**E-Cadherin, NFATC3, and PLP2 Are Differentially Methylated in Multiple Cancers**

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ABSTRACT: It is well documented that cancer cells have abnormal methylation patterns often caused by faulty methylating machinery. Specifically, E-cadherin, NFATC3, and PLP2 are 3 genes known to be aberrantly methylated in cancer cells. These genes are well documented for their role in signaling pathways involved with cell proliferation, adhesion, migration, and other signs of tumor progression. Therefore, changes in gene expression of CDH1, NFATC3, and PLP2 due to aberrant methylation can lead to profound changes in cellular function and tumor formation. In order to ensure that previous in vitro and in vivo methylation studies match what is observed in the clinic, we utilized a bioinformatics approach to complete an extensive analysis of methylation patterns of these 3 genes, analyzing over 5000 patient samples, across all cancers for which both normal and tumor tissues were available. Specifically, we analyzed overall and site-specific methylation patterns, at CpG islands and shores, of all 3 genes across 14 cancer types. Furthermore, we compared these methylation levels in normal and tumor samples of both matched and unmatched patient samples in order to determine any differences between groups. Finally, we examined whether an aberrant DNA methyltransferase, DNMT3B7, known to be expressed in cancer cells and to alter methylation patterns in vitro correlated with altered overall and site-specific methylation of CDH1, NFATC3, and PLP2 in these patient samples. Our results indicate that methylation patterns of CDH1 and NFATC3 were unexpectedly varied across tumors, contrary to previous studies performed in vitro, while PLP2 showed the expected hypomethylation pattern in tumor tissues. We also observed some correlation between DNMT3B7 expression and methylation patterns of these genes, but patterns were inconsistent. Taken together, these results emphasize the necessity for in vitro data and provide multiple areas of future research.

**KEYWORDS:** E-cadherin, NFATC3, PLP2, DNMT3B7, DNA methylation, cancer

**INTRODUCTION**

Cancer is the second-leading cause of death globally and while treatments have improved and 5-year survival has increased for most cancers, there is still much that is unknown regarding the molecular mechanisms of tumor progression in patients. Extensive studies have been performed in vitro and in vivo to answer these questions. However, there are limitations of in vitro work involving cancer cell lines, including the lack of interactions with other cell types which can affect drug delivery and metabolism. Moreover, the likelihood of a phase I clinical trial leading to approval of a drug is, unfortunately, just 5.1%. Therefore, it is imperative that we ensure that our in vitro and in vivo findings are translatable. Fortunately, with the development of The Cancer Genome Atlas and Genomic Data Commons (GDC), it is becoming much easier to validate in vitro and in vivo findings in patient samples.

It is well documented that cancer cells display abnormal methylation patterns. Specifically, cancer cells tend to show an overall global hypomethylation leading to increased gene expression compared to normal cells. However, we also see examples of specific tumor suppressor genes which have been hypermethylated leading to a corresponding loss of gene expression. While most studies have focused on methylation of CpG dinucleotides within a larger CpG island near the promoter region, more recent studies have shown that methylation patterns at CpG shores, found approximately 2 kb from an island, are more strongly associated with gene expression than CpG island methylation. Therefore, it is imperative that any methylation study that occurs in patient samples include an analysis of not just overall methylation, but also site-specific methylation in order to form a better understanding of methylation patterns in patients.

Three specific genes that have been shown to have altered methylation patterns in cancer cells are Epithelial(E)-cadherin (CDH1), nuclear factor of activated T cells 3 (NFATC3), and proteolipid protein 2 (PLP2). E-cadherin is known for its role in maintaining cell-cell adhesions in normal epithelial cells. However, the literature is clear that hypermethylation of CDH1, and corresponding loss of RNA/protein expression, promotes an epithelial-to-mesenchymal transition leading to tumor progression in epithelial cancers. While the primary role of NFATC3 is to function as a transcription factor responsible for promoting T-cell development and proliferation, as well as COX-2 dependent migration and angiogenesis within the immune system, studies have shown altered methylation patterns across a variety of cancers. Most studies have shown hypomethylation, and increased expression of NFATC3, corresponded with tumor progression using in vitro and animal models. However, one study utilizing ovarian cancer patient sample data from the GDC showed hypermethylation and decreased expression of NFATC3 correlated with decreased survival. Therefore, while it was assumed that NFATC3 is
hypo-hypomethylated in cancer, which would correspond with its known functional role, conflicting studies indicate a need for a more in-depth analysis in patient samples across cancer types. Finally, while not as well studied as CDH1 and NFATC3, PLP2 has an important role in tumor progression due to its ability to promote activation of the PI3K/Akt pathway. Because of this association, hypomethylation and increased expression of PLP2 in cancer cells has been shown to promote cell proliferation, adhesion, and invasion.25–29 Taken together, these 3 genes represent examples of genes which are typically hypermethylated (CDH1) or hypomethylated (PLP2) in cancer cells, as well as an example of a gene with conflicting data (NFATC3). However, virtually all of these methylation studies have been performed in vitro or in animal models with very little patient data to verify the results. Therefore, it is imperative that we complete a more thorough analysis in patients to determine if these methylation patterns are maintained in patient samples. Furthermore, once we understand the methylation changes that are occurring in patients, we can better identify the role of faulty DNA methylating machinery in cancer in order to develop novel treatments.

