RESEARCH NOTE

Epigenetic silencing of IncRNA MORT in 16 TCGA cancer types
[version 1; peer review: 3 approved]

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1,2

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Abstract

We have previously described a hominid-specific long non-coding RNA, MORT (also known as ZNF667-AS1, Gene ID: 100128252), which is expressed in all normal cell types, but epigenetically silenced during cancer-associated immortalization of human mammary epithelial cells. Initial analysis of The Cancer Genome Atlas (TCGA) showed that 15 of 17 cancer types, which represent the 10 most common cancers in women and men, display DNA methylation associated silencing in a large fraction of their tumors. In this study we analyzed MORT expression and DNA methylation state in the remaining 16 TCGA cancer types not previously reported. Seven of the 16 cancer types showed DNA methylation linked MORT silencing in a large fraction of their tumors. These are carcinomas (cervical cancer, and cancers of esophagus, stomach, and bile duct), and the non-epithelial tumors mesothelioma, sarcoma, and uterine carcinosarcoma. Together with the findings from our previous report, MORT expression is silenced by aberrant DNA methylation in 22 of 33 of TCGA cancer types. These 22 cancers include most carcinoma types, blood derived cancers and sarcomas. In conclusion, results suggest that the MORT gene is one of the most common epigenetic aberrations seen in human cancer. Coupled with the timing of MORT gene silencing during in vitro epithelial cell immortalization and its occurrence early in the temporal arc of human carcinogenesis, this provides strong circumstantial evidence for a tumor suppressor role for MORT.

Keywords
DNA Methylation, Gene Silencing, IncRNA, ncRNA, lncRNA, MORT, ZNF667-AS1, Epigenetics
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Author roles: Vrba L: Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Software, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; Futscher BW: Funding Acquisition, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

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Introduction

*MORT* was originally found as a transcript silenced during *in vitro* immortalization of human mammary epithelial cells\(^1\). Like a significant majority of lncRNAs, *MORT*’s molecular function remains enigmatic. The *MORT* gene is specific to higher primates, is expressed in all normal human cell types, and *MORT* RNA is located predominantly in the cytoplasm\(^1\). Analysis of *MORT* expression and the DNA methylation state of its promoter in 17 cancer types from The Cancer Genome Atlas (TCGA)\(^2\), which represent the 10 most frequent cancers in males and females, showed *MORT* is epigenetically silenced in 15 of 17 these cancers\(^1\). Based on the data from the original *in vitro* study\(^1\), we predicted epigenetic *MORT* silencing occurs early in human carcinogenesis and therefore could be seen in premalignant lesions, such as ductal carcinoma *in situ* of the breast and colonic adenomas. We used data from clinical samples from published genomic data sets\(^3\) to address this possibility, and indeed, *MORT* loss occurs prior to or at the stage of pre-malignancy and not thereafter\(^6\). Taken together these facts suggest that *MORT* transcript has a tumor suppressive role and is not simply an epigenetic “passenger error.”

Since our previous analysis of *MORT* in TCGA datasets was not exhaustive and only reported on 17 out of 33 TCGA cancer types, the goal of this short study was to extend our earlier work and complete the analysis of *MORT* DNA methylation associated gene silencing in the final 16 TCGA cancer types.

Methods

We integrated the *MORT* expression level and the DNA methylation state of its promoter region using TCGA data as described before\(^1\). The Illumina HiSeq RNA-seq and HumanMethylation450 DNA methylation data for samples of 16 TCGA cancer types listed in Table 1 were downloaded from the GDC data portal. The data were analyzed in the R programming environment, version 3.4.2\(^10\). The mean RNA-Seq rpkm values for the two exons constituting the *MORT* RNA were plotted against the mean DNA methylation beta value of the 7 CpGs from the *MORT* promoter region for the individual samples of each cancer type. The Spearman correlation coefficient rho between *MORT* expression and promoter DNA methylation in the remaining nine cancer types is also negative; however, the maximum level of the DNA methylation

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Table 1. The 16 TCGA cancer types analyzed in this study. The numbers of primary tumor and normal samples for which both the *MORT* RNA expression and the *MORT* promoter DNA methylation data were available are listed. *DNA methylation data from HumanMethylation27 platform that covers 2 CpGs out of 7 CpGs covered by HumanMethylation450 were used.*

