

Correlation of MTDH/AEG-1 and HOTAIR Expression with Metastasis and Response to Treatment in Sarcoma Patients

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Abstract

Background: Chemoresistance and metastasis are the main reasons for the failure of current treatments with sarcoma patients. Novel biomarkers are required to predict metastasis and response to treatment. The oncogene MTDH/AEG1 and the long noncoding RNA (lincRNA) HOTAIR are two novel factors involved in drug resistance and metastasis in various types of solid tumors. However, the correlation between MTDH/AEG-1 and HOTAIR expression with metastasis and drug resistance in sarcoma is unknown.

Methods: Expression of MTDH protein or HOTAIR was detected by Western blotting or qRT-PCR, respectively, in primary and metastatic sarcoma patient tissue samples.

Results: High individual or co-expression of MTDH/AEG1 and HOTAIR was observed in three of four primary and six of eight metastatic sarcoma patient tumor samples. High level expression of both of MTDH/AEG1 and HOTAIR in the primary tumor correlated with a likelihood to metastasize. MTDH expression was lower in samples pre-treated with irradiation and/or chemotherapy as compared to those that had not been treated. HOTAIR expression seemed to correlate with the percent necrosis seen in different sarcoma samples.

Conclusions: High levels of both MTDH/AEG-1 and HOTAIR in primary sarcoma are correlated with a high probability of metastasis. By contrast, reduced expression of both MTDH/AEG-1 and HOTAIR is correlated with a good response to treatment in terms of necrosis, suggesting that levels of MTDH and HOTAIR are potential biomarkers for treatment efficacy. Whether we can predict disease progression in sarcoma remains to be seen. Additional study is needed to better define the best clinical application of MTDH/AEG-1 and HOTAIR expression with metastasis and outcome.

Keywords: MTDH/AEG-1; HOTAIR; Sarcoma; Metastasis

Introduction

Sarcomas comprise a heterogeneous group of soft tissue and bone malignant tumors and are generally resistant to chemotherapy in the metastatic setting [1]. Although all sarcomas share a common origin of the embryonal mesoderm, they exhibit a wide array of histologic subtypes, biological diversity, and responsiveness to treatment [2]. Together sarcomas account for just over 10,600 new cases in the United States annually, amounting to less than 1% of all new cancer diagnoses; however, soft tissue sarcoma is more deadly, possibly because the lack of specific symptoms at early disease stages may lead to delays in diagnosis. Surgery is the primary effective treatment for localized disease. For locally advanced and metastatic disease, the vast majority of soft tissue sarcomas have benefited minimally from treatment with traditional chemotherapy, with chemoresistance and metastasis significantly contributing to the failure of sarcoma therapy [3]. Identification of novel biomarkers to predict response to treatments will facilitate the selection of optimal therapeutic regimens that improve patient survival.

MTDH/AEG-1 (also known as AEG-1 or LYRIC) has recently been identified as a causative factor and potentially crucial dual mediator of both cancer cell metastasis and resistance to chemotherapy [4-6]. MTDH/AEG-1 was identified as a potential mediator of breast cancer metastasis to the lung [7]. Later, the genomic copy number gain at chromosome 8q22, which contains the MTDH/AEG-1 gene, was recognized as a defining event in both metastasis and chemoresistance

in breast cancer patients [8], providing evidence for a role for MTDH in both of these oncogenic processes. Furthermore, MTDH/AEG-1 overexpression at the protein level has been correlated with poor outcomes in a number of types of solid tumors, including gliomas [9,10], melanomas, neuroblastomas, carcinomas of the breast [11], prostate cancer [12], hepatocellular cancer [13] and lung cancer [14]. MTDH/AEG-1 is also now known to mediate broad-spectrum resistance to various chemotherapeutics, including 5-fluorouracil (5-FU), doxorubicin, paclitaxel, and cisplatin, by increasing gene expression of drug-metabolizing enzymes and augmenting translation of multidrug resistance gene 1 (MDR1) [15].