DNA methyltransferases (DNMTs) are responsible for maintaining normal methylation patterns in cells.22–24 Specifically, DNMT1 is responsible for maintaining methylation patterns in adult cells while DNMT3A and DNMT3B are de novo methylases responsible for adding new methyl groups to CpG dinucleotides during early development. Abrerrant versions of DNMT3B have been identified in both cancer cell lines and patient tumors, but not normal cells, and their functional role continues to be determined.25–30 One of these aberrant transcripts, DNMT3B7, has been shown to be expressed in multiple cancer types.25,30 Several studies have shown that expression of DNMT3B7 promotes lymphomagenesis, breast cancer, and neuroblastoma progression through altered methylation patterns in these tumors both in vitro and in vivo using animal models.27–29 Specifically, our laboratory and others have shown that DNMT3B7 expression in cell lines led to increased methylation, and down-regulation of protein expression, of E-cadherin and decreased methylation of NFATC3 and PLP2.25,30 While these findings are very intriguing, to date they have only been performed in vitro and there are no patient studies verifying the results. Therefore, it is imperative that we determine a clinical role for DNMT3B7 and other aberrant DNMTs with regard to altered methylation patterns.

Taken together, it is clear that there is much that we still do not know about aberrant methylation patterns of genes and the role of aberrant DNMTs in patient samples. To that end, we have utilized a bioinformatics approach with patient data from the GDC to examine methylation patterns of CDH1, NFATC3, and PLP2. We analyzed over 5000 patient samples across 14 cancer types for which normal and tumor samples were available in order to examine changes in overall methylation as well as methylation at CpG islands and shores. This analysis was completed in both matched and unmatched normal and tumor samples. We also assessed whether there was any correlation between aberrant DNMT3B7 expression and methylation of these 3 genes. To our knowledge, this is the first analysis of this magnitude to occur and the results of this work provide important information regarding the future direction of methylation studies in patients.

Materials and Methods

Collection of data from Genomic Data Commons

RNAseqV2 and clinical data were obtained from the GDC Legacy data portal (https://portal.gdc.cancer.gov/legacy-archive/search/f) as previously described.28,30,31 Methylation data were collected from the GDC Data Portal (https://portal.gdc.cancer.gov/repository) and matched with corresponding DNMT3B7 RNAseqV2 expression and clinical data using the unique “Case-UUID” number for each patient. All data were organized and processed using a custom C# script and Microsoft Excel (Redmond, Washington).

Statistical analysis

All statistical analysis was performed using SigmaStat software (Systat, Chicago, IL). Methylation values were obtained by either averaging all methylation sites for 1 gene together or separating by type of methylation site (Island, N_Shore, S_Shore) and then averaging all measures within a specific group. Primary tumor versus normal tissue methylation was compared using a T-test. A Mann-Whitney Rank Sum Test was used in situations where a normal distribution was not observed based on a Shapiro-Wilk Normality Test. Matched tissues were compared with a Paired T-Test or Signed Rank Test in the absence of a normal distribution. A Bonferroni correction was utilized for each cancer data set (0.05/24 = 0.00208) to avoid type I errors due to multiple comparisons. Correlation studies were performed using a Pearson Correlation Coefficient for normally distributed data or a Spearman Rank Order Correlation for data that did not have a normal distribution pattern. A Bonferroni correction was also utilized for each cancer data set (0.05/12 = 0.00417) to avoid type I errors.

Results

E-cadherin methylation patterns are varied across cancers

To begin our large-scale analysis of CDH1 methylation, we utilized data from 14 cancer types and 5782 patient samples for which normal and tumor tissues were available on GDC. We compared methylation in all tumor samples to all normal tissues available as well as matched samples where we had normal and tumor tissue from the same patient. It was expected that we would observe increased methylation of CDH1 in tumor tissues compared to normal samples based on the results of
previous studies. Of the 14 cancers tested, when we averaged all methylation sites across the entire gene for each patient, significant changes in $CDH1$ methylation were only observed in 5 of them (BLCA, COAD, HNSC, LIHC, READ; Table 1). Interestingly, in 3 out of the 5 significant cancers, we observed the opposite methylation pattern from what was expected—hypomethylation of $CDH1$ in tumor tissues. In cancers where matched normal and tumor tissues were available, we did not observe any significant difference in expression in the direction expected. All other cancers with matched samples either had no difference in methylation or hypomethylation of $CDH1$ in tumor tissues compared to normal controls.

Because an average methylation value for a patient’s specific gene does not provide site-specific information on methylation, and previous studies have shown that CpG shores are more likely to correspond to gene expression than any other methylation region, we undertook a more detailed analysis of methylation across all patient samples. Specifically, we calculated an average methylation value for every patient at the CpG island, N_Shore (the CpG shore located 5’ to the island), and S_Shore (the CpG shore located 3’ to the island; Table 2). Once again, only 5 cancers (BLCA, COAD, KIRC, KIRP, LIHC) out of 14 showed an altered methylation pattern in at least 1 methylation site, but they were not the same 5 cancers in which we observed significant results above. Interestingly, we still observed the same unexpected hypomethylation pattern seen in Table 1 in 3 cancers (BLCA, COAD, and LIHC) when we completed the in-depth analysis (Table 2). However, the cancers which showed the expected hypermethylation pattern changed between the 2 analyses.

