



BRIEF COMMUNICATION

Epigenetic Silencing of *MORT* Is an Early Event in Cancer and Is Associated with Luminal, Receptor Positive Breast Tumor Subtypes

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Immortality is an essential characteristic of cancer cells; a recent transcriptomic study of epithelial cell immortalization has linked epigenetic silencing of the long noncoding RNA *Mortal Obligate RNA Transcript (MORT; alias ZNF667-AS1)* to this process. This study evaluated the epigenetic and transcriptional state of *MORT* in two premalignant conditions—ductal carcinomas *in situ* and colon adenomas. Results show that *MORT* silencing is an early epigenetic event in human carcinogenesis, likely occurring near the point where premalignant cells gain immortality; this epigenetic silencing is maintained throughout malignant transformation and metastatic growth. Additional associations

between *MORT* loss and clinical and molecular features of breast tumors showed that silencing of *MORT* occurs predominantly in luminal, receptor-positive breast cancer; is associated with overexpression of *CCND1* and mutations of *GATA3*; and is negatively correlated with *TP53* mutations. Taken *in toto*, *MORT* silencing occurs early in breast carcinogenesis, probably during cellular immortalization, and precedes the development of invasive luminal breast cancer.

Key Words: Breast neoplasms, Carcinogenesis, DNA methylation, Gene silencing, Long noncoding RNA

Immortality is an essential characteristic of cancer cells; our recent transcriptomic study of *in vitro* immortalization of human mammary epithelial cells (HMEC) reported on the epigenetic silencing of the long noncoding RNA (lncRNA) *Mortal Obligate RNA Transcript (MORT; alias ZNF667-AS1)* during the transition of the cells from mortal to immortal [1]. *MORT* is expressed in all normal, mortal human cell types analyzed to date, but is epigenetically silenced in the majority of human cancers and human cancer cell lines [1]; there is little evidence suggesting genetic mechanisms of inactivation. The experimental finding that *MORT* is silenced at the mortal-immortal boundary of HMEC, prior to and independent of malignant conversion, suggests that *MORT* silencing is an early epigenetic event in human carcinogenesis that occurs prior to malignant transformation. Since a large fraction of clinical carcinoma *in situ* lesions—including ductal carcinoma *in situ* (DCIS)

[2]—show evidence of immortality in the absence of a frank malignancy [3-5], it is predicted that the epigenetic silencing of *MORT* is a common feature of this premalignant state. Breast cancer is a heterogeneous disease, but our previous analysis was not focused on *MORT* silencing in individual breast cancer subtypes and could not reveal if *MORT* silencing was an early event in clinical carcinogenesis.

Using molecular and clinical data from publicly accessible databases, we set out to evaluate the epigenetic and transcriptional status of *MORT* in premalignant tumors. Consistent with previous laboratory studies, our data analysis shows that DNA methylation-mediated silencing of *MORT* is found in both breast DCIS and colonic adenomas; this moves the timing of *MORT* epigenetic silencing further back to clinical premalignant conditions. The epigenetic silencing of *MORT* in premalignancy is maintained throughout progression towards the invasive and metastatic disease; no evidence was found for genetic mechanisms of *MORT* silencing. The DNA methylation-mediated silencing of *MORT* is significantly associated with luminal, receptor positive breast cancer; in contrast, basal triple negative breast cancers do not generally display *MORT* methylation or gene silencing. These early epigenetic events and the cellular mechanisms that they influence may provide new targets for cancer prevention or treatment.

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DNA methylation (Illumina HumanMethylation450), gene expression (RNASeqV2), genetic (copy number variation, mutation), and available clinical data for breast tumor samples were downloaded from The Cancer Genome Atlas (TCGA). All data were analyzed with the R programming software [6] using custom scripts. *MORT* mean β values and mean reads per kilobase per million (RPKM) values were calculated as described previously [1]. Only nonsilent mutations were considered in the gene mutation analysis. The Spearman's rank correlation coefficient (ρ) was calculated using the `cor.test` function. All tests between groups were performed using the `wilcox.test` function. When appropriate, multiple testing corrections were performed according to Benjamini and Hochberg [7]. PAM50 tumor subtype classification was used as described previously [8]. In addition to TCGA, further Illumina HumanMethylation450 datasets for breast (GSE60185 [9], GSE66313 [10], and GSE58999 [11]) and colon (GSE48684 [12] and GSE77954 [13]) cancer samples were downloaded from Gene Expression Omnibus (GEO). Where necessary, M

