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Semin Hematol. Author manuscript; available in PMC 2013 November 19.

Published in final edited form as:
Semin Hematol. 2009 January ; 46(1): . doi:10.1053/j.seminhematol.2008.09.008.

Epigenetics of acute lymphocytic leukemia

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Abstract

The term epigenetics refers to the study of a number of biochemical modifications of chromatin that have an impact on gene expression regulation. Aberrant epigenetic lesions, in particular DNA methylation of promoter associated CpG islands, are common in acute lymphocytic leukemia (ALL). Recent data from multiple laboratories indicates that several hundred genes, involving dozens of critical molecular pathways, are epigenetically suppressed in ALL. Because these lesions are potentially reversible, the reactivation of these pathways using, for instance, hypomethylating agents may have therapeutic potential in this disease. Furthermore, the analysis of epigenetic alterations in ALL may allow: 1) the identification of subsets of patients with poor prognosis when treated with conventional therapy; 2) development of new techniques to evaluate minimal residual disease; 3) better understanding of the differences between pediatric and adult ALL; and 4) new therapeutic interventions by incorporating agents with hypomethylating activity to conventional chemotherapeutic programs. In this review, we desribe the role of epigenetic alterations in ALL from a translational perspective.

Keywords

Acute lymphocytic leukemia; DNA methylation; epigenetics

Introduction

Epigenetics is the study of biochemical modifications of chromatin. These modifications do not alter the primary sequence of DNA but have an impact on gene expression regulation, most frequently gene suppression. The field of epigenetics is rapidly expanding from DNA methylation[1] to the realization that histone modifications[2] cross talk with DNA methylation[3], and the most recent discovery of microRNA[4] as having a role in DNA methylation control[5]. Data from multiple laboratories and for all types of human malignancies has clearly demonstrated that epigenetic alterations, or at least aberrant DNA methylation, are very prevalent in cancer[6]. Epigenetic lesions complement genetic alterations in oncogenesis. Epigenetic lesions are reversible and it is postulated that a number of chemotherapeutic agents (DNA hypomethylating agents[7,8], histone deacetylase inhibitors[9]) act by reversing aberrant epigenetic marks and inducing physiologic gene expression. In this short review, we focus on current knowledge of the epigenetics of acute lymphocytic leukemia (ALL) from a translational perspective and on the use of this

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information to develop biomarkers and new therapeutic alternatives. At the present time, the field mainly relates to aberrant DNA methylation, and little is known in terms of abnormal histone code modifications or microRNA gene expression profiles.

Aberrant DNA methylation in ALL

The analysis of DNA methylation in ALL has paralleled the continuous development of simpler and more powerful techniques to assess multiple promoters and many samples in parallel. Most current techniques use bisulfite treatment of DNA[10]. This method has allowed the development of simple PCR assays to study methylation, including the more recent development of pyrosequencing assays[11]. Initial studies in ALL consisted of the analysis of single genes in limited number of samples; these investigations (Table 1) focused on genes such as *calcitonin*[12–15], *p15*[16–18], *p73*[19,20], *E-cadherin*[21], *ER*[22]. Other single gene studies have included *Dkk-3*[23], *LATS2/KPM*[24], *Hck*[25], *DBC1*[26], *BNIP3*[27], among many others. By the time of these reports, it became apparent that human cancer was characterized by the concomitant methylation of multiple genes[28]. The studies in solid tumors[28] were confirmed in leukemia [29], prompting experiments in ALL[30]. In the initial profiling study of adult ALL, ten genes (*MDR1, THSBS2, THSBS1, MYF3, ER, P15, CD10, c-ABL, p16, p73)* were analyzed in a cohort of 80 patients with ALL (Table 1). Results of this profiling analysis were reminiscent of those described in acute myelogenous leukemia (AML)[29] or colon cancer[28]. First, close to 85% of patients had methylation of at least 1 gene and 40% of them of 3 or more genes. Distribution of DNA methylation followed a bimodal pattern, with a group of patients with significant increase in the number of methylated genes.; these patients also had “denser” methylation. There was a strong correlation between methylation of most of these genes indicating the presence of a molecular defect (ie hypermethylator phenotype) leading to the concomitant aberrant methylation of multiple genes. Expression of CD10 was inversely associated with methylation of *CD10.* At the genetic level, methylation of *c-ABL* was only observed in patients with Philadelphia chromosome alterations (Ph), and only in those patients with the p210 isoform, a reproducible result[31]. In the exploratory study, an association was observed between methylation and worse prognosis (for *p73* and *p15).* These data indicated that aberrant DNA methylation of multiple promoter CpG islands is a common feature of adult ALL. A number of large profile studies were subsequently reported [32] (Table 1). When 15 genes were analyzed in more than 250 patients in both adult and pediatric ALL, methylation of at least 1 gene was observed in 77% of patients and 35% of them had methylation of 4 or more genes. Importantly, increased number of methylated genes was associated with a worse outcome. These results confirmed the prevalence and clinical relevance of aberrant DNA methylation of multiple promoter CpG islands in ALL.