**NEATC3 is hypermethylated in most tumor tissues**

To continue our analysis of methylation patterns across cancer patient samples, we measured average methylation of $NEATC3$, a gene for which the literature showed conflicting reports about methylation patterns. Because the majority of the literature showed that $NEATC3$ was hypomethylated, and its known function implies that increased expression should correlate with tumor progression, we hypothesized that $NEATC3$ would be hypomethylated in tumor tissues. Surprisingly, our analysis of average overall methylation shows that $NEATC3$ is hypermethylated in 5 cancers (BRCA, COAD, KIRP, LUAD, READ) and hypomethylated in only 1 cancer (LIHC; Table 1), indicating that $NEATC3$ is typically turned “off” in patients despite its role in promoting angiogenesis and chemoresistance. When we completed further analysis of methylation at the CpG island, N_Shore, and S_Shore, we found 3 additional cancers (HNSC, KIRC, LUSC; Table 3) that were also hypermethylated as well as some alterations in the methylation patterns observed in Table 1. For example, while we observed a significant difference in COAD overall methylation in unmatched samples (Table 1), we did not observe that same change at any specific site in our more detailed analysis (Table 3). Interestingly, we did not observe hypomethylation at any site in any cancer when we completed our detailed analysis, even though LIHC had shown an overall hypomethylated pattern (Table 1).

**PLP2 is hypomethylated in all cancers tested**

Because of the unexpected findings observed in our analysis of $CDH1$ and, especially, $NFATC3$, we examined an additional gene known to be hypomethylated in cancer—$PLP2$. As expected, overall $PLP2$ methylation levels were reduced in all tumor types tested compared to normal tissues (Table 1). Furthermore, upon completion of our in-depth analysis of site-specific methylation, we observed the expected hypomethylation pattern across all cancers with significant results (Table 4).

**DNMT3B7 expression is correlated to CDH1, NEATC3, or PLP2 methylation in some patient samples**

Finally, to determine if $DNMT3B7$ expression correlated with methylation of $CDH1$, $NFATC3$, and $PLP2$, we matched both overall methylation data, as well as methylation at individual islands and shores, to corresponding $DNMT3B7$ expression. Pearson Correlation Coefficient or Spearman Rank Order Correlation analyses were conducted to determine if there was any correlation between $DNMT3B7$ expression and overall, or site-specific, gene methylation in tumor tissue samples across cancers (Tables 5–8). Overall, correlation results were inconsistent across all cancers, and cancers which showed significant methylation changes between normal and tumor samples did not necessarily correlate with $DNMT3B7$ expression. However, some interesting trends did arise. First of all, the only significant findings for $CDH1$, whether looking at overall or site-specific methylation, indicated a negative correlation which would imply that $CDH1$ methylation decreases as $DNMT3B7$ expression increases. Conversely, when $NFATC3$ methylation correlated to $DNMT3B7$ expression, it was always in a positive relationship. Finally, $PLP2$, the only gene that had shown a consistent methylation pattern above, showed an extremely inconsistent correlation pattern across cancers. In some cases it was negatively correlated with $DNMT3B7$ (BRCA) and other times it was positively correlated in other cancers (BLCA, HNSC; Tables 5 and 8). In the case of site-specific methylation of $PLP2$ in LUAD, there is a negative correlation with $DNMT3B7$ at the CpG island and a positive correlation at the S_Shore (Table 8), indicating a need for future analysis.

**Discussion**

The results presented here clearly indicate that it is imperative that we utilize patient data as often as possible to verify results observed in *in vitro* and animal models. Fortunately, databases
Table 1. Statistical results of average overall methylation patterns for 3 genes across cancer types.

| CANCER NAME                                                | ABBREVIATION | SAMPLE SIZE | E-CADHERIN (CDH1) | NFATC3 | PLP2 |
|------------------------------------------------------------|--------------|-------------|--------------------|--------|------|
| Bladder urothelial carcinoma                               | BLCA         | 427         | (-) $P < .001$     | $P = .141$ | (-) $P < .001$ |
| Breast invasive carcinoma                                  | BRCA         | 1062        | $P = .448$         | $P = .448$ | (-) $P < .001$ |
| Cervical squamous cell carcinoma and endocervical adenocarcinoma | CESC         | 307         | $P = .832$         | $P = .699$ | $P = .420$ |
| Colon adenocarcinoma                                       | COAD         | 332         | $P = .651$         | $P = .688$ | $P = .021$ |
| Esophageal carcinoma                                       | ESCA         | 192         | $P = .652$         | $P = .016$ | (-) $P < .001$ |
| Head/neck squamous cell carcinoma                          | HNSC         | 542         | $P = .388$         | $P = .633$ | $P = .331$ |
| Kidney renal clear cell carcinoma                          | KIRC         | 342         | $P = .189$         | $P = .071$ | $P = .076$ |
| Kidney renal papillary cell carcinoma                      | KIRP         | 293         | $P = .048$         | $P = .016$ | (+) $P < .001$ |
| Liver hepatocellular carcinoma                             | LIHC         | 413         | $P = .022$         | $P = .123$ | (-) $P < .001$ |
| Lung adenocarcinoma                                        | LUAD         | 533         | $P = .312$         | $P = .956$ | $P = .009$ |
| Lung squamous cell carcinoma                               | LUSC         | 509         | $P = .400$         | $P = .250$ | $P = .040$ |
| Rectum adenocarcinoma                                      | READ         | 94          | N/A                | N/A    | N/A |
| Thyroid cancer                                             | THCA         | 554         | $P = .206$         | $P = .516$ | $P = .072$ |
| Uterine corpus endometrial carcinoma                       | UCEC         | 182         | $P = .040$         | $P = .844$ | N/A |

Normal versus tumor includes all patient samples available while matched normal versus tumor only includes matched samples. Statistically significant data ($P < .00208$ based on a Bonferroni correction of $0.05/24$) shaded yellow. (-) indicates that the gene is hypomethylated in tumor tissues compared to normal samples. (+) indicates that the gene is hypermethylated in tumor tissues compared to normal samples.
Table 2. Statistical results for average methylation of CDH1 at CpG island, N_Shore, and S_Shore.