<table>
<thead>
<tr>
<th>TCGA Cancer Type Name</th>
<th>Abbreviation</th>
<th>Tumor samples</th>
<th>Normal samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>adrenocortical carcinoma</td>
<td>ACC</td>
<td>79</td>
<td>0</td>
</tr>
<tr>
<td>cervical squamous cell carcinoma and endocervical adenocarcinoma</td>
<td>CESC</td>
<td>304</td>
<td>3</td>
</tr>
<tr>
<td>cholangiocarcinoma</td>
<td>CHOL</td>
<td>36</td>
<td>9</td>
</tr>
<tr>
<td>esophageal carcinoma</td>
<td>ESCA</td>
<td>184</td>
<td>9</td>
</tr>
<tr>
<td>glioblastoma multiforme</td>
<td>GBM</td>
<td>51</td>
<td>1</td>
</tr>
<tr>
<td>kidney chromophobe</td>
<td>KICH</td>
<td>66</td>
<td>0</td>
</tr>
<tr>
<td>brain lower grade glioma</td>
<td>LGG</td>
<td>516</td>
<td>0</td>
</tr>
<tr>
<td>mesothelioma</td>
<td>MESO</td>
<td>87</td>
<td>0</td>
</tr>
<tr>
<td>ovarian serous cystadenocarcinoma</td>
<td>OV*</td>
<td>295</td>
<td>0</td>
</tr>
<tr>
<td>pheochromocytoma and paraganglioma</td>
<td>PCPG</td>
<td>179</td>
<td>3</td>
</tr>
<tr>
<td>sarcoma</td>
<td>SARC</td>
<td>259</td>
<td>0</td>
</tr>
<tr>
<td>stomach adenocarcinoma</td>
<td>STAD</td>
<td>373</td>
<td>0</td>
</tr>
<tr>
<td>testicular germ cell tumors</td>
<td>TGCT</td>
<td>150</td>
<td>0</td>
</tr>
<tr>
<td>thymoma</td>
<td>THYM</td>
<td>120</td>
<td>2</td>
</tr>
<tr>
<td>uterine carcinosarcoma</td>
<td>UCS</td>
<td>57</td>
<td>0</td>
</tr>
<tr>
<td>uveal melanoma</td>
<td>UVM</td>
<td>80</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 1. Integration of the MORT expression and the MORT promoter DNA methylation TCGA data for 16 tumor types. The x-axis shows the MORT expression level according to RNA-seq and y-axis shows the level of MORT promoter DNA methylation according to Illumina HumanMethylation450 microarray. The correlation coefficient rho between the MORT expression and the DNA methylation of MORT promoter for each tumor type is displayed. The OV has a very low number (10) of samples analyzed by the HumanMethylation450 platform, therefore the data from the HumanMethylation27 platform that covers 2 CpGs out of 7 CpGs covered by HumanMethylation450 were used.
of MORT promoter in some of these cancers is either very low (UVM), or a very few tumor samples have MORT silenced (ACC, KICH, OV, and THYM), and some of the cancer types (GBM, LGG, PCPG, and TGCT) do not appear to display MORT gene silencing (Figure 1).

The analysis presented shows DNA methylation associated MORT gene silencing in 7 of 16 TCGA cancer types. Compared to the 17 TCGA cancer types presented in our original study, most the 16 cancer types presented here lack their respective normal tissues samples and some of them have lower amounts of tumor samples (Table 1). Nevertheless, the distribution of MORT expression and DNA methylation data in tumor samples clearly indicates MORT silencing in multiple cancer types (Figure 1).

Cervical tumors (CESC) have high proportion of MORT silencing (Figure 1); more than 75% of 304 cervical tumor samples have MORT promoter DNA hypermethylated and MORT silenced. Using TCGA data, a recent study found MORT downregulated in cervical cancer\(^1\), but surprisingly did not report on or hypothesize potential mechanisms for this transcriptional repression. Here we confirm and extend their initial analysis of MORT silencing in cervical cancer and show further that this silencing is strongly linked to aberrant DNA methylation of the MORT promoter.

Combined together with the findings from our previous report\(^1\), Table 2 shows MORT is silenced by DNA methylation in a super majority of TCGA cancer types (22 of 33). MORT loss occurs predominantly due to epigenetic silencing and increased DNA methylation of its promoter in breast cancer\(^1\). This could likely be extended to all 22 cancer types with the high fraction of MORT negative samples and the high correlation between MORT RNA level and MORT promoter DNA methylation, where MORT likely plays a tumor suppressive role. The other 11 cancer types, with a little to no MORT silencing, might have tumor suppressive pathway, where MORT is involved, interrupted elsewhere and/or MORT may play some additional vital role in tissues these tumors originate from - e.g. prostate, thyroid, brain, testes, or ovary - since these tissues typically have the highest levels of MORT RNA\(^1\).

