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

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Abstract

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

Experimental Design: Quantitative reverse transcriptase PCR (qRT-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 reexpression and MTDH knockdown were assessed using viability (MTS), clonogenic survival, cell migration/invasion, as well as in vivo tumor formation assays. The prognostic significance of miR-375 or MTDH in nasopharyngeal carcinoma (NPC) was determined by comparing low versus 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. qRT-PCR, Western blots, and luciferase assays corroborated MTDH as a target of miR-375. Reexpression of miR-375 and siRNA knockdown of MTDH both decreased cell viability and clonogenic survival, cell migration/invasion, as well as in vivo tumor formation. NPC patients whose tumors 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 fifth most common malignancy worldwide (1). Patients with locally advanced HNSCC have 5-year overall survival (OS) rates hovering around 30% to 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 and neck cancers (HNC) due to their unique etiologic, clinical/biological, and epidemiologic characteristics. Locally advanced NPC patients have a 5-year OS rate of approximately 70% (3), also showing 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, 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 downregulation of miR-375 as one of the most frequently detected aberrations (5). Preliminary functional analysis showed a potential tumor suppressive role for miR-375 in HNSCC (5). In this study, we further investigated the role of miR-375 downregulation 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 overexpression 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 and Methods

Patient information and tissues
With approval from the Institutional Research Ethics Board, 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 and 1995 (11). Normal epithelial tissues derived from 3 FFPE blocks of individuals who underwent a tonsillectomy (University Health Network), plus 3 additional FFPE blocks derived from normal laryngeal squamous epithelial tissues post-laryngectomy (commercially purchased from Asterand) served as controls. In addition, 94 primary FFPE biopsy samples from NPC patients diagnosed from 1993 to 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 more than 70% tumor cells, a representative section from each patient was stained with hematoxylin and eosin stain, then reviewed by a HNC pathologist (B P-O) to ascertain regions with malignant epithelial cells for macrodissection. 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 and cultured according to specifications. The Epstein–Barr virus–positive NPC cell line C666-1 (12) was maintained in RPMI-1640, supplemented with 10% FBS (Wisent, Inc.) and 100 mg/L penicillin/streptomycin. The 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 3 μmol/L 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 quantitative reverse transcriptase PCR (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. S1A), and 3 amplicons spanning these regions (Supplementary Fig. S1B) 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
miRNA expression was assessed by qRT-PCR analysis using TaqMan microRNA Assays (Applied Biosystems) as previously described (13). qRT-PCR was also utilized to analyze expression changes of 4 previously published miR-375 targets: MTPN, USP1, PDK1, ADIPOR2, plus 5 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 microgram of total RNA was reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen) as specified by the manufacturer. qRT-PCR was done using SYBR Green PCR Master Mix (Applied Biosystems) and an ABI PRISM 7900 Sequence Detection System (PerkinElmer Biosystems). Primers for PCR amplifications (Supplementary Table S1) were designed using Primer 3 Input (version 0.4.0). Relative mRNA levels were calculated using the 2^-ΔΔCt method (14).

Construction of plasmids and luciferase assays
Wild-type and/or mutant fragments (150–200 bp) from the 3' UTR (untranslated region) of MTDH, MTPN, and USP1 containing predicted binding sites for miR-375 were individually amplified with AmpliTaq gold DNA polymerase (Applied Biosystems) using the primers listed in Supplementary Table S2. The PCR products were subsequently purified, digested with SFI 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 1 day and 3 days before transfection, respectively. Cells were then transfected with 100 nmol/L of pre-miR-NEG or pre-miR-375 in the morning; 6 hours later, cells were cotransfected 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 posttransfection 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 reseeded at low density in 6-well plates in triplicate and incubated at 37°C under 5% CO₂ at 3 days posttransfection. After 10 to 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 done as previously described (15). Briefly, cells were harvested and washed with fluorescence-activated cell sorting (FACS) buffer (PBS/0.5% BSA), then resuspended and fixed with ice-cold 70% ethanol. After washing, cells were resuspended in FACS buffer and incubated with propidium iodide in the dark before analysis in the BD FACS-calibur 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 monoclonal antibodies (mAb; Cell Signaling Technology), anti-Akt (pan) (C67E7) rabbit mAb (Cell Signaling Technology), anti-MTDH polyclonal (clone L-19; 1:1,000 dilution; Invitrogen, Inc.), anti-β-actin (8H10D10) mouse mAb (1:20,000 dilution; Cell Signaling Technology), or anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mAbs (1:15,000 dilution; Abcam, Inc.), followed by secondary antibodies conjugated to horseradish peroxidase (1:2,000 dilution; Abcam, Inc.) or IRDye

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

<table>
<thead>
<tr>
<th>Clinical descriptors for the 94 NPC patients</th>
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**Clinical descriptors for the 20 HNSCC patients**

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</tr>
<tr>
<td>Radiation #2 (T)</td>
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<td>55</td>
</tr>
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(T = twice daily) and (D = standard).

