation only: 69 (73%)
- Radiation + chemo: 25 (27%)

### Clinical Descriptors for the 20 HNSCC Patients

#### Age
- Median: 59
- Range: 35-75

#### Stage
<table>
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<tbody>
<tr>
<td>III</td>
<td>8</td>
</tr>
<tr>
<td>IV</td>
<td>12</td>
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</tbody>
</table>

#### Relapse
- No relapse: 4 (20%)
- Local relapse: 6 (30%)
- Regional relapse: 4 (20%)
- Local + regional relapse: 6 (30%)

#### Treatment
- Radiation #1 (D): 9 (45%)
- Radiation #2 (T): 11 (55%)

(T = twice daily) and (D = standard)
FIGURE 1

A

B

C

D

E

Amplicon 1:

FaDu_V
FaDu_AZA
C666-1_V
C666-1_AZA

Amplicon 2:

FaDu_V
FaDu_AZA
C666-1_V
C666-1_AZA

Amplicon 3:

FaDu_V
FaDu_AZA
C666-1_V
C666-1_AZA

Relative Viability (%)

Proportion of Cells (%)

Relative miR-375 Expression (Log2)

Relative miR-375 Expression (Log2)

0% Not Analyzed:

100%

Time Post-Transfection (days)

Cell Cycle Phase

Normal
NPC

miR-Neg
miR-375

Vehicle alone
Lipo alone

p = 0.002

p = 0.01

p = 0.002
FIGURE 3

A

C666-1

miR-Neg  miR-375  siNEG  siMTDH

FaDu

B

miR-Neg  miR-375  siNEG  siMTDH

FaDu
FIGURE 5

A

Relative MTDP Expression (Log2)

p=0.048

B

Relative MTDP Expression (Log2)

p<0.0001

C

D

E

20 x

40 x

Overall Survival Probability (%)

Distant Relapse Free Survival Probability (%)

Survival Time (months)

Survival Probability (%)

Low MTDP
High MTDP

P=0.05 (log-rank test)

Low MTDP
High MTDP

P=0.0046 (log-rank test)

Low MTDP
High MTDP

P=0.05 (log-rank test)
Clinical Cancer Research

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