| CANCER NAME                                                      | ABBREVIATION | SAMPLE SIZE | ISLAND | MATCHED NORMAL VERSUS TUMOR | N_SHORE | MATCHED NORMAL VERSUS TUMOR | S_SHORE | MATCHED NORMAL VERSUS TUMOR |
|-----------------------------------------------------------------|--------------|-------------|--------|-------------------------------|---------|-------------------------------|---------|-------------------------------|
| Bladder urothelial carcinoma                                    | BLCA         | 427         |        | (-) P < .001                  | (-) P < .001 | (-) P < .001                  | (-) P < .001 | (-) P < .001                  |
| Breast invasive carcinoma                                        | BRCA         | 1062        |        | P = .155                      | P = .017 | P = .927                      | P = .532 | P = .006                      | P = .061 |
| Cervical squamous cell carcinoma and endocervical adenocarcinoma | CESC         | 307         |        | P = .372                      | P = .011 | P = .246                      | P = .254 | P = .842                      | P = .613 |
| Colon adenocarcinoma                                             | COAD         | 332         |        | (-) P = .001                  | (-) P < .001 | (-) P < .001                  | (-) P < .001 | P = .874                      | P = .078 |
| Esophageal carcinoma                                             | ESCA         | 192         |        | P = .773                      | P = .119 | P = .320                      | P = .149 | P = .907                      | P = .566 |
| Head/neck squamous cell carcinoma                                | HNSC         | 542         |        | P = .006                      | P = .349 | P = .013                      | P = .368 | P = .006                      | P = .073 |
| Kidney renal clear cell carcinoma                                | KIRC         | 342         |        | (+) P < .001                  | (+) P < .001 | (+) P < .001                  | (+) P < .002 | (+) P < .001                  | (+) P < .001 |
| Kidney renal papillary cell carcinoma                            | KIRP         | 293         |        | P = .059                      | P = .004 | (+) P < .001                  | (+) P < .001 | P = .209                      | P = .010 |
| Liver hepatocellular carcinoma                                   | LIHC         | 413         |        | P = .020                      | P = .014 | P = .266                      | P = .056 | (-) P < .001                  | (-) P < .001 |
| Lung adenocarcinoma                                              | LUAD         | 533         |        | P = .033                      | P = .806 | P = .971                      | P = .695 | P = .557                      | P = .786 |
| Lung squamous cell carcinoma                                     | LUSC         | 509         |        | P = .237                      | P = .774 | P = .671                      | P = .813 | P = .200                      | P = .498 |
| Rectum adenocarcinoma                                            | READ         | 94          |        | P = .238                      | N/A     | P = .933                      | N/A     | P = .461                      | N/A     |
| Thyroid cancer                                                   | THCA         | 554         |        | P = .157                      | P = .251 | P = .171                      | P = .243 | P = .594                      | P = .601 |
| Uterine corpus endometrial carcinoma                             | UCEC         | 182         |        | P = .280                      | N/A     | P = .006                      | N/A     | P = .184                      | N/A     |

Normal versus tumor includes all patient samples available while matched normal versus tumor only includes matched samples. Statistically significant data (P < .00208 based on a Bonferroni correction of 0.05/24) shaded yellow.

(-) indicates that CDH1 is hypomethylated in tumor tissues compared to normal samples.

(+) indicates that CDH1 is hypermethylated in tumor tissues compared to normal samples.
Table 3. Statistical results for average methylation of NFATC3 at CpG island, N_Shore, and S_Shore.

| CANCER NAME                        | ABBREVIATION | SAMPLE SIZE | ISLAND               | MATCHED NORMAL VERSUS TUMOR | N_Shore                 | MATCHED NORMAL VERSUS TUMOR | S_Shore                 | MATCHED NORMAL VERSUS TUMOR |
|-----------------------------------|--------------|-------------|----------------------|-----------------------------|-------------------------|-----------------------------|-------------------------|-----------------------------|
|                                   |              |             | NORMAL VERSUS TUMOR  | MATCHED NORMAL VERSUS TUMOR | NORMAL VERSUS TUMOR      | MATCHED NORMAL VERSUS TUMOR | NORMAL VERSUS TUMOR      | MATCHED NORMAL VERSUS TUMOR |
| Bladder urothelial carcinoma      | BLCA         | 427         | P = .457             | P = .927                    | P = .760                 | P = .290                     | P = .883                 | P = .022                     |
| Breast invasive carcinoma         | BRCA         | 1062        | (+) P < .001         | (+) P < .001                | (+) P < .001             | (+) P < .001                 | P = .779                 | P = .006                     |
| Cervical squamous cell carcinoma  | CESC         | 307         | P = .904             | P = .344                    | P = .251                 | P = .326                     | P = .013                 | P = .278                     |
| Colon adenocarcinoma              | COAD         | 332         | P = .078             | P = .274                    | P = .784                 | P = .437                     | P = .692                 | P = .117                     |
| Esophageal carcinoma              | ESCA         | 192         | P = .258             | P = 1.000                   | P = .015                 | P = .027                     | P = .641                 | P = .008                     |
| Head/neck squamous cell carcinoma | HNSC         | 542         | (+) P < .001         | P = .076                    | P = .011                 | P = .295                     | P = .773                 | P = .985                     |
| Kidney renal clear cell carcinoma | KIRC         | 342         | (+) P < .001         | P = .011                    | (+) P < .001             | (+) P < .001                 | P = .020                 |                             |
| Kidney renal papillary cell carcinoma | KIRP       | 293         | P = .571             | P = .173                    | (+) P < .001             | (+) P < .001                 | (+) P < .001             | P = .922                     |
| Liver hepatocellular carcinoma    | LIHC         | 413         | (+) P < .001         | (+) P < .001                | P = .079                 | P = .412                     | (+) P < .001             | (+) P = .001                 |
| Lung adenocarcinoma               | LUAD         | 533         | P = .191             | P = .546                    | (+) P < .001             | P = .072                     | P = .162                 | P = .314                     |
| Lung squamous cell carcinoma      | LUSC         | 509         | P = .003             | P = .641                    | (+) P < .001             | P = .024                     | P = .059                 | P = .578                     |
| Rectum adenocarcinoma             | READ         | 94          | P = .113             | N/A                         | N/A                     | N/A                         | N/A                     | N/A                         |
| Thyroid cancer                    | THCA         | 554         | P = .523             | P = .006                    | P = .017                 | P = .315                     | P = .004                 | P = .060                     |
| Uterine corpus endometrial carcinoma | UCEC       | 182         | P = .755             | N/A                         | P = .975                 | N/A                         | P = .206                 | N/A                         |

