The DNA methylation landscape of five pediatric-tumor types

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Fewer DNA mutations have been identified in pediatric tumors than adult tumors, suggesting that alternative tumorigenic mechanisms, including aberrant DNA methylation, may play a prominent role in pediatric tumors. Methylation is an epigenetic process of regulating gene expression in which methyl groups are attached to DNA molecules, often in promoter regions. In Wilms tumors and acute myeloid leukemias, increased levels of epigenetic silencing have been associated with worse patient outcomes. However, to date, researchers have studied methylation primarily in adult tumors and for specific genes but not on a pan-pediatric cancer scale. We addressed these gaps first by aggregating methylation data from 309 noncancerous samples and establishing baseline expectations for each gene. Even though these samples represent diverse tissue types and population ancestral groups, methylation levels were highly consistent for most genes. Second, we compared tumor methylation levels against these baseline values for five pediatric cancer types—Wilms tumors, clear cell sarcomas of the kidney, rhabdoid tumors, neuroblastomas, and osteosarcomas. Hypermethylation was more common than hypomethylation—as many as 11.8% of genes were hypermethylated in a given tumor, compared to a maximum of 4.8% for hypomethylated genes. For each cancer type, genes with the highest variance exhibited consistently divergent methylation patterns for distinct patient subsets. We evaluated whether genomic and epigenomic abnormalities contribute to pediatric tumorigenesis in a mutually exclusive manner but did not find evidence of this phenomenon. Furthermore, even though oncogenes are commonly upregulated in tumors, and tumor-suppressor genes are commonly downregulated in tumors, we did not find statistical evidence that methylation drives such patterns on a broad scale in pediatric tumors.

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6 Abstract

7 Fewer DNA mutations have been identified in pediatric tumors than adult tumors, suggesting that

8 alternative tumorigenic mechanisms, including aberrant DNA methylation, may play a prominent role in

9 pediatric tumors. Methylation is an epigenetic process of regulating gene expression in which methyl

10 groups are attached to DNA molecules, often in promoter regions. In Wilms tumors and acute myeloid

11 leukemias, increased levels of epigenetic silencing have been associated with worse patient outcomes.

12 However, to date, researchers have studied methylation primarily in adult tumors and for specific genes

13 but not on a pan-pediatric cancer scale. We addressed these gaps first by aggregating methylation data

14 from 309 noncancerous samples and establishing baseline expectations for each gene. Even though these

15 samples represent diverse tissue types and population ancestral groups, methylation levels were highly

16 consistent for most genes. Second, we compared tumor methylation levels against these baseline values

17 for five pediatric cancer types—Wilms tumors, clear cell sarcomas of the kidney, rhabdoid tumors,

18 neuroblastomas, and osteosarcomas. Hypermethylation was more common than hypomethylation—as

19 many as 11.8% of genes were hypermethylated in a given tumor, compared to a maximum of 4.8% for

20 hypomethylated genes. For each cancer type, genes with the highest variance exhibited consistently

21   divergent methylation patterns for distinct patient subsets. We evaluated whether genomic and

22   epigenomic abnormalities contribute to pediatric tumorigenesis in a mutually exclusive manner but did

23   not find evidence of this phenomenon. Furthermore, even though oncogenes are commonly upregulated in

24   tumors, and tumor-suppressor genes are commonly downregulated in tumors, we did not find statistical

25   evidence that methylation drives such patterns on a broad scale in pediatric tumors.

Introduction

27   Pediatric tumors are the leading cause of disease-related death for children in developed countries[1], and

28   those who survive pediatric cancer often experience adverse health challenges later in life[2]. Many

29   mutations and structural variants have been associated with adult forms of cancer[3]; however, significantly

30   fewer genomic abnormalities have been identified in pediatric tumors[4]. The mutation rate in pediatric

31   tumors is 14 times less than the mutation rate in adult tumors, implying that different mechanisms may be

32   involved in pediatric-cancer development than in adult cancers[5]. Of the mutations that have been

33   identified in pediatric tumors, many are associated with epigenetic regulation[1]. In many pediatric tumors,

34   molecular profiling does not identify genomic abnormalities but does show abnormal DNA methylation

35   patterns[6,7], suggesting the DNA methylation may play a critical role in tumorigenesis in these cases.

36   Gene-expression levels in cancer cells often vary from those in normal cells[8]. Such abnormalities alter

37   cellular environments and manipulate cellular processes, leading to increased survival, rapid proliferation,

38   and metastasis[9]. Oncogenes are one type of genes that contribute to the development of these abnormal

39   features. These genes are often expressed at higher levels in cancer cells than in normal cells[10]. Another

40   type of genes known as tumor suppressor genes counteracts cellular changes that lead to cancer. These

41   genes are often expressed at lower levels in tumor cells than in normal cells[11], potentially leading to rapid

42   cellular proliferation. Methylation of the promoter region is often negatively correlated with gene

43   expression levels, suggesting that DNA methylation plays a role in regulating gene expression[12,13].

44   However, relatively little is known about global methylation patterns in pediatric tumors, the interplay

45   between methylation events and mutations in pediatric tumors, or how these observations may differ

46   between known cancer genes (oncogenes and tumor suppressors) and other genes. Prior studies have

47   focused on cancer cell lines, a single tumor type at a time, or adult cancers[14–16].

48   Computational models have been developed to identify differentially expressed genes across large sets of

49   methylation data[15,17]. Applications of these methods have found several genes with highly variant

50   methylation in adult tumors[17]. Many of the genes that exhibited highly variant methylation in tumors were

51   not previously associated with cancer, and genomic aberrations in these genes were not characteristic of

52   tumors. A small-scale study of Wilms tumors (a common pediatric cancer) showed similar results[17]. It has

53   been shown that several distinct types of cancer, including endometrioid adenocarcinomas and

54   glioblastomas, share many differentially methylated regions, suggesting that these epigenetic markers

55   may be a universal feature of cancer[18].

56   While these findings hint that aberrant methylation patterns may also be characteristic of pediatric cancer,

57   most pediatric cancers have not been analyzed for DNA methylation patterns. One study investigated

58   genomic, transcriptomic, and epigenomic patterns in acute myeloid leukemia and identified dozens of

59   hypermethylated genes and age-specific patterns[19]. Additionally, structural variations were found to be

60   more common than single nucleotide polymorphisms. A separate analysis of acute lymphoblastic

61   leukemia identified a number of genes on chromosome 3, including PPP2R3A, THRB and FBLN2, that

62   were frequently hypermethylated[16].

63   Little work has been done to specifically investigate aberrant methylation of oncogenes and tumor

64   suppressor genes in cancer. One study about endometrial cancer identified seven oncogenes that were

65   hypomethylated and upregulated and twelve tumor suppressor genes that were hypermethylated and

66   downregulated[14], suggesting that changes in DNA methylation impact gene expression and may target

67   oncogenes and tumor suppressor genes.

68   We address these gaps by analyzing methylation data for five types of pediatric cancers: Wilms tumors,

69   clear cell sarcomas of the kidney, rhabdoid tumors, neuroblastomas, and osteosarcomas. We compare

70   these cancer types against each other. Furthermore, as a baseline reference, we compare the tumor data

71   against methylation levels for fetuses and children representing normal conditions for diverse cell types

72   and human populations. Because methylation events tend to be gene specific[20], we evaluate gene-level

73   patterns. But we also consider global methylation patterns. For many of the tumors, we identify genomic

74   alterations—single-nucleotide variants, small indels, and structural variants—in the tumors and evaluate

75   whether these somatic mutations exhibited gene-specific mutual exclusivity with hypo- or

76   hypermethylation events. In addition, we evaluate the consistency of these findings for oncogenes and

77   tumor suppressor genes.

Results

79   Our goal was to evaluate gene-level DNA methylation patterns for pediatric-tumor cells and normal cells.

80   We used publicly available data to characterize five types of pediatric cancers as well as baseline

81   methylation levels for normal cells. First, we evaluated the consistency of methylation levels representing

82   non-cancerous states in fetuses and children. Second, we compared tumor methylation levels against the

83   normal values and identified genes and tumor samples that exhibited patterns of hypomethylation or

84   hypermethylation. Next, under the assumption that tumors with aberrant methylation would be subject to

85   evolutionary constraints that are redundant with those resulting from somatic mutations, we evaluated

86   whether these two event types were mutually exclusive in a given tumor. Finally, we examined these

87   patterns within known oncogenes and tumor-suppressor genes.

88   **Consistency of methylation levels in normal samples and in pediatric tumors**

89   To aid in understanding how methylation levels change in cancer cells, we first characterized baseline

90   methylation levels for individual genes. We obtained Illumina Infinium 450K data for four normal

91   datasets. We used data from diverse cell types and human populations with the goal of identifying

92   methylation patterns that broadly represent baseline methylation states for healthy children. The datasets

93   were from chorionic villi, kidney, spinal cord, brain, muscle, nasal epithelial, and blood cells and included

94   data for a total of 309 patients of North American (n = 94), African American (n = 36), and Australian

95   ancestry (n = 179).

96   Because DNA methylation contributes to regulating gene expression, we expected most genes to exhibit a

97   consistent baseline methylation range. We anticipated that many genes (such as tumor suppressor genes)

98   would have consistently low methylation levels because those genes must be consistently expressed to

99   properly regulate cellular division, growth, and other proliferation activities. We anticipated that other

100 genes (such as oncogenes) would have consistently high levels of methylation because proper cellular

101 functioning requires that these genes remain relatively inactive. We expected to see some variance across

102 samples because we included data from multiple cell types and because methylation levels change as cells

103 respond to internal and external cues. We also anticipated that some genes would deviate from these

104 patterns, perhaps in part because they are regulated by mechanisms other than DNA methylation. Based

105 on a preliminary inspection of the data, we identified thresholds for categorizing genes based on the

106 magnitude and variance of methylation levels. We considered genes with a median value less than 0.2

107 across all samples in a given dataset to be methylated at "low" levels, genes with a median greater than

108 0.6 to be methylated at "high" methylation levels and the remaining genes as having "medium"

109 methylation levels. We categorized genes with a coefficient of variation (CV) less than 0.5 in a given

110 dataset as having "low" variance and the remaining genes as having "high" variance. In the largest normal

111 dataset (GSE89278), most genes exhibited low (58.9%) or medium methylation levels (25.6%).