Increased expression of HOTAIR in primary breast, liver, and colon tumors has recently emerged as another powerful predictor of eventual metastasis and chemoresistance [16]. HOTAIR is a long

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non-coding RNA (lincRNA) residing in the HOXC locus [17]. The lincRNA HOTAIR serves as a scaffold for at least two distinct histone modification complexes: a 5' domain of HOTAIR binds to the polycomb repressive complex 2 (PRC2), whereas a 3' domain of HOTAIR associates with the LSD1/CoREST/REST complex [18]. Overexpression of HOTAIR in epithelial cancer cells alters histone H3 lysine 27 methylation to promote gene expression and thereby increase cancer invasiveness and metastasis [18]. Conversely, loss of HOTAIR reduces cancer cell viability and invasion and increases sensitivity to cisplatin and doxorubicin. To date, however, the significance of MTDH and HOTAIR expression in sarcoma has not been evaluated. In this pilot study of primary and metastatic sarcoma patients, we explore the expression of MTDH/AEG-1 and HOTAIR in primary and secondary lesions and determine how their levels correlate with metastasis and response to treatment.

Materials and Methods

Ethics statement

Primary and metastatic tumor tissues from sarcoma subjects were collected under a University of Iowa Institutional Review Board approved protocol. Informed written consent was obtained from all participants involved in this study. De-identified sarcoma samples, disease status, and treatments were recorded in Table 1.

Antibodies and Western blotting

The following antibodies were used: anti-MTDH and anti- β -actin antibodies from Sigma (St Louis, MO, USA). Whole-cell protein lysates were prepared and analyzed by Western blotting as previously described [20].

Table 1A

	Control	Group A			
Sample ID	MAST 1035	MAST 1261	MAST 1194	MAST 0931	MAST 0227
Date Collected	01/04/11	05/20/11	04/05/11	10/27/10	06/10/09
Diagnosis Date	12/28/10	4/28/11	3/18/11	8/15/07	5/29/09
Sarcoma pathological subtype	Myxofibro-sarcoma	Synovial Sarcoma	Myxofibro-sarcoma	PNST	Leiomyo-sarcoma
Primary Lesion Site	L Forearm	Thigh	R Thigh	Thigh	L Calf
Sample Lesion Site	L Forearm	L Thigh	R Thigh	Lung	L Calf
Disease Status (Local vs. Metastatic)	Local	Local	Local	Metastatic	Local
Prior Radiation (XRT) to Sample	No	No	No	Yes	No
% Necrosis of Sample	0	0	0	0	0
PRE Collection Treatment	None	1. Excision	None	Excision & XRT to primary	None
Status Post Collect	NED	+ lung met 6/21/11	+ lung met 5/5/11	NED	NED
POST Collection Treatment	2. XRT arm; 3. Obs	3. Ifos/Adria	2. XRT	Obs	2. Gem/Tax
Ratio of MTDH/ β -actin	0	1.04	1.23	0.18	1.22
HotAir (RNA)	1	846	10.5	112	77.2

Table 1B

	Control	Group B			
Sample ID	MAST 1035	MAST 0850	MAST 0703	MAST 1139	MAST 0796
Date Collected	01/04/11	09/17/10	05/18/10	03/29/11	08/13/10
Diagnosis Date	12/28/10	6/15/10	7/9/09	10/4/07	2/17/06
Sarcoma pathological subtype	Myxofibro-sarcoma	Leiomyo-sarcoma	Leiomyo-sarcoma	Leiomyo-sarcoma	Leiomyo-sarcoma
Primary Lesion Site	L Forearm	R Thigh	Uterus	Uterus	L Groin
Sample Lesion Site	L Forearm	R Thigh	Colon	Abdomen	Adrenal gland
Disease Status (Local vs. etastatic)	Local	Local	Metastatic	Met, recurr	Metastatic
Prior Radiation (XRT) to Sample	No	Yes	No	No	Yes
% Necrosis of Sample	0	70%	100%	85%	5%
PRE Collection Treatment	None	1. Cis/Adria/ Mito w/ XRT	1. Hysterect 2. Gem/Tax	1. Hysterect 2. Gem/Tax	1. Gem/ tax; 2.Clin Trial; 3.Doxuribicin; 4.Dacarbazine
Status Post Collect	NED	PD	Concurr Gem/Tax	NED	SD--slow growing
POST Collection Treatment	2. XRT arm; 3. Obs	3.Ifos	3. Gem/Tax; 4. Excision; 5. VPA	4. None, PD	Obs
Ratio of MTDH/ β -actin	0	0.38	0.17	0	0

