

STUDIES OF METHYLATION CHANGES DURING HUMAN OVARIAN CANCER DEVELOPMENT

by

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(Under the Direction of John F. McDonald)

ABSTRACT

Previous epigenetic studies have shown that gene expression is inversely correlated with promoter methylation status. DNA methylation changes are a hallmark of cancer development. Two seemingly opposite events have been reported in almost all types of cancers, a global decrease of methylation (hypomethylation) and a gene specific increase of methylation (hypermethylation).

The current thesis provides a study of methylation changes in ovarian cancer development. The hypothesis that hypomethylation of retroelements may contribute significantly to global hypomethylation in cancer was tested by examining two families of retrotransposons. Retrotransposons are repetitive sequences widespread through the genome and generally methylated in non-malignant cells. A decrease of methylation levels of HERV-W and LINE1 was detected in ovarian adenocarcinoma compared with benign adenomas.

Gene hypermethylation and hypomethylation were also studied at the specific gene level. To study gene hypermethylation changes, the ovarian cancer cell line OVCAR3 was globally demethylated by 5-aza-deoxycytidine treatment. Comparison of those genes with genes

downregulated in ovarian adenocarcinoma provides a list of candidate genes hypermethylated during ovarian cancer development.

The presence of a CpG island in the promoter region of HLA-G and its upregulation in multiple cancer tissues make this gene a likely candidate for hypomethylation changes. Previous studies conducted in cancer cell lines have demonstrated a correlation between hypomethylation of a CpG rich sequence in the HLA-G promoter and increased expression. We studied the expression and methylation levels of HLA-G in ovarian tissue, and found no apparent correlation. Opposite results were obtained from an ovarian cancer cell line (BG-1), for which loss of methylation correlated with an increase of expression. Results suggest a different control mechanism for HLA-G expression between the ovarian cancer cell lines and ovarian tissue.

INDEX WORDS: Genetics, Hypomethylation, Hypermethylation, Ovarian cancer, Transposable elements, HLA-G

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Ovarian cancer

Ovarian cancer was the fourth leading cause of cancer deaths of women in 2005 in the United States, with 20,180 new cases and 15,310 related deaths estimated for 2006 [1]. High mortality related to ovarian cancer is mainly due to the lack of early symptoms and the subsequent detection of the cancer at advance stages.

Ovarian tumors can be derived from the epithelial cells (~85-90% of cases), the germ cells (~5% of cases) or the stromal cells (~5% of cases) [2]. Epithelial ovarian cancers, or adenocarcinomas, are classified according to their features as well as to the tissue they most closely resemble, into serous adenocarcinomas (fallopian tube epithelium), mucinous (GI tract or endocervical epithelium), endometrioid (proliferative endometrium), clear cell (gestational endometrium), and Brenner tumors (urinary tract epithelium). Stromal ovarian cancers include granulosa cell tumors, Sertoli-Leydig tumors, fibromas and thecomas. Germ cell tumors include dysgerminomas, yolk sac tumors (endodermal sinus tumors), teratomas, choriocarcinomas and embryonal carcinomas.

Ovarian cancers are also classified by grade, from 1 to 3, with 1 being the closest to normal cells and therefore having a better prognosis, and by stage, from I to IV, according to the following criteria:

Stage I: ovarian tumors are confined to the ovaries.

Stage II: ovarian cancer has spread from the ovaries to other organs within the pelvis (fallopian tubes, uterus, bladder, sigmoid colon or rectum).

Stage III: ovarian cancer involves one or both ovaries and has either spread beyond the pelvis or is found in the lymph nodes.

Stage IV: distant metastasis are found inside the liver, the lungs or other organs outside the peritoneal cavity.

Only 5-10% of all cases of ovarian cancers are hereditary, while the majority of cases are sporadic. Germline mutations on genes like *BRCA1*, *BRCA2*, *p53*, *PTEN*, and *NOEY*, overexpression of oncogenes like *HER-2*, *MYC* and *PI3*, and loss of heterozygosity for chromosomes 17, 13q, 18q and 22q, are amongst the most common genetic alterations found in ovarian cancer [2, 3]. An alternative mechanism of gene inactivation involves methylation of promoter of genes (discussed later in this introduction). In the case of ovarian cancer methylation is known to affect multiple genes including *hMLH1*, *LOT1*, *p21* and *RASSF1A* [4]. In general, mutations and epigenetic modifications occur only at low rates in ovarian cancer and are not present in every patient, making it difficult to establish which genes could be real targets for gene therapy or good detection markers. This fact, as well as the lack of clear symptoms, makes the detection of ovarian cancer at early stages very challenging. Nowadays the only marker accepted for ovarian cancer detection is carcinogenic antigen 125 (CA-125) [5]. CA-125 levels are elevated in patients with ovarian cancer, although level changes are normally not detectable at early stages. Also false positives might result from other conditions like endometriosis [6]. This lack of good markers highlights the need to better understand ovarian cancer development and progression.

Methylation in the human genome

The genomic DNA of higher eukaryotes, including humans, is susceptible to epigenetic modifications such as phosphorylation and methylation. Between 3-5% of cytosine nucleotides are methylated at the 5' carbon in human DNA, and 70-80% of these 5-methylcytosines are associated with guanines, forming CpG dinucleotides [7-9].

In the typical human genome, between 60 and 90% of CpG dinucleotides are methylated, and approximately 70% of these sites are in repetitive elements [10]. Since methylated cytosines are susceptible to deamination and subsequent conversion to thymines, methylation has been viewed as a host defense mechanism against retrotransposons [11].

Regions with a GC content higher than 50% and a 0.6 or greater ratio of observed versus expected CpG dinucleotides are called CpG islands [12]. They can vary in size from several hundreds of bases to several kilobases. It has been estimated there are between 28,000 to 45,000 CpG islands in the human genome, and almost half of them are in repetitive elements [10, 13, 14].

Around 40% of human genes contain CpG islands [14]. CpG islands in promoter regions of expressed genes are generally unmethylated (both housekeeping and tissue-specific genes), while methylated CpG islands are associated with silenced genes [8, 15].

Silencing of expression due to methylation is mediated through the action of methyl binding proteins. MeCP2 (Methyl-CpG-Binding Protein 2) was the first protein described to bind to DNA containing a single methylated CpG dinucleotide [14]. Later, MeCP2 was also shown to bind to histone deacetylases and to the chromatin remodeling complex SWI/SNF [16, 17], resulting in condensation of chromatin and consequent gene silencing. Besides MeCP2, at least

four more proteins have been found to have a methyl-CpG-binding domain [18] and two of them are also associated with histone deacetylases. An additional mechanism by which methylation may affect gene expression directly is through the binding inhibition of transcription factors or repressors, due to the presence of the protruding methyl group on binding sites (reviewed in [19]).

In mammals, DNMTs (DNA methyltransferases) are responsible for establishing *de novo* methylation patterns (DNMT3a and DNMT3b) and for methylation maintenance (DNMT1) (reviewed in [20]). Mutations in any of these genes are lethal in mice, reinforcing the importance of methylation for correct development and cell function [21]. DNA demethylation is much less well understood than methylation. Some studies suggest that MBD2, a known methyl binding protein, and DNA glycosylase might have demethylase activity, but this subject remains controversial [22-24].

Incorrect methylation patterns are associated with multiple human diseases. Mutations of DNMT3b in ICF syndrome (immunodeficiency, centromeric region instability, and facial anomalies) cause genomic hypomethylation and severe chromosomal instability [25, 26] and mutations in MeCP2 are associated with Rett syndrome (reviewed in [20]). Methylation alterations and consequent defects in imprinting (mono-allelic gene expression caused by the inactivation of either the maternal or paternal allele generally through methylation) are also common in patients with Beckwith-Wiedemann, Prader-Willi and Angelman syndromes (reviewed in [20]).

Methylation changes associated with cancer

Studies of early staged cancer cells indicated that one of the first genomic events during oncogenesis is a global hypomethylation of previously methylated sequences, accompanied by regional hypermethylation of tumor suppressor genes and other genes involved in DNA repair, apoptosis and the immune response. It is believed that these early events initiate a cascade of subsequent events that lead to cellular transformation [15].

The global reduction of methylation (hypomethylation) is estimated to be between 8-25% in many cancers including prostate, liver, ovarian and breast cancers (reviewed in [27]). Several studies suggest that a decrease in methylation correlates with increase of malignancy in breast, cervical and ovarian tumors [14, 28, 29]. In ovarian cancer, studies reported an average decrease of 20 to 24% of genomewide methylation in ovarian adenocarcinomas compared with normal ovaries [14, 28].

CpG demethylation mechanisms are currently unknown. Some studies have shown that DNA demethylation activity of glycosylase is altered in cancer cells, changing preferences from hemimethylated (methylation of only one DNA strand) to fully methylated DNA, and suggest glycosylase might then act as an active demethylating enzyme [23]. Even though current studies have shown a global hypomethylation in cancer, the precise sequences affected by this loss of methylation have not been localized. Some reports have shown hypomethylation of microsatellites close to centromeres [28, 30] and of some retroelements (reviewed later in this introduction). Consequently, in general there is relatively scarce information about the hypomethylation process and the effects it has on cancer cells.

Regional hypermethylation changes have been widely studied. Costello et al. estimated that around 1.3% of all CpG islands were hypermethylated in a study of 98 tumors that included breast, head, neck, testicular and colon samples [31]. The percentage of hypermethylation and the affected genes varies depending on the type of tissue. Genes involved in numerous pathways have been reported to be hypermethylated during cancer progression, including genes involved in apoptosis (*DAPK*, *TMS1*), DNA repair (*hMLH1*, *MGMT*), cell cycle (*p16*, *p15*, *p14*, *Rb*), cell adherence (*CDH1*, *CDH13*), growth factor response (*ER*, *RASSF1A*), drug resistance, angiogenesis, metastasis and differentiation [8, 18, 30]. It was hypothesized that hypermethylation might be a consequence of overexpression of DNA methyltransferases (DNMTs), but even though some experiments have shown an upregulation of DNMT1 in cancer cells, this did not seem to be correlated with a higher hypermethylation of CpG islands [30].

The identification of hypermethylated genes in cancer is normally monitored on an individual basis. Global studies of gene hypermethylation can be performed by using demethylating agents and microarray analysis of gene expression. Treatment of cancer cell lines with the demethylating agent 5-aza-deoxycytidine (5-aza-dC) releases hypermethylated genes from transcriptional repression. This cytosine analogue inhibits the action of DNMTs, reducing the methyl content of DNA after every cell division. Changes in expression caused by 5-aza-dC can then be verified by microarray analysis of the cancer cell line before and after treatment. An example of this technique is depicted in Lodygin et al. [32]. Demethylation of prostate cancer cell lines provided hundreds of genes with differential expression after 5-aza-dC treatment. Several of these genes were found to be methylated before the treatment, as well as in prostate cancer samples, proving the technique effective at identifying hypermethylated genes in cancer

tissue. Similar studies have also been done in colorectal carcinoma cell lines [33, 34], breast cancer cell lines [35] and endometrial cancer cell lines [17].

Besides global hypomethylation and gene specific hypermethylation, gene specific hypomethylation has also been reported in cancer cells although to a much less extent. Hypomethylated genes during cancer progression include *Synuclein- γ* , *BRAC2*, *Claudin4*, *Lipocalin2*, *Trefoil factor2*, *S100A4*, mesothelin, prostate stem cell antigen, *XAGE-1* and members of the *MAGE* gene family [28, 36-40]. Loss of methylation was correlated with an increase of gene expression, indicating hypomethylation may be a possible activation mechanism of oncogenes and other upregulated genes during cancer development. *HLA-G* is an example of an upregulated gene in multiple cancers, and contains a large CpG island in its promoter region that can be susceptible to methylation modification. *HLA-G* has been found to have lower levels of methylation in cell lines with higher gene expression, making it a likely candidate for gene hypomethylation in cancer cells expressing *HLA-G* [41, 42].

Transposable elements

Around 45% of the human genome is comprised of transposable elements (TEs) [8]. In humans, TEs are associated with the coding regions of 4% of the genes [14, 43] and with around 25% of gene regulatory regions [44]. TEs are classified into two major groups, DNA transposons and retrotransposons.

DNA transposons use the enzyme transposase to excise themselves and move to another position in the genome. In the human genome, there are around 300,000 copies of these elements, which can be subdivided into 7 major sub-classes.

Retrotransposons use the enzyme reverse transcriptase to make an RNA copy and insert it in a new location in the genome. In humans, retrotransposons can be subdivided into non-LTR and LTR retroelements. Non-LTR retrotransposons include autonomous long interspersed elements (LINEs), and non-autonomous short interspersed elements (SINEs). There is an estimated number of 850,000 LINE elements (21% of the human genome), most of them truncated and mutated, and almost 1.5 million copies of SINE elements (13% of the human genome) [14]. LTR retrotransposons have long terminal repeats (LTRs) flanking two open reading frames (ORFs), *gag* and *pol*. Retrotransposons that contain a third ORF, *env*, are called retroviruses [45]. LTR retrotransposons and retroviruses comprise around 5% of the human genome [14], and they can be divided into Human Endogenous Retroviruses (HERVs) and non-autonomous retroelements [MER4 elements and Mammalian apparent LTR elements (MaLRs)].

In general, intragenic or near-gene insertions of retrotransposons are not favorable for the host genome, and there are numerous examples of their deleterious effects in the human genome. Some harmful retrotransposons insertions include disruption of ribosomal S6 kinase gene in Coffin-Lowry syndrome [46], disruption of *Myc* gene in breast carcinoma [47], disruption of *APC* gene in colon cancer [48], and insertions in the *Factor VIII* gene in hemophilia A [49] amongst many others.

On the contrary, some retrotransposons insertions have become necessary for correct gene function. Their effects vary widely and include restriction of the expression of genes to specific tissues [50], translational control [51], activation of genes [52, 53], polyadenylation signaling [54, 55] and alternative splicing [56, 57]. An important example of coevolution of HERVs and the human genome is the case of the HERV-W envelope protein (syncytin),

expressed in placental tissue [58]. This membrane glycoprotein has been shown to be involved in trophoblast cell fusion, mediating the formation of syncytiotrophoblast [59].

Retrotransposons are mostly methylated and transcriptionally silenced in non-cancerous tissues. Hypomethylation might result in higher retroelement transcription, and in a subsequent increase of insertion rates. It has also been proposed that reduced levels of methylation and the consequent opening of chromatin structure might contribute to ectopic pairing/recombination among elements on different chromosomes resulting in translocations and other forms of genomic instability [10, 30]. Because genome hypomethylation is known to occur during cancer development, retroelements provide multiple targets for loss of methylation and subsequent genomic alterations. Loss of methylation in retroelements has been reported for HERV-K elements in testicular tumors and renal cell carcinoma [60, 61] and for LINE1 elements in renal cell carcinoma [61], hepatocellular carcinomas, urothelial tumors [62, 63] and colorectal carcinomas [64], as well as in diverse cancer cell lines (bladder carcinoma cell lines [62], breast cancer cell lines [65], and gastric cell lines [66]).

Studies of methylation changes during human ovarian cancer development

This dissertation provides an analysis of different aspects of DNA methylation changes in ovarian adenocarcinoma.

Chapter 2 is an analysis of the methylation level of retroelements HERV-W and LINE1 in ovarian adenocarcinomas, adenomas and normal ovaries. For both families of retroelements there was a loss of methylation in the cancer samples, accompanied by an increase of expression in the samples analyzed.

Chapter 3 is an analysis of the methylation status of the HLA-G in an ovarian cancer cell line as well as in ovarian adenocarcinomas and benign adenomas. The ovarian cancer cell line BG-1 displayed extensive methylation of the promoter of HLA-G and silencing of its expression, and treatment with demethylating agents caused a decrease of methylation and a subsequent increase of HLA-G expression. In contrast, tissue samples displayed variable levels of HLA-G expression that did not correlate with the methylation level of the same promoter region or Exon1.

Chapter 4 utilizes a global demethylation assay in the ovarian cancer cell line OVCAR3 to identify candidate genes that may be controlled by methylation. An increase of gene expression in cell lines after treatment with demethylating agents suggests those genes were transcriptionally repressed through hypermethylation. Comparison of upregulated genes in the ovarian cancer cell OVCAR3 with down-regulated genes in ovarian cancer tissue provides a system to identify potential candidate genes hypermethylated in ovarian cancer.

REFERENCES

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C, Thun MJ: Cancer statistics, 2006. *CA Cancer J Clin* 2006, 56:106-130.
2. Bartlett JMS: *Ovarian Cancer. Methods and Protocols*. Totowa: Humana Press; 2000.
3. Edlich RF, Winters KL, Lin KY: Breast cancer and ovarian cancer genetics. *J Long Term Eff Med Implants* 2005, 15:533-545.
4. Balch C, Huang TH, Brown R, Nephew KP: The epigenetics of ovarian cancer drug resistance and resensitization. *Am J Obstet Gynecol* 2004, 191:1552-1572.
5. Fields MM, Chevlen E: Ovarian cancer screening: a look at the evidence. *Clin J Oncol Nurs* 2006, 10:77-81.

6. Barbieri RL, Niloff JM, Bast RC, Jr., Scaetzel E, Kistner RW, Knapp RC: Elevated serum concentrations of CA-125 in patients with advanced endometriosis. *Fertil Steril* 1986, 45:630-634.
7. Bird AP: CpG-rich islands and the function of DNA methylation. *Nature* 1986, 321:209-213.
8. Esteller M: CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. *Oncogene* 2002, 21:5427-5440.
9. Ehrlich M, Gama-Sosa MA, Huang LH, Midgett RM, Kuo KC, McCune RA, Gehrke C: Amount and distribution of 5-methylcytosine in human DNA from different types of tissues of cells. *Nucleic Acids Res* 1982, 10:2709-2721.
10. Ponger L, Duret L, Mouchiroud D: Determinants of CpG islands: expression in early embryo and isochore structure. *Genome Res* 2001, 11:1854-1860.
11. Yoder JA, Soman NS, Verdine GL, Bestor TH: DNA (cytosine-5)-methyltransferases in mouse cells and tissues. Studies with a mechanism-based probe. *J Mol Biol* 1997, 270:385-395.
12. Gardiner-Garden M, Frommer M: CpG islands in vertebrate genomes. *J Mol Biol* 1987, 196:261-282.
13. Antequera F, Bird A: Number of CpG islands and genes in human and mouse. *Proc Natl Acad Sci U S A* 1993, 90:11995-11999.
14. Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, Smith HO, Yandell M, Evans CA, Holt RA, et al: The sequence of the human genome. *Science* 2001, 291:1304-1351.
15. Robertson KD, Jones PA: DNA methylation: past, present and future directions. *Carcinogenesis* 2000, 21:461-467.
16. Harikrishnan KN, Chow MZ, Baker EK, Pal S, Bassal S, Brasacchio D, Wang L, Craig JM, Jones PL, Sif S, El-Osta A: Brahma links the SWI/SNF chromatin-remodeling complex with MeCP2-dependent transcriptional silencing. *Nat Genet* 2005, 37:254-264.
17. Takai N, Kawamata N, Walsh CS, Gery S, Desmond JC, Whittaker S, Said JW, Popoviciu LM, Jones PA, Miyakawa I, Koeffler HP: Discovery of epigenetically masked tumor suppressor genes in endometrial cancer. *Mol Cancer Res* 2005, 3:261-269.
18. Das PM, Singal R: DNA methylation and cancer. *J Clin Oncol* 2004, 22:4632-4642.
19. Momparler RL, Bovenzi V: DNA methylation and cancer. *J Cell Physiol* 2000, 183:145-154.

20. Costello JF, Plass C: Methylation matters. *J Med Genet* 2001, 38:285-303.
21. Okano M, Bell DW, Haber DA, Li E: DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 1999, 99:247-257.
22. Bhattacharya SK, Ramchandani S, Cervoni N, Szyf M: A mammalian protein with specific demethylase activity for mCpG DNA. *Nature* 1999, 397:579-583.
23. Vairapandi M: Characterization of DNA demethylation in normal and cancerous cell lines and the regulatory role of cell cycle proteins in human DNA demethylase activity. *J Cell Biochem* 2004, 91:572-583.
24. Cervoni N, Bhattacharya S, Szyf M: DNA demethylase is a processive enzyme. *J Biol Chem* 1999, 274:8363-8366.
25. Tuck-Muller CM, Narayan A, Tsien F, Smeets DF, Sawyer J, Fiala ES, Sohn OS, Ehrlich M: DNA hypomethylation and unusual chromosome instability in cell lines from ICF syndrome patients. *Cytogenet Cell Genet* 2000, 89:121-128.
26. Yu Y, Xu F, Peng H, Fang X, Zhao S, Li Y, Cuevas B, Kuo WL, Gray JW, Siciliano M, et al: NOEY2 (ARHI), an imprinted putative tumor suppressor gene in ovarian and breast carcinomas. *Proc Natl Acad Sci U S A* 1999, 96:214-219.
27. Ehrlich M, Jiang G, Fiala E, Dome JS, Yu MC, Long TI, Youn B, Sohn OS, Widschwendter M, Tomlinson GE, et al: Hypomethylation and hypermethylation of DNA in Wilms tumors. *Oncogene* 2002, 21:6694-6702.
28. De Smet C, Lurquin C, Lethe B, Martelange V, Boon T: DNA methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG-rich promoter. *Mol Cell Biol* 1999, 19:7327-7335.
29. Shimizu K, Nagamachi Y, Tani M, Kimura K, Shiroishi T, Wakana S, Yokota J: Molecular cloning of a novel NF2/ERM/4.1 superfamily gene, ehm2, that is expressed in high-metastatic K1735 murine melanoma cells. *Genomics* 2000, 65:113-120.
30. Rountree MR, Bachman KE, Herman JG, Baylin SB: DNA methylation, chromatin inheritance, and cancer. *Oncogene* 2001, 20:3156-3165.
31. Costello JF, Fruhwald MC, Smiraglia DJ, Rush LJ, Robertson GP, Gao X, Wright FA, Feramisco JD, Peltomaki P, Lang JC, et al: Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. *Nat Genet* 2000, 24:132-138.
32. Lodygin D, Epanchintsev A, Menssen A, Diebold J, Hermeking H: Functional epigenomics identifies genes frequently silenced in prostate cancer. *Cancer Res* 2005, 65:4218-4227.