In general, gene specific studies are limited by selection bias. Genome-wide analysis allows unmasking of unanticipated genes and molecular pathways. Several efforts have been made to create an unbiased methylation profile in ALL[33,34]. Kuang et al used the Methylated CpG Island Amplification (MCA) technique coupled with an established promoter array[33]: in excess of 400 genes were uncoverd as potential methylation targets in ALL[33]. Using bisulfite pyrosequencing, 26 of these genes were validated in leukemia cell lines and 15 (Table 1) in primary ALL samples. Genes were evenly distributed through all autosomes (Figure 1) and could be clustered in specific molecular pathways encompassing multiple functions[33]. There was overrepresentation of Wnt related genes and kinases such as the Ephrin family of ligands and receptors. Independent studies have already shown the importance of Wnt signaling epigenetic alterations in ALL[35]. The inactivation of Ephrin (a large family of kinases) mediated signaling also seems common in ALL (Kuang et al in preparation)[36] and may be related to altered Akt signaling[36]. In a parallel study, Taylor et al[34] also reported results of a large scale methylation analysis in ALL using a different type of array. Using this technique, this group identified 262 differentially methylated genes in

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ALL and validated 10 of them in a small cohort of patients (Table 1). Of interest, there was little overlap between the results of the 2 array experiments[33,34], with only 9 genes concordant between both studies. These differences are probably related to the CpG islands covered by each array platform and the results are likely complementary. Recently, Taylor et al also have shown the feasibility of performing "deep-sequencing" methylation studies in acute leukemia[37].

Analysis of aberrant DNA methylatyion as a tool to predict prognosis in ALL

Methylation of multiple genes/pathways is common in ALL. Because aberrant DNA methylation can suppress tumor suppressor genes, it is possible that the methylation/suppression of these genes may confer distinct clinical pathological characteristics to these patients, including worse prognosis. One of the first studies to demonstrate such an effect, an analysis of 3 genes (*p15, p73* and *p57*)[38], is illustrative, in that none of these genes when analyzed individually showed clear prognostic value. For instance, *p57* was first found to be methylated in leukemia[39]. Subsequent studies showed the gene to be methylated in close to 50% of adult patients and to be correlated with methylation of *p73* and *p15*, but not with any other significant patient characteristic. *p73* is a *p53* homologue that is upstream of *p57*[40]; these three genes have a role in cell cycle progression. Patients who showed methylation of more than 1 gene of this triad had a median survival of 52 weeks (the equivalent of Ph + ALL in the preimatinib era) that was significantly worse than that of patients with methylation of only 0 or 1 gene of this triad (Figure 2). The inference from these data was that the cell cycle control check point controlled by *p73/p57/p15* had evolved requiring redundant systems (ie *p15* and *p57)*and therefore the need of complete epigenetic suppression to have an effect on survival. Indeed, in a limited set of patients, there was clear cell cycle dysregulation in patients with methylation of this pathway[38]. Because it is presumed that methylation results in silencing, the same group of investigators analyzed the prognostic value of protein expression (the reverse of methylation) of the *p73/p15/p57* triad in ALL[41] using a tissue microarray platform constructed with the bone marrow samples that had been used for methylation analysis[38]. Methylation of these genes inversely correlated with protein expression, and those patients with evidence of protein expression had a significant better outcome by multivariate analysis. These results have several implications: 1) that methylation and expression studies can be complementary and 2) that the identification of methylation marks may result in the development of widely available clinical assays.