(T = twice daily) and (D = standard).
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 Pixel Quantification Plug-In (Richard Rosenman Advertising & Design), or the Odyssey Application Software 2.1 (Li-cor Biosciences).

**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, 1 × 10^5 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 chemoattractant. After 48 hours, 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- to 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 hours before they were harvested, and their viability was assessed by the trypan blue exclusion method. Subsequently, 2.5 × 10^5 viable cells were suspended in 100 μL of growth medium and injected intramuscularly into the left gastrocnemius muscle of female SCID mice. Tumor growth was monitored by measuring tumor plus leg diameter (TLD) 3 times a week. Mice were euthanized by CO2 once TLDs reached 14 mm.

**Immunohistochemical detection of MTDH expression**

Protein expression of MTDH was evaluated in 94 NPC patient samples using immunohistochemistry (IHC), which was done on 5-μm FFPE sections of tumor of each patient 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 immunooexpression.

**Statistical analyses**

All experiments were conducted at least 3 independent times, with the data presented as the mean ± SEM. The statistical differences between treatment groups were determined using a Student t test when comparing 2 treatment groups, or a 1-way ANOVA followed by Tukey’s method when comparing more than 2 treatment groups. Statistical analyses and graphing were done 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 on the basis of several clinical parameters: OS, 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 less than 0.05 was considered significant.

**Results**

**Downregulation of miR-375 in HNC cell lines and primary NPC patient samples**

We have previously reported a high frequency of miR-375 downregulation in HNSCC samples (5). To further validate the importance of this miRNA in HNC, expression of miR-375 was measured in an additional 7 HNC cell lines and compared with that of the NOE cell line (Supplementary Fig. S2). Downregulation of miR-375 was noted in 5 of the 7 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 showed that miR-375 transfection in C666-1 cells caused a similarly significant reduction in viability to approximately 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 with cells transfected with pre-miR-NEG (11.6% vs. 1.5%; P = 0.002); no other phases of the cell cycle seemed to be affected (Fig. 1B). The expression level of miR-375 was then examined in 92 FFPE NPC compared with 8 normal nasopharyngeal epithelial samples. This analysis showed significantly lower miR-375 expression in NPC versus 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 underexpressed in 69 of 92 (75%) NPCs; its downregulation however, was not significantly associated with clinical outcome (Supplementary Fig. S3).

**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; ref. 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 3 amplicons spanning the genomic region previously reported to be methylated in HICC (17) showed that approximately 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 demethylating agent 5-aza-2'-deoxycytidine (5-aza) significantly reduced methylation at all CpG sites (Fig. 1D), which was associated with the subsequent reexpression of miR-375 (Fig. 1E), clearly indicating that one mechanism for miR-375 suppression in HNC cells is hypermethylation 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 done comparing pre-miR-375–transfected HNC cells with that of pre-miR-NEG–transfected cells (data not shown). Transcripts with more than 2-fold downregulation in pre-miR-375–transfected cells were compared with in silico predicted targets from 7 publicly available databases (miRanda, miRDB, RNA22, PITA, miR-Walk, RNAHybrid, TargetScan), generating a list of 9 candidate target mRNAs (Supplementary Table S3). As confirmation of the validity of our methodology, 2 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 2 previously reported miR-375 targets (PDK1, ADIPOR2; refs. 19, 20). Transfection of FaDu and C666-1 cells with pre-miR-375 led to downregulation of all potential mRNA targets, with the exception of ADIPOR2 in FaDu cells (Supplementary Fig. S4). Of note, MTDH was consistently downregulated to the lowest level in both HNC cell lines; moreover, MTDH was the only target predicted by all 7 in silico databases (Supplementary Table S3).

Downregulation 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. S5 depicts the in silico predicted binding sites between the miR-375 seed region with the MTDH transcript. The predicted binding site located at nucleotides 3,518 to 3,539 in the 3' UTR of the MTDH transcript was supported by all 7 in silico databases and was, therefore, selected for downstream functional analysis.
plasmid construction. In comparison with control cells transfected with pMIR-REPORT, FaDu and C666-1 cells transfected with pMIR-REPORT-MTDH showed a significant reduction in luciferase activity of 18% and 20%, respectively, when cotransfected 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 shown to be potential targets of miR-375 in both HNC cell lines (Supplementary Fig. S6).

Effects of MTDH knockdown 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 knockdown 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, showing a similar effect (Supplementary Fig. S7A). As yet another level of corroboration, a rescue plasmid expressing a MTDH transcript refractory to siRNA was cotransfected with siMTDH, which completely abrogated any siMTDH-mediated cytotoxicity, confirming that this was indeed an MTDH knockdown-specific effect (Supplementary Fig. S7B).