HotAir (RNA)	1	149	-2.7	78	780
Table 1C					
	Control	Group C			
Sample ID	MAST 1035	MAST 0006	MAST 0268	MAST 0510	
Date Collected	01/04/11	11/19/08	07/10/09	01/22/10	
Diagnosis Date	12/28/10	11/5/08	4/14/09	5/6/09	
Sarcoma pathological subtype	Myxofibro-sarcoma	Lipo-sarcoma, Myxoid	Malignant Fibrous Histiocytoma	Leiomyo-sarcoma	
Primary Lesion Site	L Forearm	Buttock	Thigh	L Leg	
Sample Lesion Site	L Forearm	Buttock	Lung	Lung	
Disease Status (Local vs. Metastatic)	Local	Metastatic	Metastatic	Metastatic	
Prior Radiation (XRT) to Sample	No	No	No	No	
% Necrosis of Sample	0	0	0	0	
PRE Collection Treatment	None	None	1. Above knee amp	1. Amp; 2. Gem/ Tax; 3. Lung resection	
Status Post Collect	NED	+ other met sites	+ lung met 8/25/09	+ lung met 4/30/11	
POST Collection Treatment	2. XRT arm; 3. Obs	None, PD	None, PD	None, PD	
Ratio of MTDH/β-actin	0	0	2.1	1.62	
HotAir (RNA)	1	36.4	0.8	0.7	

Abbreviations: NED: No Evidence of Disease; XRT: Radiation; Obs: Observation; Amp: Amputation; L: Left; R: Right; PD: Progressive Disease; SD: Stable Disease; Gem: Gemcitabine; Ifos: Ifosfamide; Tax: Taxotere (Docetaxel Injection); Adria: Adriamycin

Table 1: Detailed description of disease status and treatments before and after sample collection in three groups of sarcoma subjects. Primary sarcoma subjects were shown in 1A, metastatic sarcoma subjects with reduced MTDH and high HOTAIR were shown in 1B, metastatic sarcoma subjects with variable MTDH and HOTAIR were shown in 1C.

Detection of HOTAIR by real-time PCR

Expression analysis of HOTAIR was carried out by quantitative RT-PCR (q-RT-PCR). RNA was isolated from frozen cancer tissues from sarcoma samples using the *mir*Vana microRNA isolation kit (Ambion, Austin) per the manufacturer's instructions. qRT-PCR was performed with total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad) and Syber Green PCR master mix (Applied Biosystems, CA). The following primers for HOTAIR and 18S rRNA (IDT, Coralville, IA), were used:

HOTAIR Forward primer	5'-GGTAGAAAAAGCAACCACGAAGC-3'
HOTAIR Reverse primer	5'-ACATAAACCTCTGTCTGTGAGT-GCC-3'
18S rRNA Forward primer	5'-GCTTAATTTGACTCAACACGGGA-3'
18S rRNA Reverse primer	5'-AGCTATCAATCTGTCAATCCTGTC-3'

Results

High expression of MTDH and HOTAIR in primary sarcoma may predict high probability of metastasis