33. Cameron EE, Bachman KE, Myohanen S, Herman JG, Baylin SB: Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nat Genet* 1999, 21:103-107.
34. Suzuki H, Gabrielson E, Chen W, Anbazhagan R, van Engeland M, Weijenberg MP, Herman JG, Baylin SB: A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer. *Nat Genet* 2002, 31:141-149.
35. Rivenbark AG, Jones WD, Risher JD, Coleman WB: DNA methylation-dependent epigenetic regulation of gene expression in MCF-7 breast cancer cells. *Epigenetics* 2006, 1:e1-12.
36. Liu H, Liu W, Wu Y, Zhou Y, Xue R, Luo C, Wang L, Zhao W, Jiang JD, Liu J: Loss of epigenetic control of synuclein-gamma gene as a molecular indicator of metastasis in a wide range of human cancers. *Cancer Res* 2005, 65:7635-7643.
37. Sato N, Maitra A, Fukushima N, van Heek NT, Matsubayashi H, Iacobuzio-Donahue CA, Rosty C, Goggins M: Frequent hypomethylation of multiple genes overexpressed in pancreatic ductal adenocarcinoma. *Cancer Res* 2003, 63:4158-4166.
38. Chan KY, Ozcelik H, Cheung AN, Ngan HY, Khoo US: Epigenetic factors controlling the BRCA1 and BRCA2 genes in sporadic ovarian cancer. *Cancer Res* 2002, 62:4151-4156.
39. Wang Z, Zhang J, Zhang Y, Lim SH: SPAN-Xb expression in myeloma cells is dependent on promoter hypomethylation and can be upregulated pharmacologically. *Int J Cancer* 2006, 118:1436-1444.
40. Sigalotti L, Coral S, Nardi G, Spessotto A, Cortini E, Cattarossi I, Colizzi F, Altomonte M, Maio M: Promoter methylation controls the expression of MAGE2, 3 and 4 genes in human cutaneous melanoma. *J Immunother* 2002, 25:16-26.
41. Moreau P, Mouillot G, Rousseau P, Marcou C, Dausset J, Carosella ED: HLA-G gene repression is reversed by demethylation. *Proc Natl Acad Sci U S A* 2003, 100:1191-1196.
42. Mouillot G, Marcou C, Rousseau P, Rouas-Freiss N, Carosella ED, Moreau P: HLA-G gene activation in tumor cells involves cis-acting epigenetic changes. *Int J Cancer* 2005, 113:928-936.
43. Nekrutenko A, Li WH: Transposable elements are found in a large number of human protein-coding genes. *Trends Genet* 2001, 17:619-621.
44. Jordanova ES, Riemersma SA, Philippo K, Schuurin E, Kluin PM: Beta2-microglobulin aberrations in diffuse large B-cell lymphoma of the testis and the central nervous system. *Int J Cancer* 2003, 103:393-398.

45. Patience C, Wilkinson DA, Weiss RA: Our retroviral heritage. *Trends Genet* 1997, 13:116-120.
46. Martinez-Garay I, Ballesta MJ, Oltra S, Orellana C, Palomeque A, Molto MD, Prieto F, Martinez F: Intronic L1 insertion and F268S, novel mutations in RPS6KA3 (RSK2) causing Coffin-Lowry syndrome. *Clin Genet* 2003, 64:491-496.
47. Morse B, Rotherg PG, South VJ, Spandorfer JM, Astrin SM: Insertional mutagenesis of the myc locus by a LINE-1 sequence in a human breast carcinoma. *Nature* 1988, 333:87-90.
48. Miki Y, Nishisho I, Horii A, Miyoshi Y, Utsunomiya J, Kinzler KW, Vogelstein B, Nakamura Y: Disruption of the APC gene by a retrotransposal insertion of L1 sequence in a colon cancer. *Cancer Res* 1992, 52:643-645.
49. Kazazian HH, Jr., Wong C, Youssoufian H, Scott AF, Phillips DG, Antonarakis SE: Haemophilia A resulting from de novo insertion of L1 sequences represents a novel mechanism for mutation in man. *Nature* 1988, 332:164-166.
50. Schon U, Seifarth W, Baust C, Hohenadl C, Erfle V, Leib-Mosch C: Cell type-specific expression and promoter activity of human endogenous retroviral long terminal repeats. *Virology* 2001, 279:280-291.
51. Kowalski PE, Mager DL: A human endogenous retrovirus suppresses translation of an associated fusion transcript, PLA2L. *J Virol* 1998, 72:6164-6168.
52. Di Cristofano A, Strazzullo M, Parisi T, La Mantia G: Mobilization of an ERV9 human endogenous retroviral element during primate evolution. *Virology* 1995, 213:271-275.
53. Liu AY, Abraham BA: Subtractive cloning of a hybrid human endogenous retrovirus and calbindin gene in the prostate cell line PC3. *Cancer Res* 1991, 51:4107-4110.
54. Mager DL, Hunter DG, Schertzer M, Freeman JD: Endogenous retroviruses provide the primary polyadenylation signal for two new human genes (HHLA2 and HHLA3). *Genomics* 1999, 59:255-263.
55. Baust C, Seifarth W, Germaier H, Hehlmann R, Leib MoC: HERV-K-T47D-Related long terminal repeats mediate polyadenylation of cellular transcripts. *Genomics* 2000, 66:98-103.
56. Baust C, Seifarth W, Schon U, Hehlmann R, Leib-Mosch C: Functional activity of HERV-K-T47D-related long terminal repeats. *Virology* 2001, 283:262-272.
57. Kapitonov VV, Jurka J: The long terminal repeat of an endogenous retrovirus induces alternative splicing and encodes an additional carboxy-terminal sequence in the human leptin receptor. *J Mol Evol* 1999, 48:248-251.

58. Blond JL, Beseme F, Duret L, Bouton O, Bedin F, Perron H, Mandrand B, Mallet F: Molecular characterization and placental expression of HERV-W, a new human endogenous retrovirus family. *J Virol* 1999, 73:1175-1185.
59. Frendo JL, Olivier D, Cheynet V, Blond JL, Bouton O, Vidaud M, Rabreau M, Evain-Brion D, Mallet F: Direct involvement of HERV-W Env glycoprotein in human trophoblast cell fusion and differentiation. *Mol Cell Biol* 2003, 23:3566-3574.
60. Gotzinger N, Sauter M, Roemer K, Mueller-Lantzsch N: Regulation of human endogenous retrovirus-K Gag expression in teratocarcinoma cell lines and human tumours. *J Gen Virol* 1996, 77 (Pt 12):2983-2990.
61. Florl AR, Lower R, Schmitz-Drager BJ, Schulz WA: DNA methylation and expression of LINE-1 and HERV-K provirus sequences in urothelial and renal cell carcinomas. *Br J Cancer* 1999, 80:1312-1321.
62. Jurgens B, Schmitz-Drager BJ, Schulz WA: Hypomethylation of L1 LINE sequences prevailing in human urothelial carcinoma. *Cancer Res* 1996, 56:5698-5703.
63. Takai D, Yagi Y, Habib N, Sugimura T, Ushijima T: Hypomethylation of LINE1 retrotransposon in human hepatocellular carcinomas, but not in surrounding liver cirrhosis. *Jpn J Clin Oncol* 2000, 30:306-309.
64. Suter CM, Martin DI, Ward RL: Hypomethylation of L1 retrotransposons in colorectal cancer and adjacent normal tissue. *Int J Colorectal Dis* 2004, 19:95-101.
65. Alves G, Tatro A, Fanning T: Differential methylation of human LINE-1 retrotransposons in malignant cells. *Gene* 1996, 176:39-44.
66. Kaneda A, Tsukamoto T, Takamura-Enya T, Watanabe N, Kaminishi M, Sugimura T, Tatematsu M, Ushijima T: Frequent hypomethylation in multiple promoter CpG islands is associated with global hypomethylation, but not with frequent promoter hypermethylation. *Cancer Sci* 2004, 95:58-64.

CHAPTER 2

L1 AND HERV-W RETROTRANSPOSONS ARE HYPOMETHYLATED IN HUMAN OVARIAN CARCINOMAS¹

¹ Menendez L., B.B Benigno , and J.F. McDonald 2004. Molecular Cancer: Reprinted here with permission of the publisher

Abstract

Wide-spread hypomethylation of CpG dinucleotides is characteristic of many cancers. Retrotransposons have been identified as potential targets of hypomethylation during cellular transformation. We report the results of a preliminary examination of the methylation status of CpG dinucleotides associated with the L1 and HERV-W retrotransposons in benign and malignant human ovarian tumors. We find a reduction in the methylation of CpG dinucleotides within the promoter regions of these retroelements in malignant relative to non-malignant ovarian tissues. Consistent with these results, we find that relative L1 and HERV-W expression levels are elevated in representative samples of malignant vs. non-malignant ovarian tissues.

Findings

In the genome of differentiated somatic human cells, it is estimated that ~80% of all CpG dinucleotides are methylated [1]. Methylation of CpG dinucleotides located in or near promoter regions is typically associated with transcriptional repression [e.g, 2]. Most retrotransposons, satellite DNA sequences, imprinted genes, X-chromosome inactivated genes, and many genes that are expressed tissue-specifically and that are transcriptionally silenced in differentiated human cells are associated with highly methylated (hypermethylated) CpG dinucleotides [3, 4]. In contrast, CpG dinucleotides associated with the promoter regions of genes normally expressed in differentiated human cells, including tumor suppressor genes and genes involved in DNA repair and apoptosis, are associated with under-methylated (hypomethylated) CpG dinucleotides [e.g, 5]. A number of recent studies have demonstrated that dramatic changes in these

characteristic patterns of methylation are typically associated with cellular transformation [3]. For example, wide-spread (global) hypomethylation of up to 25% of CpG dinucleotides normally methylated in differentiated cells has been shown to be characteristic of many cancer cells, including those isolated from prostate, liver, ovarian and breast carcinomas [6]. In many instances, these increases in levels of global hypomethylation have been observed to be correlated with an increase in more localized patterns of hypermethylation associated with a subset of genes that includes tumor suppressor genes and other genes involved in DNA repair and apoptosis. The consequent reduction in expression of these genes is believed to initiate a cascade of events leading to cellular transformation [7].

Relatively little is currently known concerning the specific targets of global increases in hypomethylation associated with cellular transformation, although they frequently include repetitive sequences located in proximity to centromeres [8]. One major class of middle repetitive sequences believed to be targets of hypomethylation during cellular transformation are retrotransposons [9, 10].

At least 40% of the human genome is comprised of retrotransposons [11]. Moreover, retrotransposons are widely dispersed throughout the human genome and, as alluded above, these elements are generally hypermethylated and transcriptionally silenced in most postnatal somatic cells. Retrotransposons are classified into three subtypes: long interspersed elements (LINEs) comprising 16-21% of the genome, short interspersed elements (SINEs) comprising ~13% of the genome and LTR [long terminal repeat] retrotransposons comprising 5-8% of the genome [12].

A significant increase in the expression of a variety of retrotransposons has been previously observed in a number of carcinomas including lymphocytic leukemia [13, 14], lung [15], renal cell [9], colorectal [16] and breast [17] carcinomas. Direct evidence for the

hypomethylation of retrotransposons has been observed in hepatocellular [18], prostate [19], lymphocytic leukemia [20], renal cell [9], and urinary bladder [21] carcinomas. In this paper, we report the results of a preliminary examination of the methylation status of CpG dinucleotides associated with two representative families of retrotransposons in benign and malignant human ovarian tumors. L1 is the most abundant family of human LINE elements comprising about 17% of the genome [22]. Human Endogenous Retrovirus-W (HERV-W) is a family LTR retrotransposons consisting of ~140 full-length or truncated elements randomly dispersed throughout the human genome [23]. Our results demonstrate that large numbers of both families of retrotransposons are hypomethylated in ovarian carcinomas. We further demonstrate that relative levels of both L1 and HERV-W expression are elevated in representative samples of malignant vs. non-malignant ovarian tissues. Our findings are consistent with the hypothesis that retrotransposons are a major target of global hypomethylation associated with cellular transformation.

To test the hypothesis that L1 and HERV-W elements may experience reduced methylation in malignant ovarian carcinomas, we utilized a restriction-enzyme based assay to compare the methylation status of CpG dinucleotides located within the promoter regions of these elements in a series of malignant and non-malignant ovarian tissues. The restriction enzymes *MspI* and *HpaII* both recognize the sequence CCGG but *HpaII* only cuts when the recognition sequence is unmethylated at the inner cytosine (i.e., CCGG) while *MspI* is indifferent to the methylation status of the inner cytosine

Figure1 displays Southern blots of *HpaI* and *MspI* digested genomic DNA isolated from tissue samples and hybridized against probes homologous to regions encompassing the promoter regions of each family of elements. The *HpaII/MspI* restriction sites located within the promoter

regions of both L1 and HERV-W elements are polymorphic among family members. By aligning the promoter regions of both families of elements present in the consensus human genome [<http://genome.ucsc.edu/>] and identifying the *HpaII/MspI* sites present, we estimated that the expected size range of restriction fragments within the elements to be between ~100 - 700 bp and ~1500 - 3000 bp for L1 elements and between ~100 - 500 bp for HERV-W elements. Larger sized fragments representing partial digestions and/or polymorphic *HpaII/MspI* sites located within the elements or in regions flanking the elements are also visible.

The results presented in the figure show that *MspI*-generated bands within the expected size range of internal fragments were visible in digestions of DNA from all tissue samples. In contrast, *HpaII*-generated fragments within the expected size range were only visible in digestions of DNA from the malignant samples. These results are indicative of a consistent reduction in the methylation of CpG dinucleotides within the promoter regions of both L1 and HERV-W elements in the malignant tissue. The fact that the number and intensity of *HpaII* generated bands in the malignant samples is significantly less than generated by *MspI* digestion indicates that some L1 and HERV-W elements remain hypermethlyated in the malignant samples. The reasons for this variable response among members of the same retrotransposon family are currently under investigation. Regardless, so far as we know, this is the first report of the hypomethylation of L1 elements in ovarian carcinomas and of the hypomethylation of HERV-W in any human cancer.

As noted above, hypomethylation of retroelement promoter regions may be expected to result in a localized relaxation of chromatin structure and a corresponding increased element expression [e.g., 10]. In order to test this prediction in our samples, we extracted total RNA from representative samples of two malignant and two non-malignant ovarian tissues and conducted

quantitative Real Time RT-PCR. Two replicate assays were run for each tissue sample. The results shown in Table 2 indicate a significant average increase in both L1 and HERV-W expression in the malignant vs. non-malignant ovarian tissues examined.

If further work supports the generality of these and other similar findings [10, 19-22], the implications would be important. Hypomethylation is generally associated with the relaxation of chromatin structure, an increased accessibility of transcription factors and a consequent elevation in levels of expression [24]. Our findings are generally consistent with these prior results. Since transcription is a rate limiting step in retrotransposition [11], hypomethylation might be expected to result in an increase in retrotransposon insertion mutations. While there have been occasional reports of L1 and other retrotransposon insertion mutations implicated in cancer development in humans [e.g, 25], this may not be as significant a factor as it apparently is in the mouse [26], perhaps because most L1 and other retrotransposon sequences in the human genome are believed to be truncated or otherwise transpositionally defective [27].

Another possible consequence of the hypomethylation of retroelements in humans is the opportunity it provides for ectopic pairing and recombination among homologous elements dispersed throughout the genome. The unequal-crossover events typically associated with ectopic recombination might well account for at least some of the various chromosomal aberrations and aneuploid events characteristic of human malignancies. Indeed, direct evidence of such an effect has recently been documented in mice [28, 29]. In humans, L1 retrotransposition events have been shown to induce various forms of chromosomal instabilities [30] and L1 and other retrotransposon sequences have frequently been linked with a variety of chromosomal aberrations associated with human cancers [e.g, 31].

A third possible consequence of the hypomethylation of retroelements in cancer cells is the potential regulatory impact of the release of methylation complexes known to be bound to these elements in post-embryonic somatic cells [e.g, 32]. Although little is currently understood concerning the factors that determine the relative affinity of methylation complexes for DNA target sequences, retrotransposons are known to be high affinity targets [e.g, 10]. Complexes released from retroelements may initiate a cascade of regulatory changes by binding to other lower affinity target sites and possibly resulting in the down regulation of genes essential for DNA repair and genome stability.

Methods

Tissue samples, DNA extraction, Southern hybridization

Bulk ovarian tissue samples were surgically removed and placed in RNA later (Ambion, Austin, TX) in the operating room within 1 minute of removal from the patients. The pathological and clinical information of each sample is as follows: Sample #11 (Age 43), Adenocarcinoma (papillary serous, poorly differentiated, Stage IIc); Sample #18 (Age 34), Adenocarcinoma (endometrioid, well differentiated, Stage IIb); Sample #19 (Age 57), Adenocarcinoma (papillary serous, poorly differentiated, Stage IIc); Sample #21 (Age 80), Malignant mixed mullerian; Sample #23 (Age 52), Adenocarcinoma (papillary serous, poorly differentiated, Stage IIa); Sample #29 (Age 66), Adenocarcinoma (papillary serous, poorly differentiated, Stage III); Sample #15 (Age 54), Serous borderline /low-malignancy potencial; Sample #31 (Age 40), Benign cystic masses; Sample #16 (Age 53), Normal ovary; Sample #89 (Age 53), Normal ovary. . This study was approved by the Institutional Review Board of the

University of Georgia and of Northside Hospital (Atlanta), from which the samples were obtained.

Genomic DNA was extracted by proteinase K digestion of 20-25 mg of bulk ovarian tissue and phenol-chlorophorm extraction. DNA was ethanol precipitated and re-suspended in water. Ten micrograms of genomic DNA were digested overnight at 37°C with 10 to 16 excess amount of either *HpaII* [methylation sensitive restriction enzyme] or *MspI* [not sensitive for methylation at internal cytosine]. These enzymes recognize the sequence CCGG, which is found in diverse positions in the promoter regions of these retroelements. Digested DNA was resolved on an agarose gel and transferred to a nylon membrane (Hybond N; Amersham-Biosciences, Piscataway, NJ) with NaOH. Membranes were prehybridized for 1 hour with 10 mg/ml of herring sperm DNA in Church buffer [0.5M NaH₂PO₄, 7% SDS and 10M EDTA] and hybridized overnight at 65°C in the same buffer with 100-200ng of probe DNA labeled with [α -³²P]dCTP using a Nick Translation Kit (Roche, Indianapolis, IN). Filters were washed twice for 15 min in 2xSSC and 0.1% SDS and then twice for 30 min in 1x SSC and 0.1% SDS at 65°C and exposed to Phosphorimager screens (Molecular Dynamics, Sunnyvale, CA).

The HERV-W probe was designed in the LTR region, downstream of the putative TTAAAT box. PCR was performed on genomic DNA with forward primer HERVF 5'-CCACCACTGCTGTTTGCCAC-3' and reverse primer HERVR 5'-GCCTCGTGTTCTCTGACCTGGGG-3', producing a 304 bp fragment. The LINE1 probe for the promoter region was designed according to Takai et al [18]. PCR was performed on genomic DNA with forward primer L1F 5'-CGGGTGATTCTGCATTTC-3' and reverse primer L1R 5'-GACATTTAAGTCTGCAGAGG-3', giving a product of 540 bp. PCR products were cloned into pCR2.1-TOPO and transformed into TOP10 E.coli cells (Invitrogen, Carlsbad, CA).

Plasmids were extracted (Qiaprep Spin Miniprep Kit, Qiagen, Valencia, CA) and sequenced. Subsequent PCR reactions were performed on cloned plasmid DNA for both HERV-W and LINE1, and gel extracted PCR products were used as hybridization probes.

RNA extraction, Quantitative real time RT-PCR

Total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA) and 2-5 µg of total RNA were reverse transcribed into first-strand cDNA using the Thermoscript RT-PCR system (Invitrogen, Carlsbad, CA) in a final volume of 20 µl. The HERV-W primers used were: forward; 5'-TTGGCGGTATCACAACCTCT-3' reverse; 5'-GTGACGATTCCGGATTGA-3'; (product size:230 bp) based on the HERV-W sequence (GeneBank accession no. AC000064). The LINE-1 primers were: forward 5'-TCATAAAGCAAGTCCTCAGTGACC-3'; reverse 5'-GGGGTGGAGAGTTCTGTAGATGTC-3' (product size:165 bp) based on the LINE-1 sequence (GeneBank accession no. M80343). Real-time monitoring of PCR reactions was performed using the DNA Engine Opticon 2 System (MJ Research, Waltham, MA) and the SYBR Green iQ dye (BioRad, Hercules, CA) [33]. For each reaction, the amount of a target and of an endogenous control (Ribosomal Protein S27A) were determined using a calibration curve and the amount of target molecule was divided by the amount of endogenous reference to obtain a normalized target value [34, 35]. RPS27A has been previously identified as a valid control gene in expression studies conducted among human malignant and control tissues [26]. In addition, we independently verified by microarray analyses that RPS27A expression levels are constant among the samples examined in this study (data not shown). Separate calibration (standard) curves for RPS27A, HERV-W and LINE-1 were constructed using serial dilutions of total cDNA from normal human ovarian tissue (purchased from Ambion, Austin, TX). Standards for HERV-

W, LINE-1 and RPS27A were defined to contain an arbitrary starting concentration, and serial dilutions were used to construct the standard curve. Standard curve calibrations were included in each assay.