values were converted to β values using the formula $2^M / (1+2^M)$. The reduced representation bisulfite sequencing (RRBS) and RNA-seq DCIS datasets (GSE69993 and GSE69240 [14]) were downloaded from GEO. For RRBS, the mean methylation value for the 19 covered CpGs within the *MORT* CpG island promoter was used for analysis.

We have previously reported that *MORT* is epigenetically silenced by DNA methylation in breast cancer and 14 out of 16 other common human tumors investigated. To determine the potential timing of *MORT* silencing during human breast carcinogenesis, we analyzed the DNA methylation state of the *MORT* promoter in DCIS samples from two published, independent cohorts [9,10]. The results show a significant increase in DNA methylation of the *MORT* promoter in DCIS compared with normal samples (Figure 1A, B); *MORT* methylation did not appear to increase further during progression from DCIS to invasive breast cancer (IBC) (Figure 1A), nor during progression to the metastatic disease, as illustrated by data from a cohort [11] of pairs of primary and metastatic

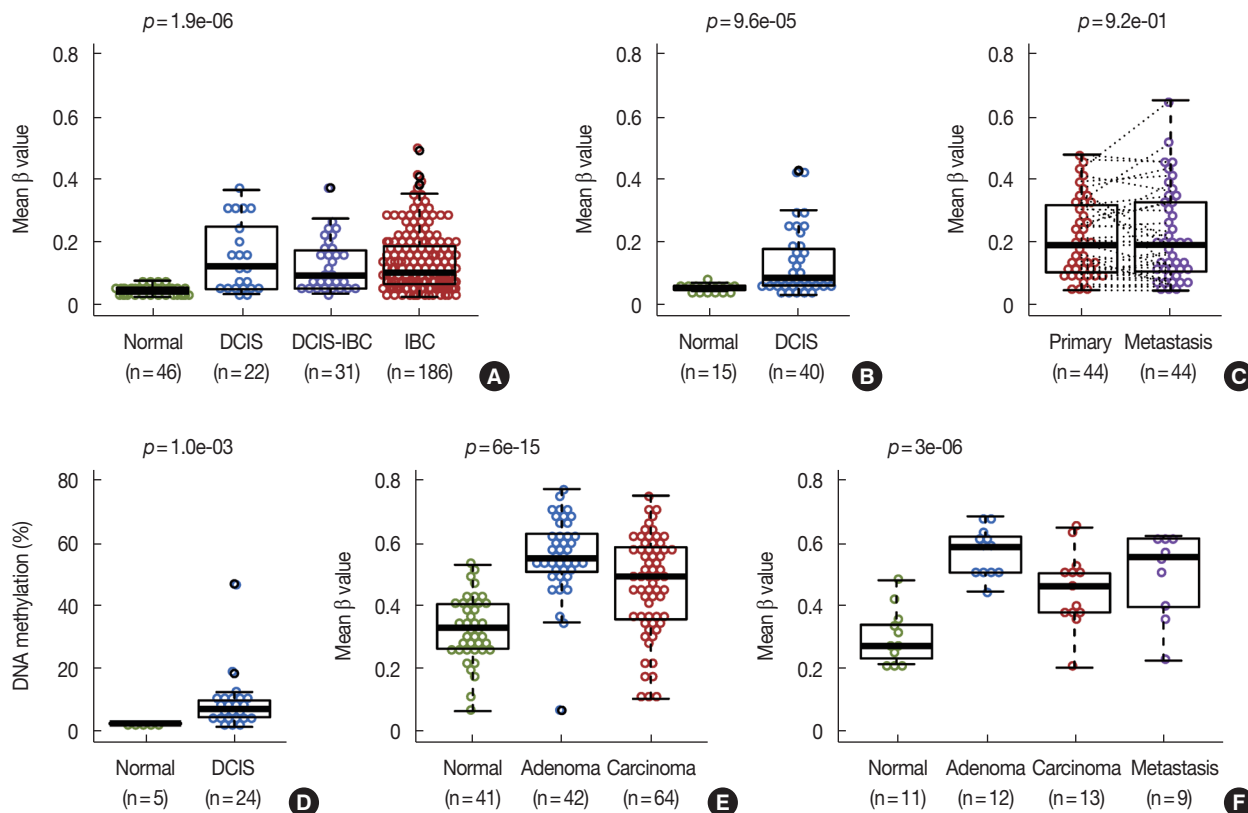


Figure 1. *MORT* gene silencing is early event in cancer. (A, B) DNA methylation of the *MORT* promoter in two independent cohorts of breast ductal carcinoma *in situ* (DCIS) patients. (C) DNA methylation of the *MORT* promoter in 44 pairs of primary breast tumors and lymph node metastasis. (D) DNA methylation of the *MORT* promoter as determined by reduced representation bisulfite sequencing in a DCIS cohort. (E, F) DNA methylation of the *MORT* promoter in two independent cohorts of colon adenomas and carcinomas. All data except (D) were obtained using the Illumina Human Methylation 450 platform. Where more than two groups are presented the p -value is for *in situ* lesions versus normal samples. IBC=invasive breast cancer.

breast tumors (Figure 1C). A third cohort of DCIS, analyzed by RRBS and RNA-seq [14], also showed a significant increase in *MORT* methylation (Figure 1D) and *MORT* silencing in a substantial portion of the samples (Supplementary Figure 1, available online). Together, these results show that *MORT* silencing is an early epigenetic event that occurs during human breast carcinogenesis.

We extended our analysis of premalignant lesions and analyzed the DNA methylation state of the *MORT* promoter in two independent cohorts of colon adenoma samples [12,13]. Although not equivalent to DCIS, colonic adenomas are early premalignant lesions in colorectal cancer and therefore might display *MORT* silencing if *MORT* silencing occurs early in carcinogenesis. The DNA methylation level of the *MORT* promoter in both adenoma