Others have also shown that the methylation of multiple genes and pathways is associated with worse outcome in ALL[32] and that the larger the number of genes methylated, the worse the outcome[32,33]. Although in general results of most groups have been concordant or complementary, there has been some controversy surrounding the *p21* gene. In the original study, *p21* methylation was shown to be a strong prognostic marker in ALL[42]. Subsequent investigations failed to find this association[43], a finding confirmed by several other groups[44]. [unclear syntax: what is being confirmed, the original or the second study?] The most likely reason for this discrepancy is probably use in the original study of a non-bisulfite assay, prone to false positives and in some cases difficult to interpret.

Are there methylation differences between pediatric and adult ALL?

An obvious question was whether differences in prognosis known to differentiate adult from pediatric ALL could be related, in part, to DNA methylation: quantitatively (number of genes methylated) or qualitatively (differences in methylation patterns). The first report[45] indicated that there were no obvious differences in terms of the frequency of methylation

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observed in children and adult ALL. However, there were several limitations to this study, as the number of patients with pediatric ALL studied was small and insufficient genotypic subsets of patients were studied. However, aberrant methylation of multiple genes is common in pediatric ALL[45,46]: the Spanish group also demonstrated that the high frequency of methylation in the younger group of patients and a potential correlation between methylation and prognosis[32]. These data were against expectations, founded on the concept that methylation increases with aging[47] and therefore older patients should have a higher frequency of aberrant methylation. Prognostic differences between children and adults possibly are not related to quantitative methylation but to the inactivation of specific pathways. One example may be the *p73/p15/p57* pathway[38,48]: epigenetic inactivation was observed in close to 25% of adult patients but was extremely rare in the younger patients[48], despite that individual methylation of any of these three genes was not significantly different from the adult subgroup[48]. Although these results need to be confirmed in other larger cohort of patients, they suggest that prognostic differences between age groups could be related in part to differences in methylation patterns. Methylation of *p16*, a rare event in primary ALL[30,49], has been shown to be present in pediatric cases with *MLL* alterations[50]. The same phenomenon has also been the cases for the *FHIT* gene[51,52]. Specific patterns and genetic associations may have a role in the prognosis of pediatric patients with ALL.

Can the analysis of DNA methylation be used as a marker of minimal residual disease?

Another question is whether methylation patterns at the time of relapse are stable in ALL (Figure 3); the answer would have important implications. If stable, it could be proposed that these methylation alterations are a key molecular component of the malignant clone, and the detection of residual levels of methylation might usefully indicate presence of residual leukemia in patients in morphological remission (Figure 3A). Methylation profiles of 5 genes (*ER, MDR1, p73, p15 and p16)* was determined in a group of patients before therapy and at the time of morphological relapse[53]. Overall, methylation patterns were stable in about 70% of patients. Genes such as *p73* (92%), *ER* (88%) and *MDR1* (72%) were stable at the time of relapse, whereas *p15* was only concordant in around 60% of cases. Of interest, there was an increase in *p16* methylation accompanied by gain of *p15* methylation (Figure 3B), suggesting that gain of methylation may have a role in relapse/resistance mechanisms in ALL. Overall, this study indicated that methylation patterns are stable in a large fraction of patients with ALL, and therefore it might be possible to design minimal residual disease assays using detection of aberrant DNA methylation in ALL. Whether the methylation changes observed in the 30% of patients at relapse was the result of the emergence of a new clone or epigenetic alterations in the original clone remains uncertain but of with potential implications for our understanding of relapse dynamics and patterns of resistance.