List of abbreviations

HERV-W, human endogenous retrovirus-W; LINEs, L1, long interspersed elements; LTR, long terminal repeat; RPS27A, ribosomal protein S27A; PCR, polymerase chain reaction; RT-PCR, reverser transcription PCR

Authors' contributions

LM performed all assays. LM and JM collectively designed the study, analyzed the results and drafted the manuscript. BB provided tissue samples and suggestions for finalization of the manuscript. All authors read and approved the manuscript.

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References

1. Bird AP, Taggart MH: **Variable patterns of total DNA and rDNA methylation in animals.** *Nucleic Acids Res* 1980, **8**(7):1485-1497.
2. Whitelaw E, Martin DI: **Retrotransposons as epigenetic mediators of phenotypic variation in mammals.** *Nat Genet* 2001, **27**(4):361-365.
3. Robertson KD, Jones PA: **DNA methylation: past, present and future directions.** *Carcinogenesis* 2000, **21**(3):461-467.

4. Esteller M, Herman JG: **Cancer as an epigenetic disease: DNA methylation and chromatin alterations in human tumours.** *J Pathol* 2002, **196**(1):1-7.
5. Tycko B: **DNA and alterations in cancer: genetic and epigenetic alterations.** In. Edited by M E: Natick: Eaton Publishing; 2000: 333-349.
6. Ehrlich M: **DNA methylation in cancer: too much, but also too little.** *Oncogene* 2002, **21**(35):5400-5413.
7. Jones PA, Baylin SB: **The fundamental role of epigenetic events in cancer.** *Nat Rev Genet* 2002, **3**(6):415-428.
8. Qu G, Dubeau L, Narayan A, Yu MC, Ehrlich M: **Satellite DNA hypomethylation vs. overall genomic hypomethylation in ovarian epithelial tumors of different malignant potential.** *Mutat Res* 1999, **423**(1-2):91-101.
9. Florl AR, Lower R, Schmitz-Drager BJ, Schulz WA: **DNA methylation and expression of LINE-1 and HERV-K provirus sequences in urothelial and renal cell carcinomas.** *Br J Cancer* 1999, **80**(9):1312-1321.
10. Lorincz MC, Schubeler D, Groudine M: **Methylation-mediated proviral silencing is associated with MeCP2 recruitment and localized histone H3 deacetylation.** *Mol Cell Biol* 2001, **21**(23):7913-7922.
11. Deininger PL, Batzer MA: **Mammalian retroelements.** *Genome Res* 2002, **12**(10):1455-1465.
12. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W *et al*: **Initial sequencing and analysis of the human genome.** *Nature* 2001, **409**(6822):860-921.
13. Patzke S, Lindeskog M, Munthe E, Aasheim HC: **Characterization of a novel human endogenous retrovirus, HERV-H/F, expressed in human leukemia cell lines.** *Virology* 2002, **303**(1):164-173.
14. Depil S, Roche C, Dussart P, Prin L: **Expression of a human endogenous retrovirus, HERV-K, in the blood cells of leukemia patients.** *Leukemia* 2002, **16**(2):254-259.
15. Andersson AC, Svensson AC, Rolny C, Andersson G, Larsson E: **Expression of human endogenous retrovirus ERV3 (HERV-R) mRNA in normal and neoplastic tissues.** *Int J Oncol* 1998, **12**(2):309-313.
16. Debniak T, Gorski B, Cybulski C, Jakubowska A, Kurzawski G, Kladny J, Lubinski J: **Comparison of Alu-PCR, microsatellite instability, and immunohistochemical**

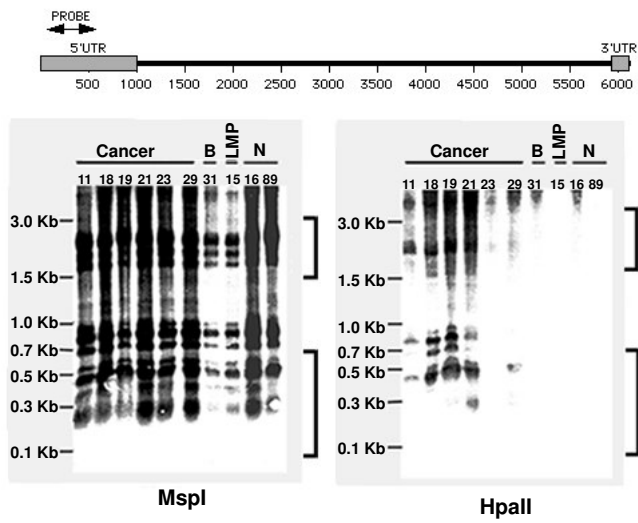
- analyses in finding features characteristic for hereditary nonpolyposis colorectal cancer.** *J Cancer Res Clin Oncol* 2001, **127**(9):565-569.
17. Wang-Johanning F, Frost AR, Jian B, Epp L, Lu DW, Johanning GL: **Quantitation of HERV-K env gene expression and splicing in human breast cancer.** *Oncogene* 2003, **22**(10):1528-1535.
 18. Takai D, Yagi Y, Habib N, Sugimura T, Ushijima T: **Hypomethylation of LINE1 retrotransposon in human hepatocellular carcinomas, but not in surrounding liver cirrhosis.** *Jpn J Clin Oncol* 2000, **30**(7):306-309.
 19. Santourlidis S, Florl A, Ackermann R, Wirtz HC, Schulz WA: **High frequency of alterations in DNA methylation in adenocarcinoma of the prostate.** *Prostate* 1999, **39**(3):166-174.
 20. Dante R, Dante-Paire J, Rigal D, Roizes G: **Methylation patterns of long interspersed repeated DNA and alphoid repetitive DNA from human cell lines and tumors.** *Anticancer Res* 1992, **12**(2):559-563.
 21. Jurgens B, Schmitz-Drager BJ, Schulz WA: **Hypomethylation of L1 LINE sequences prevailing in human urothelial carcinoma.** *Cancer Res* 1996, **56**(24):5698-5703.
 22. Ostertag EM, Kazazian HH, Jr.: **Biology of mammalian L1 retrotransposons.** *Annu Rev Genet* 2001, **35**:501-538.
 23. Kim HS, Lee WH: **Human endogenous retrovirus HERV-W family: chromosomal localization, identification, and phylogeny.** *AIDS Res Hum Retroviruses* 2001, **17**(7):643-648.
 24. Chandler LA, Jones PA: **Hypomethylation of DNA in the regulation of gene expression.** *Dev Biol (N Y 1985)* 1988, **5**:335-349.
 25. Miki Y, Nishisho I, Horii A, Miyoshi Y, Utsunomiya J, Kinzler KW, Vogelstein B, Nakamura Y: **Disruption of the APC gene by a retrotransposal insertion of L1 sequence in a colon cancer.** *Cancer Res* 1992, **52**(3):643-645.
 26. Kuff EL: **Intracisternal A particles in mouse neoplasia.** *Cancer Cells* 1990, **2**(12):398-400.
 27. Sassaman DM, Dombroski BA, Moran JV, Kimberland ML, Naas TP, DeBerardinis RJ, Gabriel A, Swergold GD, Kazazian HH, Jr.: **Many human L1 elements are capable of retrotransposition.** *Nat Genet* 1997, **16**(1):37-43.
 28. Eden A, Gaudet F, Waghmare A, Jaenisch R: **Chromosomal instability and tumors promoted by DNA hypomethylation.** *Science* 2003, **300**(5618):455-455.

29. Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Dausman J, Gray JW, Leonhardt H, Jaenisch R: **Induction of tumors in mice by genomic hypomethylation.** *Science* 2003, **300**(5618):489-492.
30. Symer DE, Connelly C, Szak ST, Caputo EM, Cost GJ, Parmigiani G, Boeke JD: **Human II retrotransposition is associated with genetic instability in vivo.** *Cell* 2002, **110**(3):327-338.
31. Kolomietz E, Meyn MS, Pandita A, Squire JA: **The role of Alu repeat clusters as mediators of recurrent chromosomal aberrations in tumors.** *Genes Chromosomes Cancer* 2002, **35**(2):97-112.
32. Hakimi MA, Bochar DA, Schmiesing JA, Dong Y, Barak OG, Speicher DW, Yokomori K, Shiekhhattar R: **A chromatin remodelling complex that loads cohesin onto human chromosomes.** *Nature* 2002, **418**(6901):994-998.
33. Wittwer CT, Herrmann MG, Moss AA, Rasmussen RP: **Continuous fluorescence monitoring of rapid cycle DNA amplification.** *Biotechniques* 1997, **22**(1):130-131, 134-138.
34. Bieche I, Onody P, Laurendeau I, Olivi M, Vidaud D, Lidereau R, Vidaud M: **Real-time reverse transcription-PCR assay for future management of ERBB2-based clinical applications.** *Clin Chem* 1999, **45**(8 Pt 1):1148-1156.
35. Lee PD, Sladek R, Greenwood CM, Hudson TJ: **Control genes and variability: absence of ubiquitous reference transcripts in diverse mammalian expression studies.** *Genome Res* 2002, **12**(2):292-297.

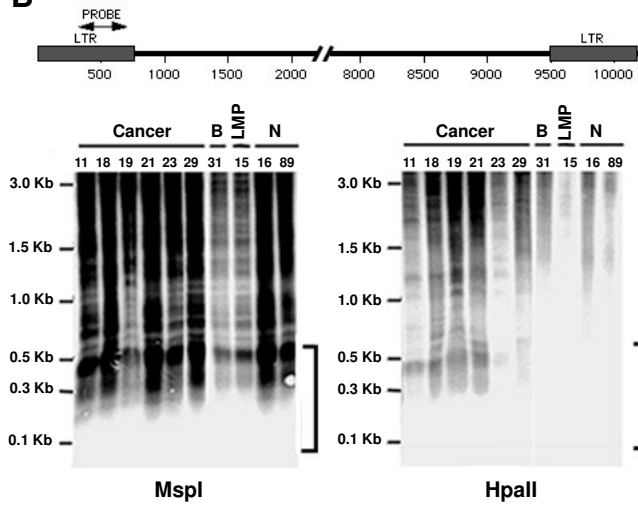
Figure 2.1 Hypomethylation and expression of L1 and HERV-W elements in ovarian cancer

Genomic DNA was digested either with *MspI* (left) or *HpaII* (right), and hybridized with probes specific for the promoter regions of L1 (A) or HERV-W (B) elements. The restriction enzymes *MspI* and *HpaII* recognize the sequence CCGG but *HpaII* only cuts when the recognition sequence is unmethylated at the inner cytosine (i.e., CCGG) while *MspI* is indifferent to the methylation status of the inner cytosine. Brackets indicate bands from restriction cut sites internal to the elements (*B* = benign cystic mass; *LMP* = low-malignancy potential or borderline tumor; *N* = normal ovary). (C) Real time RT-PCR was performed to determine expression levels of LINE-1 and HERV-W elements in representative malignant and non malignant samples. Normalized values (retroelement expression value divided by expression value of the RPS27A control gene). Shown is the average of 3 replicate assays per sample $\pm$ SE. RPS27A expression has been previously determined to be unchanged between the malignant and non-malignant samples examined in this study (see text for details).

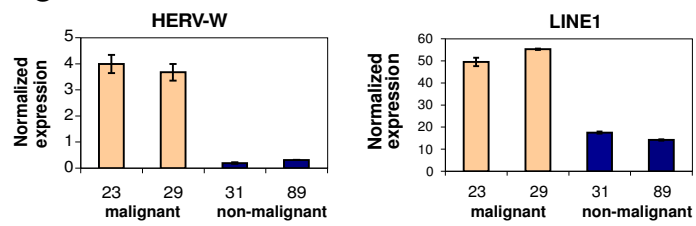
A



B



C



CHAPTER 3

HLA-G EXPRESSION IN OVARIAN TISSUE IS NOT CORRELATED WITH METHYLATION OF THE PROMOTER REGION OR EXON 1

Background

Classic and non-classic HLA (human leukocyte antigen) class I genes play a central role in the regulation of the immune response. The non-classic *HLA-G* gene is expressed in the fetal-maternal interface on the extravillous cytotrophoblast and has been postulated to help protect the fetus from maternal allorecognition [1]. This hypothesis is supported by subsequent studies demonstrating that *HLA-G* proteins can suppress a variety of immune functions including natural killer (NK) cell-mediated cytotoxicity and the T-cell proliferative response [2]. Recent findings have demonstrated that HLA-G antigens are present in various types of malignant cells and tissues. These findings have led to the hypothesis that induction of *HLA-G* expression in tumor cells may contribute to their avoidance of immunosurveillance by the host [3-11]. Regulatory sequences known to be involved in the transcriptional regulation of most HLA class I genes are disrupted in the *HLA-G* gene [12, 13] raising questions as to the mechanisms underlying *HLA-G* expression [14-16]. Studies conducted in a variety of human cancer cell lines suggest that epigenetic mechanisms may play an important role in *HLA-G* expression [17]. To explore the potential role of DNA methylation on *HLA-G* expression in ovarian cancer, we tested the effect of the demethylating agent 5-aza-deoxycytidine (5-aza-dC) on *HLA-G* expression and methylation patterns in an ovarian cancer cell line. In addition, *HLA-G* expression levels were compared with methylation patterns in eighteen malignant and benign ovarian tissue samples.

The results demonstrate that although 5-aza-dC-induced hypomethylation of a previously identified CpG-enriched 5' regulatory region of the *HLA-G* gene was correlated with a significant increase in expression in the ovarian cancer cell line, no such correlation was detected among the ovarian tissue samples examined. Further studies of the methylation status of Exon1 of *HLA-G* also showed no correlation with expression, although some specific CpG dinucleotides displayed significant differences in methylation between normal surface epithelium and adenomas or adenocarcinomas.

Material and Methods

Tissue samples

Bulk ovarian samples were collected at Northside Hospital (Atlanta, GA) during surgery and stored in RNA later (Ambion, Austin, GA). Patient consent and approval from the Institutional Board of the University of Georgia and of Northside Hospital were obtained. Pathological and clinical information of samples are presented in Table 1.

Human cell-line culture

The BG-1 ovarian adenocarcinoma cell line was kindly provided from JM Hall and KS Korach (NIH). Cells were propagated in DMEM: F12/50:50 (Invitrogen , Carlsbad, CA), supplemented with 2 mM L-glutamine (Mediatech Inc., Herndon, VA), 10% heat inactivated FBS (Invitrogen , Carlsbad, CA), 1 mM Sodium Pyruvate (Mediatech Inc., Herndon, VA), and 1% penicillin and streptomycin (Mediatech, Inc., Herndon, VA) at 37°C, 5% CO² and ~80% relative humidity.

Cell Treatments

BG-1 cells were treated with 50 μ M 5-aza-deoxycytidine (Sigma, St. Louis, MO) for 5 days. Cells were plated at low density 16 hours before treatment. Fresh media with 5-aza-dC was changed on the third day of treatment. Control cells treated with PBS were grown for the same amount of time.

Laser Capture Microdissection (LCM)

Fresh frozen tissues were cut into seven-micron sections, fixed in 75% ethanol for 30 seconds, stained and dehydrated using the HistoGene LCM Frozen Section Staining Kit (Arcturus, Mountain View, CA). LCM was performed with an AutoPix Automated Laser Capture Microdissection System using the CapSure Macro Caps or HS Caps (Arcturus, Mountain View, CA). Approximately 10,000 cells were captured on each cap. Subsequently the DNA was extracted from captured cells using the Pico Pure DNA Extraction Kit (Arcturus, Mountain View, CA). RNA extraction from capture cells was done using the Pico Pure RNA Extraction Kit.

RNA extraction from ovarian tissue

Ovarian tissue (75 ± 25 mg) was homogenized in Trizol (Invitrogen, Carlsbad, CA) using a polytron homogeneizer and RNA extracted following manufacturers suggestions.

DNA and RNA extraction from ovarian epithelium surface

Epithelial brushes were obtained from normal ovaries and kept in RNA-later (Qiagen, Valencia, CA). For DNA extraction, cells were spun down and resuspended in lysis buffer (10 mM TRIS

HCl, 10 mM EDTA, 0.2% SDS and 50 mM NaCl), with 50 ug/ml RNase A (Invitrogen, Carlsbad, CA) and 100 ug/ml Proteinase K (EM Science, Gibbstown, NJ) overnight at 37° C. DNA was phenol-extracted, ethanol precipitated, and 500 ng of DNA were modified with sodium bisulfite using the EZ DNA Methylation-Gold Kit (Zymo, Orange, CA). For RNA extraction, cells were spun down and Trizol was added, following the manufacturers recommended instructions (Invitrogen, Carlsbad, CA).

Semi-quantitative RT-PCR

Total RNA was extracted from BG-1 cells by adding 1 ml of Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations, and further purified using RNEasy mini kit (Qiagen, Valencia, CA). Four to six µg of DNase-treated RNA (DNA-free; Ambion, Houston, TX) were used to generate cDNA by oligo-dT reverse transcription using Superscript RT-PCR (Invitrogen, Carlsbad, CA). Primers that recognize all HLA-G variants were used for PCR. Using the HLA-G RNA sequence (X12273) as a reference, primers were designed at 257F 5' GGAAGAGGAGACACGGAACA-3' and 1200R 5' TCCTGTTCCAGAAAAGGGG3'. GAPDH (NM_002046) was used as an endogenous control, using primers 287F 5'-GAAATCCCATCACCATCTTCCAG-3' and 599R 5' ATGAGTCCTTCCACGATACAAAAG-3'

Real time RT-PCR

Four to six µg of DNase-treated RNA obtained from bulk ovarian tissue (DNA-free; Ambion, Houston, TX) were used to generate cDNA by oligo-dT reverse transcription using Superscript RT-PCR (Invitrogen, Carlsbad, CA). RNA from ovarian epithelium surface and from cells

obtained from LCM was amplified and cDNA was synthesized using the RiboAmpHS RNA Amplification kit (Arcturus, Mountain View, CA).

Quantitative real-time RT-PCR was performed with cDNA and TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA). Expression of *HLA-G* (Hs00365950_g1) and of the housekeeping gene *GAPDH* as an endogenous control (Hs99999905_m1) were analyzed using the DNA Engine Opticon System (MJ Research, Waltham, MA). A standard curve of serial dilutions of a plasmid containing either *HLA-G* or *GAPDH* was established for each assay to obtain a relative quantification of *HLA-G* or *GAPDH* gene expression. For each sample normalized *HLA-G* expression values are presented as the ratio between the target gene (*HLA-G*) and the endogenous control (*GAPDH*).

Sodium bisulfite genomic sequencing

Five hundred nanograms to one microgram of genomic DNA from the BG-1 cell line or epithelium cells from normal ovaries was modified with Sodium bisulfite according to Herman et al. [18]. For LCM samples, sodium bisulfite modification was performed by pooling DNA extracted from ~30,000 cells, and adding only 15 µl of Hydroquinone and 200 µl of Sodium bisulfite, as well as 1 µg of Herring Sperm (Promega, Madison, WI) as a carrier.

Modified DNA was used for PCR using primers for the CpG-enriched 5' regulatory region of *HLA-G* [19]. Position of the primers from the ATG codon of *HLA-G* gene are: Primer F (-456) 5'-AAGAGTATAGGAGGATAGGTAAGG-3' and primer R (-16) 5'-AACACCATAACCACCATCCTTAAC-3'.

A second region of *HLA-G* from -7 to +392 from the transcriptional start site was amplified performing two rounds of PCR. First round of PCR was done with primers F-5'-

GGATTTATTTTTTTTAGATGTTAAG-3' and R-5'

ATCTACAAATTCATTCTATCAATCTATAC-3' -and then nested PCR was performed with F-5'-GTTAAGGATGGTGGTTATGGTGTT-3' and R-5'-TATCTCCTCTTCCCAATACTCCAA-3'.

PCR products were purified using the QIAquick Gel extraction Kit (Qiagen, Valencia, CA), and the DNA cloned using TOPO TA Cloning for Sequencing Kit (Invitrogen, Carlsbad, CA). DNA plasmids from individual colonies were extracted using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). Automatic sequencing was performed by Integrated Biotech Laboratories (Athens, GA).

Results

HLA-G gene expression in (BG-1) ovarian tumor cells is increased following demethylating 5-Aza-dC treatment

Previous studies have shown that demethylating treatment (5-aza-deoxycytidine) of a variety of tumor (glioma, choriocarcinoma, B-lymphoma and melanoma) cell lines results in a significant increase in *HLA-G* expression correlated with significant hypomethylation of a CpG-rich region located within 450 bp 5' of the *HLA-G* start codon [17]. To determine if ovarian tumor cells would show a similar response, we selected an ovarian cancer cell line (BG-1) that did not display *HLA-G* expression prior to treatment. Consistent with previous results, we found that BG-1 cells treated with 5-aza-dC displayed a significant increase in *HLA-G* expression relative to controls (Figure 1).

To determine if this induced increase in *HLA-G* expression was correlated with hypomethylation of the CpG-rich region previously implicated in 5-aza-dC induced *HLA-G*

expression in other tumor cell lines, we performed sodium bisulfite genomic sequencing of several cloned fragments from treated and untreated BG-1 cells. The results demonstrate that while all 19 CpG sites located within the sequenced region were methylated in untreated cells, there was a 35% decrease of methylation after 5-aza-dC treatment (Figure 2).