In summary, our findings show that the MORT gene is one of the most common epigenetic aberrations seen in human cancer. Coupled together with MORT silencing occurring early in the

### Table 2. Summary of MORT silencing in all 33 TCGA cancer types.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>TCGA cancer type name</th>
<th>MORT silencing</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC</td>
<td>adrenocortical carcinoma</td>
<td>No*</td>
</tr>
<tr>
<td>BLCA</td>
<td>bladder urothelial carcinoma</td>
<td>Yes**</td>
</tr>
<tr>
<td>BRCA</td>
<td>breast invasive carcinoma</td>
<td>Yes**</td>
</tr>
<tr>
<td>CESC</td>
<td>cervical squamous cell carcinoma and endocervical adenocarcinoma</td>
<td>Yes*</td>
</tr>
<tr>
<td>CHOL</td>
<td>cholangiocarcinoma</td>
<td>Yes*</td>
</tr>
<tr>
<td>COAD</td>
<td>colon adenocarcinoma</td>
<td>Yes**</td>
</tr>
<tr>
<td>DLBC</td>
<td>lymphoid neoplasm diffuse large b-cell lymphoma</td>
<td>Yes**</td>
</tr>
<tr>
<td>ESCA</td>
<td>esophageal carcinoma</td>
<td>Yes*</td>
</tr>
<tr>
<td>GBM</td>
<td>glioblastoma multiforme</td>
<td>No*</td>
</tr>
<tr>
<td>HNSC</td>
<td>head and neck squamous cell carcinoma</td>
<td>Yes**</td>
</tr>
<tr>
<td>KICH</td>
<td>kidney chromophobe</td>
<td>No*</td>
</tr>
<tr>
<td>KIRC</td>
<td>kidney renal clear cell carcinoma</td>
<td>Yes**</td>
</tr>
<tr>
<td>KIRP</td>
<td>kidney renal papillary cell carcinoma</td>
<td>Yes**</td>
</tr>
<tr>
<td>LAML</td>
<td>acute myeloid leukemia</td>
<td>Yes**</td>
</tr>
<tr>
<td>LGG</td>
<td>brain lower grade glioma</td>
<td>No*</td>
</tr>
<tr>
<td>LIHC</td>
<td>liver hepatocellular carcinoma</td>
<td>Yes**</td>
</tr>
<tr>
<td>LUAD</td>
<td>lung adenocarcinoma</td>
<td>Yes**</td>
</tr>
<tr>
<td>LUSC</td>
<td>lung squamous cell carcinoma</td>
<td>Yes**</td>
</tr>
<tr>
<td>MESO</td>
<td>mesothelioma</td>
<td>Yes*</td>
</tr>
<tr>
<td>OV</td>
<td>ovarian serous cystadenocarcinoma</td>
<td>No*</td>
</tr>
<tr>
<td>PAAD</td>
<td>pancreatic adenocarcinoma</td>
<td>Yes**</td>
</tr>
<tr>
<td>PCPG</td>
<td>pheochromocytoma and paraganglioma</td>
<td>No*</td>
</tr>
<tr>
<td>PRAD</td>
<td>prostate adenocarcinoma</td>
<td>No**</td>
</tr>
<tr>
<td>READ</td>
<td>rectum adenocarcinoma</td>
<td>Yes**</td>
</tr>
<tr>
<td>SARC</td>
<td>sarcoma</td>
<td>Yes*</td>
</tr>
<tr>
<td>SKCM</td>
<td>skin cutaneous melanoma</td>
<td>Yes**</td>
</tr>
<tr>
<td>STAD</td>
<td>stomach adenocarcinoma</td>
<td>Yes*</td>
</tr>
<tr>
<td>TGCT</td>
<td>testicular germ cell tumors</td>
<td>No*</td>
</tr>
<tr>
<td>THCA</td>
<td>thyroid carcinoma</td>
<td>No**</td>
</tr>
<tr>
<td>THYM</td>
<td>thymoma</td>
<td>No*</td>
</tr>
<tr>
<td>UCEC</td>
<td>uterine corpus endometrial carcinoma</td>
<td>Yes**</td>
</tr>
<tr>
<td>UCS</td>
<td>uterine carcinosarcoma</td>
<td>Yes*</td>
</tr>
<tr>
<td>UVM</td>
<td>uveal melanoma</td>
<td>No*</td>
</tr>
</tbody>
</table>
temporal arc of human carcinogenesis it strongly supports a tumor suppressive role for MORT.

Data availability
Illumina HiSeq RNA-seq and HumanMethylation450 DNA methylation data for TCGA cancer types used in the present study can be downloaded from the GDC data portal.

Competing interests
No competing interests were disclosed.