112 Relatively few genes exhibited high methylation (15.5%), meaning that under normal conditions, most

113 genes appear not to be subject to strong expression constraints as a result of DNA methylation. Nearly all

114 genes (95.1%) exhibited low variance. The other normal datasets reflected similar patterns.

115   By combining these two ways of categorizing the genes (Additional Data File S1), we found that the most

116   common combination across all datasets was low methylation / low variance (56.9-57.2% per dataset).

117   For GSE89278, the following numbers of genes fell into each category: low methylation / low variance:

118   12,874; low methylation / high variance: 534; medium methylation / low variance: 3,898; medium

119   methylation / high variance: 14; high methylation / low variance: 5,246; high methylation / high variance:

120   0. These results were similar for the other normal datasets. To explore the consistency of these patterns

121   across the normal datasets, we classified each gene into the following consistency levels: consistent (same

122   category in all four datasets), semiconsistent (same category in three datasets), and inconsistent (same

123   category in two or fewer datasets). Of the 22,253 genes that we profiled, 20,089 were consistent, 1,512

124   were semiconsistent, and 652 were inconsistent (Figure 1). Thus even though the normal datasets differed

125   based on cell type and population ancestral groups, gene-level methylation levels were largely consistent.

126   The gene with the smallest variance across the normal datasets was BTG3 (variance = 2.09E-5). In each

127   of the normal datasets, BTG3 exhibited low methylation, suggesting that a biological constraint

128   suppresses methylation of this gene under normal circumstances. The process that normally keeps BTG3

129   methylation low may be disrupted in tumors. Indeed, hypermethylation of the promoter region of BTG3

130   has previously been associated with several types of cancer, including breast[21], prostate[22], and renal[23]. The

131   gene with the largest variance across the normal datasets was DOCK11. Information about this gene is

132   sparse in the literature. Its role in cancer[24] and hypercholesterolemia[25] has been discussed. Its high

133   variance in normal cells suggests that consistent expression of DOCK11 is inessential to normal cellular

134   function and/or that processes other than promoter methylation regulate its expression.

135   **Tumor methylation relative to normal levels**

136   Under the hypothesis that tumorigenesis alters local and global methylation patterns, we evaluated the

137   extent to which methylation levels and variances differed for a given gene between normal and tumor

138   conditions. For each gene, we compared the most common median / variance category across the four

139   normal datasets against the most common category across the five cancer datasets. Typically genes stayed

140   under the same category. For example, of the 12,368 genes that were categorized as low methylation / low

141   variance for the normal datasets, only 549 (4.4%) changed categories: either to low methylation / high

142   variance (n = 462) or to medium methylation / low variance (n = 87) (Table 1). The 227 genes in the

143   medium methylation / high variance category were most likely to change categories, with 137 genes

144   (60.0%) changing to low methylation / high variance and 19 genes (8.4%) changing to medium

145   methylation / low variance. For the 512 genes that changed median methylation levels, approximately half

146   (n = 259, 50.6%) increased (from low to medium or medium to high). For the 562 genes that changed

147   variance categories, 519 (92.3%) increased from low to high variance. These increased variances suggest

148   that the factors that normally keep methylation levels stable under normal conditions often become

149   dysregulated in tumors. Altered expression of these genes may play a role in tumor development or may

150   lead to downstream effects that affect tumor development. For example, if methylation levels of an

151   oncogene are decreased, higher expression of the gene may result, leading to increased cellular growth,

152   proliferation, or survival[9]. On the other hand, increased methylation levels of a tumor suppressor gene

153   could cause lower gene expression and prevent it from performing regulatory functions[13].

154   To identify genes that may influence pediatric tumorigenesis, we compared tumor methylation levels for

155   each gene against the respective normal values on a per-cancer basis. We used a two-sided, Mann-

156   Whitney U test and adjusted for multiple tests using the Benjamini-Hochberg False Discovery Rate

157   (FDR)[26]. We considered genes with an FDR < 0.05 and an absolute methylation change > 0.02 to be

158   statistically significant. Out of the 22,253 genes we analyzed, 37 exhibited differential methylation for at

159   least one cancer type. Of these genes, 19 had decreased methylation in tumors, and 18 had increased

160   methylation, including 15 genes that were differentially methylated for Wilms tumors, 0 for clear cell

161   sarcomas, 6 for rhabdoid tumors, 17 for neuroblastomas, and 4 for osteosarcomas; 4 genes were

162   statistically significant for 2 or more cancer types (Table S1; Figure 2). We note that the number of

163   significant genes was smaller for cancer types with relatively small sample sizes.

164   We performed a pathway analysis for the significant genes from each cancer type using reactome.org[27,28].

165   For Wilms tumors, rhabdoid tumors, and osteosarcomas, pathways associated with ubiquitination,

166   transcriptional regulation, and growth signaling were significant; for neuroblastomas, growth-signaling,

167   integrin-signaling, and blood-biosynthesis pathways were among the most significantAdditional Data

168   Files S2-S5. These functions have plausible connections to tumor biology because they are central to

169   cellular function in general,[29,30] but it is difficult to make precise inferences due to the relatively small

170   numbers of significant genes.

171   To characterize differences in methylation across the five cancer types, independent of normal

172   methylation levels, we performed a one-way ANOVA test for each gene and adjusted the p-values using

173   the FDR correction. Methylation levels of most genes were consistent across the 5 cancer types, including

174   for genes that we had identified as being differentially methylated in tumors compared to normal cells.

175   Methylation levels for 19 genes differed significantly across the cancer types (FDR < 0.05; Table S2;

176   Figure 3). But in most cases, the mean differences were small. For example, the largest absolute

177   difference in mean methylation between tumor types for any of the significant genes was an increase of

178   0.24 in CCSK compared to RT (KCNQ1OT1 gene). Of the 19 genes, 10 fell into the medium methylation

179   / low variance category, a higher proportion (0.526) than the overall proportion of genes in this category

180   (0.175).

181   Next we focused on the 20 genes for which methylation levels varied most across all tumor types and

182   plotted the methylation values, relative to normal levels, for each patient (Figure 4). Some tumors

183   exhibited relatively high methylation levels for nearly all of these genes, whereas other tumors exhibited

184   relatively low methylation levels for the same genes. This was especially true for Wilms tumors, rhabdoid

185   tumors, and osteosarcomas. For example, the average methylation difference (relative to normals) for

186   these 20 genes was 0.13 for 58 (44.3%) of the Wilms tumors but -0.11 for the remaining tumors. For the

187   osteosarcomas and rhabdoid tumors, we observed a similar pattern in which subsets of 49 (57.0%) and 35

188   (51.5%) tumors, respectively, showed markedly higher methylation levels than the remaining tumors.

189   These patterns of consistently divergent methylation might be useful for identifying tumor subtypes in a

190   precision-medicine context[31] and may be driven by factors such as the presence of somatic mutations and

191   structural alterations that affect global methylation in characteristic ways.

192   To investigate global methylation patterns, we calculated gene-level z-scores for each tumor using the

193   normal data as a reference. In cases where a tumor's methylation value was more than three standard

194   deviations higher than the mean normal value for a particular gene, we classified that gene as being

195   hypermethylated in that tumor. In cases where a tumor's methylation value was more than three standard

196   deviations lower than the mean normal value for a particular gene, we classified that gene as being

197   hypomethylated in that tumor. Then we calculated the proportion of hypomethylated and hypermethylated

198   genes for each patient. We categorized tumors for which greater than 1% of genes were hypermethylated

199   as being "frequently hypermethylated" and tumors for which greater than 1% of genes were

200   hypomethylated as being "frequently hypomethylated." Frequent hypermethylation was more common

201   (17.5% of tumors) than frequent hypomethylation (3.4%)(Figure 5). The maximum percentage of

202   hypermethylated genes for any particular patient was 11.8%, compared to a maximum of 4.8% for

203   hypomethylated genes.

204   **Cancer mutation data analysis**

205   Although they occur less frequently in pediatric tumors than in adult tumors, somatic mutations often

206   contribute to pediatric tumorigenesis[4,5]. To evaluate the frequency of and interplay between somatic

207   mutations and methylation events, we examined pediatric tumors for which both data types were

208   available. Mutation data were available for somatic single nucleotide variants (SNVs),

209   insertions/deletions (indels), and RNA fusions. We used the RNA fusion data as indicators of structural

210   DNA variants. Copy-number data were available for only a small number of tumors, so we did not

211   include this data type in the analysis. Methylation, SNV, indel, and RNA fusion data were available for

212   Wilms tumors (n = 41), neuroblastomas (n = 65), and osteosarcomas (n = 66) but not for the other tumor

213   types. For a given tumor, we considered genes with at least one SNV, indel, or RNA fusion event to be

214   "mutated." Across all tumor types, aberrant methylation—via either hypomethylation or

215   hypermethylation of a given gene—occurred less frequently (in 1.1% of tumors) than mutations (2.8%);

216   the largest disparity occurred for neuroblastomas (Table S3). Wilms tumors and osteosarcomas were

217   aberrantly methylated nearly twice as often as neuroblastomas (1.4% vs. 0.8%; Table S3). In contrast,

218   Wilms tumors and osteosarcomas were mutated less than half as often as neuroblastomas (1.7-1.8%

219   vs. 4.6%).