Based on these data, detection of residual methylation as a predict or of relapse in ALL has been sought (Yang et al submitted)[54], DNA was extracted from 199 patients with Ph negative ALL at the time of morphological remission (around day 14 to 21 after standard hyperCVAD based chemotherapy[55]). Three genes were analyzed: *p15, p73* and *p57,* using a real-time bisulfite PCR assay especially developed for this analysis. Residual methylation of *p73* was detected in 10% of patients, *p15* in 17%, and *p57* in 4%. In all, 25% of the patients had evidence of residual methylation. The presence of residual *p73* methylation was associated with a significantly worse outcome (HR 2.68, p=−.003) (Figure 3C). Although these results are exploratory due to the limited number of genes analyzed, they show the feasibility of methylation based assays to detect residual leukemia, which could complement other flow or molecular assays. A more systematic analysis of genes in ALL may provide a useful tool to predict outcome in patients in remission with ALL. These results also allow

the consideration of incorporating hypomethylating agents as consolidation/maintenance strategies in ALL.

Incorporating hypomethylating-based therapy in ALL

At the present time, two drugs with hypomethylating activity are approved in the US for patients with myelodysplastic syndrome[7,8]. These agents also have activity in AML[56]. In vitro data using cellular systems has indicated that the selective reactivation of a gene specifically inactivated in ALL (in this case *p57)* results in ALL cell death, only in cells in which the gene is epigenetically silenced and not in leukemia cells in which the gene is not methylated[57]. These results indicate that reactivation of epigenetically silenced genes can have an anti-leukemia effect, perhaps selective, and they reinforces consideration ofhypomethylating therapy in ALL, either as single agent or in combination with other standard forms of therapy exploiting possible synergistic effects[58]. There is only limited experience with the hypomethylating agents in ALL. In one trial[59], relative high doses of 5-azacitidine (150 mg/m^2 as a continous infusion daily x 5) were combined with cytarabine in patients that had previously failed cytarabine. The hypothesis was that treatment with 5-azacitidine could induce expression of deoxycytidine kinase. Two complete responses (CR) were observed in 17 patients treated. In another study[60], decitabine was combined with either amsacrine or idarubicin in patients with acute leukemia. There was a CR rate of 36% (23 out 63) with a median disease free survival of 8 months. An ongoing trial is evaluating the role of decitabine in patients with advanced relapsed/refractory ALL[61]. In this study two phase 1 trials are conducted in parallel. In the first phase, patients are treated with a 5-day schedule of decitabine every other week. In the second phase, patients who had received decitabine in the first phase but did not respond or relapsed to single agent decitabine can then participate in a phase 1 study of decitabine and hyper-CVAD[62] combination. The reasons for the more frequent schedule of decitabine in ALL were twofold: first, data from in vitro modeling indicating the activity of decitabine in ALL cell lines especially when using chronic exposure[63], and, second, the rapid proliferative nature of the leukemia clone in patients with relapse disease. To date, doses up to 100 mg/m^2 IV daily x 5 every other week have been safely administered, without excess toxicity and with evidence of clinical activity in patients with multiple relapse/refractory leukemia.

Summary and future research

The study of epigenetic alterations in ALL is transitioning from a relative obscure field of research to the potential development of new biomarkers and therapeutic alternatives for patients with this disease. Here, we have summarized data that multiple tumor suppressor genes and molecular pathways are inactivated in ALL. This information potentially can be utilized to predict response to therapy, detect patients at risk that are in morphological relapse, and to target the incorporation of hypomethylating agents in ALL. Large scale validation studies and trials are needed to confirm these early data and to allow its into clinical practice. The analysis of specific histone modification patterns, and the role of histone deacetylase inhibitors[9] in combination with conventional chemotherapeutic agents[58] or hypomethylating agents[64,65], as well as the role of microRNAs[4], should be studied in conjunction in ALL.