Levels of *HLA-G* expression are variable among ovarian adenocarcinomas and benign ovarian adenomas and do not correlate with methylation patterns

To determine if levels of *HLA-G* expression are significantly different among malignant and benign ovarian tumors, we determined relative expression levels in 18 malignant and benign ovarian tumor samples using real time RT-PCR. The results presented in Table 2 indicate that although the two highest levels of *HLA-G* expression were associated with adenocarcinomas (samples 212 and 229), there was no consistent correlation between *HLA-G* expression and sample pathology. To determine if relative levels of *HLA-G* expression among our tissue samples was correlated with methylation pattern differences in the CpG-rich region previously associated with 5-Aza-dC-induced expression in tumor cell lines, we performed sodium bisulfite genomic sequencing of DNA isolated from laser capture microdissected (LCM) tumor cells from each of our 18 tissue samples. Five to eight clones were sequenced from each DNA sample and the results are presented in Figure 3. Methylation levels were variable amongst samples, ranging from 36.8 % to 55.3%.

Percentage of methylation computed overall 19 CpG sites was plotted against the *HLA-G* expression for all 18 samples (Figure 4). While the 3 samples displaying the highest level of *HLA-G* expression had some of the lowest percentage of methylation over this region, overall there was no significant correlation between expression and levels of methylation ($R^2=0.069$).

We also looked for a correlation between *HLA-G* expression and methylation patterns at each of the 19 individual CpG sites. CpG dinucleotides at positions –188, -186 and –39 were consistently unmethylated in the 3 samples having the highest levels of *HLA-G* expression (212, 229 and 382). However, two of the samples associated with the lowest levels of *HLA-G* expression (383 and 242) were also unmethylated at these sites in 4 out of 5 of their sequenced clones. In general, there is no consistent correlation between levels of *HLA-G* expression and patterns of methylation at individual CpG sites among the 18 samples examined in this study.

The CpG Island of *HLA-G* spans from the promoter region to Exon3, therefore we examined the methylation levels of a second region of the island of 399 bp in Exon 1, which contained 42 CpG dinucleotides. Methylation levels were also highly variable amongst samples and no significant correlation was found with sample pathology or *HLA-G* expression ($R^2=0.1374$) (Figure 5).

The RNA used for these studies had been obtained from bulk tissue samples, therefore representing a mixture of ovarian cell types, which could have different patterns of *HLA-G* expression. To test this hypothesis, we extracted and amplified RNA from adenocarcinomas or adenomas using Laser Capture Microdissection (LCM) for 14 out the previous 18 samples. This technique allows the selection of specific cells, therefore eliminating the presence of stromal or other non-neoplastic cells. We also extracted and amplified RNA from four samples obtained from epithelial cells from normal ovaries. Results for real time RT-PCR display lower levels of *HLA-G* expression than those obtained from bulk tissue for all samples, but with a similar ratio between samples (Table 4); for example, samples 212 (adenocarcinoma) and 382 (adenoma) still show the highest expression levels of *HLA-G*. Normal samples also showed variable expression of *HLA-G*, which did not correlate with the methylation level of the promoter or exon1 (Figure

6). There was no significant correlation between *HLA-G* expression and sample pathology, or between expression and levels of methylation of either the promoter region or exon1 (Figure 6).

HLA-G promoter region is hypermethylated in tumor samples compared to normal epithelium cells

Even though there was no correlation between expression and methylation of *HLA-G*, we looked for possible methylation differences between the three types of samples (adenomas, adenocarcinomas and normal epithelium). As mentioned before, there was no significant difference between adenomas and adenocarcinomas ($p>0.05$), but t-test revealed significant overall hypermethylation when comparing the promoter regions from normal epithelium to tumor cells, both benign ($p=0.019$) and malignant ($p=0.038$) sample. Comparison of the methylation status of Exon1 showed no significant methylation differences between any of the histopathological types of samples ($p>0.05$). To determine if the methylation differences affected specific dinucleotides, we calculated the average methylation for each CpG dinucleotides in the three types of samples (adenocarcinomas, adenomas and normal epithelial cells). Table 4 shows that for the promoter region, five CpG dinucleotides are significantly hypomethylated (-394, -356, -134, -74, -69) and five are significantly hypermethylated (-299, -281, -270, -200, -159) in adenomas and adenocarcinomas when comparing to epithelial cells from normal. An additional site was also hypermethylated in adenomas when comparing with normal ovarian cells (-230). In the case of Exon1, 5 out of 42 CpG dinucleotides were significantly hypomethylated (+40, +59, +154, +156, +159) when comparing epithelial cells from normal ovaries to adenomas (Table 5). These sites could therefore be used as markers for neoplasm development, although they did not correlate with expression of *HLA-G*.

Reports show that for some genes, the methylation status of SP1 binding sites or nearby CpG dinucleotides is correlated with gene expression [20-22]. The *HLA-G* promoter has two SP1 binding sites, consequently we looked for methylation differences CpG sites in them as well as in nearby CpG sites, to examine if any of them were significantly differentially methylated in adenomas, adenocarcinomas and normal epithelium cells. The first SP1 binding site is located in the κ B2 box (-198 to -188). Both CpG sites at positions -188 bp and -186 bp, have low or no methylation in all of our samples regardless of the pathological state and having no correlation with expression values. The CpG site at position -211 showed a significant hypermethylation when comparing normal epithelium to adenomas and adenocarcinomas, although it had no correlation with expression. The second SP1 binding site is located in the X1 box (-138 to -127). The closest CpG dinucleotide (-125 bp) to this SP1 site is significantly hypomethylated when comparing normal epithelium to adenomas, therefore showing an inverse correlation to the sites close to the SP1 site in the κ B2 box.

Therefore we have found several positions significantly ($p < 0.05$) differentially methylated as well as a general decrease of methylation levels between normal epithelium cells and adenomas or adenocarcinomas, although neither of those changes correlated with *HLA-G* expression or with possible hindrance of SP1 binding.

Discussion

Contrary to other HLA molecules, *HLA-G* regulation is only partially understood. Studies of the methylation levels of 450 bp of the promoter region of *HLA-G* in melanoma and choriocarcinoma cell lines showed a correlation between expression and low levels of methylation [17, 18]. In our study we looked at the same region in the ovarian cancer cell line

BG-1, which showed almost complete methylation of all CpG dinucleotides and no expression of *HLA-G*. Treatment of the cells with the demethylating agent 5-aza-dC resulted in high expression of *HLA-G* as well as a decrease of the methylation level of the 450 bp promoter region (Figure 1 and 2). These results suggest that in ovarian cancer, this region is important for the control of *HLA-G* expression. To confirm these results we analyzed the same region in 18 ovarian adenocarcinomas and benign adenomas. Methylation levels were variable ranging from 36.8 % to 55.3%, and did not correlate with histopathological state ($p>0.05$) (Figure 3). Expression of *HLA-G* was variable amongst samples and did not correlate with stage of the disease or methylation levels (Table 2). High mRNA levels were only found in 2 out of 5 high-grade adenocarcinomas, while the rest of adenocarcinomas had low levels of expression. Furthermore, one benign sample also showed high levels of *HLA-G* RNA. Thus, contrary to the results obtained from the BG-1 cell line, high levels of *HLA-G* expression did not inversely correlate with overall low levels of methylation of the promoter region (Figure 4). Because of the global action of 5-aza-deoxycytide, one cannot exclude the possibility that there might be another region controlling *HLA-G* expression that is affected by the chemical, causing the increase of expression of *HLA-G* in the cancer tissue. In *HLA-G*, the CpG island extends from the promoter further downstream into Exon1, therefore we looked at the methylation levels of 399 bp of Exon1 of *HLA-G*, but still found no correlation with expression levels (Figure 5).

Since RNA had been obtained from bulk tissue, it derives from a mixture of cell types that could have different expression of *HLA-G*. Therefore the presence of non-tumorigenic cells could be skewing the *HLA-G* expression data. To eliminate this possible source of variation we also extracted and amplified RNA from 14 adenocarcinomas and adenomas through LCM as well as from 4 normal ovarian epithelial cell samples obtained through surface brushings.

Expression was still variable in all of those samples and followed a similar pattern that those results obtained from bulk tissue RNA extraction (Table 4). No significant correlation was found between expression and methylation of the promoter and exon1 of *HLA-G*.

Even though there was no correlation between expression and methylation of *HLA-G*, we looked for methylation differences in the three types of samples. Total methylation levels were significantly lower in normal epithelium than in adenomas and adenocarcinomas ($p < 0.05$) suggesting a change of methylation content during ovarian cancer development. We also looked for differences in specific CpG dinucleotides. In the promoter region we found several sites differentially methylated between normal and neoplasm (five hypermethylated and five hypomethylated CpG dinucleotides), but couldn't correlate any of them with expression or possible inhibition of SP1 binding.

Our results indicate that for ovarian cancer there are marked differences between tissue and cell lines. While in cell lines *HLA-G* expression was correlated with methylation, we could not corroborate this relationship in tissue. It is also possible that because of long-term propagation of the cell lines, the mechanism of *HLA-G* control has been altered from that of ovarian tissue. In that case it might be possible that epigenetic effects have no relation with expression in vivo, or once more, that they affect another region of the gene. Regardless of this lack of inverse correlation, both the ovarian cancer cell line and the tumor samples had higher levels of *HLA-G* methylation than normal epithelium samples. Even though other studies have looked at *HLA-G* expression in ovarian cancer [8], and have found a higher protein concentration than in normal samples, this is the first study of methylation of *HLA-G* in ovarian tissue.

In conclusion, we have shown that the methylation of the promoter region or Exon1 of *HLA-G* does not correlate with the expression of this gene in ovarian tissue, even though there is an increase of methylation in tumor samples compared with normal epithelium cells.

References

1. Kovats S, Main EK, Librach C, Stubblebine M, Fisher SJ, DeMars R: A class I antigen, HLA-G, expressed in human trophoblasts. *Science* 1990, 248:220-223.
2. Carosella ED, Moreau P, Le Maoult J, Le Discorde M, Dausset J, Rouas-Freiss N: HLA-G molecules: from maternal-fetal tolerance to tissue acceptance. *Adv Immunol* 2003, 81:199-252.
3. Ferguson AT, Evron E, Umbricht CB, Pandita TK, Chan TA, Hermeking H, Marks JR, Lambers AR, Futreal PA, Stampfer MR, Sukumar S: High frequency of hypermethylation at the 14-3-3 sigma locus leads to gene silencing in breast cancer. *Proc Natl Acad Sci U S A* 2000, 97:6049-6054.
4. Lefebvre S, Antoine M, Uzan S, McMaster M, Dausset J, Carosella ED, Paul P: Specific activation of the non-classical class I histocompatibility HLA-G antigen and expression of the ILT2 inhibitory receptor in human breast cancer. *J Pathol* 2002, 196:266-274.
5. Fukushima Y, Oshika Y, Nakamura M, Tokunaga T, Hatanaka H, Abe Y, Yamazaki H, Kijima H, Ueyama Y, Tamaoki N: Increased expression of human histocompatibility leukocyte antigen-G in colorectal cancer cells. *Int J Mol Med* 1998, 2:349-351.
6. Ibrahim EC, Guerra N, Lacombe MJ, Angevin E, Chouaib S, Carosella ED, Caignard A, Paul P: Tumor-specific up-regulation of the nonclassical class I HLA-G antigen expression in renal carcinoma. *Cancer Res* 2001, 61:6838-6845.
7. Bukur J, Malenica B, Huber C, Seliger B: Altered expression of nonclassical HLA class Ib antigens in human renal cell carcinoma and its association with impaired immune response. *Hum Immunol* 2003, 64:1081-1092.
8. Singer G, Rebmann V, Chen YC, Liu HT, Ali SZ, Reinsberg J, McMaster MT, Pfeiffer K, Chan DW, Wardelmann E, et al: HLA-G is a potential tumor marker in malignant ascites. *Clin Cancer Res* 2003, 9:4460-4464.
9. Amiot L, Onno M, Drenou B, Monvoisin C, Fauchet R: HLA-G class I gene expression in normal and malignant hematopoietic cells. *Hum Immunol* 1998, 59:524-528.

10. Urosevic M, Kurrer MO, Kamarashev J, Mueller B, Weder W, Burg G, Stahel RA, Dummer R, Trojan A: Human leukocyte antigen G up-regulation in lung cancer associates with high-grade histology, human leukocyte antigen class I loss and interleukin-10 production. *Am J Pathol* 2001, 159:817-824.
11. Paul P, Rouas-Freiss N, Khalil-Daher I, Moreau P, Riteau B, Le Gal FA, Avril MF, Dausset J, Guillet JG, Carosella ED: HLA-G expression in melanoma: a way for tumor cells to escape from immunosurveillance. *Proc Natl Acad Sci U S A* 1998, 95:4510-4515.
12. Gobin SJ, Keijsers V, van Zutphen M, van den Elsen PJ: The role of enhancer A in the locus-specific transactivation of classical and nonclassical HLA class I genes by nuclear factor kappa B. *J Immunol* 1998, 161:2276-2283.
13. Solier C, Mallet V, Lenfant F, Bertrand A, Huchencq A, Le Bouteiller P: HLA-G unique promoter region: functional implications. *Immunogenetics* 2001, 53:617-625.
14. Gobin SJ, Keijsers V, Cheong C, van Zutphen M, Van den Elsen PJ: Transcriptional regulation of HLA-G. *Transplant Proc* 1999, 31:1857-1859.
15. Rousseau P, Paul P, O'Brien M, Dausset J, Carosella ED, Moreau P: The X1 box of HLA-G promoter is a target site for RFX and Sp1 factors. *Hum Immunol* 2000, 61:1132-1137.
16. Lefebvre S, Berrih-Aknin S, Adrian F, Moreau P, Poeta S, Gourand L, Dausset J, Carosella ED, Paul P: A specific interferon (IFN)-stimulated response element of the distal HLA-G promoter binds IFN-regulatory factor 1 and mediates enhancement of this nonclassical class I gene by IFN-beta. *J Biol Chem* 2001, 276:6133-6139.
17. Moreau P, Mouillot G, Rousseau P, Marcou C, Dausset J, Carosella ED: HLA-G gene repression is reversed by demethylation. *Proc Natl Acad Sci U S A* 2003, 100:1191-1196.
18. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB: Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci U S A* 1996, 93:9821-9826.
19. Mouillot G, Marcou C, Rousseau P, Rouas-Freiss N, Carosella ED, Moreau P: HLA-G gene activation in tumor cells involves cis-acting epigenetic changes. *Int J Cancer* 2005, 113:928-936.
20. Cowled P, Kanter I, Leonardos L, Jackson P: Uroplakin Ib gene transcription in urothelial tumor cells is regulated by CpG methylation. *Neoplasia* 2005, 7:1091-1103.

21. Mulero-Navarro S, Carvajal-Gonzalez JM, Herranz M, Ballestar E, Fraga MF, Ropero S, Esteller M, Fernandez-Salguero PM: The dioxin receptor is silenced by promoter hypermethylation in human acute lymphoblastic leukemia through inhibition of Sp1 binding. *Carcinogenesis* 2006, 27:1099-1104.
22. Zhu WG, Srinivasan K, Dai Z, Duan W, Druhan LJ, Ding H, Yee L, Villalona-Calero MA, Plass C, Otterson GA: Methylation of adjacent CpG sites affects Sp1/Sp3 binding and activity in the p21(Cip1) promoter. *Mol Cell Biol* 2003, 23:4056-4065.

Table 3.1. Clinicopathologic data of patients

Patient	Histology	Age	FIGO Stage	Grade
228	Clear cell adenocarcinoma	55	Ia	N/A
317	Serous papillary adenocarcinoma	59	Ic	3
170	Mixed endometrioid/serous papillary adenocarcinoma	57	IIa	2
413	Serous papillary adenocarcinoma	50	IIb	3
212	Mixed endometrioid/serous papillary adenocarcinoma	59	IIIc	3
369	Serous papillary adenocarcinoma	52	IIIc	3
242	Serous papillary adenocarcinoma	64	IIIc	3
229	Serous papillary adenocarcinoma	58	IIIc	3
183	Serous papillary adenocarcinoma	66	IIIc	2
412	Mucinous cystadenoma	65		
377	Mucinous cystadenoma	42		
383	Mucinous cystadenoma with focal borderline features	75		
382	Serous cystadenofibroma	67		
386	Serous cystadenofibroma	59		
388	Serous cystadenofibroma	53		
371	Serous cystadenofibroma	60		
400	Serous cystadenofibroma	43		
416	Serous cystadenoma	65		
492	Normal surface epithelium	38		
499	Normal surface epithelium	69		
500	Normal surface epithelium	25		
525	Normal surface epithelium	70		

Table 3.2. Real-time RT-PCR analysis of HLA-G expression in ovarian samples (bulk tissue)

ADENOCARCINOMAS			ADENOMAS	
Patient	Expression	FIGO Stage	Patient	Expression
228	1.38	Ia	383	0.00
317	19.56	Ic	400	1.61
413	11.76	IIb	386	3.16
170	2.82	IIa	377	4.70
242	0.47	IIIc	371	8.72
369	13.50	IIIc	416	14.20
183	23.82	IIIc	388	23.14
229	391.00	IIIc	412	38.30
212	3401.75	IIIc	382	176.00
AVERAGE	429.08		AVERAGE	29.95
SD	1121.84		SD	55.88

Table 3.3. Real-time RT-PCR analysis of HLA-G expression in ovarian samples (LCM)

ADENOCARCINOMAS			ADENOMAS		NORMAL	
Patient	Expression	FIGO Stage	Patient	Expression	Patient	Expression
228	ND	Ia	377	ND	499	ND
317	ND	Ic	388	ND	500	3.50
413	ND	IIb	412	ND	492	20.53
242	0.42	IIIc	386	0.67	525	53.71
369	3.05	IIIc	371	0.83		
183	4.03	IIIc	416	3.54		
212	177.01	IIIc	382	13.36		
AVE			AVE	4.60	AVE	25.91
SD			SD	5.98	SD	25.53

ND, expression of HLA-G not detectable

Table 3.4. Methylation averages for 19 CpG sites in the 450 bp promoter region of HLA-G

	-394	-356	-290	-272	-261	-241	-211	-188	-186	-150	-125	-121	-68	-65	-62	-60	-39	-27	-8
212	0	0	1	1	1	1	1	0.00	0.00	0.25	0.00	0.25	0.75	0.00	0.75	0.00	0.00	0.00	0.75
229	0	0	1	1	1	1	1	0.00	0.00	1.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
183	0	0	1	1	1	1	1	0.00	0.00	0.38	0.00	0.38	0.63	0.00	0.63	0.00	0.00	0.00	0.63
317	0	0	1	1	1	1	1	0.60	0.60	0.80	0.00	0.80	0.20	0.20	0.80	0.00	0.60	0.00	0.40
369	0	0	1	1	1	1	1	0.40	0.40	0.60	0.20	0.60	0.40	0.00	1.00	0.20	0.60	0.00	0.20
413	0	0	1	1	1	1	1	0.60	0.60	1.00	0.00	0.80	0.00	0.00	0.80	0.00	0.60	0.00	0.20
170	0	0	1	1	1	1	1	0.20	0.20	1.00	0.00	1.00	0.00	0.00	0.20	0.00	0.20	0.00	0.00
228	0	0	0.8	0.8	0.8	0.8	0.8	0.60	0.60	0.60	0.00	0.60	0.20	0.20	0.80	0.00	0.60	0.00	0.20
242	0	0	0.8	1	1	1	1	0.20	0.20	0.40	0.00	0.40	0.60	0.00	0.80	0.00	0.20	0.00	0.80
AVE	0.000	0.000	0.956	0.978	0.978	0.978	0.978	0.289	0.289	0.670	0.022	0.648	0.308	0.044	0.642	0.022	0.311	0.000	0.353

382	0	0	1	1	1	1	1	0.00	0.00	0.40	0.00	0.40	0.60	0.00	0.40	0.00	0.00	0.00	0.80
412	0	0	1	1	1	1	1	0.50	0.80	0.80	0.00	0.80	0.33	0.00	0.83	0.00	0.50	0.00	0.17
388	0	0	1	1	1	1	1	0.17	0.17	0.17	0.00	0.17	0.67	0.00	1.00	0.00	0.33	0.00	0.83
416	0.2	0	1	1	1	1	1	0.40	0.40	0.60	0.00	0.60	0.40	0.00	0.80	0.00	0.40	0.00	0.60
371	0.1	0	1	1	1	1	1	0.14	0.14	0.43	0.00	0.43	0.57	0.00	0.71	0.00	0.25	0.00	0.71
377	0.2	0.2	0.6	1	1	1	1	0.40	0.60	0.60	0.20	0.60	0.20	0.00	0.60	0.00	0.60	0.00	0.00
386	0.2	0	1	1	1	1	1	0.00	0.00	0.60	0.00	0.60	0.40	0.00	0.40	0.00	0.00	0.00	0.40
400	0	0	1	1	1	1	1	0.20	0.50	0.50	0.00	0.50	0.50	0.00	1.00	0.00	0.50	0.00	0.50
383	0	0	1	1	1	1	1	0.10	0.10	0.90	0.00	0.90	0.10	0.00	0.30	0.00	0.10	0.00	0.13
AVE	0.078	0.022	0.956	1.000	1.000	1.000	1.000	0.212	0.301	0.556	0.022	0.556	0.419	0.000	0.671	0.000	0.298	0.000	0.460