To determine the relative expression of MTDH at the protein level (Figure 1) and the lincRNA HOTAIR (Figure 2), we first determined the baseline expression of these factors in a primary sarcoma sample, #1035, that consisted of a high grade primary myxofibrosarcoma which was completely resected without any prior treatment and no evidence

of metastasis at the time of resection or thereafter. This sample was used as the control to assess MTDH and HOTAIR expression in other sarcoma samples. Importantly, there was no detectable MTDH and low levels of HOTAIR (Figures 1,2). Overexpression of MTDH and a greater than 800-fold increase in HOTAIR was detected in a high grade synovial sarcoma sample, #1261. Metastatic disease was detected one month after resection of this sample in the lung. MTDH overexpression and a nearly 10-fold increase in HOTAIR was also observed in a high grade myxofibrosarcoma sample, #1194 with lung metastasis present at diagnosis. Interestingly, a lung metastasis, #0931, in a subject with malignant peripheral nerve sheath tumor that was resected previously demonstrated an overexpression of MTDH and HOTAIR. A sample of the primary lesion was not available for comparison. Sarcoma sample, #0227, from a high grade leiomyosarcoma showed high expression of both MTDH and HOTAIR. However, this subject received adjuvant chemotherapy (Gemcitabine and Taxotere) and thus far shows no evidence of metastasis. A Pearson's product-moment coefficient between MTDH with HOTAIR is 0.259, indicating the correlation of MTDH with HOTAIR in primary sarcoma samples. Based on these it is probable that high grade sarcomas that overexpress MTDH and HOTAIR will likely metastasize. This population could be the target of more aggressive therapy (Table 1A).

Samples from select sarcoma subjects pretreated with radiation and/or chemotherapy demonstrated a reduction in MTDH expression and maintained a relatively high HOTAIR expression compared to the control that correlated with the percent necrosis on hematoxylin and eosin (HE) stain

Sample #0850, a high grade pleomorphic leiomyosarcoma,

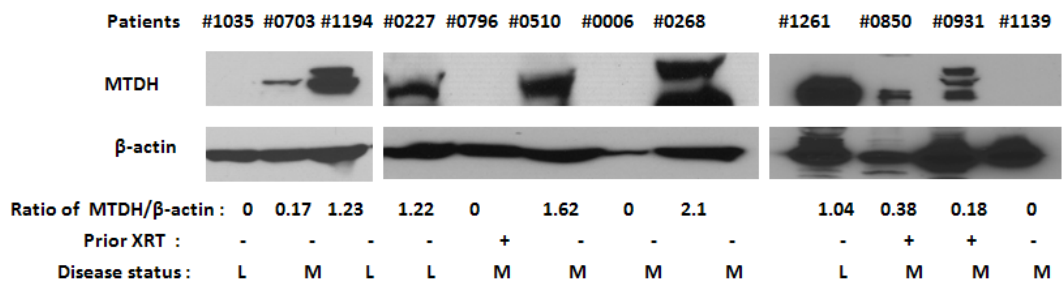


Figure 1: Expression of MTDH in primary or metastatic cancer tissues from sarcoma samples. Expression of MTDH at the protein level was detected by Western blotting in cancer tissues from twelve subjects with local (L) or metastatic (M) sarcoma. β -actin serves as a loading control.