Acknowledgments

This work was supported by the Leukemia and Lymphoma Society of America, a Physician-Scientist Award from MD Anderson Cancer Center and NIH grants CA 100067, CA105771 and CA126457 (all to GGM) and by a Leukemia SPORE (P50 CA100632) Career Development Award (S-Q K)

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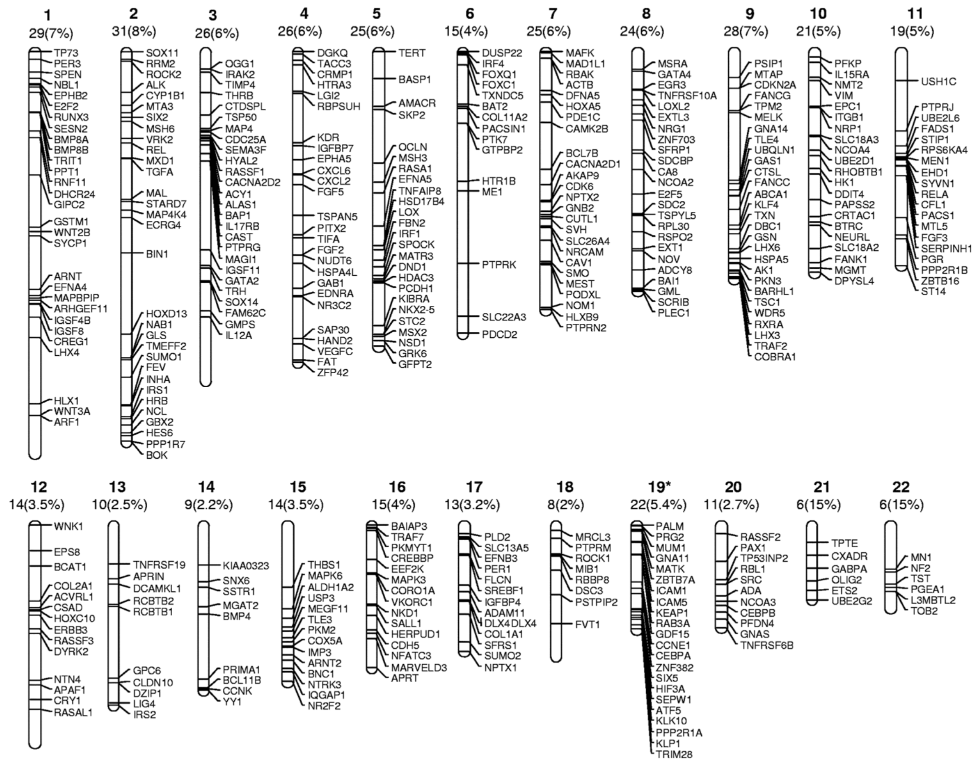

Figure 1. Chromosomal distribution of potential methylation targets uncovered by MCA/Agilent array[33]

The figures on top of each chromosome indicate the number of the chromosome and the number of genes (an percentage) located in each individual chromosome.

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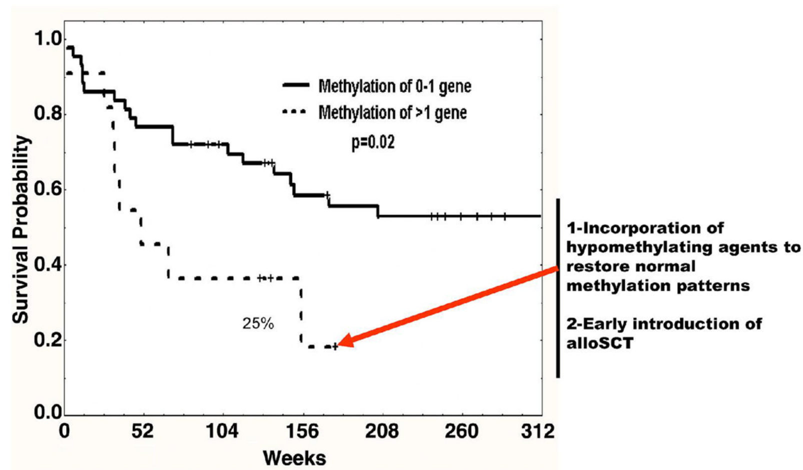