NL492	0.4	0.4	0.4	0.6	0.8	1	0.8	0	0.20	0.20	0.20	0.20	0.40	1.00	0.20	0.20	0.00	0.00	0.20
NL499	0.17	0.33	0.50	0.67	0.83	0.67	0.83	0.50	0.00	0.33	0.50	0.50	0.50	0.50	0.67	0.17	0.00	0.00	0.17
NL500	0.00	1.00	0.00	0.50	0.50	0.50	1.00	0.00	0.00	0.00	0.50	0.50	1.00	1.00	1.00	1.00	0.50	0.00	0.00
NL525	0.33	0.33	0.00	1.00	1.00	1.00	0.67	0.00	0.00	0.00	0.33	0.33	0.33	0.33	0.67	0.00	0.00	0.33	0.33
AVE	0.225	0.517	0.225	0.692	0.783	0.792	0.825	0.125	0.050	0.133	0.383	0.383	0.558	0.708	0.633	0.342	0.125	0.083	0.174

P-VALUES

CR VS NL	0.002	4E-04	9E-06	0.003	0.023	0.0523	0.018	0.321	0.116	0.0061	6E-05	0.0976	0.191	0.0001	0.965	0.0492	0.2853	0.139	0.296
BN VS NL	0.076	7E-04	3E-05	9E-04	0.007	0.0224	0.002	0.488	0.122	0.0054	6E-05	0.1776	0.329	4E-05	0.826	0.0332	0.2406	0.139	0.106

Red samples:adenocarcinomas; green samples:adenomas; black samples: normal epithelium. Yellow highlight, CpG significantly (p<0.05) lower methylation in normal epithelium cells; green highlight, CpG significantly (p<0.05) higher methylation in normal epithelium cells

Table 3.5 Methylation averages for 42 CpG dinucleotides in Exon1 of HLA-G

	+18	+40	+59	+70	+80	+97	+106	+108	+124	+129	+131	+137	+147	+154	+156	+159	+171
212	0.63	0.50	0.50	0.50	0.50	0.75	0.63	0.63	0.50	0.00	0.50	0.50	0.50	0.50	0.50	0.50	0.50
183	0.50	0.50	0.50	0.50	0.50	0.00	0.50	0.50	0.50	0.00	0.50	0.50	0.50	0.50	0.50	0.50	0.50
369	0.80	0.90	0.90	0.80	0.80	0.80	0.80	0.80	0.58	0.00	0.80	0.70	0.68	0.80	0.68	0.68	0.58
242	0.38	0.39	0.45	0.39	0.32	0.25	0.13	0.32	0.32	0.25	0.32	0.32	0.32	0.32	0.32	0.32	0.32
170	1.00	0.60	1.00	1.00	1.00	1.00	1.00	1.00	0.88	0.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
413	1.00	1.00	1.00	1.00	0.93	0.50	1.00	0.88	1.00	0.00	1.00	1.00	1.00	0.88	1.00	1.00	1.00
317	0.88	0.88	0.88	0.88	0.88	0.75	0.88	0.88	0.88	0.00	0.88	0.88	0.88	0.88	0.88	0.88	0.88
228	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	0.50	1.00	1.00	1.00	1.00	1.00	1.00
AVE	0.77	0.72	0.78	0.76	0.74	0.63	0.74	0.75	0.71	0.03	0.69	0.74	0.73	0.73	0.73	0.73	0.72

382	0.58	0.71	0.58	0.58	0.46	0.34	0.58	0.58	0.73	0.00	0.58	0.58	0.58	0.58	0.58	0.58	0.58
412	1.00	1.00	1.00	1.00	1.00	0.75	1.00	1.00	0.75	0.13	0.63	0.75	1.00	1.00	1.00	1.00	0.63
388	1.00	1.00	1.00	1.00	1.00	0.50	1.00	0.63	1.00	0.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
416	0.50	0.67	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.00	0.50	0.50	0.17	0.50	0.50	0.50	0.50
371	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.83	0.17	1.00	1.00	1.00	1.00	1.00	1.00	1.00
377	1.00	1.00	1.00	1.00	1.00	0.38	1.00	0.88	0.75	0.17	0.88	0.88	0.88	1.00	1.00	1.00	1.00
386	1.00	1.00	1.00	1.00	0.88	0.88	1.00	1.00	0.71	0.50	0.83	0.83	0.83	1.00	1.00	1.00	1.00
400	1.00	1.00	1.00	1.00	1.00	1.00	0.67	0.67	0.70	0.00	0.50	1.00	1.00	1.00	1.00	1.00	1.00
383	1.00	1.00	1.00	1.00	1.00	0.00	1.00	1.00	1.00	0.00	0.00	1.00	1.00	1.00	1.00	1.00	1.00
AVE	0.90	0.93	0.90	0.90	0.87	0.59	0.86	0.81	0.78	0.11	0.66	0.84	0.83	0.90	0.90	0.90	0.86

500	1.00	0.83	0.83	0.83	1.00	0.67	0.83	0.83	1.00	0.00	0.83	0.83	0.50	0.50	0.33	0.50	0.50
525	0.75	0.75	0.63	0.75	0.75	0.50	0.75	0.88	0.75	0.00	0.75	0.75	0.75	0.75	0.75	0.63	0.75
492	0.63	0.50	0.50	0.50	0.50	0.25	0.63	0.63	0.63	0.00	0.63	0.50	0.63	0.63	0.63	0.75	0.75
499	0.75	0.71	0.58	0.71	0.58	0.29	0.88	0.88	0.54	0.00	0.71	0.71	0.88	0.58	0.71	0.88	0.88
AVE	0.78	0.70	0.64	0.70	0.71	0.43	0.77	0.80	0.73	0.00	0.73	0.70	0.69	0.61	0.60	0.69	0.72

P-VALUES

CR VS NL	0.946	0.869	0.322	0.673	0.838	0.317	0.857	0.699	0.879	0.506	0.766	0.795	0.762	0.403	0.415	0.762	0.986
BN VS NL	0.332	0.018	0.041	0.105	0.255	0.385	0.446	0.976	0.660	0.235	0.671	0.220	0.380	0.025	0.032	0.096	0.283

	+175	+227	+230	+233	+240	+245	+249	+251	+261	+269	+281	+287	+292	+299	+303	+308	+314
212	0.50	0.50	0.50	0.50	0.50	0.50	0.38	0.00	0.50	0.50	0.50	0.50	0.50	0.38	0.50	0.50	0.50
183	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
369	0.68	0.80	0.80	0.80	0.80	0.80	0.90	0.80	0.70	0.80	0.80	0.80	0.80	0.80	0.80	0.68	0.80
242	0.32	0.46	0.39	0.39	0.39	0.32	0.32	0.32	0.39	0.46	0.32	0.32	0.32	0.20	0.32	0.39	0.32
170	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.63	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
413	1.00	0.88	0.88	0.88	0.88	0.88	0.75	0.88	0.75	0.88	0.88	0.63	1.00	1.00	1.00	1.00	1.00
317	0.38	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.50	1.00	1.00	1.00	1.00
228	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
AVE	0.67	0.77	0.76	0.76	0.76	0.75	0.73	0.69	0.68	0.77	0.75	0.72	0.70	0.73	0.77	0.76	0.77
382	0.58	0.58	0.58	0.58	0.58	0.58	0.58	0.46	0.58	0.71	0.58	0.46	0.58	0.58	0.58	0.58	0.58
412	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
388	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.75	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
416	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.67	0.50
371	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.17	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
377	0.88	1.00	1.00	1.00	1.00	1.00	0.88	1.00	0.88	1.00	1.00	0.88	1.00	1.00	1.00	1.00	1.00
386	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.83	1.00	1.00	1.00	1.00	1.00	1.00	0.88	1.00
400	1.00	1.00	1.00	1.00	0.70	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
383	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
AVE	0.88	0.90	0.90	0.90	0.86	0.90	0.88	0.88	0.75	0.91	0.90	0.87	0.90	0.90	0.90	0.90	0.90
500	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.83	0.50	0.50	0.50	0.50	0.50
525	0.63	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.88	0.63	0.63	0.75	0.75	0.75	0.75
492	0.88	0.75	0.75	0.63	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.63	0.75	0.63	0.75
499	0.88	0.88	0.88	0.88	0.88	0.88	0.88	0.88	0.88	0.88	0.88	0.88	0.88	0.88	0.88	0.88	0.88
AVE	0.72	0.72	0.72	0.69	0.72	0.72	0.72	0.72	0.72	0.72	0.75	0.77	0.69	0.69	0.72	0.69	0.72

P-VALUES

CR VS NL	0.776	0.725	0.784	0.627	0.784	0.840	0.941	0.877	0.788	0.725	0.998	0.720	0.923	0.799	0.769	0.642	0.769
BN VS NL	0.188	0.148	0.148	0.096	0.241	0.148	0.173	0.222	0.865	0.094	0.235	0.427	0.096	0.096	0.148	0.051	0.148

	+319	+322	+328	+340	+342	+346	+349
212	0.50	0.50	0.50	0.50	0.50	0.50	0.50
183	0.50	0.50	0.50	0.50	0.50	0.50	0.50
369	0.80	0.80	0.80	0.80	0.80	0.80	0.80
242	0.39	0.32	0.45	0.45	0.45	0.45	0.45
170	1.00	1.00	1.00	1.00	1.00	1.00	1.00
413	1.00	1.00	1.00	1.00	1.00	1.00	1.00
317	1.00	1.00	1.00	1.00	1.00	1.00	1.00
228	1.00	1.00	1.00	1.00	1.00	1.00	1.00
AVE	0.77	0.77	0.78	0.78	0.78	0.78	0.78

382	0.58	0.58	0.58	0.58	0.58	0.58	0.58
412	1.00	1.00	1.00	1.00	1.00	1.00	1.00
388	1.00	1.00	1.00	1.00	1.00	1.00	1.00
416	0.50	0.50	0.50	0.50	0.50	0.50	0.50
371	1.00	1.00	1.00	1.00	1.00	1.00	1.00
377	1.00	1.00	1.00	1.00	1.00	1.00	1.00
386	1.00	1.00	1.00	1.00	1.00	1.00	1.00
400	1.00	1.33	1.00	1.00	1.00	1.00	1.00
383	1.00	1.00	1.00	1.00	1.00	1.00	1.00
AVE	0.90	0.94	0.90	0.90	0.90	0.90	0.90

500	0.50	0.50	0.33	0.50	0.50	0.50	0.50
525	0.75	0.75	0.75	0.75	0.75	0.75	0.75
492	0.75	0.75	0.75	0.75	0.75	0.75	1.00
499	0.88	0.88	0.88	0.88	0.88	0.88	0.88
AVE	0.72	0.72	0.68	0.72	0.72	0.72	0.78

P-VALUES

CR VS NL	0.714	0.769	0.515	0.671	0.671	0.671	0.998
BN VS NL	0.148	0.142	0.112	0.148	0.148	0.148	0.365

Red samples:adenocarcinomas; green samples:adenomas; black samples: normal epithelium. Yellow highlight, CpG significantly (p<0.05) lower methylation in normal epithelium cells; green highlight, CpG significantly (p<0.05) higher methylation in normal epithelium cells

Figure 3.1. Activation of HLA-G gene transcription in the BG-1 cell line treated with 5-aza-deoxycytidine (5-aza-dC)

Results of semi-quantitative RT-PCR on the ovarian carcinoma cell line BG-1, either untreated (control) or treated with 50 μ M of 5-aza-deoxycytidine (5-aza-dC). + and - indicate samples with and without reverse transcriptase respectively. GAPDH was used as an endogenous control.

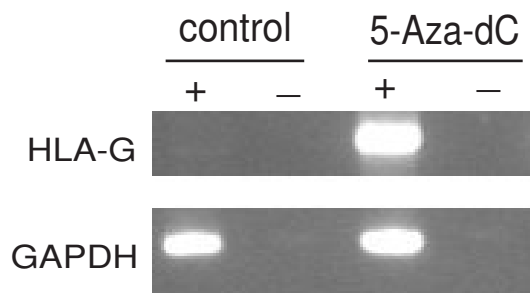
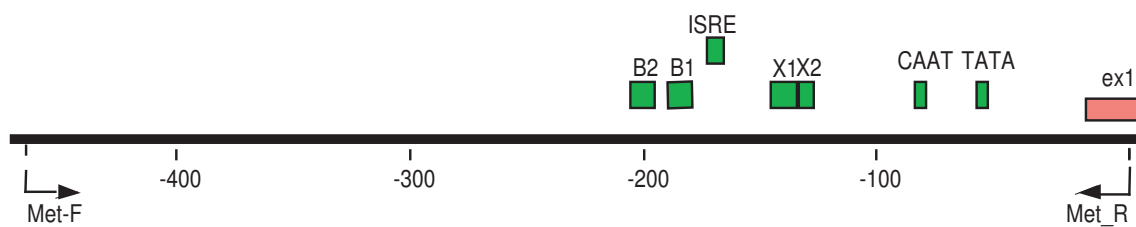


Figure 3.2. Methylation analysis of the HLA-G promoter in control and 5-aza-dC treated BG-1 cell line

(A) Schematic map of 450 bp of the HLA-G promoter region. Green boxes represent enhancers and regulator binding sites: B2, enhancer κ B2 box.; B1, enhancer κ B1 box; X1, conserved X1 regulatory box; X2, X2 box; CAAT, CAAT box; TATA, TATA box.; Met-F and Met-R, primer binding sites (B) Bisulfite genomic sequencing of 19 CpG dinucleotides of the region from –450 to ATG. Individual CpG dinucleotides are depicted as circles. Each line of circles represents an individual sequenced clone, either untreated (PBS) or treated with 50 μ M of 5-aza-dC (AZA) (open circle, 100% unmethylated; filled circle, 100% methylated). %MET, percentage of methylation of each individual clone; %CONV, efficiency of sodium bisulfite treatment.

(A)



(B)

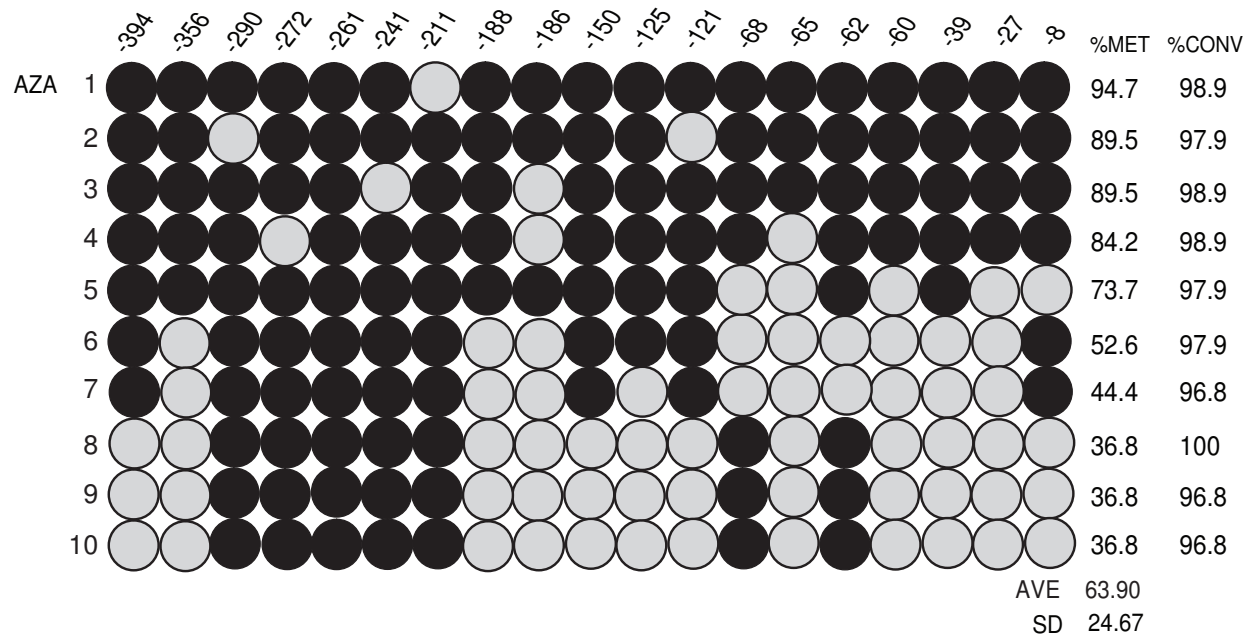
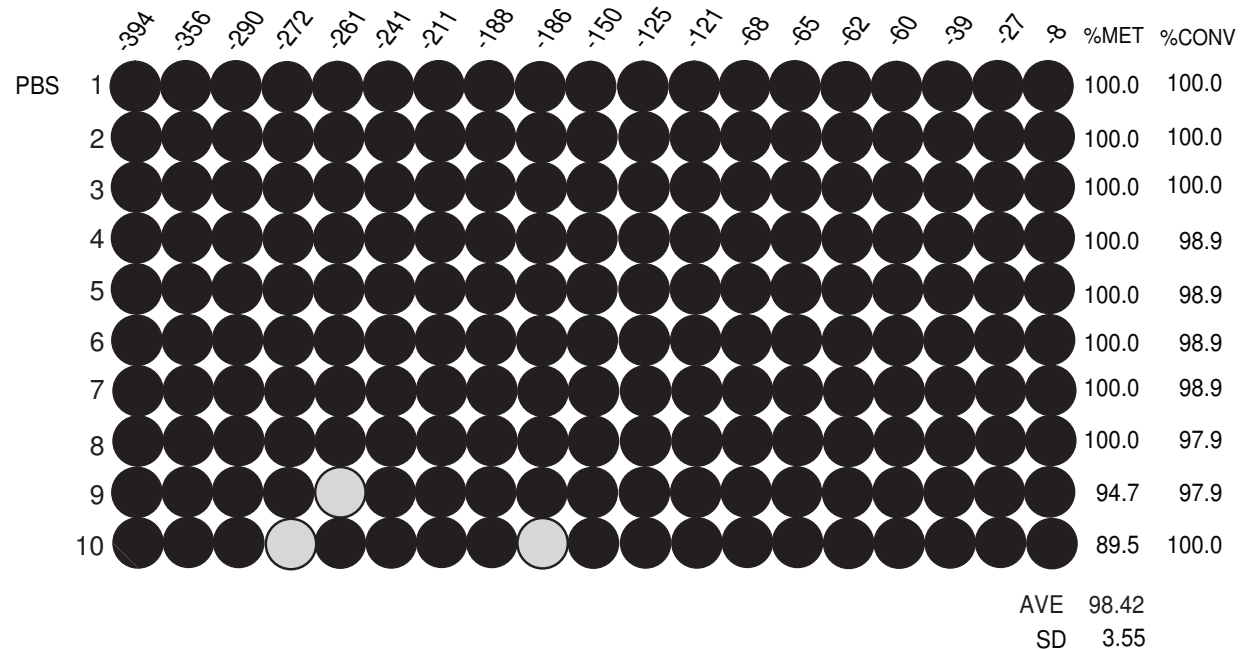


Figure 3.3. Methylation analysis of the HLA-G promoter in ovarian cancer samples

(A) Schematic map of 450 bp of the HLA-G promoter region. Green boxes represent enhancers and regulator binding sites: B2, enhancer-B2 box; B1, enhancer-B1 box; X1, conserved X1 regulatory box; X2, X2 box; CAAT, CAAT box; TATA, TATA box.; Met-F and Met-R, primer binding sites (B) Bisulfite genomic sequencing of 19 CpG dinucleotides of the region from –450 to the transcription start site is analyzed for 18 ovarian adenocarcinomas (red) and benign adenomas (green). Eight to ten clones were sequenced for each sample and the methylation percentage for each site was calculated. Each circle represents the average methylation for a single CpG dinucleotide (open circle, 100% unmethylated; filled circle, 100% methylated).%M, percentage of total methylation per sample.