Normal versus tumor includes all patient samples available while matched normal versus tumor only includes matched samples. Statistically significant data (P < .00208 based on a Bonferroni correction of 0.05/24) shaded yellow. (+) indicates that NFATC3 is hypermethylated in tumor tissues compared to normal samples.
Table 4. Statistical results for average methylation of PLP2 at CpG island, N_Shore, and S_Shore.

| CANCER NAME                                               | ABBREVIATION | SAMPLE SIZE | ISLAND         | MATCHED NORMAL VERSUS TUMOR | N_SHORE          | MATCHED NORMAL VERSUS TUMOR | S_SHORE          | MATCHED NORMAL VERSUS TUMOR |
|-----------------------------------------------------------|--------------|-------------|----------------|----------------------------|-----------------|----------------------------|-----------------|----------------------------|
| Bladder urothelial carcinoma                              | BLCA         | 427         | P = .011        | P = .008                    | (-) P < .001    | (-) P < .001                | (-) P < .001    | P = .101                    |
| Breast invasive carcinoma                                 | BRCA         | 1062        | P = .462        | P = .198                    | (-) P < .001    | (-) P < .001                | (-) P < .001    | (-) P < .001               |
| Cervical squamous cell carcinoma and endocervical adenocarcinoma | CESC         | 307         | P = .179        | P = .080                    | P = .042        | P = .250                    | P = .003        | P = .055                    |
| Colon adenocarcinoma                                      | COAD         | 332         | P = .012        | P = .298                    | P = .045        | P = .012                    | P = .770        | P = .518                    |
| Esophageal carcinoma                                      | ESCA         | 192         | P = .050        | P = .820                    | P = .006        | P = .649                    | (-) P < .001    | P = .316                    |
| Head/neck squamous cell carcinoma                         | HNSC         | 542         | P = .835        | P = .812                    | P = .386        | P = .783                    | P = .082        | P = .008                    |
| Kidney renal clear cell carcinoma                         | KIRC         | 342         | P = .242        | P = .920                    | P = .138        | P = .411                    | (-) P < .001    | (-) P < .001               |
| Kidney renal papillary cell carcinoma                     | KIRP         | 293         | P = .230        | P = .581                    | P = .600        | P = 1.000                   | P = .010        | P = .030                    |
| Liver hepatocellular carcinoma                            | LIHC         | 413         | P = .059        | P = .162                    | (-) P < .001    | (-) P < .001                | (-) P < .001    | (-) P < .001               |
| Lung adenocarcinoma                                       | LUAD         | 533         | P = .657        | P = .522                    | P = .086        | (-) P < .001                | P = .864        | P = .031                    |
| Lung squamous cell carcinoma                              | LUSC         | 509         | P = .027        | P = .710                    | P = .024        | (-) P < .001                | P = .008        | (-) P < .001               |
| Rectum adenocarcinoma                                     | READ         | 94          | P = .005        | N/A                        | P = .037        | N/A                        | N/A             | N/A                        |
| Thyroid cancer                                            | THCA         | 554         | P = .285        | P = .082                    | P = .354        | P = 3.90                    | P = .106        | P = .032                    |
| Uterine corpus endometrial carcinoma                      | UCEC         | 182         | P = .119        | N/A                        | P = .117        | N/A                        | P = .025        | N/A                        |

Normal versus tumor includes all patient samples available while matched normal versus tumor only includes matched samples. Statistically significant data (P < .00208 based on a Bonferroni correction of 0.05/24) shaded yellow. (-) indicates that PLP2 is hypomethylated in tumor tissues compared to normal samples.
Table 5. Correlation results for overall methylation of all 3 genes versus DNMT3B7 across cancer types.