Figure 2. Translational implications of using analysis of DNA methylation in adult Ph negative ALL

The impact on survival of methylation of a triad of genes composed of *p73/p15* and p57 was evaluated in a cohort of adult patients with Ph negative ALL treated homogenously with hyper-CVAD therapy[38]. Patients with methylation of 2 or 3 genes had a significantly worse prognosis compared with those with methylation of 0 or 1 genes[38]. This data was confirmed at the protein level[41]. This information could be used to incorporate hypomethylating agents or to consider the introduction of allogeneic stem cell transplantation.

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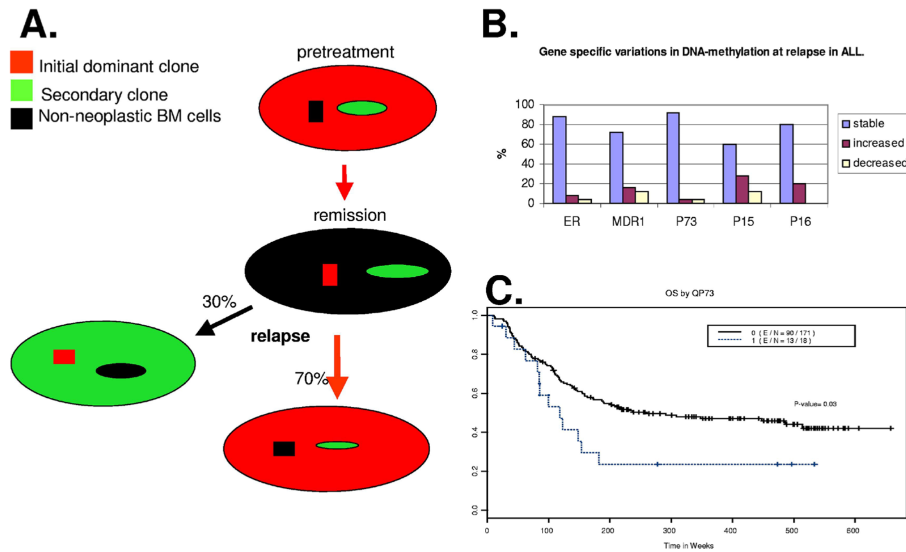

Figure 3. Analysis of DNA methylation as a tool to detect minimal residual disease

A. A model of methylation dynamics in ALL. Pretreatment bone marrow of patients with ALL should be populated by two or three clones: a dominant malignant clone (red), residual normal hematopoiesis (black) and potential a subdominant malignant clone (green). With intensive chemotherapy, most patients achieve complete remission and the marrow is now dominated by the normal clone. But because a large fraction of adult patients will relapse with conventional therapy it is possible that residual molecular levels of both the dominant and possibly the subdominant clone are present during remission. At the time of relapse approximately 70% of patients relapse with a methylation pattern similar to that of the initial presentation and 30% with a different profile[53]. **B.** Gene specific dynamics of 5 genes at the time of relapse[53]. Stable indicates that the methylation status of the gene does not change from initial presentation to the time of relapse. **C.** The data presented in A and B allows the hypothesis that detection of residual levels of methylation at the time of remission could predict worse outcomes. This has been shown for the *p73* gene[54].