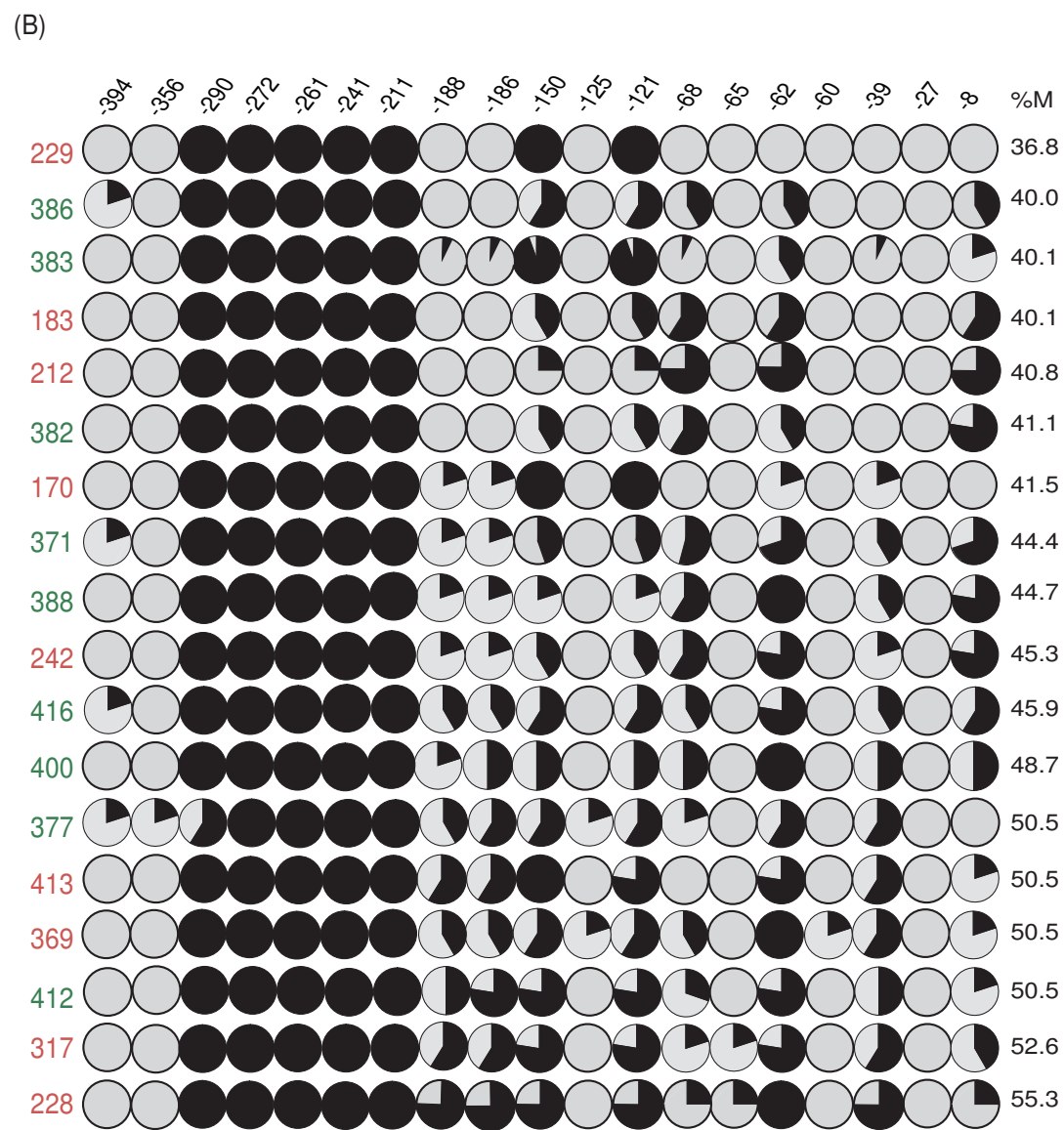
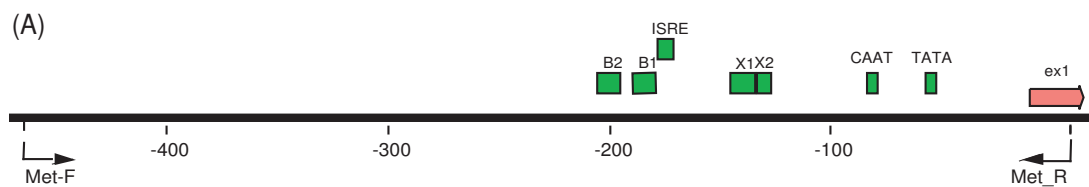


Figure 3.4. Correlation of expression and methylation of HLA-G in the 450 bp promoter region

Methylation percentages of sequenced clones for every sample are plotted versus expression of HLA-G detected through relative real time RT-PCR, and normalized to GAPDH expression. (A) Scatter plot graph of total methylation levels versus expression. No correlation was found between these two variables as shown by the R^2 value of 0.0695. (B) Bar graph representation of total methylation levels versus expression. Samples are ordered in increasing amount of expression. Values for methylation as well as expression are shown below each sample. Red samples, adenocarcinomas. Green samples, adenomas

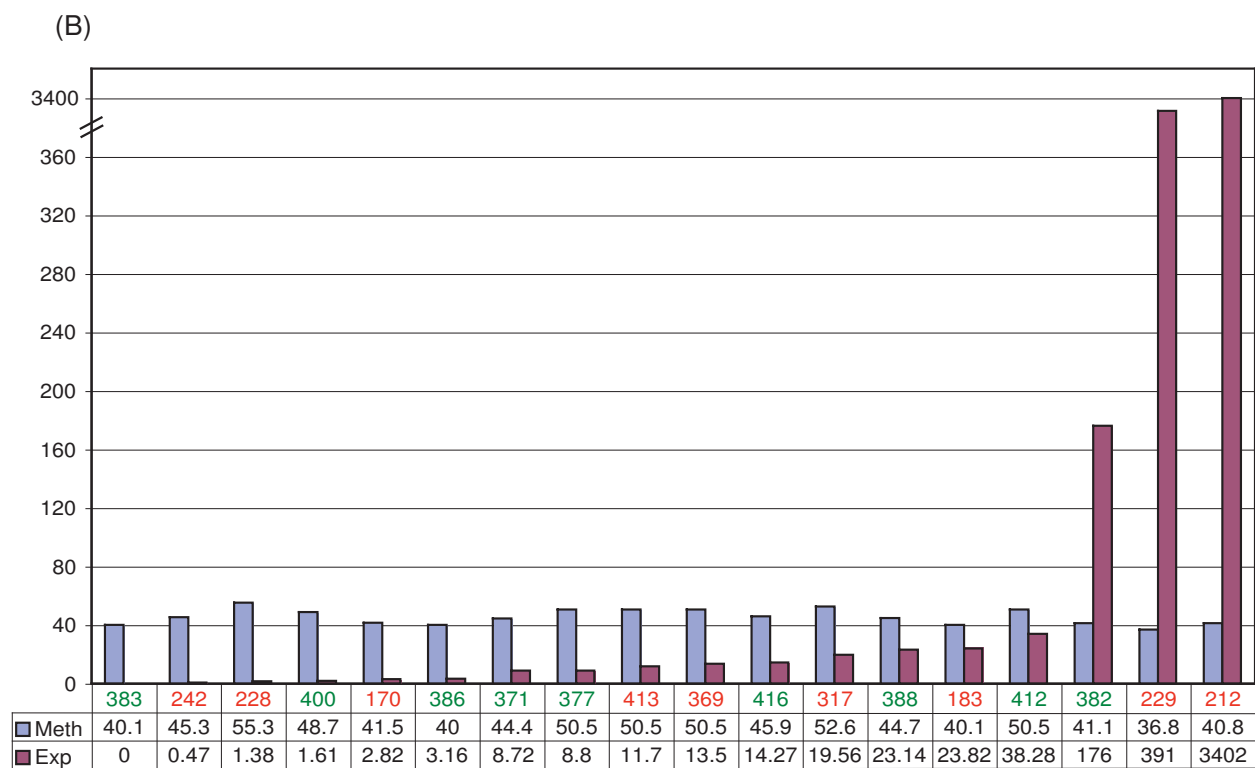
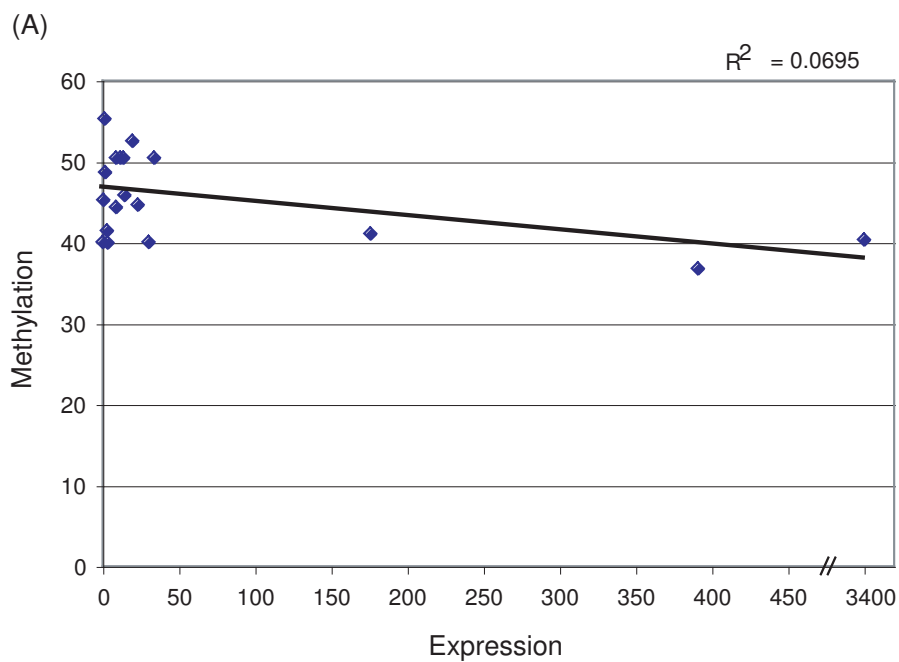


Figure 3.5. Correlation of expression and methylation of Exon1 of HLA-G

Methylation percentages of sequenced clones for every sample are plotted against the expression of HLA-G detected through relative real time RT-PCR (HLA-G expression normalized to GAPDH expression). Samples are ordered in increasing amounts of expression. Values for methylation as well as expression are shown below each sample. Red samples, adenocarcinomas. Green samples, adenomas

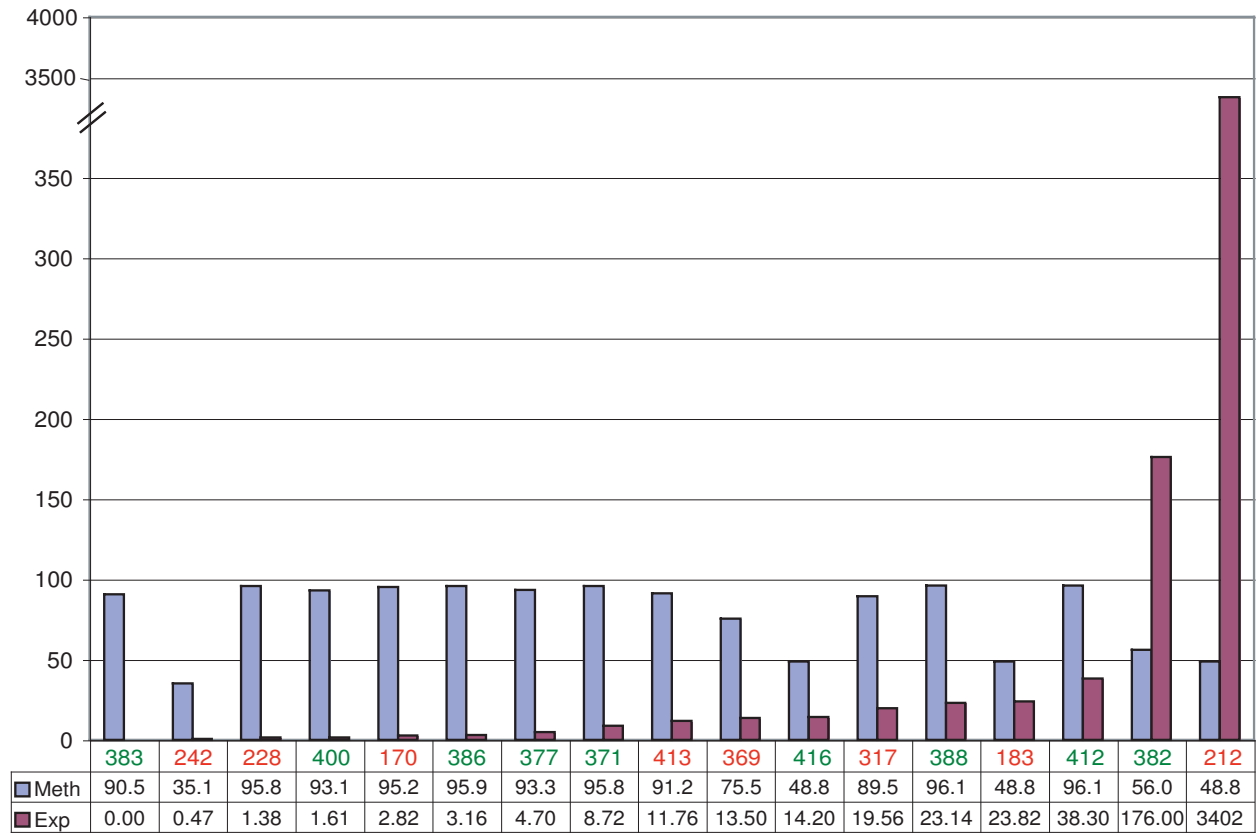
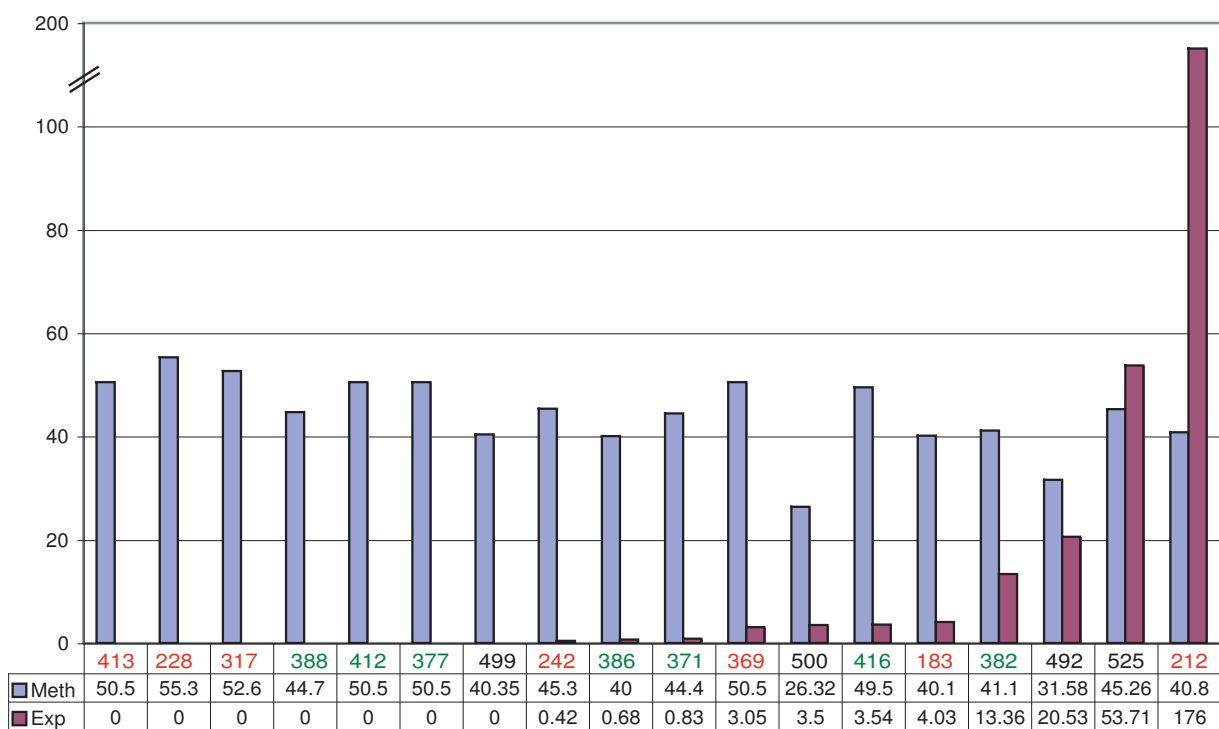


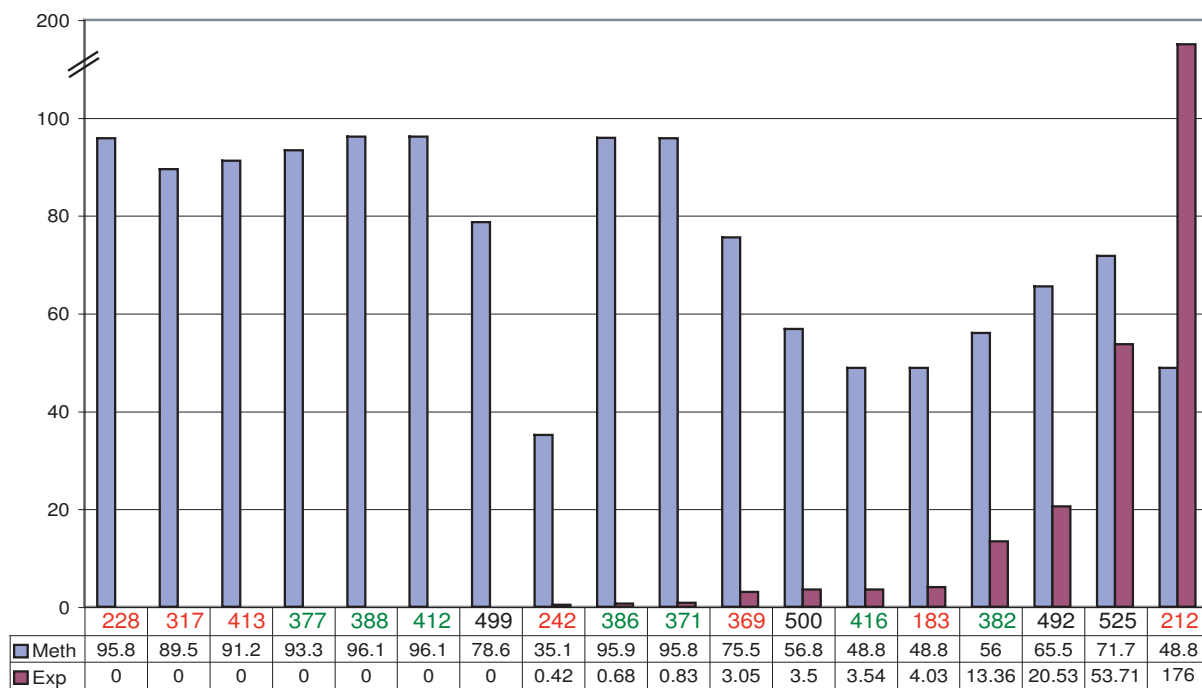
Figure 3.6. Correlation between HLA-G expression (LCM samples) and methylation of the promoter region and Exon1

Methylation percentages of sequenced clones for every sample are plotted against the expression of HLA-G detected through relative real time RT-PCR (HLA-G expression normalized to GAPDH expression). A, promoter region methylation. B, exon1 methylation. Samples are ordered in increasing amounts of expression. Values for methylation as well as expression are shown below each sample.

(A)



(B)



CHAPTER 4

GENOMIC SCREEN FOR DNA METHYLATION-DEPENDENT GENE EXPRESSION INHIBITION IN OVARIAN CANCER

Background

Ovarian cancer was the fourth leading cause of cancer deaths of women in 2005, with an estimated 20,180 new cases and 15,310 deaths in 2006 in the United States [1]. The high mortality rate is primarily due to the fact that most cases are not detected until late in the progression of the disease [2].

Gene expression profiling is now a common first approach toward the characterization of molecular changes occurring over cancer development [3]. While recurrent changes in patterns of gene expression are beginning to emerge for a variety of cancers, the causal basis of the observed differences in expression, by and large, remain to be delineated. One characteristic change associated with many cancers is the down regulation of genes involved in suppressing the tumor phenotype. Down regulation of these “tumor-suppressor genes” may occur, not only by genetic (i.e., nucleotide substitution) changes but also by epigenetic modifications, such as DNA methylation [4, 5]. DNA methylation largely occurs at cytosines associated with CpG dinucleotides. CpG rich regions are also known as CpG Islands (CGIs). A CpG Island is defined as a fragment of at least 200 bp with a GC content of 50% or more, and an observed/expected ratio of CpGs higher than 0.6 [6]. Methylation of CpG islands in the promoter region of genes is known to transcriptionally repress those genes [5]. Numerous reports have shown that many genes are silenced during cancer progression through hypermethylation of the CpG islands.

Some examples of such gene silencing in ovarian cancer include OPCML, RASSF1A, BRAC1 and p16 amongst others [7-9].

Treatment of cancer cell lines with the demethylating agent 5-aza-deoxycytidine (5-aza-dC) has been shown to lead to changes in gene expression due to the loss of methylation in gene regulatory regions [10-13]. In this study, an ovarian cancer cell line (OVCAR3) was treated with 5-aza-dC to identify genes regulated in cancer cells by methylation. We compared changes in gene expression patterns in the 5-aza-dC treated cancer cell line with observed differences in patterns of gene expression between normal and cancerous ovarian tissue in order to identify genes that may have experienced epigenetic changes over ovarian cancer development. Our results indicate that the gene expression response of 5-aza-dC treated ovarian cancer cell lines may be a good predictor of genes subject to epigenetic mediated changes in expression over ovarian cancer development.

Materials and Methods

Cell lines and cell culture

OVCAR3 cells were grown in RPMI 1640 supplemented with 20% v/v heat-inactivated FBS (Invitrogen, Carlsbad, CA), 2 mM L-glutamine (Mediatech Inc., Herndon, VA), 10 mM HEPES buffer (Sigma, St. Louis, MO) penicillin (100 U/ml), streptomycin (100 µg/ml), 1 mM sodium pyruvate (Mediatech Inc., Herndon, VA), and 0.01 mg/ml bovine insulin (Sigma, St. Louis, MO).

5-aza-dC Treatment

OVCAR3 cells were plated at a concentration of 1×10^6 in 10 cm^3 cell culture dishes. Cells were allowed to adhere to the plates overnight, and the medium was replaced the next morning. $5 \text{ }\mu\text{M}$ 5-aza-deoxycytidine (Sigma, St. Louis, MO) or PBS as a control, was added to the medium and the plates were incubated for 3 days at 37°C in a $5\% \text{ CO}_2$ atmosphere. Each treatment was done in triplicate.

Microarray Analysis

OVCAR3 cells were washed once in the plate with HBSS prior to the extraction of RNA and DNA by established techniques (Trizol, Invitrogen, Carlsbad, CA). Integrity of RNA was verified using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Biotin labeled cRNA was synthesized, hybridized to Affymetrix Human U133 Plus 2.0 oligonucleotide arrays and analyzed with a GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA). Gene expression analysis was performed using Spotfire DecisionSite for Microarray Analysis software (Spotfire, Somerville, MA).

RT-PCR analysis

$1 \text{ }\mu\text{g}$ of total RNA was reverse transcribed using oligo (dT) primers using the Superscript III cDNA synthesis kit (Invitrogen, Carlsbad, CA). PCR was performed using $1 \text{ }\mu\text{l}$ of synthesized cDNA. Primer sequences and expected length of the products are provided in Table 1. Gene expression was normalized to the expression of the housekeeping gene GAPDH.