220   Aberrant methylation levels and damaging mutations can have similar downstream effects in tumors[32].

221   After one of these alteration types has occurred in a given gene, it may be less likely for cells with a

222   second alteration in the same gene to gain an additional selective advantage. Thus, having both a mutation

223   and aberrant methylation in one gene may be less likely than expected by random chance. This mutual-

224   exclusivity hypothesis has been examined extensively for pairs of genes in which somatic mutations

225   might occur across diverse types of cancers[33–36]. It has also been investigated for DNA methylation

226   events, though to a lesser extent[37]. Little is known about mutual exclusivity between methylation events

227   and somatic mutations in pediatric tumors.

228   Treating each combination of tumor and gene as an independent observation, we examined whether DNA

229   methylation events are mutually exclusive with somatic mutations. Mutations and aberrant methylation

230   co-occurred rarely (0.03%) in the same gene and the same tumor (Table S3). Because both of these event

231   types are rare, we reduced the data to the 962 genes for which at least 5 mutation events and at least 5

232   aberrant methylation events had occurred across the cancer types. We used a permutation test to evaluate

233   whether mutations and aberrant methylation in the same gene and tumor co-occurred less frequently than

234   would be expected by random chance. Across all tumors and the 962 genes, we observed 318 co-

235   occurrences, whereas the average number of co-occurrences in the permuted data was 341.6. However,

236   this difference was not statistically significant (p = 0.092).

237   **Oncogenes and tumor suppressor genes**

238   Because oncogenes are typically expressed at relatively high levels and tumor suppressor genes are

239   typically expressed at relatively low levels, we expected that oncogenes would have higher methylation

240   levels than tumor suppressor genes. We identified 80 "tier 1" oncogenes and 141 "tier 1" tumor

241   suppressor genes in the Cancer Genome Census[38] and calculated the mean methylation value for each

242   gene in the normal datasets and used a two-sample t-test to evaluate whether these mean values differed

243   between the oncogenes and tumor suppressor genes. Under the hypothesis that oncogenes would be

244   methylated at higher levels than tumor-suppressor genes, we used a one-sided test. The mean of the

245   means for oncogenes was 0.02 higher than for tumor-suppressor genes; however, this difference was not

246   statistically significant (p = 0.109). Many tumor-suppressor genes were highly methylated, and many

247   oncogenes were methylated at low levels (Figure S1).

248   Next we evaluated the extent to which methylation levels differed for oncogenes and tumor-suppressor

249   genes between tumor and normal conditions. First, we filtered the results from the two-sided Mann-

250   Whitney U tests described above to include only oncogenes and tumor suppressor genes. Using FDR <

251   0.05 and an absolute mean difference > 0.02 as constraints, only 1 tumor suppressor gene was statistically

252   significant (CTCF in Wilms tumors). No oncogenes reached statistical significance. We relaxed the

253   threshold to FDR < 0.2 and removed the mean difference constraint. In this case, CTCF was significant

254   for all cancer types except CCSK (Figure S2). DNM2 (a tumor suppressor gene) was significant for OS,

255   and GNA11 (an oncogene) was significant for NBLTable S4. Mean methylation values for none of the

256   oncogenes or tumor suppressor genes differed across the tumor types (FDR < 0.2; Table S2).

257   Across the tumor types, mutation rates for oncogenes and tumor suppressor genes (0.031-0.089) were

258   approximately twice the mutation rates for other genes (0.017-0.046) (Table 2), which aligns with prior

259   evidence that mutations in these genes are associated with tumorigenesis. However, aberrant methylation

260   rates for oncogenes and tumor suppressor genes (0.007-0.014) were approximately equal to methylation

261   rates for other genes (0.008-0.014). In Wilms tumors and osteosarcomas, mutation rates for oncogenes

262 and tumor suppressor genes were approximately three times higher than aberrant methylation rates, while

263 mutation and methylation rates were approximately equal for other genes. In neuroblastomas, mutation

264 rates for oncogenes and tumor suppressor genes were approximately twelve times higher than aberrant

265 methylation rates but only six times higher for other genes.

266 We performed a modified version of the permutation-based, mutual-exclusivity analysis in which we

267 searched for co-occurrences of either 1) a mutation in an oncogene and aberrant hypomethylation of the

268 same gene or 2) a mutation in a tumor suppressor gene and hypermethylation of the same gene. Because

269 few genes met these criteria, we performed this analysis with the oncogenes (n = 4) and tumor-suppressor

270 genes (n = 29) that were mutated in at least 2 tumors and aberrantly methylated in at least 2 tumors. These

271 event combinations were not mutually exclusive for oncogenes (p = 0.90) nor for tumor suppressors (p =

272 0.54).

Discussion

273

274 Using publicly available data, we examined methylation patterns for five childhood cancers. We

275 summarized baseline methylation levels for healthy children, identified deviations from these baseline

276 levels in tumors, and evaluated mutual exclusivity of methylation events and somatic mutations.

277 Subsequently, we evaluated these patterns for oncogenes and tumor-suppressor genes specifically. In the

278 309 healthy samples we studied, DNA methylation levels were highly consistent, suggesting that

279 biological processes maintain this consistency in diverse tissue types and ancestral groups. In the 531

280 tumors we studied, hypermethylation or hypomethylation of promoter regions was a common feature,

281 providing additional evidence that the tumor epigenome contributes to pediatric tumorigenesis.

282 Hypermethylation was more common than hypomethylation. Hypermethylation of promoter regions has

283 been associated with decreased gene expression[12]—tumors with frequent hypermethylation may result in

284 broad silencing of proteins necessary for normal cellular function. Hypermethylation affecting multiple

285   genes in the same tumor has been identified in adult thyroid neoplasms[39] and colorectal tumors[40] and has

286   been associated with multidrug resistance in cell cultures[41]. However, global hypomethylation has

287   garnered more attention than multigene hypermethylation[42]. Yet in the pediatric-tumor samples that we

288   examined, widespread hypomethylation in a given tumor was uncommon. We also observed tumor

289   subsets that exhibited divergent methylation—methylation levels for specific genes were consistently

290   either high or low in many tumors of a given cancer type. While methylation dysregulation may be a

291   common feature of pediatric tumors in general, specific mechanisms may drive these divergent changes in

292   each tumor subset, and there may be overlap in these mechanisms across cancer types. Identifying such

293   mechanisms may be useful for developing targeted treatments and informing patients.

294   Our mutual-exclusivity analysis did not provide evidence that somatic mutations and aberrant methylation

295   are mutually exclusive with each other, whether for oncogenes and tumor-suppressor genes specifically or

296   across all genes. However, we had access to a limited number of tumors (n = 172) for which both

297   mutation data and methylation data were available; a larger-scale analysis is warranted. Furthermore, we

298   evaluated single-nucleotide variants, indels, and structural variants because data were available for these

299   mutation types. But large-scale amplifications and deletions occur regularly in tumors[43], so including

300   copy-number data in this type of analysis would also be useful in future research.

301   In normal cells, methylation levels did not differ significantly between known cancer genes (oncogenes

302   and tumor suppressors) and other genes. In tumors, few oncogenes and tumor suppressors had high rates

303   of aberrant methylation, suggesting that while aberrant methylation occurs in pediatric tumors,

304   dysregulation likely does not occur disproportionately in these known gene categories. Global

305   hypermethylation and/or hypomethylation patterns may be more important indicators of pediatric-tumor

306   biology than local events. However, CTCF may be one exception. CTCF promoter methylation was

307   significantly increased relative to normal conditions in every cancer type that we examined, except clear

308   cell sarcoma of the kidney, which had a small sample size. CTCF codes for a zinc finger nuclease that is

309   highly conserved in eukaryotes[44]. As a regulatory protein involved in DNA methylation[45], improper

310   function of this gene could lead to epigenetic abnormalities. Mutations in CTCF have previously been

311   associated with several cancer types, including breast cancers, prostate cancers, and Wilms tumors[46,47].

312   Our findings across multiple pediatric cancer types suggest that CTCF hypermethylation—and

313   consequent inhibition of gene expression— may influence pediatric tumorigenesis broadly and cause

314   downstream effects.

315   In performing these analyses, we needed to choose arbitrary thresholds at times. For example, we

316   specified three standard deviations above or below the mean in the normal data as a conservative

317   threshold to indicate aberrant methylation. Using this threshold, we found that tumors were mutated more

318   frequently than they were aberrantly methylated. However, if we had relaxed this threshold to two

319   standard deviations, the average aberrant methylation rate would have been 0.045 rather than 0.012 (the

320   average mutation rate was 0.027). Thus, our conclusions would have been moderately different. The use

321   of arbitrary thresholds in research is common and cannot be completely avoided, especially when

322   discipline-specific precedents have yet to be specified by the research community. In this scenario, we

323   have introduced a new way of identifying aberrant methylation events. Future quantitative, experimental,

324   and translational work will be necessary to improve our ability to determine when a particular gene in a

325   given biological sample is methylated to an extent that alters that gene's behavior and may in turn have

326   clinical relevance.

327   A better understanding of DNA methylation's role in pediatric tumors could shed light on mechanisms of

328   tumorigenesis and eventually lead to insights about patient care and treatments. DNA methylation

329   inhibitors have proven effective in some adult cancers, especially hematologic malignancies[20] and may

330   prove beneficial in pediatric cases. However, these therapies primarily target hypermethylation in a broad

331   sense and thus may not be suitable for targeting specific genes[48,49]. Furthermore, as we have shown,

332   hypermethylation events may be more common than hypomethylation events in pediatric tumors. Little is

333   understood about how to reverse hypomethylation in vivo; however, global-hypomethylation patterns

334   may be useful as biomarkers for therapies for targeting genes that have been activated as a result of

335   hypomethylation.