MTDH 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 with 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 knockdown on PI(3)K-Akt signaling**

Western blot analyses were done to investigate the signaling pathways downstream of miR-375 overexpression or MTDH knockdown in FaDu and C666-1 cells. The PI(3)K-Akt pathway has been previously reported to be activated by MTDH overexpression; 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 and B), 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 knockdown and miR-375 transfection delayed tumor formation in vivo**

To investigate the effects of miR-375 overexpression and MTDH knockdown on head and neck tumor forming ability, FaDu and C666-1 cells transfected with siMTDH, pre-miR-375, or corresponding negative controls were injected intramuscularly into SCID mice. The results showed that both miR-375 overexpression and MTDH knockdown resulted in a significant delay in tumor formation, wherein such transfected tumors attained the humane endpoint (TLD = 14 mm) approximately 10 to 15 days later than tumors transfected with the corresponding negative controls (Fig. 4C and D). Tumors from both C666-1 and FaDu xenografts were FFPE for immunohistochemical analyses for CD31 and Ki-67, which did not show any significant difference in their expressions (data not shown).

**Overexpression 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 with corresponding normal controls as measured by qRT-PCR (Fig. 5A and B). Using a 2-fold cut-off, MTDH mRNA overexpression was detected in 65% and 93% of HNSCC and NPC samples, respectively. Overexpression of MTDH at the protein level was corroborated in 94 NPC patient samples (including the 92 previously investigated cases) using IHC, illustrating that MTDH expression was predominantly localized to the cell membrane or cytoplasm, with...
little to no immunostaining observed in the surrounding stroma (Fig. 5C). The vast majority of NPC samples had cytoplasmic MTDH immunoeexpression (91 of 94 cases, or 97%), with only 1 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 with patients with low MTDH (<median) expression (Fig. 5E). The association of MTDH expression with 2 additional factors that are potentially predictive of distant relapse in NPC patients was also explored; namely treatment and stage. First, MTDH expression was not different as a function of treatment (chemoradiation vs. radiation alone; Supplementary Fig. S8A). In addition, when the radiation-only group of patients was analyzed, MTDH remained a powerful predictor of distant relapse (Supplementary Fig. S8B).

A similar trend was observed for the chemoradiation patients, but the difference was not statistically significant (P = 0.17), likely because of the small sample size (Supplementary Fig. S8B). Second, MTDH expression was interestingly inversely related to stage, being lower in stage III/IV versus stage I/II patients (P = 0.04; Supplementary Fig. S8C). Importantly, however, when patients were further categorized according to both stage (I/II vs. III/IV), as well as MTDH expression (<median vs. ≥ median), there was a highly significant association with distant relapse (P = 0.006; Supplementary Fig. S8D). 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.

Because of the small sample size and maldistribution of outcome of the HNSCC patients (the majority of the 20
samples had relapsed disease), survival analysis was not done on this cohort.

Discussion

Our recent study profiling miRNA expression in locally advanced HNSCC reported a high frequency of miR-375 underexpression in these tumors and suggested its potential tumor suppressor function (5). This article shows that miR-375 is also downregulated in 75% of NPCs and confirms a tumor 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 article, 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 shown a tumor 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 tumor suppressor and an oncogene, probably dependent upon context and cancer type.

Chromosomal loss and promoter hypermethylation are 2 common mechanisms for inactivation of tumor suppressor genes. Deletion of chromosome 2q35 has indeed been reported in HNSCC (37). In this work, bisulfite sequencing showed 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 reexpression of miR-375 (Fig. 1E), corroborating that hypermethylation is indeed one important mechanism for miR-375 underexpression in HNC, similar to the previous report in HCC (17). Hence, chromosomal loss and hypermethylation-mediated silencing are 2 likely mechanisms leading to miR-375 downregulation in HNC.

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

The human MTDH gene was first cloned in astrocytes, in which it was shown to be upregulated in response to treatment with TNF-α, gp120, or HIV infection, therefore initially denoted as “Astrocyte elevated gene 1” (AEG-1; ref. 6). The mouse homolog was cloned shortly thereafter in a murine model for metastatic breast cancer, discovered because of 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 overexpression has already been associated with poor outcome for breast (8), liver (42), esophageal (44), renal cell (45), colorectal (46), and nonsmall 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 signaling events have included activation of PI3K-Akt (21), NF-κB (47), mitogen-activated protein kinase and Wnt (42), upregulation of MMP-9 (48), and FOXO3a (22, 44), suppression of FOXO1 (23), as well as induction of EMT (47).

Herein, we report the overexpression of MTDH in both HNSCC and NPC. Due to small sample size (n = 20), the significance of MTDH overexpression 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 and colleagues (49), we identified that MTDH was also significantly overexpressed in their HNSCC samples by 2.1- to 4.2-fold (P < 0.08). Furthermore, another group has also recently reported the significant overexpression of MTDH in HNSCC using 20 paired HNSCC/normal samples (24); hence, we are confident about MTDH overexpression 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 shown 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, the 5-year local control rates for NPC are excellent, at approximately 90% (3). However, the 5-year OS for NPC remains modest at approximately 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, showing some preliminary success in reducing tumor 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 seems to be predominantly cytoplasmic in this disease (Fig. 5C).

In conclusion, a novel pathway of miR-375 downregulation, leading to MTDH overexpression, 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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