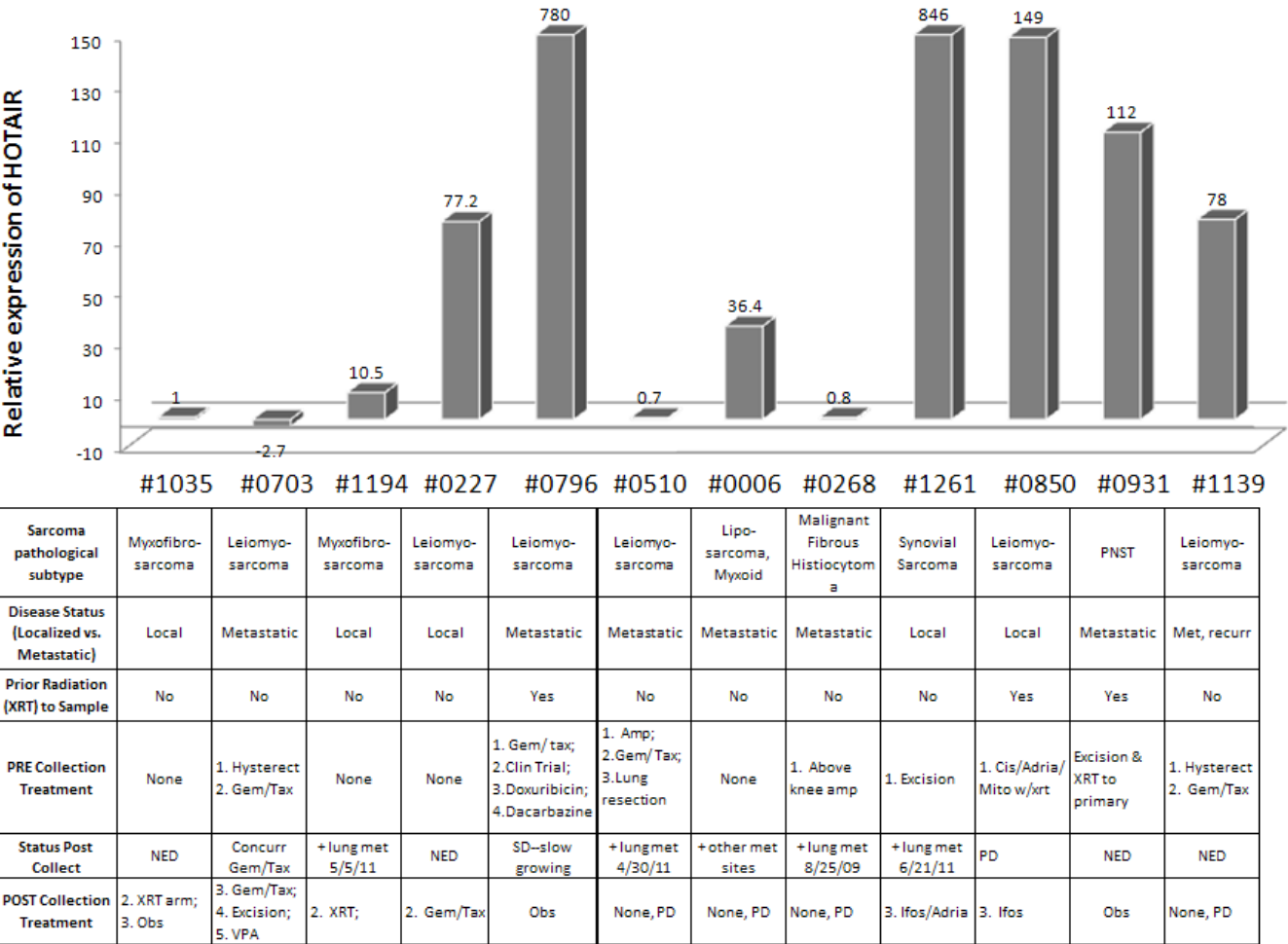


Figure 2: Expression of HOTAIR in primary or metastatic cancer tissues from sarcoma subjects. Expression of the long noncoding RNA HOTAIR was detected by qRT-PCR in cancer tissues from twelve subjects with primary or metastatic sarcoma. Samples are normalized to 18S rRNA.

treated with radiation and chemotherapy (Mitomycin C, Adriamycin, Cisplatin) showed a low MTDH expression and persistent HOTAIR expression. This subject later developed lung metastasis. Sample #0703, a high grade uterine leiomyosarcoma that received chemotherapy (Gemcitabine, Taxotere) showed an absence of MTDH and HOTAIR

expression and a 100% necrosis. Sample #1139, a high grade uterine leiomyosarcoma, was resected after the subject received chemotherapy (Gemcitabine, Taxotere) and showed absent MTDH expression and persistent HOTAIR expression. Sample #0796 was a high grade leiomyosarcoma metastasis in an adrenal gland that received radiation

therapy prior to resection and again demonstrated absent MTDH with robust HOTAIR expression. Interestingly there was only 5% necrosis in this sample. As shown in Figure 3, expression of HOTAIR is reverse correlated with the percentage of necrosis after treatments in group B samples. The pearson's product-moment coefficient is -0.992 (Table 1B).