References

2. NCi, NHGRI, NIH: The Cancer Genome Atlas. NIH, National Cancer Institute, National Human Genome Research Institute, 2016. Reference Source

Grant information
This work was supported by the Maynard Chair in Breast Cancer Epigenomics at the University of Arizona Cancer Center and the Cancer Center Support Grant (P30 CA023074).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgments
The results shown here are based upon data generated by the TCGA Research Network: http://cancergenome.nih.gov/.
Open Peer Review

Current Peer Review Status: ✔️ ✔️ ✔️

Version 1

Reviewer Report 03 April 2018

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Alexander Dobrovic

Translational Genomics and Epigenomics Laboratory, Olivia Newton-John Cancer Research Institute, Heidelberg, Vic, Australia

This brief article provides an analysis of MORT epigenetic silencing across 16 TCGA tumour types not previously reported by the authors enabling assessment across all TCGA tumour types. MORT silencing seems to be a key early event in carcinogenesis. This indicates that further studies of MORT biology are required as well as investigation of MORT methylation as a potential biomarker.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: DNA methylation biomarkers, liquid biopsies, digital PCR
I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 03 April 2018

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Gwen Lomberk
Division of Research, Department of Surgery, Medical College of Wisconsin, Milwaukee, WI, USA

STRENGTHS: The authors utilize the TCGA database to extend their previous studies on MORT that has been primarily characterized in breast cancer, but also observed to undergo silencing in 15 out of the 17 most common cancers. The current work takes this analysis deeper into the 33 TCGA cancer types and perform a more thorough analysis of the DNA methylation associated with the 16 cancer types evaluated here.

SUGGESTIONS FOR IMPROVEMENT:

- The authors make a bold statement that “the MORT gene is one of the most common epigenetic aberrations seen in human cancer”. This statement is not supported by the data presented. In order to make a statement of this level, the authors would have to provide comparisons to other documented genes that undergo a high frequency of epigenetic alteration.
- This statement becomes more difficult to make when considering that several of the tumors present do not have normal counterparts for comparison. We therefore do not know if those tissue types whether the DNA methylation is abnormal or typical for the tissue type.
- Authors should consider revising some of the scales on the graphs in Figure 1 to highlight the lack of methylation (for example, in testicular germ cell tumors). Similarly, the cutoff for methylation could be shown by a dotted line or similar feature.
- The location of methylation relative to the MORT gene would be informative. Of the tumor types that are methylated, are the same regions methylated across types?
- The authors should be cautious not to overstate their conclusions throughout the text since the conclusions are speculative without experiments to support their statements. Nevertheless, these correlations present interesting speculation and avenues for further investigations.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes
Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

**Competing Interests:** No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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**Reviewer Report 01 March 2018**

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Adam Karpf

Eppley Institute and Fred & Pamela Buffett Cancer Center, University of Nebraska Medical Center, Omaha, NE, USA

The authors present a straightforward study of DNA methylation and RNA expression of the lncRNA MORT in 16 TCGA cancer types not previously reported. Most of the paper is clearly presented and the conclusions are reasonable. Suggestions for improvement are presented below.

1) Statements are made regarding MORT being “one of the most common” epigenetic aberrations seen in human cancer. No support for this statement is presented.
2) The classification of some tumor types as showing “strong” MORT silencing by DNA methylation, and other tumor types as not showing this, is arbitrary. A quantitative definition is needed.
3) Fig 1 might be clearer if presented in two panels, subdivided by whether the quantitative definition of “strong silencing” is met. The same is true for Table 2, which could be subdivided into two parts.
4) Since normal samples were not available for many of the tumors profiled, a more accurate statement might be “DNA methylation regulation” rather than “DNA methylation silencing,” as reduced expression in tumors as compared to normal tissues was not shown.
5) A diagram showing the MORT gene and the position of the methylation sites examined by the TCGA would be helpful.
6) Can the authors reference any data, e.g. from other publications, showing that DNA methylation actively suppresses MORT expression, by for example turning on MORT expression using treatment with a DNA methyltransferase inhibitor?
7) In the introduction, the statement “prior to or at the stage of pre-malignancy and not thereafter” is
confusing, and gives the impression that MORT becomes hypomethylated, or its expression is elevated, in tumors as compared to pre-malignant lesions. A better way to phrase this would be to say e.g. that “MORT is repressed in pre-malignant lesions and remains repressed in tumors.”

8) The final sentence in the discussion uses the wording “strongly supports” when “suggests” would be more accurate, given the absence of functional data showing tumor suppression by MORT.

Is the work clearly and accurately presented and does it cite the current literature?  
Yes

Is the study design appropriate and is the work technically sound?  
Yes

Are sufficient details of methods and analysis provided to allow replication by others?  
Yes

If applicable, is the statistical analysis and its interpretation appropriate?  
Partly

Are all the source data underlying the results available to ensure full reproducibility?  
Yes

Are the conclusions drawn adequately supported by the results?  
Partly

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.