DNA methylation analysis

Genomic DNA, from both 5-aza-dC treated and control cells, was treated with sodium bisulfite to convert unmethylated cytosines to thymines. EZ DNA Methylation-Gold Kit (Zymo, Orange, CA) was used to modify 500 to 1 µg of DNA. PCR conditions and primer sequences are provided in Table 1. PCR fragments were gel extracted (Qiagen, Valencia, CA) and cloned into pCR 2.1-TOPO TA cloning vectors (Invitrogen, Carlsbad, CA). Plasmids from five clones were extracted (Qiaprep Spin Miniprep Kit, Qiagen, Valencia, CA) and sequenced to verify methylation differences.

Results and Discussion

Gene expression changes in OVCAR3 in response to 5-aza-treatment

The ovarian cancer cell line OVCAR3 was treated with 5 µM 5-aza-deoxycytidine to identify genes whose expression is responsive to changes in methylation. After 72 hours of treatment, RNA was extracted, used to synthesize biotinylated cRNA, and hybridized to Affymetrix Human U133 Plus 2.0 oligonucleotide arrays representing approximately 47,000 transcripts. GC Robust Multiarray Analysis (GCRMA) signal values were obtained from the .CEL files and normalized data was obtained in log2 format. Analysis of variance (ANOVA) was applied to identify differentially expressed genes among the three control and the three 5-aza-dC treated samples. A total of 831 genes were differentially expressed ($p < 0.01$) between the control and the 5-aza-dC treated cells of which 465 were upregulated and 366 were downregulated. Tables 2 and 3 display the 30 genes with the highest fold changes in expression (increase or decrease). A higher fold change in expression was observed for genes upregulated after the treatment possibly due to the direct effect of loss of methylation in gene regulatory regions.

Among the genes upregulated after 5-aza-dC treatment were imprinted genes (e.g., *H19*) and other genes previously reported to be transcriptionally repressed due to hypermethylation in other malignant (e.g., *SPANX*, *CST6*) and non-malignant cells/tissue (e.g., *MAGEA1*) [14-16]. It is also interesting that thirteen out of the highest upregulated genes are in chromosome X, suggesting a possible reactivation of the silenced X chromosome or a change in chromatin structure affecting this group of genes. Confirmation of the microarray results was carried out by RT-PCR of 7 randomly selected genes found to have been significantly upregulated in response to the 5-aza-dC treatment by the microarray analysis [*Cystatin 6 (CST6)*, *Caveolin-1 (CAV1)*, *Melanoma antigen family A3 (MAGEA3)*, *Chemokine (C-X-C motif) ligand 6 (CXCL6)*, *Aquaporin3 (AQP3)*, *Stratifin (SFN)* and *Epithelial membrane protein 3 (EMP3)*] (Figure 1).

Methylation pattern of candidate genes

Genes displaying altered expression levels after 5-aza-dC treatment could have been directly or indirectly affected by demethylation. In order to be directly affected by 5-aza-dC, genes would be expected to have a methylated CpG island in or in proximity to promoter regions. Indirect effects might, for example, include the altered expression of loci regulated by genes containing CpG islands. To identify genes associated with CpG islands, we utilized the CpG island identification feature of the USCS Human Genome Browser Gateway. Of the 831 genes differentially expressed after 5-aza-dC treatment, 74% are known to be associated with CpG islands. In contrast, only 67.5% of all the genes present in the Affymetrix oligoarray are associated with CpG islands.

The methylation pattern of the promoter region of five genes associated with CpG islands and found to be up-regulated after 5-aza-dC treatment (*CAV1*, *CST6*, *MAGEA3*, *EMP3* and

CXCL6) was examined. With the exception of *CXCL6*, all have been previously shown to be hypermethylated in other cancers or cancer cell lines [14, 17-19].

Figure 2 presents the results of sodium bisulfite sequencing analysis of CpG rich regulatory regions in each of the five genes. All genes showed extensive methylation of their promoter region in the untreated ovarian cancer cells (OVCAR 3) and a variable decrease of methylation after 5-aza-dC treatment. *CST6*, *CAVI*, *EMP3* and *MAGEA3* displayed a significant decrease of methylation ranging from 13% to 61% while *CXCL6* exhibited no methylation change in the promoter region examined in response to 5-aza-dC treatment. Variability in the response of different CpG regions to 5-aza-dC has been previously observed, therefore we can not exclude the possibility that expression and *CXCL6* is controlled by the methylation levels of a different region than the one studied here [11, 20].

Genes responsive to 5-aza-dC in the OVCAR 3 cell line display decreased expression in ovarian adenocarcinomas

Since OVCAR3 is an ovarian cancer cell line, we hypothesized that if epigenetic mediated changes in gene expression is occurring in ovarian cancer development, at least a subset of the genes that were up regulated in response to 5-aza-dC treatment in the OVCAR3 cells might be expected to display lower levels of expression in ovarian adenocarcinomas relative to controls. Of the 831 genes displaying a significant change in expression in response to 5-aza-dC treatment of OVCAR 3 cells, 123 were found to display a significant difference in expression between normal ovarian epithelial cells and adenocarcinoma cells isolated from ovarian tumors via laser capture microdissection (LCM) (Table 4). Of these 123 genes, 102 were upregulated and 21 were downregulated in response to the 5-aza-dC treatment in the OVCAR 3

cell line. Included in the list of 102 genes are genes previously identified as being hypermethylated and down-regulated in cancer (e.g., the zinc finger protein 185 in prostate cancer [21], *CAVI* in breast cancer [22], and *DIRAS3* in ovarian cancer [23]). We thus predicted that the 102 genes upregulated in 5-aza-dC treated cells were, at least in part, downregulated in ovarian adenocarcinomas due to hypermethylation. To determine if these 102 genes contained CpG islands as our model predicts, we examined sequences in and immediately flanking each of the 102 genes. Ninety-six of the 102 genes (94 %) were found to be directly associated with CpG islands. We found that 3 of the 6 genes not directly associated with CpG islands, mapped in close proximity to genes associated with CpG islands (Table 5). In contrast, it has been estimated that only 40% of all human genes are associated with CpG islands [24].

We also look for the function of the genes with the highest fold change of expression after 5-aza-dC treatment to look for genes associated with cancer development (Table 6). We found genes involved in cell growth like *DIRAS3* [25], *RAB31* [26] and *HSPA1* [27]. We also found several genes involved in the immune response to be de-regulated after 5-aza-dC treatment, including *GEM* [28], *NT5E* [29], and *CXCL6* [30]. In addition genes involved in cell adherence and migration like *TRAF5* [31], *GALTN12* [32], *CRIMI* [33], *ITGAV* [34], *EPB41LAB* [35] and *CST6* [36] were also responsive to the treatment. *TACCI*, a gene required for normal maintenance of epithelium surfaces, with proposed functions in cell division and transcriptional control [37] also had altered expression after the 5-aza-dC treatment. All of these genes are therefore good candidates for the study of ovarian cancer progression.

We conclude that the gene expression response of 5-aza-dC treated ovarian cancer cell lines may be a good predictor of genes subject to epigenetic mediated changes in expression over ovarian cancer development.

References

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C, Thun MJ: Cancer statistics, 2006. *CA Cancer J Clin* 2006, 56:106-130.
2. Holschneider CH, Berek JS: Ovarian cancer: epidemiology, biology, and prognostic factors. *Semin Surg Oncol* 2000, 19:3-10.
3. Crijns AP, Duiker EW, de Jong S, Willemse PH, van der Zee AG, de Vries EG: Molecular prognostic markers in ovarian cancer: toward patient-tailored therapy. *Int J Gynecol Cancer* 2006, 16 Suppl 1:152-165.
4. Esteller M: Epigenetics provides a new generation of oncogenes and tumour-suppressor genes. *Br J Cancer* 2006, 94:179-183.
5. Herman JG, Baylin SB: Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med* 2003, 349:2042-2054.
6. Gardiner-Garden M, Frommer M: CpG islands in vertebrate genomes. *J Mol Biol* 1987, 196:261-282.
7. Ibanez de Caceres I, Battagli C, Esteller M, Herman JG, Dulaimi E, Edelson MI, Bergman C, Ehya H, Eisenberg BL, Cairns P: Tumor cell-specific BRCA1 and RASSF1A hypermethylation in serum, plasma, and peritoneal fluid from ovarian cancer patients. *Cancer Res* 2004, 64:6476-6481.
8. Makarla PB, Saboorian MH, Ashfaq R, Toyooka KO, Toyooka S, Minna JD, Gazdar AF, Schorge JO: Promoter hypermethylation profile of ovarian epithelial neoplasms. *Clin Cancer Res* 2005, 11:5365-5369.
9. Teodoridis JM, Hall J, Marsh S, Kannall HD, Smyth C, Curto J, Siddiqui N, Gabra H, McLeod HL, Strathdee G, Brown R: CpG island methylation of DNA damage response genes in advanced ovarian cancer. *Cancer Res* 2005, 65:8961-8967.
10. Lodygin D, Epanchintsev A, Menssen A, Diebold J, Hermeking H: Functional epigenomics identifies genes frequently silenced in prostate cancer. *Cancer Res* 2005, 65:4218-4227.

11. Mund C, Hackanson B, Stresemann C, Lubbert M, Lyko F: Characterization of DNA demethylation effects induced by 5-Aza-2'-deoxycytidine in patients with myelodysplastic syndrome. *Cancer Res* 2005, 65:7086-7090.
12. Suzuki H, Gabrielson E, Chen W, Anbazhagan R, van Engeland M, Weijenberg MP, Herman JG, Baylin SB: A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer. *Nat Genet* 2002, 31:141-149.
13. Takai N, Kawamata N, Walsh CS, Gery S, Desmond JC, Whittaker S, Said JW, Popoviciu LM, Jones PA, Miyakawa I, Koeffler HP: Discovery of epigenetically masked tumor suppressor genes in endometrial cancer. *Mol Cancer Res* 2005, 3:261-269.
14. Riverbark AG, Wendell, D.J., Risher, J.D., Coleman, W.B.: DNA Methylation-Dependent Epigenetic Regulation of Gene Expression in MCF-7 Breast Cancer Cells. *Epigenetics* 2006, 1:e1-12.
15. Wang Z, Zhang J, Zhang Y, Lim SH: SPAN-Xb expression in myeloma cells is dependent on promoter hypomethylation and can be upregulated pharmacologically. *Int J Cancer* 2006, 118:1436-1444.
16. De Smet C, Lurquin C, Lethe B, Martelange V, Boon T: DNA methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG-rich promoter. *Mol Cell Biol* 1999, 19:7327-7335.
17. Sunaga N, Miyajima K, Suzuki M, Sato M, White MA, Ramirez RD, Shay JW, Gazdar AF, Minna JD: Different roles for caveolin-1 in the development of non-small cell lung cancer versus small cell lung cancer. *Cancer Res* 2004, 64:4277-4285.
18. Sigalotti L, Coral S, Nardi G, Spessotto A, Cortini E, Cattarossi I, Colizzi F, Altomonte M, Maio M: Promoter methylation controls the expression of MAGE2, 3 and 4 genes in human cutaneous melanoma. *J Immunother* 2002, 25:16-26.
19. Alaminos M, Davalos V, Ropero S, Setien F, Paz MF, Herranz M, Fraga MF, Mora J, Cheung NK, Gerald WL, Esteller M: EMP3, a myelin-related gene located in the critical 19q13.3 region, is epigenetically silenced and exhibits features of a candidate tumor suppressor in glioma and neuroblastoma. *Cancer Res* 2005, 65:2565-2571.
20. Cameron EE, Bachman KE, Myohanen S, Herman JG, Baylin SB: Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nat Genet* 1999, 21:103-107.
21. Vanaja DK, Cheville JC, Iturria SJ, Young CY: Transcriptional silencing of zinc finger protein 185 identified by expression profiling is associated with prostate cancer progression. *Cancer Res* 2003, 63:3877-3882.

22. Chen ST, Lin SY, Yeh KT, Kuo SJ, Chan WL, Chu YP, Chang JG: Mutational, epigenetic and expressional analyses of caveolin-1 gene in breast cancers. *Int J Mol Med* 2004, 14:577-582.
23. Yu Y, Xu F, Peng H, Fang X, Zhao S, Li Y, Cuevas B, Kuo WL, Gray JW, Siciliano M, et al: NOEY2 (ARHI), an imprinted putative tumor suppressor gene in ovarian and breast carcinomas. *Proc Natl Acad Sci U S A* 1999, 96:214-219.
24. Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, Smith HO, Yandell M, Evans CA, Holt RA, et al: The sequence of the human genome. *Science* 2001, 291:1304-1351.
25. Lu Z, Luo RZ, Peng H, Rosen DG, Atkinson EN, Warneke C, Huang M, Nishimoto A, Liu J, Liao WS, et al: Transcriptional and posttranscriptional down-regulation of the imprinted tumor suppressor gene ARHI (DRAS3) in ovarian cancer. *Clin Cancer Res* 2006, 12:2404-2413.
26. Bao X, Faris AE, Jang EK, Haslam RJ: Molecular cloning, bacterial expression and properties of Rab31 and Rab32. *Eur J Biochem* 2002, 269:259-271.
27. Rohde M, Daugaard M, Jensen MH, Helin K, Nylandsted J, Jaattela M: Members of the heat-shock protein 70 family promote cancer cell growth by distinct mechanisms. *Genes Dev* 2005, 19:570-582.
28. Vanhove B, Hofer-Warbinek R, Kapetanopoulos A, Hofer E, Bach FH, de Martin R: Gem, a GTP-binding protein from mitogen-stimulated T cells, is induced in endothelial cells upon activation by inflammatory cytokines. *Endothelium* 1997, 5:51-61.
29. Gordon JL: Extracellular ATP: effects, sources and fate. *Biochem J* 1986, 233:309-319.
30. Gijssbers K, Gouwy M, Struyf S, Wuyts A, Proost P, Opdenakker G, Penninckx F, Ectors N, Geboes K, Van Damme J: GCP-2/CXCL6 synergizes with other endothelial cell-derived chemokines in neutrophil mobilization and is associated with angiogenesis in gastrointestinal tumors. *Exp Cell Res* 2005, 303:331-342.
31. Ye X, Mehlen P, Rabizadeh S, VanArsdale T, Zhang H, Shin H, Wang JJ, Leo E, Zapata J, Hauser CA, et al: TRAF family proteins interact with the common neurotrophin receptor and modulate apoptosis induction. *J Biol Chem* 1999, 274:30202-30208.
32. Guo JM, Chen HL, Wang GM, Zhang YK, Narimatsu H: Expression of UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase-12 in gastric and colonic cancer cell lines and in human colorectal cancer. *Oncology* 2004, 67:271-276.
33. Glienke J, Sturz A, Menrad A, Thierauch KH: CRIM1 is involved in endothelial cell capillary formation in vitro and is expressed in blood vessels in vivo. *Mech Dev* 2002, 119:165-175.

34. Sims MA, Field SD, Barnes MR, Shaikh N, Ellington K, Murphy KE, Spurr N, Campbell DA: Cloning and characterisation of ITGAV, the genomic sequence for human cell adhesion protein (vitronectin) receptor alpha subunit, CD51. *Cytogenet Cell Genet* 2000, 89:268-271.
35. Shimizu K, Nagamachi Y, Tani M, Kimura K, Shiroishi T, Wakana S, Yokota J: Molecular cloning of a novel NF2/ERM/4.1 superfamily gene, ehm2, that is expressed in high-metastatic K1735 murine melanoma cells. *Genomics* 2000, 65:113-120.
36. Vigneswaran N, Wu J, Nagaraj N, James R, Zeeuwen P, Zacharias W: Silencing of cystatin M in metastatic oral cancer cell line MDA-686Ln by siRNA increases cysteine proteinases and legumain activities, cell proliferation and in vitro invasion. *Life Sci* 2006, 78:898-907.
37. Lauffart B, Vaughan MM, Eddy R, Chervinsky D, DiCioccio RA, Black JD, Still IH: Aberrations of TACC1 and TACC3 are associated with ovarian cancer. *BMC Womens Health* 2005, 5:8.
38. Rowland BD, Bernards R, Peeper DS: The KLF4 tumour suppressor is a transcriptional repressor of p53 that acts as a context-dependent oncogene. *Nat Cell Biol* 2005, 7:1074-1082.
39. Ferguson AT, Evron E, Umbricht CB, Pandita TK, Chan TA, Hermeking H, Marks JR, Lambers AR, Futreal PA, Stampfer MR, Sukumar S: High frequency of hypermethylation at the 14-3-3 sigma locus leads to gene silencing in breast cancer. *Proc Natl Acad Sci U S A* 2000, 97:6049-6054.

Table 4.1. Primers used for RT-PCR and Sodium Bisulfite sequencing

Gene	Primer	Sequence	Size	Tm	Ref.
CAV1	RT-PCR_F	5'-CGACCCTAAACACCTCAACGATG-3'	278	55°C	[14]
	RT-PCR-R	5'-GCAGACAGCAAGCGGTAAAACC-3'			
	MOD_F	5'-TGTGTATTTTGTAAATATGGTATAATTTG-3'	537	58°C	
	MOD_R	5'-CATTTTCCCTACTCTAAACCAC-3'			
SFN	RT-PCR_F	5'-GTGTGTCCCCAGAGCCATGG-3'	161	60°C	[39]
	RT-PCR-R	5'-ACCTTCTCCCGGTACTCACG-3'			
CST6	RT-PCR_F	5'-AAGACCAGGGTCACTGGAGA-3'	163	60°C	[14]
	RT-PCR-R	5'-CGGGGACTTATCACATCTGC-3'			
	MOD_F	5'-GGTTGGAATGTTGTAGTGGT-3'	430	55°C	
	MOD_R	5'-CCCAACAACAAATACCAA-3'			
MAGEA3	RT-PCR_F	5'-TGGAGGACCAGAGGCCCCC-3'	725	68°C	[18]
	RT-PCR-R	5'-GGACGATTATCAGGAGGCCTGC-3'			
	MOD_F	5'-AGATTTGGTTTGAGGGGAGTAGAAGT-3'	405	60°C	
	MOD_R	5'-AACCC(G/A)ACAACAAAAACAACACTAAA-3'			
EMP3	RT-PCR-F	5'-TGCTCTCCCTCATCTCTGCTGTC-3'	274	60°C	[19]
	RT-PCR-R	5'-CGCTTCCGTAGGTGGATGTAGATG-3'			
	MOD-F	5'-TAGTATATATTGAGAGGAGGAGAG-3'	340	62°C	
	MOD-R	5'-CTTCCCAAACACTACTACATTCCCA-3'			
CXCL6	RT-PCR-F	5'-CCTGAAGAACGGGAAGC-3'	135	60°C	
	RT-PCR-R	5'-GACTGGGCAATTTTATGATG-3'			
	MOD-F	5'-GAGGGATGAATGTAGATAAAGGGAGT-3'	616	60°C	
	MOD-R	5'-AACTTCCAAATCCAAACAACTTACTT-3'			
GAPDH	RT-PCR-F	5'-GAAATCCCATCACCATCTTCCAG-3'	312	56°C	
	RT-PCR-R	5'-ATGAGTCCTTCCACGATACAAAAG-3'			

Size, product size in bp; Tm, annealing temperature

Table 4.2. Upregulated genes after 5-aza-dC treatment of OVCAR cells

Accession No.	Symbol	Gene Name	Chr.	Signal log ratio
NM_001351	DAZL	Deleted in azoospermia-like	3p24	4.78
NM_052957	ACRC	Acidic repeat containing	Xq13	4.76
NM_144594	FLJ32942	Hypothetical protein FLJ32942	12q13	4.51
NM_032858	MAEL	Maelstrom homolog	1q24	4.23
NM_002364	MAGEB2	Melanoma antigen family B, 2	Xp21	4.23
NM_005363	MAGEA6	Melanoma antigen family A, 6	Xq28	4.03
NM_012253	TKTL1	Transketolase-like 1	Xq28	3.91
NM_023930	KCTD14	Potassium channel tetramerisation domain	11q14	3.52
NM_013453	SPANX	Sperm protein associated with the nucleus, X-linked	Xq27	3.46
NM_005362	MAGEA3	Melanoma antigen family A, 3	Xq28	3.40
NM_005335	HCLS1	Hematopoietic cell-specific Lyn substrate 1	3q13	3.38
NM_006228	PNOC	Prepronociceptin	8p21	3.31
NM_152578	FMR1NB	Fragile X mental retardation 1 neighbor	Xq27	3.21
NM_005367	MAGEA12	Melanoma antigen family A, 12	Xq28	3.14
NM_003289	TPM2	Tropomyosin 2 (beta)	9p13	2.81
NM_080618	CTCFL	CCCTC-binding factor (zinc finger protein)-like	20q13	2.68
NM_001011544	MAGEA11	Melanoma antigen family A, 11	Xq28	2.66
NM_005213	CSTA	Cystatin A	3q21	2.54
NR_002196	H19	H19, imprinted maternally expressed untranslated mRNA	11p15	2.51
NM_032048	EMILIN2	Elastin microfibril interfacer 2	18p11	2.44
NM_173571	LOC255313	Hypothetical protein LOC255313	Xq24	2.40
NM_003480	MFAP5	Microfibrillar associated protein 5	12p13	2.26
NM_001323	CST6	Cystatin E/M	11q13	2.26
NM_001327	CTAG1B&A	Cancer-testis antigen 1B / cancer-testis antigen 1A	Xq28	2.20
NM_001011548	MAGEA4	Melanoma antigen family A, 4	Xq28	2.16
NM_020826	SYT13	Synaptotagmin XIII	11p11	2.13
NM_013238	DNAJC15	DnaJ (Hsp40) homolog, subfamily C, member 15	13q14	2.12
NM_005602	CLDN11	Claudin 11	3q26	2.08
NM_001002915	IGFL2	Insulin growth factor-like family member 2	19q13	2.03
NM_004988	MAGEA1	Melanoma antigen family A, 1 (directs expression of antigen MZ2-E)	Xq28	1.90