Three sarcoma samples expressed variable levels of both MTDH and HOTAIR

Sample #0006 is a low grade myxoid liposarcoma that was metastatic at diagnosis and showed an absent expression of MTDH and a slightly high expression of HOTAIR. This is the only low grade metastatic lesion assessed and may explain the low MTDH level, implying that grade may predict different levels of expression of MTDH. Interestingly two sarcoma samples, #0268 and #0510, obtained from lung metastasis demonstrated very high MTDH and low HOTAIR. One sample from the lung, #0931, demonstrated both overexpression of MTDH and HOTAIR. These cases imply that lung metastatic lesions persistently express high levels of MTDH (Table 1C).

Discussion

MTDH/AEG1 and HOTAIR are two novel metastasis-related oncogenes overexpressed in breast cancer and other solid tumors [20,21], yet their roles in sarcoma metastasis and response to treatment was previously not assessed. Our pilot study shows that overexpression of both MTDH/AEG1 and HOTAIR correlates with a high probability for metastasis in primary sarcoma subjects. The fact that, in some subjects, either MTDH or HOTAIR is overexpressed in metastatic tissue samples indicates that it is possible for either MTDH/AEG1 or HOTAIR alone to serve as an independent prognostic marker for metastasis. However, some subjects with high MTDH/AEG-1 or HOTAIR expression in the collected sample demonstrated a favorable response to treatment or no evidence of disease after treatment. It will be interesting to explore whether these post-collection treatments efficiently inhibit MTDH-and HOTAIR-mediated metastasis and cancer development in these subjects.

Compared to samples from subjects who did not receive any form of treatment, be it chemotherapy or irradiation, undetectable or reduced levels of MTDH was observed in samples from subjects that had been treated. Our clinical observations indicate that both chemotherapy and/or radiation may promote down-regulation of MTDH in sarcoma

subjects. However, without evaluation of the levels of MTDH in tumors before and after treatment, we cannot definitively conclude that radiation or chemo alters MTDH expression. Future *in vitro* cell culture and *in vivo* animal model studies of the effect of irradiation on the levels of MTDH will help to address this important question. It was interesting to see an expression level of HOTAIR that correlated with the percent necrosis in tumor samples exposed to chemotherapy and/or radiation, being high when minimal necrosis is present (#0796) or low with maximum necrosis (#0703) present. Long-term follow-up to evaluate response to treatment as well as to monitor the expression of MTDH/AEG-1 and HOTAIR in recurrent disease will determine the effect of MTDH/AEG-1 and HOTAIR levels on success of therapeutic treatments. Study of a large number of subjects paired with long-term evaluation is critical to elucidate whether MTDH/AEG1 and HOTAIR levels correlate with metastasis and response to therapeutic treatments.

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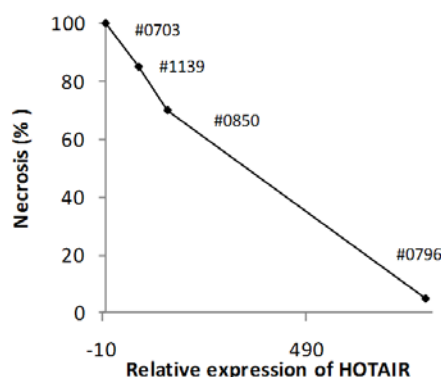


Figure 3: Correlation of the expression level of HOTAIR with the percent necrosis in group B tumor samples. The pearson's product-moment coefficient is -0.992.

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