Methods

336

Normal methylation datasets

337

338   We downloaded datasets containing DNA methylation levels for cohorts of normal patients as a way to

339   establish a baseline against which tumor methylation could be compared for pediatric samples. We

340   selected four datasets from Gene Expression Omnibus that used Illumina Infinium

341   HumanMethylation450K arrays to profile normal cells in healthy fetuses and children aged 18 or under

342   for which raw (.idat) files were available. Illumina Infinium HumanMethylation450K is a microarray

343   platform that detects DNA methylation at over 450,000 locations in the human genome. Beta values from

344   these arrays indicate the ratio of methylated signals to unmethylated signals[50]. Values closer to one

345   indicate relatively high methylation levels; values closer to zero indicate relatively low methylation

346   levels. Below we describe each dataset included in the study and provide accession identifiers from Gene

347   Expression Omnibus.

348   GSE69502 originated from a study of Canadian second trimester fetuses[51]. Tissue was collected from

349   chorionic villi, kidney, spinal cord, brain, and muscle. The original study analyzed DNA methylation

350   differences in fetuses with spina bifida and anencephaly compared to normal fetuses. We included only

351   data from the 65 normal fetuses.

352   GSE65163 originated from a study of nasal epithelial cells from African American children aged 10-12[52].

353   The original study analyzed DNA methylation differences in children with or without asthma. We

354   included only data from the 36 children without asthma.

355 GSE109446 originated from a study of children aged 5-18 living in Cincinnati, Ohio (USA)[53]. Similar to

356 GSE65163, nasal epithelial cells were used, and the goal of the original study was to understand

357 methylation differences in children with or without asthma. We included only data from the 29 children

358 without asthma.

359 GSE89278 originated from a study of Australian infant blood spot samples taken at birth[54]. The goal of

360 the original study was to understand methylation differences in children with and without

361 docosahexaenoic acid deficiency. We included only control data from 179 infants.

362 We chose these samples because they represented a variety of cell types and ancestral groups, which we

363 hoped would make our findings more broadly generalizable than if our samples had been from a single,

364 homogeneous population. Another factor in our decision was the age of the individuals. We searched for

365 datasets representing relatively young patients to limit the possible confounding factor of methylation

366 changes accumulating throughout life. In this process, we considered one additional normal dataset

367 (GSE72556)[55]; however, we found that the beta levels from this dataset were consistently different from

368 the other four normal datasets. This dataset originated from saliva samples, which often result in

369 systematically different methylation levels than other types of samples[56–58], perhaps due to external

370 contaminants or different sample-collection procedures. As a result, we excluded this dataset from our

371 analysis.

372 **Normal data processing**

373 We processed the data using the minfi package (version 1.34.0) from R (version 4.0.2) and the

374 Bioconductor suite[59,60]. We followed a standard workflow to process the methylation array files. The steps

375 in this workflow included preprocessing, ratio conversion, and beta value calculations. We then

376 summarized the beta values at the gene level. We mapped probes to genes based on an annotation file

377 created by Price et al. (see http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GPL16304)[61]. Because

378 we were interested in methylation changes in the promoter regions of genes, we included only probes

379   within 300 base pairs of transcription start sites. We then calculated gene-level beta values for each

380   patient as the mean beta value across all remaining probes in a given gene.

381   **Methylation data acquisition and processing**

382   We obtained methylation data representing five tumor types from the Therapeutically Applicable

383   Research to Generate Effective Treatments (TARGET) Data Matrix (retrieved August 24, 2020 from

384   Website, https://ocg.cancer.gov/programs/target/data-matrix). We acquired data for 131 patients with

385   Wilms tumor, 11 patients with clear cell sarcoma of the kidney, 68 patients with rhabdoid tumor, 235

386   patients with neuroblastoma, and 86 patients with osteosarcoma. We downloaded the data from TARGET

387   using the rvest package (version 0.3.6)[62]. The data for Wilms tumors, clear cell sarcomas of the kidney,

388   neuroblastomas, and osteosarcomas were generated using the Illumina Infinium HumanMethylation450K

389   platform, and the data for rhabdoid tumors were generated using the Illumina Infinium MethylationEPIC

390   platform. The HumanMethylation450K platform produces beta values for 22,579 genes, and EPIC

391   produces values for 22,411 genes; we limited our analysis to the 22,253 genes that overlapped between

392   the two platforms.

393   We followed the same workflow that we used to process the normal data. We calculated probe-level beta

394   values using minfi and summarized values at the gene-level. We used principal component analysis to

395   assess high-level patterns across the datasets and visualized the results using a scatter plot of the first two

396   principal components. Methylation samples from each dataset generally clustered tightly with other

397   samples from the same dataset, demonstrating that batch effects were present (Figure S3). To reduce the

398   impact of these effects, we applied batch correction to a combined dataset that included all of the normal

399   datasets. We used the dataset identifier as the batch variable. In addition, we logit transformed the data

400   before performing the batch correction and inverse logit transformed the data after performing the batch

401   correction to ensure the beta values stayed between 0 and 1. We performed these transformations using

402   functions from the gtools package (version 3.8.2)[63]. Because the logit function cannot handle exact values

403   of 0 or 1, we removed genes in which the beta values were exactly 0 or 1. We also removed genes in

404   which beta values were NA. Filtering the data in this way removed 155 (0.7%) genes (out of 22,411 total

405   genes). To perform the batch correction, we used the ComBat function from the sva package (version

406   3.36.0)[64]. After completing the batch correction, we performed another principal component analysis and

407   visualized the results. The points no longer clustered by dataset (Figure S3). We used these newly

408   corrected values for all remaining analyses.

409   **Somatic-mutation data acquisition and processing**

410   We obtained somatic-mutation data for TARGET patients via the Genomic Data Commons portal[65–67].

411   We first selected the Repository section. Under the Cases tab, we specified the TARGET program. We

412   also specified Wilms tumor, neuroblastoma, and osteosarcoma as the tumor types of interest (data were

413   unavailable for the other two tumor types). Under the Files tab, we selected "simple nucleotide variation"

414   and "annotated somatic mutation" as the data category and type, and we indicated that we would use

415   variants that had been called using Mutect2[68]. The data were stored in VCF format (version 4.2)[69].

416   We wrote custom code to parse the mutation data for each patient. We included only mutations that had

417   either 1) "HIGH" impact according to the variant annotations or 2) had "MODERATE" impact and were

418   considered to be "deleterious" by SIFT[70] or either "damaging" or "probably_damaging" according to

419   Polyphen-2[71].We considered using the specified minor-allele-frequency (MAF) values for filtering, but

420   those values were unavailable for a significant portion of the variants, so we focused on functional

421   annotations.

422   **RNA-fusion data acquisition and processing**

423   We downloaded data for RNA fusions via the Genomic Data Commons portal. Under the Cases tab, we

424   applied the same filters that we used for obtaining the somatic-mutation data. Under the Files tab, we

425   selected "structural variation" and "Transcript Fusion" as the data category and type. We specified

426   "STAR-Fusion"[72] as the workflow type and "bedpe" as the data format. We wrote custom code to parse

427   the RNA fusion data for each patient. The bedpe files described RNA transcripts from each patient that

428   had genetic information originating from two separate genes. We considered an RNA fusion to have a

429   functional impact on both genes affected by the fusion.

430   **Methylation and mutation data integration**

431   Different naming conventions were used to identify neuroblastoma and osteosarcoma patients in the

432   methylation data files versus the somatic-mutation and RNA fusion data files. We used a sample sheet

433   provided by the Genomic Data Commons (gdc_sample_sheet.2021-03-11.tsv) and the sdrf metadata files

434   for neuroblastoma and osteosarcoma to map the patient identifiers between these sources (see

435   https://target-

436   data.nci.nih.gov/Public/OS/methylation_array/METADATA/TARGET_OS_MethylationArray_20161103

437   .sdrf.txt and https://target-

438   data.nci.nih.gov/Public/NBL/methylation_array/METADATA/TARGET_NBL_MethylationArray_20160

439   812.sdrf.txt). A few files did not map properly across naming conventions. One fusion data sample was

440   mapped to two patients, so we excluded this sample. There were also two pairs of single nucleotide

441   mutation samples that were mapped to single patients, so we excluded these samples.

442   **Inferring hypermethylation and hypomethylation states**

443   To determine hypermethylation and hypomethylation status, we scaled the beta values for each gene to

444   have a zero mean and a standard deviation of one across all normal samples. Then for each tumor sample,

445   we compared the beta value for a given gene against the standardized values from the normal samples. If

446   a tumor's beta value was more than three standard deviations above the mean of the normal samples, we

447   classified that tumor as being hypermethylated for that gene. If a tumor's beta value was more than three

448   standard deviations below the mean of the normal samples, we classified that tumor as being

449   hypomethylated for that gene.

Mutual exclusivity

451 We evaluated whether aberrant methylation—either hyper- or hypomethylation—was mutually exclusive

452 with somatic-mutation events for a given gene. We calculated the total number of times a gene was both

453 mutated and aberrantly methylated in the same tumor. Next we permuted methylation status for all tumors

454 and kept mutation status constant. We repeated the permutation process 10,000 times to create an

455 empirical null distribution to use as a baseline. For each permutation, we calculated the number of times a

456 gene was both mutated and aberrantly methylated. We then compared the number of times that mutations

457 co-occurred with aberrant methylation in the non-permuted data relative to the permuted data and

458 calculated a p-value based on these numbers.

Oncogenes vs tumor suppressor genes

460 We identified genes known to be oncogenes or tumor suppressor genes, based on information from The

461 Cancer Gene Census[38]. We included only tier 1 genes classified as "oncogene" or "TSG" and ignored any

462 for which it was ambiguous or unknown. To be classified as a tier 1 gene, there must be scientific

463 evidence that a gene plays a role in cancer development and that mutations in the gene modify the activity

464 of the associated protein. Several genes were listed in the Cancer Genome Census as both oncogenes and

465 tumor suppressor genes; because of this ambiguity, we excluded these from our analysis.