| CANCER NAME                                      | ABBREVIATION | SAMPLE SIZE | E-CADHERIN (CDH1) | NFATC3 | PLP2 |
|-------------------------------------------------|--------------|-------------|--------------------|--------|------|
| Bladder urothelial carcinoma                    | BLCA         | 427         | -0.0693 (rP)       | +0.0371 | +0.0794 | 0.108 |
| Breast invasive carcinoma                       | BRCA         | 1062        | -0.0421            | +0.0510 | -0.122 | 0.000150 |
| Cervical squamous cell carcinoma and endocervical adenocarcinoma | CESC         | 307         | -0.161             | -0.0872 | -0.0747 | 0.458 |
| Colon adenocarcinoma                            | COAD         | 332         | -0.0504            | -0.0346 | -0.0256 | 0.0000150 |
| Esophageal carcinoma                            | ESCA         | 192         | -0.0582 (rP)       | +0.0878 | -0.0249 | 0.738 |
| Head/neck squamous cell carcinoma               | HNSC         | 542         | -0.0241            | +0.0691 | +0.137 | 0.00173 |
| Kidney renal clear cell carcinoma               | KIRC         | 342         | -0.0576            | +0.0324 | +0.0357 | 0.525 |
| Kidney renal papillary cell carcinoma           | KIRP         | 293         | -0.0685            | +0.104  | +0.0976 | 0.109 |
| Liver hepatocellular carcinoma                  | LIHC         | 413         | -0.159             | -0.0240 | +0.0281 | 0.590 |
| Lung adenocarcinoma                             | LUAD         | 533         | +0.0281            | +0.224  | -0.0911 | 0.0226 |
| Lung squamous cell carcinoma                    | LUSC         | 509         | -0.101             | +0.116  | -0.0911 | 0.0416 |
| Rectum adenocarcinoma                           | READ         | 94          | +0.0444            | -0.0875 | -0.0416 | 0.698 |
| Thyroid cancer                                  | THCA         | 554         | -0.113             | -0.102  | +0.0242 | 0.588 |
| Uterine corpus endometrial carcinoma            | UCEC         | 182         | +0.0233            | -0.0284 | -0.0619 | 0.411 |

Statistically significant data (P < 0.00417 based on a Bonferroni correction of 0.05/12) shaded yellow. (rP), Pearson correlation coefficient.
*R measured by Spearman rank order correlation (rS) unless otherwise notated.
(−) indicates negative correlation coefficient.
(+) indicates positive correlation coefficient.
Table 6. Correlation results for CDH1 at CpG islands, N_Shore, and S_Shore compared to DNMT3B7 expression.

| CANCER NAME                                      | ABBREVIATION | SAMPLE SIZE | ISLAND   | N_SHORE   | S_SHORE   |
|--------------------------------------------------|--------------|-------------|----------|-----------|-----------|
| Bladder urothelial carcinoma                     | BLCA         | 427         | -0.124   | -0.144    | -0.167    |
| Breast invasive carcinoma                        | BRCA         | 1062        | -0.00194 | -0.00521  | -0.00817  |
| Cervical squamous cell carcinoma and endocervical adenocarcinoma | CESC | 307         | -0.167   | -0.158    | -0.162    |
| Colon adenocarcinoma                             | COAD         | 332         | -0.177   | -0.151    | -0.150    |
| Esophageal carcinoma                             | ESCA         | 192         | -0.196   | -0.141    | -0.153    |
| Head/neck squamous cell carcinoma                | HNSC         | 542         | -0.0758  | -0.0594   | -0.0453   |
| Kidney renal clear cell carcinoma                | KIRC         | 342         | +0.0502  | +0.0518   | +0.0163   |
| Kidney renal papillary cell carcinoma            | KIRP         | 293         | -0.138   | +0.0685   | -0.163    |
| Liver hepatocellular carcinoma                   | LIHC         | 413         | -0.0636  | -0.144    | -0.0538   |
| Lung adenocarcinoma                              | LUAD         | 533         | -0.0316  | -0.0672   | -0.0500   |
| Lung squamous cell carcinoma                     | LUSC         | 509         | -0.282   | -0.325    | -0.293    |
| Rectum adenocarcinoma                            | READ         | 94          | +0.0200  | -0.00746  | +0.0386   |
| Thyroid cancer                                   | THCA         | 554         | -0.115   | -0.126    | -0.167    |
| Uterine corpus endometrial carcinoma             | UCEC         | 182         | +0.0111  | -0.148    | -0.0398   |

Statistically significant data (P < .00417 based on a Bonferroni correction of 0.05/12) shaded yellow.
(RP), Pearson correlation coefficient.
*R measured by Spearman rank order correlation (rS) unless otherwise notated.
(–) indicates negative correlation coefficient.
(+) indicates positive correlation coefficient.
Table 7. Correlation results for *NFATC3* at CpG islands, N_Shore, and S_Shore compared to *DNMT3B7* expression.

| CANCER NAME                                      | ABBREVIATION | SAMPLE SIZE | ISLAND | N_SHORE | S_SHORE |
|-------------------------------------------------|--------------|-------------|--------|---------|---------|
| Bladder urothelial carcinoma                    | BLCA         | 427         | +0.0445 | .369    | -0.0901 | -0.0116 |
| Breast invasive carcinoma                       | BRCA         | 1062        | +0.112  | .000502 | +0.0328 | -0.0424 |
| Cervical squamous cell carcinoma and endocervical adenocarcinoma | CESC       | 307         | -0.0380 | .509    | -0.0726 | +0.0196 |
| Colon adenocarcinoma                            | COAD         | 332         | -0.0563 | .332    | -0.0306 | -0.0635 |
| Esophageal carcinoma                            | ESCA         | 192         | +0.151  | .0410   | +0.148  | +0.0865 |
| Head/neck squamous cell carcinoma               | HNSC         | 542         | +0.0500 | .255    | +0.0712 | +0.0421 |
| Kidney renal clear cell carcinoma               | KIRC         | 342         | -0.0306 | .587    | +0.00851 | +0.0699 |
| Kidney renal papillary cell carcinoma           | KIRP         | 293         | -0.0114 | .852    | +0.201 (rP) | .000875 |
| Liver hepatocellular carcinoma                  | LIHC         | 413         | +0.0461 | .376    | -0.0264 | +0.0499 |
| Lung adenocarcinoma                             | LUAD         | 533         | +0.0312 | .481    | +0.0879 | +0.182 | .000108 |
| Lung squamous cell carcinoma                    | LUSC         | 509         | -0.0401 | .371    | +0.0895 | +0.120 | .0214 |
| Rectum adenocarcinoma                           | READ         | 94          | -0.0990 | .355    | -0.0296 (rP) | .785 |
| Thyroid cancer                                  | THCA         | 554         | +0.152  | .000628 | +0.133  | -0.00830 | .853 |
| Uterine corpus endometrial carcinoma            | UCEC         | 182         | -0.0987 | .190    | -0.165  | +0.0229 | .789 |

Statistically significant data (P < .00417 based on a Bonferroni correction of 0.05/12) shaded yellow.