cohorts was significantly higher than in normal colon samples; there were no further increases in *MORT* methylation throughout progression of the adenoma to invasive colon cancer, similar to the results for DCIS and IBC (Figure 1E, F). These results broaden the range of premalignant conditions where *MORT* is silenced and provide additional evidence that *MORT* silencing is an early epigenetic event in human carcinogenesis.

Because breast cancer is a heterogeneous disease, we sought to identify any associations between *MORT* loss and other clinical and molecular features of breast tumors. Figure 2A presents analysis performed to assess whether gene deletion is an additional mechanism of *MORT* loss in cancer, in addition to aberrant DNA methylation. The results show that *MORT* loss rarely occurs due to gene deletion (Figure 2A). *MORT* loss is therefore predominantly due to epigenetic silencing and increased DNA methylation of the *MORT* promoter; *MORT* promoter methylation is a good indicator of *MORT* silencing that could be used as a surrogate for *MORT* loss in samples where expression data are not available. When we tested the association between *MORT* methylation and clinical variables, we found a strong link between *MORT* methylation and the presence of hormone receptors. Tumors positive for individual receptors have a much higher frequency of *MORT* promoter methylation (Figure 2B-D) and *MORT* silencing (Supplementary Figure 2A-C, available online). In contrast, triple negative tumors have a low frequency of *MORT* methylation (Figure 2E) and near normal *MORT* RNA levels (Supplementary Figure 2D, available online). These data predict that *MORT* is predominantly silenced in luminal breast cancers; indeed, when we classified the breast tumors into subtypes according to PAM50 [15], luminal B, luminal A, and human epidermal growth factor receptor 2 (HER2)-enriched tumors had a large proportion of *MORT* methylation, while basal-like tumors showed no significant difference in

MORT methylation or expression compared to normal control samples (Figure 2F, Supplementary Figure 2E, available online). Since some breast cancer subtypes are linked to age, we analyzed the relationship between *MORT* level and patient age. There is a moderate but notable increase in *MORT* methylation and decrease in *MORT* expression in tumors from patients over 60 years old (Figure 2G, Supplementary Figure 2F, available online), which is consistent with evidence of higher incidence of luminal tumors in older women [16]. Together, these results indicate that epigenetic silencing of *MORT* occurs predominantly in luminal, receptor-positive breast cancers.