466 In evaluating our hypothesis that oncogenes generally have decreased methylation levels in tumor

467 samples relative to normal samples and that tumor suppressor genes generally have increased methylation

468 levels in tumor samples relative to normal samples, we applied a two-sided, Mann-Whitney U test to each

469 oncogene and tumor suppressor gene to identify genes that did (or did not) align with these expectations.

Abbreviations

Abbreviation	Term
WT	Wilms tumor

CCSK   Clear cell sarcoma of the kidney

RT   Rhabdoid tumor

NBL   Neuroblastoma

OS   Osteosarcoma

TSG   Tumor suppressor gene

471

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477 Research to Generate Effective Treatments (TARGET) initiative, phs000218, managed by the NCI. The

478 data used for this analysis are available from the National Cancer Institute Genomic Data Commons

479 (https://gdc.cancer.gov). Information about TARGET can be found at

480 http://ocg.cancer.gov/programs/target.

481 **Ethics approval and consent to participate**

482 Brigham Young University's Institutional Review Board approved this study under exemption status.

483 This study uses data collected from public repositories only. We played no part in recruiting patients or

484 obtaining consent.

485 **Competing Interests**

486 The authors declare that they have no competing interests.

487 **Code and data availability**

488 All of the code used to perform the analysis is publicly available on Open Science Framework so that

489 others can verify and build upon our work (https://osf.io/79yfb/).

490 **Author Contributions**

491 The contributions listed below correspond to the CRediT Taxonomy[73].

492 ACP: Conceptualization, Formal Analysis, Funding Acquisition, Investigation, Methodology, Software,

493 Visualization, Writing – Original Draft Preparation, Writing – Review & Editing

494 BIQ: Conceptualization, Formal Analysis, Software, Visualization, Writing – Original Draft Preparation,

495 Writing – Review & Editing

496 SRP: Conceptualization, Funding Acquisition, Methodology, Project Administration, Resources,

497 Supervision, Writing – Review & Editing

498 References

499 1.   Filbin, M. & Monje, M. Developmental origins and emerging therapeutic opportunities for

500 childhood cancer. *Nat. Med.* **25**, 367–376 (2019).

501 2.   Oeffinger, K. C., Eshelman, D. A., Tomlinson, G. E., Buchanan, G. R. & Foster, B. M. Grading

502 of late effects in young adult survivors of childhood cancer followed in an ambulatory adult setting.

503 *Cancer* **88**, 1687–1695 (2000).

504 3.   Stratton, M. R., Campbell, P. J. & Futreal, P. A. The cancer genome. *Nature* **458**, 719–724

505 (2009).

506 4.   Yiu, T. T. & Li, W. Pediatric cancer epigenome and the influence of folate. *Epigenomics* **7**, 961–

507 973 (2015).

508   5.   Gröbner, S. N. *et al.* The landscape of genomic alterations across childhood cancers. *Nature* **555**,

509   321–327 (2018).

510   6.   Mack, S. C. *et al.* Epigenomic alterations define lethal CIMP-positive ependymomas of infancy.

511   *Nature* **506**, 445–450 (2014).

512   7.   Bayliss, J. *et al.* Lowered H3K27me3 and DNA hypomethylation define poorly prognostic

513   pediatric posterior fossa ependymomas. *Science Translational Medicine* **8**, 366ra161–366ra161 (2016).

514   8.   Ehrlich, M. DNA hypomethylation in cancer cells. *Epigenomics* **1**, 239–259 (2009).

515   9.   Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: The next generation. *Cell* **144**, 646–674

516   (2011).

517   10.   Croce, C. M. Oncogenes and Cancer. *N. Engl. J. Med.* **358**, 502–511 (2008).

518   11.   Macleod, K. Tumor suppressor genes. *Current Opinion in Genetics & Development* **10**, 81–93

519   (2000).

520   12.   Spainhour, J. C., Lim, H. S., Yi, S. V. & Qiu, P. Correlation Patterns Between DNA Methylation

521   and Gene Expression in The Cancer Genome Atlas. *Cancer Inform* **18**, 1176935119828776 (2019).

522   13.   Esteller, M. Epigenetic gene silencing in cancer: The DNA hypermethylome. *Hum. Mol. Genet.*

523   **16**, R50–R59 (2007).

524   14.   Liu, J. *et al.* Identification of aberrantly methylated differentially expressed genes and associated

525   pathways in endometrial cancer using integrated bioinformatic analysis. *Cancer Med.* **9**, 3522–3536

526   (2020).

527   15.   Shi, M., Tsui, S. K.-W., Wu, H. & Wei, Y. Pan-cancer analysis of differential DNA methylation

528   patterns. *BMC Medical Genomics* **13**, 154 (2020).

529   16.   Dunwell, T. L. *et al.* Epigenetic analysis of childhood acute lymphoblastic leukemia. *Epigenetics*

530   **4**, 185–193 (2009).

531   17.   Saghafinia, S., Mina, M., Riggi, N., Hanahan, D. & Ciriello, G. Pan-Cancer Landscape of

532   Aberrant DNA Methylation across Human Tumors. *Cell Reports* **25**, 1066–1080.e8 (2018).

533   18.   Karlow, J. A., Miao, B., Xing, X., Wang, T. & Zhang, B. Common DNA methylation dynamics

534   in endometriod adenocarcinoma and glioblastoma suggest universal epigenomic alterations in

535   tumorigenesis. *Commun Biol* **4**, 1–16 (2021).

536   19.   Bolouri, H. *et al.* The molecular landscape of pediatric acute myeloid leukemia reveals recurrent

537   structural alterations and age-specific mutational interactions. *Nat Med* **24**, 103–112 (2018).

538   20.   Issa, J.-P. J. & Kantarjian, H. M. Targeting DNA Methylation. *Clin Cancer Res* **15**, 3938–3946

539   (2009).

540   21.   Yu, J. *et al.* Methylation-mediated downregulation of the B-cell translocation gene 3 (BTG3) in

541   breast cancer cells. *Gene Expr* **14**, 173–182 (2008).

542   22.   Majid, S. *et al.* Genistein reverses hypermethylation and induces active histone modifications in

543   tumor suppressor gene B-Cell translocation gene 3 in prostate cancer. *Cancer* **116**, 66–76 (2010).

544   23.   Majid, S. *et al.* BTG3 tumor suppressor gene promoter demethylation, histone modification and

545   cell cycle arrest by genistein in renal cancer. *Carcinogenesis* **30**, 662–670 (2009).

546   24.   Almstrup, K. *et al.* Improved gene expression signature of testicular carcinoma in situ. *Int J*

547   *Androl* **30**, 292–302; discussion 303 (2007).

548   25.   Reeskamp, L. F. *et al.* Differential DNA methylation in familial hypercholesterolemia.

549   *EBioMedicine* **61**, 103079 (2020).

550   26.   Benjamini, Y. & Hochberg, Y. Controlling the False Discovery Rate: A Practical and Powerful

551   Approach to Multiple Testing. *J. R. Stat. Soc. Ser. B Methodol.* **57**, 289–300 (1995).

552   27.   Jassal, B. *et al.* The reactome pathway knowledgebase. *Nucleic Acids Res* **48**, D498–D503

553   (2020).

554   28.   Fabregat, A. *et al.* Reactome pathway analysis: A high-performance in-memory approach. *BMC*

555   *Bioinformatics* **18**, (2017).

556   29.   Futreal, P. A. *et al.* A CENSUS OF HUMAN CANCER GENES. *Nat Rev Cancer* **4**, 177–183

557   (2004).

558 30.   Goode, E. L., Ulrich, C. M. & Potter, J. D. Polymorphisms in DNA Repair Genes and

559   Associations with Cancer Risk. *Cancer Epidemiol Biomarkers Prev* **11**, 1513–1530 (2002).

560 31.   Forrest, S. J., Geoerger, B. & Janeway, K. A. Precision medicine in pediatric oncology. *Curr*

561   *Opin Pediatr* **30**, 17–24 (2018).

562 32.   Bodily, W. R. *et al.* Effects of germline and somatic events in candidate BRCA-like genes on

563   breast-tumor signatures. *PLoS One* **15**, (2020).

564 33.   Ciriello, G., Cerami, E., Sander, C. & Schultz, N. Mutual exclusivity analysis identifies

565   oncogenic network modules. *Genome Res* **22**, 398–406 (2012).

566 34.   Babur, Ö. *et al.* Systematic identification of cancer driving signaling pathways based on mutual

567   exclusivity of genomic alterations. *Genome Biol* **16**, 45 (2015).

568 35.   Kang, S. *et al.* Mutual exclusiveness between PIK3CA and KRAS mutations in endometrial

569   carcinoma. *Int J Gynecol Cancer* **18**, 1339–1343 (2008 Nov-Dec).

570 36.   Szczurek, E. & Beerenwinkel, N. Modeling mutual exclusivity of cancer mutations. *PLoS*

571   *Comput Biol* **10**, e1003503 (2014).

572 37.   Ding, W., Feng, G., Hu, Y., Chen, G. & Shi, T. Co-occurrence and Mutual Exclusivity Analysis

573   of DNA Methylation Reveals Distinct Subtypes in Multiple Cancers. *Front Cell Dev Biol* **8**, 20 (2020).

574 38.   Tate, J. G. *et al.* COSMIC: The Catalogue Of Somatic Mutations In Cancer. *Nucleic Acids Res*

575   **47**, D941–D947 (2019).

576 39.   Keelawat, S. *et al.* Detection of global hypermethylation in well-differentiated thyroid neoplasms

577   by immunohistochemical (5-methylcytidine) analysis. *J Endocrinol Invest* **38**, 725–732 (2015).