(rP), Pearson correlation coefficient.

*R measured by Spearman rank order correlation (rS) unless otherwise notated.

(−) indicates negative correlation coefficient.

(+) indicates positive correlation coefficient.
Table 8. Correlation results for PLP2 at CpG islands, N_Shore, and S_Shore compared to DNMT3B7 expression.

| CANCER NAME | ABBREVIATION | SAMPLE SIZE | ISLAND  | N_SHORE  | S_SHORE  |
|-------------|--------------|-------------|---------|----------|----------|
|             |              |             | $r^a$   | $r^p$    | $r^a$    | $r^p$    |
| Bladder urothelial carcinoma | BLCA | 427 | −0.0520 | .293 | +0.0279 | .572 | +0.234 | .0000178 |
| Breast invasive carcinoma | BRCA | 1062 | −0.0892 | .00569 | −0.155 | .00000151 | +0.129 | .000993 |
| Cervical squamous cell carcinoma and endocervical adenocarcinoma | CESC | 307 | −0.0730 | .204 | −0.0596 | .300 | −0.0208 | .718 |
| Colon adenocarcinoma | COAD | 332 | −0.152 | .00831 | −0.0361 | .534 | +0.157 | .00765 |
| Esophageal carcinoma | ESCA | 192 | −0.0686 | .355 | −0.0475 | .523 | +0.0301 | .686 |
| Head/neck squamous cell carcinoma | HNSC | 542 | +0.126 | .00389 | +0.138 | .00158 | +0.117 | .00777 |
| Kidney renal clear cell carcinoma | KIRC | 342 | +0.0253 | .654 | +0.0747 | .184 | +0.0245 | .663 |
| Kidney renal papillary cell carcinoma | KIRP | 293 | +0.112 | .0659 | +0.141 | .0200 | +0.0179 | .769 |
| Liver hepatocellular carcinoma | LIHC | 413 | +0.0475 | .361 | −0.00278 | .957 | +0.0375 | .471 |
| Lung adenocarcinoma | LUAD | 533 | −0.147 | .000878 | −0.0986 | .0257 | +0.165 | .000476 |
| Lung squamous cell carcinoma | LUSC | 509 | −0.0358 | .424 | −0.106 | .0172 | −0.113 | .0302 |
| Rectum adenocarcinoma | READ | 94 | −0.140 | .191 | −0.0486 | .651 | +0.121 | .262 |
| Thyroid cancer | THCA | 554 | −0.00358 | .936 | +0.0308 | .490 | +0.125 | .00483 |
| Uterine corpus endometrial carcinoma | UCEC | 182 | −0.0249 | .742 | −0.119 | .112 | +0.00615 | .943 |

Statistically significant data ($P < .00417$ based on a Bonferroni correction of 0.05/12) shaded yellow.
(rP), Pearson correlation coefficient.
(−) indicates negative correlation coefficient.
(+) indicates positive correlation coefficient.
like the GDC are making that work far easier to do than it was in the past. While the literature have been clear that CDH1 is hypermethylated and NFATC3 and PLP2 are hypomethylated in other cancer models, those results were not consistently seen when we examined patient data at this large scale. While scientists are aware of the biological constraints that occur when using in vitro models, the unexpected results shown here indicate that some facts that we have held to be true may not be as universal as once expected. If we are going to successfully develop novel therapeutics to improve patient outcomes, we must understand what is happening in patients, rather than rely primarily on in vitro work, to direct our long-term drug development.

Perhaps the most surprising results we obtained were from the analysis of methylation status of CDH1 across cancers. The hypermethylation of CDH1 leading to decreased E-cadherin protein levels to promote epithelial-to-mesenchymal transition, tumor progression, and metastasis is well documented.7–11 There are no conflicting results in the literature pertaining to methylation status of CDH1 in epithelial tumors, yet our results indicate that in half of the tumors for which we have significant data, CDH1 is hypomethylated, not hypermethylated (Tables 1 and 2). This cannot be explained by differences in tumor type because these results were observed in epithelial, not mesenchymal, tumors. One possible explanation is that our previous work has shown that a preponderance of patient samples available on GDC are from patients with early stage tumors rather than later stage tumors.30 Loss of E-cadherin and epithelial-to-mesenchymal transition is associated with a more aggressive, late-stage disease so it is possible that these altered results are due to an uneven balance of samples across stages rather than a new paradigm in E-cadherin research. Nevertheless, these data demonstrate the importance of continuing to collect samples from cancer patients at all stages of their disease in order to obtain the best data sets for future studies.