We investigated potential associations between *MORT* methylation and genetic features in breast tumors. When we correlated *MORT* methylation with the copy number variation data on the whole genome scale, amplification of a locus containing genes *CCND1* and *ORAOV1* on chromosome 11 showed the strongest positive correlation ($\rho = 0.28$, *adj. p* = $4.95e-11$) with *MORT* promoter methylation (Figure 2H). Of these two amplified genes, only the *CCND1* transcript level was significantly negatively correlated with the *MORT* transcript level ($\rho = -0.35$, *adj. p* = $8.01e-31$). *CCND1* is a known cancer driver linked to luminal tumors [8] and therefore its overexpression, which inactivates the RB pathway [17], might be also implicated in *MORT* silencing. Finally, we analyzed the links between *MORT* methylation and mutations in 23 genes frequently mutated in breast cancer [8]. Mutations in two of these genes were strongly linked to the level of *MORT* methylation. Mutation of *TP53* is negatively correlated to *MORT* methylation (Figure 2I)—tumors carrying a *TP53* mutation usually have normal levels of *MORT* (Supplementary Figure 2G, available online). *GATA3* is frequently mutated in tumors that have reduced *MORT* expression (Supplementary Figure 2H, available online)—there is a positive correlation between *GATA3* mutation and *MORT* methylation (Figure 2J). This is consistent with evidence showing that *TP53* mutations are enriched in basal-like triple negative tumors, while *GATA3* mutations are found predominantly in luminal tumors [8]. Overall, these findings suggest that *MORT* silencing in luminal breast tumors is connected to overexpression of *CCND1*. The lack of *MORT* silencing in basal tumors that frequently carry *TP53* mutations indicates that *MORT* might act in a pathway that requires functional *TP53*, as the cells with nonfunctional *TP53* can “afford” to express *MORT* and become immortal. The dissociation between *TP53* mutations and *MORT* silencing is consistent with the *in vitro* immortalization model used in the discovery of *MORT*, where the immortal HMEC retained wild-type *TP53* [18].