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Table 1

A list of methylated genes in ALL

Table 1 summarizes a partial list of genes reported to be methylated in ALL, as well as their chromosomal location, potential function (when known) and the number and percentage of metyhylation. It should be noted that a number of techniques were used to determine methylation and that criteria for "methylation positivity" may difer from study to study. This table is therefore only orientative.

Gene	Chromosomal location	Function	Number methylated/Number analyzed	% (Range)	References
GIPC2	1p31	Prostanoid signalling	31/31	100	33
RSPO1	1p34	Wnt signalling	46/46	100	33
P73	1p36	Transcription factor	11/35, 17/80, 45/251	20 (18–31)	19,20,30,32
KCNK2	1q41	K^+ channel	14/16	87	34
LRP1B	2q21	LDL complex	15/16	93	34
DCL-1	2q24	GTPase negative regulator	11/16	68	34
CAST1	3p14	CNS, synapsis	49/57	86	33
MAGI1	3p14	Guanylate kinase	27/45	60	33
WntA5	3p21	Wnt signaling	132/307	43	35
ADCY5	3q13		38/56	68	33
CD10	3q25	Peptidase	8/80	10	30
HSPA4L	4q28	Heat shock protein	24/35	69	33
sFRP2	4q31	Wnt signaling	42/261	16	35
OCLN	5q13	Tight junction	31/41	76	33
EFNA5	5q21	Ephrin signalling	44/58	76	33
MSX2	5q34	Transcriptional repressor	54/55	98	33
GFPT2	5q34	Aminotransferase	8/35	23	33
LATS1	6q24	Kinase	100/251	40	32
ER	6q25	Estrogen receptor	17/18, 29/80	47 (36–94)	22, 30
PARKIN	6q25	Proteosomal degradation	67/251	27	32
THSBS2	6q27	Cell adhesion	42/80	52	30
sFRP4	7p14	Wnt signaling	55/261	21	35
MDR1	7q21	Transmembrane transporter	36/80	45	30
sFRP1	8p12	Wnt signaling	99/261	38	35

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Gene	Chromosomal location	Function	Number methylated/Number analyzed	% (Range)	References
P15	9p21	Cell cycle control	17/45, 20/46, 17/34, 19/80,73/251	32 (23–50)	16–18, 30,32
GNA14	9q21	G protein	27/44	59	33
DBC1	9q32	Inhibitor of Sirt1	29/170	17	26
c-ABL	9q34	Kinase	6/80	8	30
DAPK	9q34	Apoptosis	33	13	32
PTEN	10q23	Phosphatase	50	20	32
sFRP5	10q24	Wnt signaling	73	28	35
BNIP3	10q26	Apoptosis	5/34	15	27
Calcitonin	11p15	Calcium metabolism	6/7, 13/14, 44/47, 45/105	62 (42–93)	12–15
Dkk3	11p15	Wnt signaling	60/183	33	35
P57	11p15	Cell cycle control	31/63, 45/251	30 (18–50)	30,32
SLC2A14	12p13	Glucose transport	12/16	75	34
WIF1	12q14	Wnt signaling	78	30	35
APAF-1	12q23	Apoptosis	85	34	32
DDX51	12q24		8/16	50	34
HDPR1	14q23	Wnt signaling	68/261	26	35
THSBS1	15q15	Cell adhesion	16/80	20	30
NOPE	15q22		13/16	81	34
SALL1	16q12	Zn finger protein	41/41	100	33
E-cadherin	16q22	Cell adhesion	18/33, 92/251	39 (37–54)	21, 30
CDH-13	16q24	Cell adhesion	87/251	35	32
DCC	18q21	Putative tumor suppressor gene	14/16	87	34
MY05B	18q21	Protein traffick	36/36	100	33
ZNF382	19q13	Zn finger protein	24/46	52	33
NES-1	19q13	Serine protease	143/251	57	32
Hck	20q11	Kinase	9/44	20	25
TMS-1	20q13		22/251	9	32
MN1	22q12	Involved in meningioma	45/53	85	33