578 40.   Toyota, M. *et al.* CpG island methylator phenotype in colorectal cancer. *PNAS* **96**, 8681–8686

579   (1999).

580 41.   Segura-Pacheco, B. *et al.* Global DNA hypermethylation-associated cancer chemotherapy

581   resistance and its reversion with the demethylating agent hydralazine. *Journal of Translational Medicine*

582   **4**, 32 (2006).

583   42.   Feinberg, A. P. & Tycko, B. The history of cancer epigenetics. *Nat Rev Cancer* **4**, 143–153

584   (2004).

585   43.   Shlien, A. & Malkin, D. Copy number variations and cancer. *Genome Medicine* **1**, 62 (2009).

586   44.   Ohlsson, R., Renkawitz, R. & Lobanenkov, V. CTCF is a uniquely versatile transcription

587   regulator linked to epigenetics and disease. *Trends in Genetics* **17**, 520–527 (2001).

588   45.   Wiehle, L. *et al.* DNA (de)methylation in embryonic stem cells controls CTCF-dependent

589   chromatin boundaries. *Genome Res* **29**, 750–761 (2019).

590   46.   Kemp, C. J. *et al.* CTCF Haploinsufficiency Destabilizes DNA Methylation and Predisposes to

591   Cancer. *Cell Reports* **7**, 1020–1029 (2014).

592   47.   Oh, S., Oh, C. & Yoo, K. H. Functional roles of CTCF in breast cancer. *BMB Rep* **50**, 445–453

593   (2017).

594   48.   Roberti, A., Valdes, A. F., Torrecillas, R., Fraga, M. F. & Fernandez, A. F. Epigenetics in cancer

595   therapy and nanomedicine. *Clinical Epigenetics* **11**, 81 (2019).

596   49.   Cheishvili, D., Boureau, L. & Szyf, M. DNA demethylation and invasive cancer: Implications for

597   therapeutics. *Br J Pharmacol* **172**, 2705–2715 (2015).

598   50.   Dedeurwaerder, S. *et al.* A comprehensive overview of Infinium HumanMethylation450 data

599   processing. *Brief Bioinform* **15**, 929–941 (2014).

600   51.   Price, E. M. *et al.* Profiling placental and fetal DNA methylation in human neural tube defects.

601   *Epigenetics Chromatin* **9**, 6 (2016).

602   52.   Yang, I. V. *et al.* The nasal methylome and childhood atopic asthma. *J Allergy Clin Immunol* **139**,

603   1478–1488 (2017).

604   53.   Zhang, X. *et al.* Nasal DNA methylation is associated with childhood asthma. *Epigenomics* **10**,

605   629–641 (2018).

606   54.   van Dijk, S. J. *et al.* Effect of prenatal DHA supplementation on the infant epigenome: Results

607   from a randomized controlled trial. *Clin Epigenetics* **8**, 114 (2016).

608 55. Oelsner, K. T., Guo, Y., To, S. B.-C., Non, A. L. & Barkin, S. L. Maternal BMI as a predictor of

609 methylation of obesity-related genes in saliva samples from preschool-age Hispanic children at-risk for

610 obesity. *BMC Genomics* **18**, 57 (2017).

611 56. Armstrong, D. A., Lesseur, C., Conradt, E., Lester, B. M. & Marsit, C. J. Global and gene-

612 specific DNA methylation across multiple tissues in early infancy: Implications for children's health

613 research. *FASEB J.* **28**, 2088–2097 (2014).

614 57. Godderis, L. *et al.* Global Methylation and Hydroxymethylation in DNA from Blood and Saliva

615 in Healthy Volunteers. *BioMed Res. Int.* **2015**, e845041 (2015).

616 58. Smith, A. K. *et al.* DNA extracted from saliva for methylation studies of psychiatric traits:

617 Evidence tissue specificity and relatedness to brain. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* **168**,

618 36–44 (2015).

619 59. Aryee, M. J. *et al.* Minfi: A flexible and comprehensive Bioconductor package for the analysis of

620 Infinium DNA methylation microarrays. *Bioinformatics* **30**, 1363–1369 (2014).

621 60. Amezquita, R. A. *et al.* Orchestrating single-cell analysis with Bioconductor. *Nat. Methods* **17**,

622 137–145 (2020).

623 61. Price, M. E. *et al.* Additional annotation enhances potential for biologically-relevant analysis of

624 the Illumina Infinium HumanMethylation450 BeadChip array. *Epigenetics Chromatin* **6**, 4 (2013).

625 62. Wickham, H. *Rvest: Easily harvest (scrape) web pages.* (2020).

626 63. Warnes, G. R., Bolker, B. & Lumley, T. *Gtools: Various r programming tools.* (2020).

627 64. Leek, J. T., Johnson, W. E., Parker, H. S., Jaffe, A. E. & Storey, J. D. The sva package for

628 removing batch effects and other unwanted variation in high-throughput experiments. *Bioinformatics* **28**,

629 882–883 (2012).

630 65. Grossman, R. L. *et al.* Toward a Shared Vision for Cancer Genomic Data. *N Engl J Med* **375**,

631 1109–1112 (2016).

632 66. Pugh, T. J. *et al.* The genetic landscape of high-risk neuroblastoma. *Nat Genet* **45**, 279–284

633 (2013).

634 67.   Gadd, S. *et al.* A Children's Oncology Group and TARGET initiative exploring the genetic

635 landscape of Wilms tumor. *Nat Genet* **49**, 1487–1494 (2017).

636 68.   Benjamin, D. *et al.* Calling Somatic SNVs and Indels with Mutect2. *bioRxiv* 861054 (2019)

637 doi:10.1101/861054.

638 69.   Danecek, P. *et al.* The variant call format and VCFtools. *Bioinformatics* **27**, 2156–2158 (2011).

639 70.   Vaser, R., Adusumalli, S., Leng, S. N., Sikic, M. & Ng, P. C. SIFT missense predictions for

640 genomes. *Nat. Protoc.* **11**, 1–9 (2016).

641 71.   Adzhubei, I., Jordan, D. M. & Sunyaev, S. R. Predicting Functional Effect of Human Missense

642 Mutations Using PolyPhen-2. *Curr. Protoc. Hum. Genet.* **76**, 7.20.1–7.20.41 (2013).

643 72.   Haas, B. J. *et al.* Accuracy assessment of fusion transcript detection via read-mapping and de

644 novo fusion transcript assembly-based methods. *Genome Biology* **20**, 213 (2019).

645 73.   Brand, A., Allen, L., Altman, M., Hlava, M. & Scott, J. Beyond authorship: Attribution,

646 contribution, collaboration, and credit. *Learn. Publ.* **28**, 151–155 (2015).

Figure 1

Consistency of DNA methylation levels and variances in normal cells

We assigned each gene to a category that indicated whether it was methylated at low, medium, or high levels and whether it had low or high variance across samples in a given dataset. For each gene, we counted the maximum number of datasets for which the level / variance category was consistent. For most genes, the category was consistent across all four datasets.

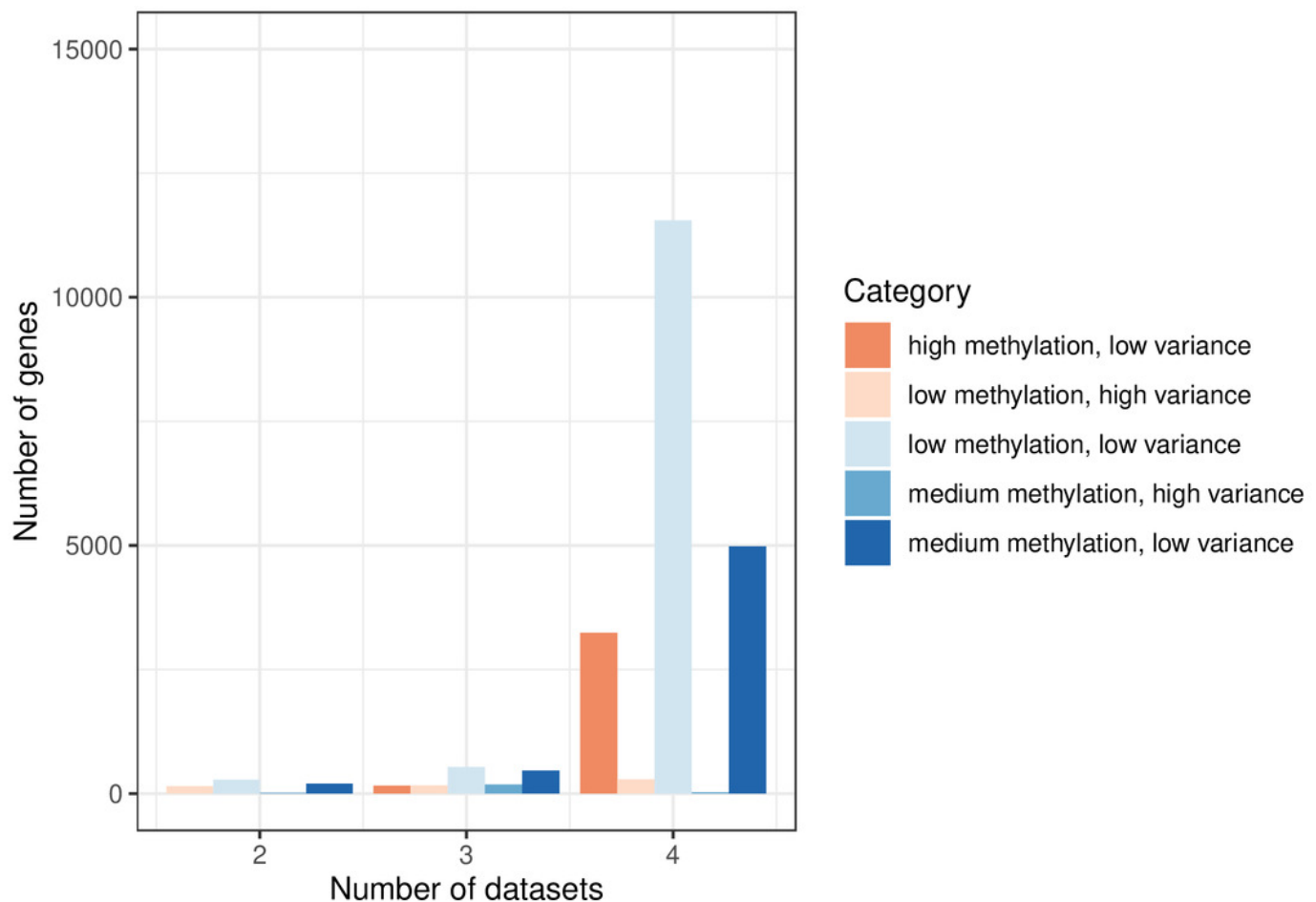



Figure 2

Volcano plots showing differentially methylated genes for each tumor type

For each tumor type, we compared methylation levels at the gene level between tumors and the normal samples. Genes showing significantly different methylation levels between tumor and normal conditions are highlighted.