Overall, our analysis found that, as predicted from the *in vitro*

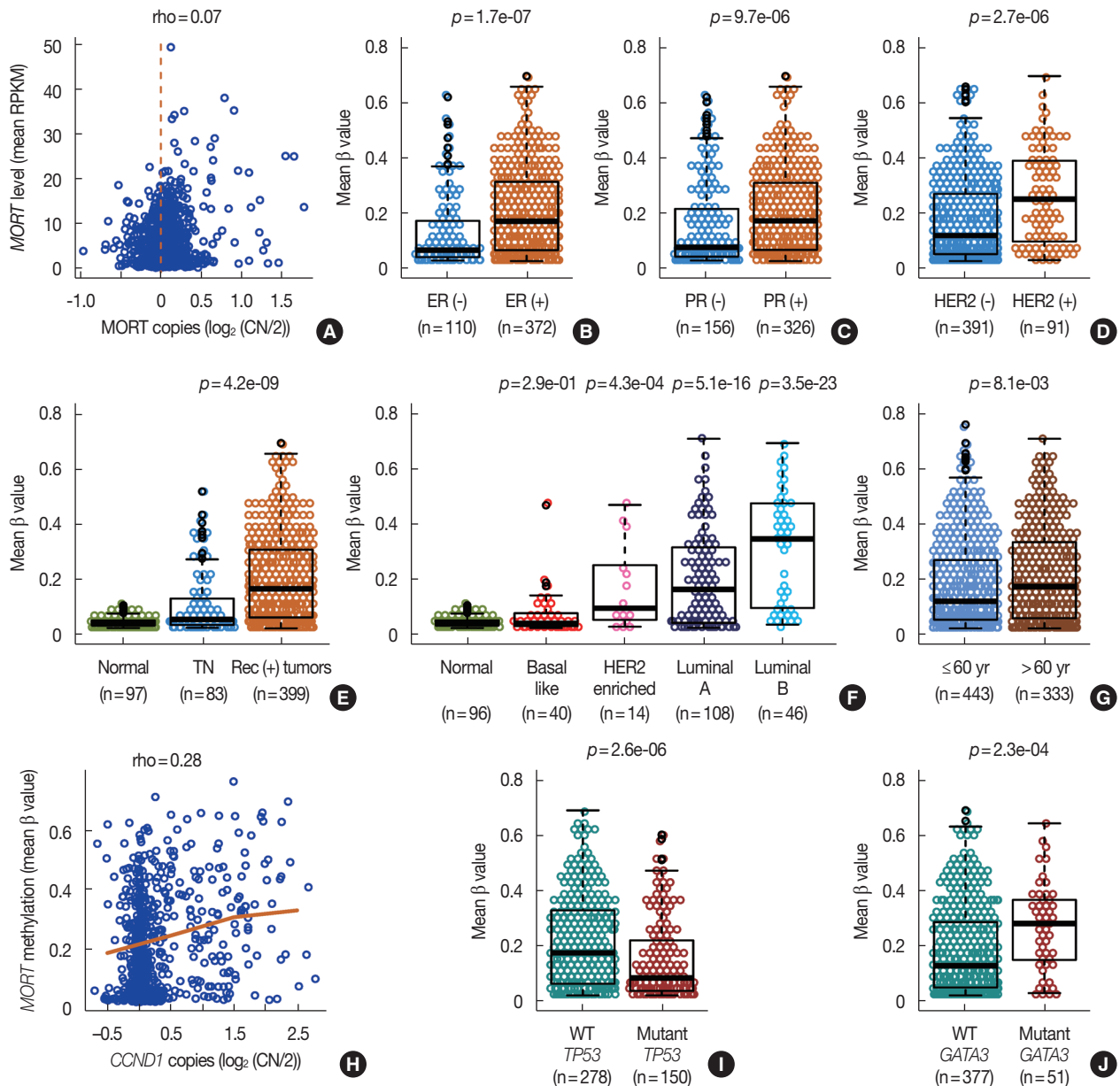


Figure 2. DNA methylation mediated *MORT* gene silencing is linked to luminal, receptor positive breast cancers. (A) *MORT* expression level plotted versus *MORT* copy number. DNA methylation of the *MORT* promoter in receptor negative and receptor positive tumors estrogen receptor (ER) (B), progesterone receptor (PR) (C), and human epidermal growth factor receptor 2 (HER2) (D). (E) DNA methylation of the *MORT* promoter in triple negative (TN) tumors, tumors positive for at least one receptor and a group of normal samples (*p*-value is for contrast TN vs. receptor (+) tumors). (F) DNA methylation of the *MORT* promoter in breast tumors classified into subtypes according to PAM50 (*p*-values are for contrast of individual tumor subtypes vs. normal). (G) DNA methylation of the *MORT* promoter in samples from patients >60 years of age compared to those from patients ≤60. (H) Association between amplification of the cyclin D1 locus and DNA methylation of the *MORT* promoter. (I) DNA methylation of the *MORT* promoter in samples with wild type *TP53* compared to samples with mutated *TP53*. (J) DNA methylation of the *MORT* promoter in samples with wild type *GATA3* compared to samples with mutated *GATA3*. RPKM=reads per kilobase per million; CN=copy number; WT=wild type.

model of HMEC immortalization, aberrant methylation of the *MORT* CpG island promoter occurs early in human breast carcinogenesis, likely at or near the point where premalignant cells gain immortality; this epigenetic silencing is maintained

throughout malignant transformation and metastatic growth. In breast cancer, *MORT* gene silencing occurs predominantly in hormone receptor positive luminal tumors, while triple negative basal-like tumors usually show normal levels of

MORT expression. Thus, taken *in toto*, these results are consistent with *MORT* silencing occurring early in breast carcinogenesis, during cellular immortalization, and before the development of luminal IBC. Delineating the molecular functions of the *MORT* lncRNA could provide new strategies for therapeutic or chemopreventive interventions.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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