In the case of NFATC3, while some studies indicated that it was hypomethylated with increased expression in cancer cells leading to proliferation, migration, angiogenesis, and chemoresistance, other studies show poor survival correlated with decreased expression and hypermethylation.12–16 It should be noted that many of the studies showing hypomethylation of NFATC3 were completed using in vitro models. However, orthotopic mouse models have also shown that silencing of NFATC3 led to tumor regression.15 Furthermore, another study indicated that increased expression of NFATC3 correlated with chemoresistance in patients.12 Taken together, these studies would indicate that hypomethylation of NFATC3 leads to tumor progression. The only study, prior to this, that showed hypermethylation of NFATC3 corresponded to decreased survival also utilized data from GDC, matching the results we obtained here (Tables 1 and 3).16 Taken together, these findings lead us to question which data are accurate. It is possible that, similar to the aforementioned unexpected E-cadherin results, the types of patient samples collected for the GDC are biasing our analysis in one direction. For example, if the GDC patient samples are primarily those in early stages of disease, we would not expect to see markers of chemoresistance and angiogenesis, such as the hypomethylation of NFATC3, in these samples. While multiple laboratories have now shown similar results when utilizing the GDC data for analysis, these results are not necessarily matching the rest of the literature. Therefore, it is imperative that we ensure a well-represented sample is available in all studies before drawing any conclusions on the role of NFATC3 in promoting tumor progression.

PLP2 was the only gene analyzed in this study for which both the in vitro and patient sample data completely corresponded (Tables 1 and 4).17–21 No matter the cancer type involved, PLP2 was clearly hypomethylated. Because of its known role in promoting tumor progression through activation of the PI3K/Akt pathway, PLP2 is an exciting target for future studies. While PLP2 is not nearly as well-known or well-studied as CDH1 or NFATC3, the results shown here that match previous in vitro work indicate its potential importance in future clinical studies.

Finally, while DNMT3B7 has been shown to be highly expressed in virtually all cancer types, and its expression promotes aberrant methylation of various genes in vitro and in animal models, we observed only a few correlations between DNMT3B7 expression and the methylation status of CDH1, NFATC3, or PLP2 in patient tumor samples across cancers (Tables 5–8).25,27–30 Specifically, we observed a negative correlation with CDH1 in 6 cancers (Tables 5 and 6), a positive correlation with NFATC3 in 4 cancers (Tables 5 and 7), and a mix of positive and negative correlations with PLP2 across 4 cancers (Tables 5 and 8). In the case of CDH1, these results are contrary to what we have previously observed in vitro showing DNMT3B7 expression leading to hypermethylation of CDH1 in breast cancer cell lines, but would match with the findings above (Tables 1 and 2) showing a hypomethylation pattern in cancers known to have significantly increased DNMT3B7 levels.30 Furthermore, the fact that significant correlations were observed at every methylation site in LUAD indicates that future research should examine this relationship more closely (Table 6). Some of the first studies identifying aberrant DNMTs were performed in lung cancers,26 so it is perhaps not surprising to see significant correlations in this particular cancer type.

While the NFATC3 findings showing positive correlation data was unexpected, it matches the data seen in Tables 1 and 3. Unlike with CDH1 we did not observe any consistent correlations across any one cancer type, but this may be because previous NFATC3 studies have focused on leukemias which were not part of this analysis. Similar to CDH1, if and when the data are available, this may be another area of future research. Finally, for PLP2, the inconsistent correlation findings are confounding, especially in the case of LUAD in which there was a negative correlation in the CpG island and a positive correlation in the S_Shore (Table 8). We do not have an explanation for these findings other than to say that it is clear that additional research.
is needed to understand what is happening not only across cancers, but also in LUAD specifically.

While these correlation results were surprising, there are some confounding factors that may also be affecting our findings. First, the data available on GDC only allow an analysis of DNMT3B7 gene expression, not protein expression. While it is known that gene expression does not always correlate with protein expression in cells, previous DNMT3B7 studies have shown that expression at the DNA/RNA level has corresponded to protein expression in cell lines and animal models. However, it should be noted that previous studies have also shown that DNMT1 is post-translationally regulated so that RNA expression does not match protein expression. While these results imply a similar process could occur in DNMT3B7, the site of post-translational regulation in the N-terminus of DNMT1 does not exist in either DNMT3A or DNMT3B. Indeed, the structure of the N-terminal region of DNMT1 is completely different from that of either DNMT3 gene. Therefore, we would not expect DNMT3B7 to undergo the same type of post-translational modification based on the known structure. Unfortunately, because DNMT3B7 is an aberrantly spliced gene and truncated protein, its protein expression is not measured by the typical methods used to supply information to databases like GDC. Therefore, while we can determine that patients express DNMT3B7, we do not know how that correlates to protein levels in these patients. While the results from in vitro and animal models are promising, our analysis of methylation patterns indicates that in vitro studies do not always match with patient data. Therefore, we cannot draw any definitive conclusions about the lack of correlation at this time. Furthermore, Ostler and colleagues have shown that when DNMT3B7 is active in cells, it is localized in the nucleus, but one hypothesis is that a normal DNMT carries it into the nucleus and DNMT3B7 may work either alone or in collaboration with that normal DNMT to regulate methylation patterns. To date, that hypothesis has not been supported by any experimental evidence, but the possibility is still there. Unfortunately, due to the nature of bioinformatics we cannot test either the localization or binding to other proteins with the data available, so there is still much we do not understand about DNMT3B7 in patient tissues.

Taken together, this study emphasizes the necessity of including patient samples with in vitro work in order to verify results observed in the closed system of cell lines. Fortunately, with the growing amount of data publicly available on various bioinformatics databases, correlating in vitro results to clinical samples is becoming easier and more cost effective than in previous decades. It is hoped that an increased reliance on patient data in the future will save researchers valuable time, money, and effort in preclinical drug development studies that will lead to better diagnosis, treatment, and survival of patients.

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Authors’ Contributions
MJL, CJW, and SLR conducted the statistical analyses, interpreted the data, and drafted the manuscript. SLR was responsible for conceptualization, methodology, data collection, and final proofreading of the manuscript.

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