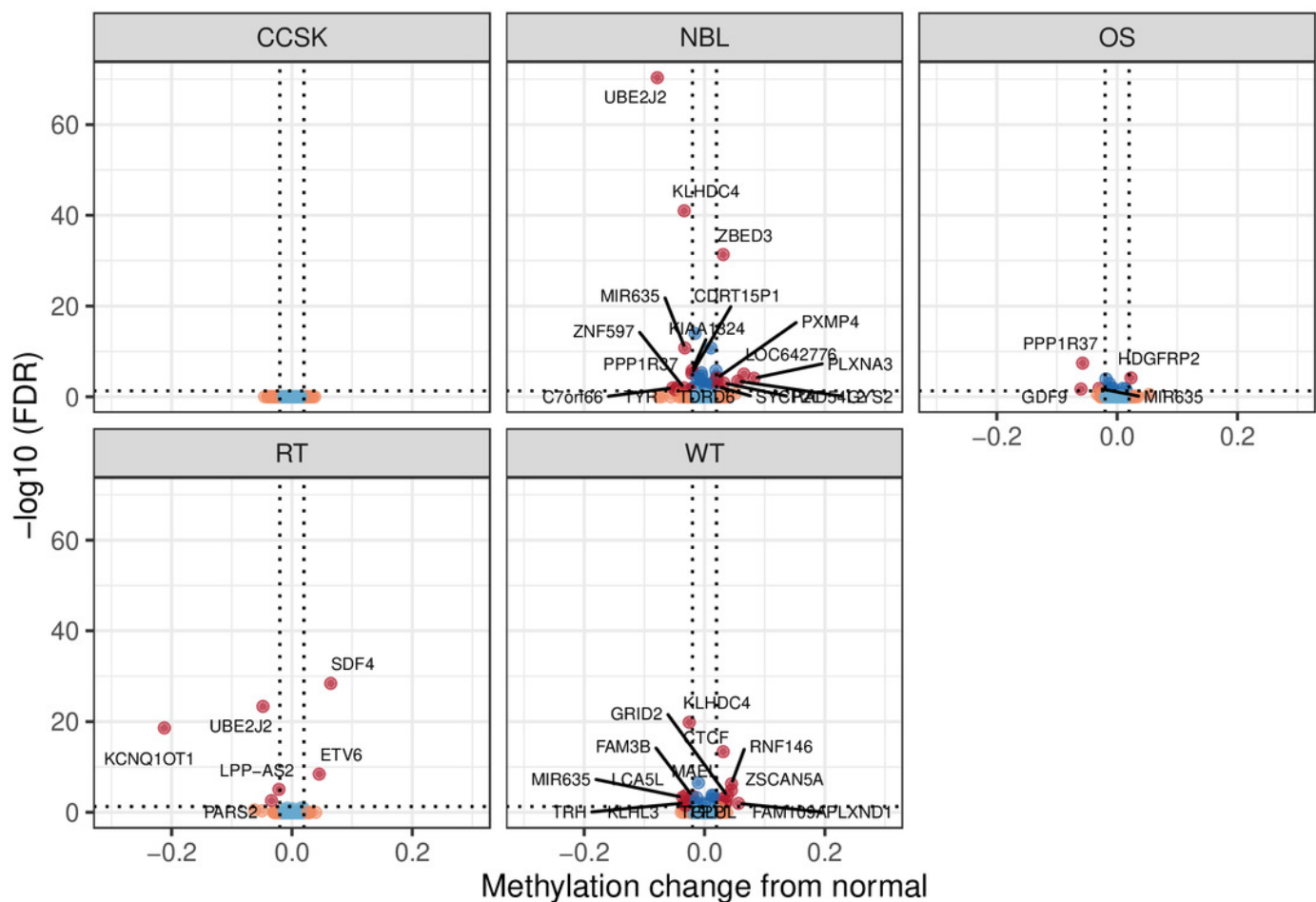



Figure 3

Methylation levels for genes that differed significantly across the tumor types

One-way ANOVA tests applied to tumor methylation levels identified 19 genes for which the means differed significantly across the tumor types. These violin plots show the range and density of the methylation values for these genes across the tumor types.

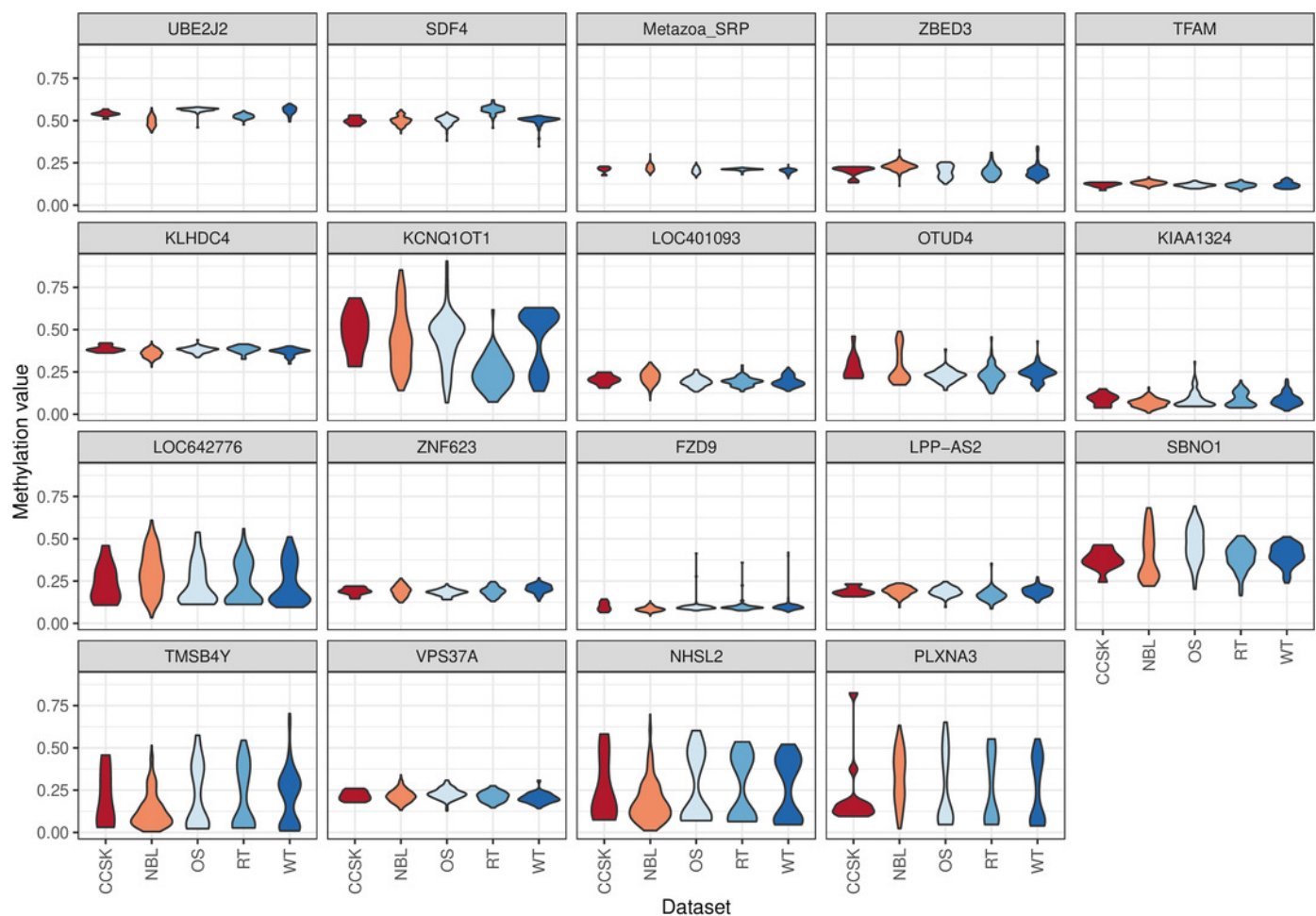



Figure 4

Gene-level DNA methylation changes for high-variance genes

Rows in these heatmaps indicate methylation levels, relative to the normal data, for the 20 genes with the largest variance across the tumor types. Columns represent individual tumors. Approximately half of all tumors exhibited consistently lower methylation levels than the remaining tumors.

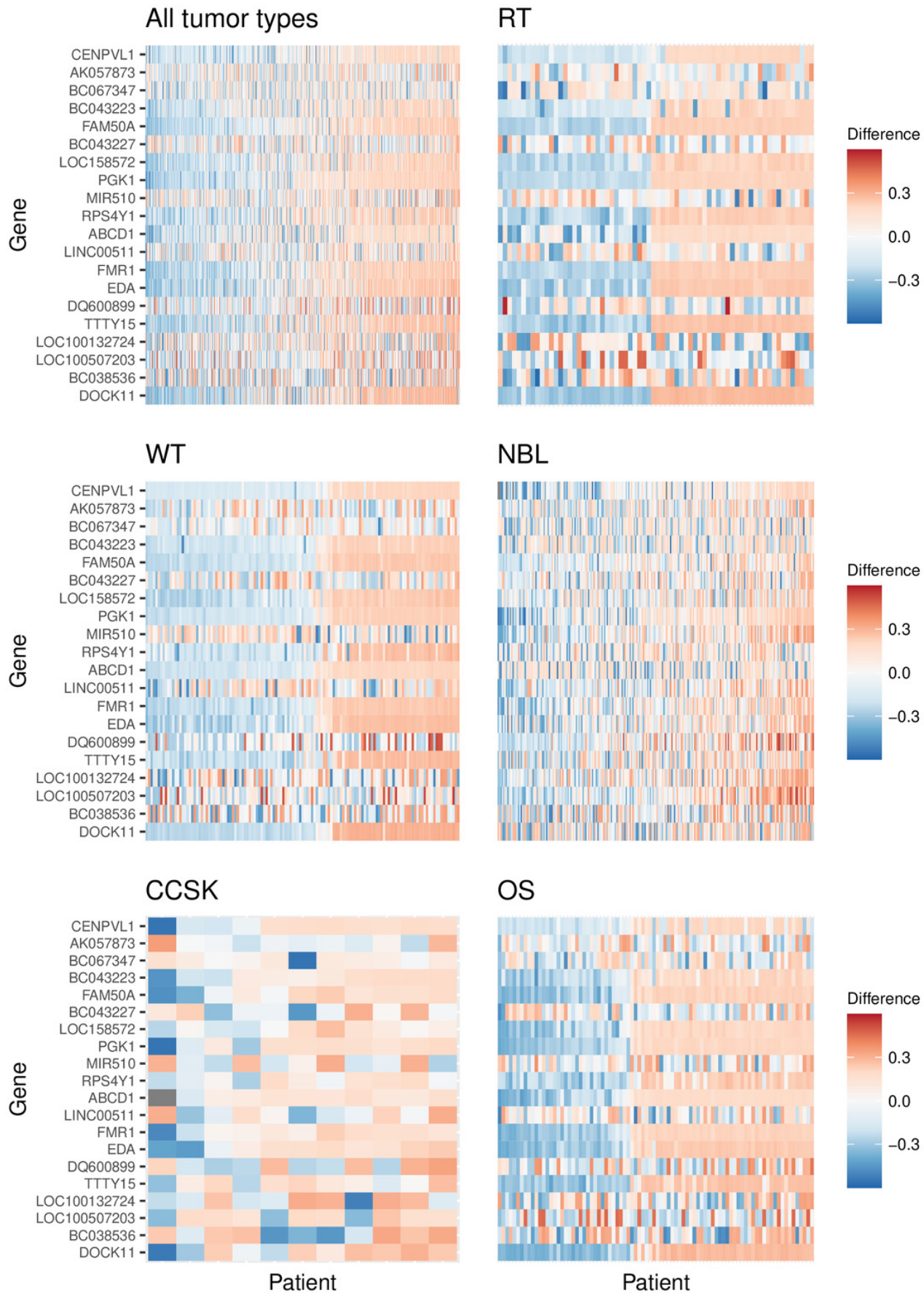
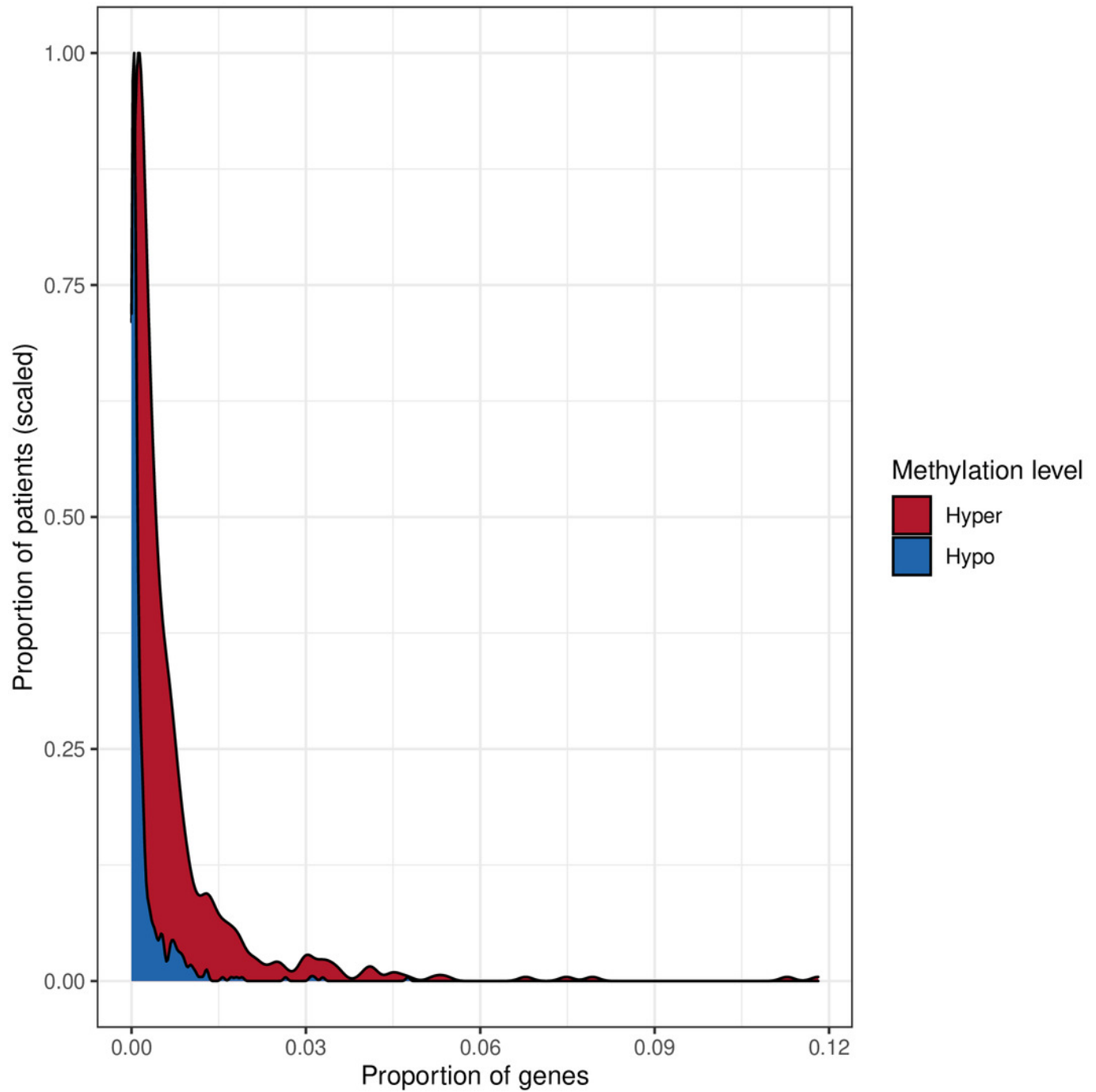



Figure 5

Distributions of the proportion of hypermethylated or hypomethylated genes in a given tumor

Using the normal data as a reference, we identified genes that were hypermethylated or hypomethylated in a given tumor. All five tumor types are represented. A relatively large number of hypermethylated genes in a given tumor was more common than a relatively large number of hypomethylated genes.

Summary of changes in methylation level/variance categories between normal and cancer datasets

We assigned each gene to a category that indicated whether it was methylated at low, medium, or high levels and whether it had low or high variance across samples in a given dataset. The table shows the total number of genes in each category for the normal datasets and the number (and percentage) of genes that changed from one category to another in the tumor datasets.

Normal category	Tumor category	Total # genes	# genes changed	% genes changed
high methylation / low variance	medium methylation / low variance	3403	41	1.2
low methylation / high variance	low methylation / low variance	604	20	3.3
low methylation / high variance	medium methylation / high variance	604	9	1.5
low methylation / high variance	medium methylation / low variance	604	4	0.66
low methylation / low variance	low methylation / high variance	12368	462	3.7
low methylation / low variance	medium methylation / low variance	12368	87	0.7
medium methylation / high variance	low methylation / high variance	227	137	60
medium methylation / high variance	medium methylation / low variance	227	19	8.4
medium methylation / low variance	high methylation / low variance	5651	159	2.8
medium methylation / low variance	low methylation / high variance	5651	23	0.41
medium methylation / low variance	low methylation / low variance	5651	52	0.92
medium methylation / low variance	medium methylation / high variance	5651	34	0.6

Aberrant methylation and mutation rates in oncogenes, tumor suppressor genes, and all other genes

For three pediatric tumor types, we identified aberrant methylation events (either hypomethylation or hypermethylation) that had occurred in a given tumor and gene. For the same tumor/gene combinations, we identified somatic single-nucleotide variants, indels, and structural variants that had occurred. These numbers indicate overall rates of aberrant methylation or mutation across all tumors of a given type. Methylation rates and mutation rates were typically similar across all three gene categories, but mutation rates for oncogenes and tumor suppressor genes were always higher than for other genes.

Tumor type	Aberration type	Oncogenes	TSG	Other
Wilms tumor	Mutation	0.04	0.034	0.017
Wilms tumor	Methylation	0.012	0.012	0.014
Neuroblastoma	Mutation	0.083	0.089	0.046
Neuroblastoma	Methylation	0.007	0.007	0.008
Osteosarcoma	Mutation	0.031	0.034	0.018
Osteosarcoma	Methylation	0.014	0.011	0.014