Table 4.3. Downregulated genes after 5-aza-dC treatment of OVCAR cells

Accession No.	Symbol	Gene Name	Chr.	Signal log ratio
NM_0010138360	MAD1L1	MAD1 mitotic arrest deficient-like 1 (yeast)	7p22	-2.09
NM_004883	NRG2	Neuregulin 2	5q31	-1.98
NM_001001331	ATP2B2	ATPase, Ca++ transporting, plasma membrane 2	3p25	-1.53
NM_001719	BMP7	Bone morphogenetic protein 7 (osteogenic protein 1)	20q13	-1.36
NM_032932	RAB11FIP4	RAB11 family interacting protein 4 (class II)	17q11	-1.3
NM_014914	CENTG2	Centaurin, gamma 2	2q37	-1.2
NM_013372	GREM1	Gremlin 1, cysteine knot superfamily, homolog	15q13	-1.19
XM_373734	---	Hypothetical LOC388388	3p13	-1.19
NM_020761	Raptor	Raptor	17q25.3	-1.18
NM_001031693	HHLA3	HERV-H LTR-associating 3	---	-1.14
NM_003598	TEAD2	TEA domain family member 2	19q13	-1.12
NM_022773	FLJ12681	Hypothetical protein FLJ12681	16p13.3	-1.12
NM_004389	CTNNA2	Catenin (cadherin-associated protein), alpha 2	2p12	-1.07
XM_032996	MICAL3	Microtubule associated monooxygenase, calponin and LIM domain containing 3	22q11	-1.06
NM_000620	NOS1	Nitric oxide synthase 1 (neuronal)	12q24	-1.04
NM_006613	GRAP	GRB2-related adaptor protein-like	17p11	-0.98
NM_022138	SMOC2	SPARC related modular calcium binding 2	6q27	-0.97
NM_017594	DIRAS2	DIRAS family, GTP-binding RAS-like 2	9q22	-0.97
NM_021018	HIST1H3F	Histone 1, H3f	6p22	-0.94
NM_004192	ASMTL	Acetylserotonin O-methyltransferase-like	Xp22	-0.94
NM_002412	MGMT	O-6-methylguanine-DNA methyltransferase	10q26	-0.93
NM_001466	FZD2	Frizzled homolog 2 (Drosophila)	17q21	-0.93
XM_498471	LOC147727	Hypothetical protein LOC147727	19p13	-0.92
NM_003562	SLC25A11	Solute carrier family 25 (mitochondrial carrier; oxoglutarate carrier), member 11	17p13	-0.91
NM_004565	PEX14	Peroxisomal biogenesis factor 14	1p36	-0.90
NM_003955	SOCS3	Suppressor of cytokine signaling 3	17q25	-0.90
NM_007037	ADAMTS8	ADAM metalloproteinase with thrombospondin type 1 motif, 8	11q24	-0.89
NM_032892	FRMD5	FERM domain containing 5	15q15	-0.88
NM_017774	CDKAL1	CDK5 regulatory subunit associated protein 1-like 1	6p22	-0.88
NM_022479	WBSCR17	Williams-Beuren syndrome chromosome region 17	7q11	-0.88

Table 4.4 .Common 123 genes between genes with significant (p<0.01) changes of expression in OVCAR3 after 5-aza-dC and genes downregulated in ovarian cancer samples

Accession No.	Symbol	Gene Name	Signal log ratio	Chr	CpG ^(a)
NM_022138	SMOC2	SPARC related modular calcium binding 2	-0.97	6q27	S
NM_005769	CHST4	Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 4	-0.67	16q22	N
NM_032314	MGC4767	Hypothetical protein MGC4767	-0.61	12q24	S
NM_014767	SPOCK2	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2	-0.59	10q22	IN(L)
NM_005407	SALL2	Sal-like 2 (Drosophila)	-0.57	14q11	N
NM_025155	WDR71	WD repeat domain 71	-0.53	11q13	S
NM_018434	RNF130	Ring finger protein 130	-0.50	5q35	L
NM_004853	STX8	Syntaxin 8	-0.49	17p13	S
NM_001015881	TSC22D3	TSC22 domain family, member 3	-0.39	Xq22	L
NM_031866	FZD8	Frizzled homolog 8 (Drosophila)	-0.37	10p11	L
NM_001014842	TM9SF1	Transmembrane 9 superfamily member 1	-0.37	14q11	L
NM_005194	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	-0.33	20q13	L
NM_001390	DTNA	Dystrobrevin, alpha	-0.32	18q12	L
NM_002998	SDC2	Syndecan	-0.30	8q22	L
NM_001457	FLNB	Filamin B, beta (actin binding protein 278)	-0.30	3p14	L
NM_019843	EIF4ENIF1	Eukaryotic translation initiation factor 4E nuclear import factor 1	-0.25	22q12	L
XM_166164	LOC219854	Hypothetical protein LOC219854	-0.23	11q24	ND
NM_000378	WT1	Wilms tumor 1	-0.22	11p13	L
NM_024026	MRP63	Mitochondrial ribosomal protein 63	-0.19	13q12	L
NM_022766	CERK	Ceramide kinase	-0.17	22q13	N
NM_001914	CYB5	Cytochrome b-5	-0.14	18q22	L
NM_002136	HNRPA1	Heterogeneous nuclear ribonucleoprotein A1	0.06	12q13	L
NM_001025	RPS23	Ribosomal protein S23	0.08	5q14	S
NM_007167	ZMYM6	Zinc finger, MYM-type 6	0.13	1p34	S
NM_002793	PSMB1	Proteasome (prosome, macropain) subunit, beta type, 1	0.14	6q27	S
NM_006367	CAP1	CAP, adenylate cyclase-associated protein 1	0.14	1p34	L
NM_000454	SOD1	Superoxide dismutase 1, soluble	0.14	21q22	L
NM_006430	CCT4	Chaperonin containing TCP1, subunit 4 (delta)	0.20	2 p15	L
NM_024077	SECISBP2	SECIS binding protein 2	0.21	9q22	L
NM_002802	PSMC1	Proteasome26S subunit, ATPase, 1	0.21	14q32	S
NM_016324	ZNF274	Zinc finger protein 274	0.21	19q13	L
NM_017750	RetSat	All-trans-13,14-dihydroretinol saturase	0.22	2p11	S
NM_002881	RALB	V-ral simian leukemia viral oncogene homolog B (ras related; GTP binding protein)	0.22	2q14	L
NM_006708	GLO1	Glyoxalase I	0.23	6p21	S
NM_001008897	TCP1	T-complex 1	0.23	6q25	L
NM_019029	CPVL	Carboxypeptidase, vitellogenic-like	0.23	7p15	N

Accession No.	Symbol	Gene Name	Signal log ratio	Chr	CpG ^(a)
NM_000712	BLVRA	Biliverdin reductase A	0.23	7p13	L
NM_001018111	PODXL	Podocalyxin-like	0.24	7q32	L
NM_016284	CNOT1	CCR4-NOT transcription complex, subunit 1	0.24	16q21	L
NM_000274	OAT	Ornithine aminotransferase (gyrate atrophy)	0.25	10q26	L
NM_001449	FHL1	Four and a half LIM domains 1	0.26	Xq26	L
NM_016355	DDX47	DEAD (Asp-Glu-Ala-Asp) box polypeptide 47	0.26	12p13	S
NM_138446	C7orf30	Chromosome 7 open reading frame 30	0.27	7p15	S
NM_014267	SMAP	Small acidic protein	0.28	11p15	S
NM_005437	NCOA4	Nuclear receptor coactivator 4	0.28	10q11	S
NM_000318	PXMP3	Peroxisomal membrane protein 3, 35kDa	0.28	8q21	L
NM_003640	IKBKAP	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein	0.29	9q31	L
NM_173515	CNKSR3	CNKSR family member 3	0.29	6q25	L
NM_033546	MRLC2	Myosin regulatory light chain MRLC2	0.30	18 p11	L
NM_004048	B2M	Beta-2-microglobulin	0.32	15q21	L
NM_016048	ISOC1	Isochorismatase domain containing 1	0.37	5q23	L
NM_017866	TMEM70	Transmembrane protein 70	0.38	8q21	L
NM_003340	UBE2D3	Threonine synthase, chloroplast	0.39	4q24	L
NM_016548	GOLPH2	Golgi phosphoprotein 2	0.39	9q21	L
NM_018428	C17orf40	Chromosome 17 open reading frame 40	0.40	17q11	S
NM_020781	ZNF398	Zinc finger protein 398	0.40	7q36	L
NM_015391	ANAPC13	Anaphase promoting complex subunit 13	0.42	3q22	L
NM_002306	LGALS3	Lectin, galactoside-binding, soluble, 3 (galectin 3)	0.43	14q22	L
NM_152435	MGC35366	Hypothetical protein MGC35366	0.44	12q23	S
NM_019592	RNF20	Ring finger protein 20	0.45	9q31	S
NM_030759	NRBF2	Nuclear receptor binding factor 2	0.45	10q21	S
NM_012406	PRDM4	PR domain containing 4	0.45	12q23	L
NM_016545	IER5	Immediate early response 5	0.45	1q25	L
NM_015251	ASCIZ	ATM/ATR-Substrate Chk2-Interacting Zn2+- finger protein	0.46	16q23	L
NM_003690	PRKRA	Protein kinase, interferon-inducible double stranded RNA dependent activator	0.47	2q31	L
NM_004105	EFEMP1	EGF-containing fibulin-like extracellular matrix protein1	0.48	2p16	L
NM_007085	FSTL1	Follistatin-like 1	0.48	3q13	L
NM_005749	TOB1	Transducer of ERBB2, 1	0.49	17q21	N
NM_013279	C11orf9	Chromosome 11 open reading frame 9	0.50	11q12	IN(L)
NM_006493	CLN5	Ceroid-lipofuscinosis, neuronal 5	0.51	13q22	L
NM_015339	ADNP	Activity-dependent neuroprotector	0.51	20q13	S
NM_007150	ZNF185	Zinc finger protein 185 (LIM domain)	0.52	Xq28	S
NM_022482	ZNF336	Zinc finger protein 336	0.53	20p11	L
NM_014817	KIAA0644	KIAA0644 gene product	0.53	7p15	L
NM_007044	KATNA1	Katanin p60 (ATPase-containing) subunit A 1	0.54	6q25	S

Accession No.	Symbol	Gene Name	Signal log ratio	Chr	CpG ^(a)
NM_014504	RABGEF1	RAB guanine nucleotide exchange factor (GEF) 1	0.54	7q11	L
NM_004235	KLF4	Kruppel-like factor 4 (gut)	0.55	9q31	L
XM_291277	DKFZp761P0423	Hypothetical protein DKFZp761P0423	0.56	8p23	3'(L)
NM_001012978	NGFRAP1L1	NGFRAP1-like 1	0.57	Xq22	N
NM_022157	RRAGC	Ras-related GTP binding C	0.57	1p34	L
NM_133636	HEL308	DNA helicase HEL308	0.57	4q21	L
NM_017526	LEPROT	Leptin receptor overlapping transcript	0.58	1p31	L
NM_007223	GPR	Putative G protein coupled receptor	0.61	15q14	L
NM_007216	HPS5	Hermansky-Pudlak syndrome 5	0.61	11p15	L
NM_031412	GABARAPL1	GABA(A) receptor-associated protein like 1	0.61	12p13	S
NM_014570	ARFGAP3	ADP-ribosylation factor GTPase activating protein 3	0.61	22q13	L
NM_004789	LHX2	LIM homeobox 2	0.62	9q33	L
NM_024616	TTMP	TPA-induced transmembrane protein	0.62	3q13	L
XM_091914	LOC162993	Hypothetical protein LOC162993	0.63	19p13	L
NM_020828	ZFP28	Zinc finger protein 28 homolog (mouse)	0.65	19q13	L
NM_006868	RAB31	RAB31, member RAS oncogene family	0.65	18p11	L
NM_000147	FUCA1	Fucosidase, alpha-L- 1, tissue	0.66	1p36	L
NM_080821	C20orf108	Chromosome 20 open reading frame 108	0.68	20q13	L
NM_006868	RAB31	RAB31, member RAS oncogene family	0.68	18p11	L
NM_003115	UAP1	UDP-N-acetylglucosamine pyrophosphorylase 1	0.69	1q23	J
NM_000345	SNCA	Synuclein, alpha	0.69	4q22	L
NM_018424	EPB41L4B	Erythrocyte membrane protein band 4.1 like 4B	0.70	9q31	L
XM_172341	ZBTB38	Zinc finger and BTB domain containing 38	0.70	3q23	IN(L)
NM_001004051	GPRASP2	G protein-coupled receptor associated sorting protein 2	0.71	Xq22	L
NM_006283	TACC1	Transforming, acidic coiled-coil containing protein 1	0.72	8p11	L
NM_152611	C20orf75	Chromosome 20 open reading frame 75	0.78	20p12	L
NM_016441	CRIM1	Cysteine rich transmembrane BMP regulator 1	0.79	2p22	L
NM_021979	HSPA2	Heat shock 70kDa protein 2	0.79	14q23	IN(L)
NM_001860	SLC31A2	Solute carrier family 31 (copper transporters), member 2	0.79	9q32	L
NM_004675	DIRAS3	DIRAS family, GTP-binding RAS-like 3	0.82	1p31	S
NM_006404	PROCR	Protein C receptor, endothelial (EPCR)	0.85	20q11	IN(S)
NM_001753	CAV1	Caveolin 1, caveolae protein, 22kDa	0.90	7q31	L
NM_002210	ITGAV	Integrin, alpha V	0.94	2q32	L
NM_001006605	FAM69A	Family with sequence similarity 69, member A	0.94	1p22	L
XM_374020	LOC375295	Hypothetical gene supported by BC013438	0.96	2q31	L
NM_021199	SQRDL	Sulfide quinone reductase-like	0.97	15q21	L
NM_001122	ADFP	Adipose differentiation-related protein	1.00	9p22	L
NM_024642	GALNT12	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylglucosaminyltransferase 12 (GalNAc-T12)	1.00	9q.22	L

Accession No.	Symbol	Gene Name	Signal log ratio	Chr	CpG ^(a)
NM_001003818	TRIM6	Tripartite motif-containing 6	1.10	11p15	N
NM_002526	NT5E	5'-nucleotidase, ecto (CD73)	1.17	6q14	L
NM_001033910	TRAF5	TNF receptor-associated factor 5	1.24	1q32	S
NM_003982	SLC7A7	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 7	1.30	14q11	N
NM_004925	AQP3	Aquaporin 3	1.33	9p13	S
NM_005261	GEM	GTP binding protein overexpressed in skeletal muscle	1.34	8q22	S
NM_001482	GATM	Glycine amidinotransferase (L-arginine:glycine amidinotransferase)	1.56	15q21	L
NM_024829	FLJ22662	Hypothetical protein FLJ22662	1.57	12p13	L
NM_002993	CXCL6	Chemokine (C-X-C motif) ligand 6	1.63	4q13	S
NM_001323	CST6	Cystatin E/M	2.26	11q13	L
NM_013453	SPANX	Sperm protein associated with the nucleus, X-linked family	3.46	Xq27	ND

^(a) CpG Island presence; S, island in the promoter to exon1 region smaller than 500 bp; L, island in the promoter to exon1 region bigger than 500 bp; IN, island further inside than Exon1 (L, bigger than 500 bp; S, smaller than 500 bp); ND, position unidentified; 3', island in the 3' end of the gene

Table 4.5. Chromosomal location of genes without a CpG island

Gene without CGI	Chr. Location	Other genes in same Chr. location
CPVL	7p15.1	C7orf30; KIAA0644
NGFRAP1L1	Xq22.1	GPRASP2
SLC7A7	14q11.2	SALL2; TM9SF1
ZBTB38	3q23	None
NGFRAP1L1	Xq22.1	None
TRIM6	11p15.4	None

Figure 4.1. RT-PCR analysis of representative genes upregulated after 5-aza-dC treatment of OVCAR3 cell line

Expression results from the microarray analysis were confirmed by RT-PCR for CAV1, SFN, EMP3, CST6, CXCL6 and MAGEA3. All the genes showed induction of expression after 5-aza-dC treatment (AZA) compared to the untreated cells (PBS). + and – indicate the presence or absence of reverse transcriptase during cDNA synthesis. GAPDH is used as an endogenous control.

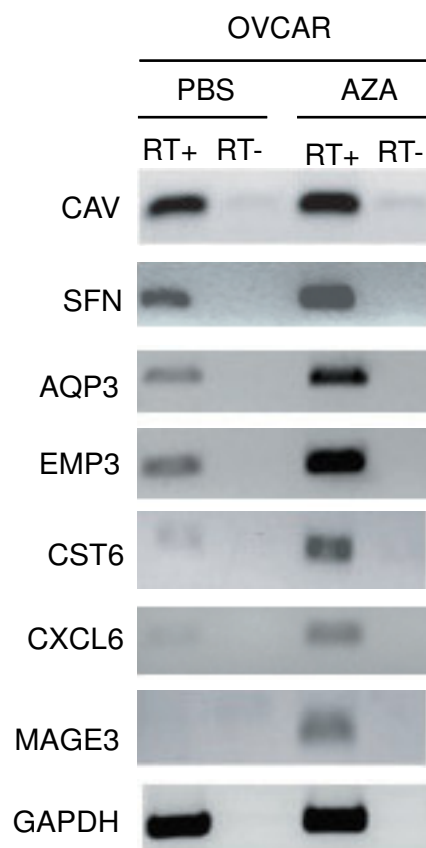
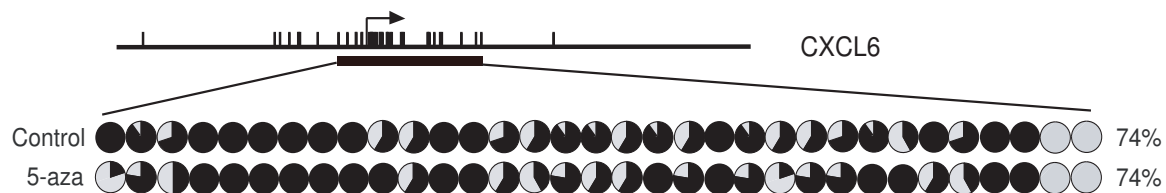
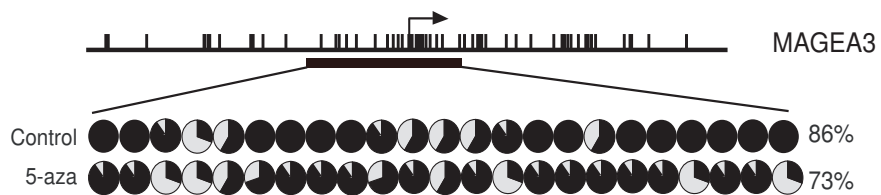
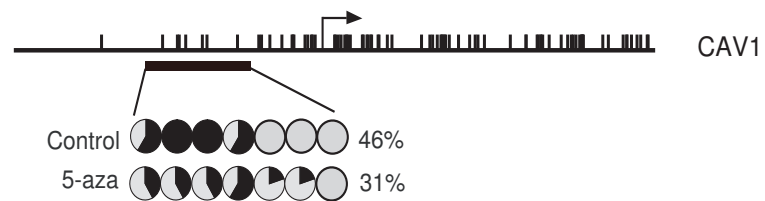
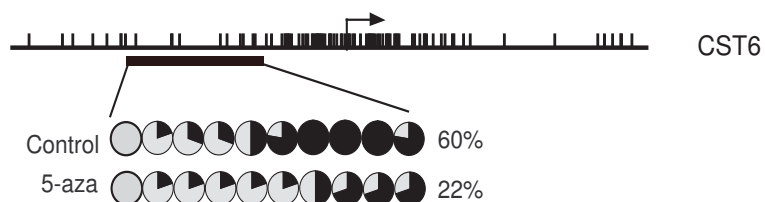
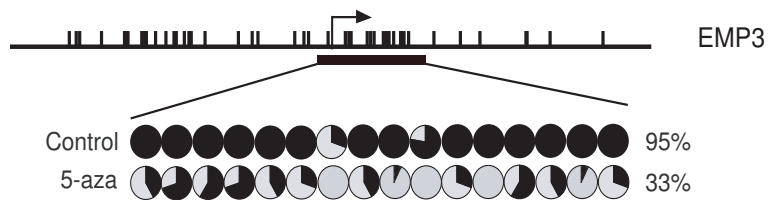


Figure 4.2. Methylation analysis of upregulated genes after 5-aza-dC treatment of OVCAR3 cell line

Results of the methylation analysis of EMP3, CST6, CAV1, MAGEA3 and CXCL6 for the control and 5-aza-dC treated OVCAR3 cells are shown. The depicted areas correspond to genomic DNA sequences of 2 kb. Five clones were sequenced for each sample, and each circle represents the average methylation for a single CpG dinucleotides (open circle, 100% unmethylated; filled circle, 100% methylated). Total methylation for untreated (control) and 5-aza-dC treated cells is shown next to each row of circles. (*Vertical bars*, CpG dinucleotides; *arrow*, position of the transcription start site; *horizontal black rectangle*, region amplified and sequenced after sodium bisulfite).



CHAPTER 5

CONCLUSION

DNA methylation changes have been studied in several cancer types. Two opposite changes in methylation have been shown to occur concurrently in some cancers. While there is a general loss of methylation at the genomic level (hypomethylation), specific genes go through an increase of the methylation within their promoter regions (hypermethylation) [1].

In this dissertation I have studied hypomethylation and hypermethylation in ovarian cancer. Ovarian cancer is the fourth leading cause of cancer deaths in women. The American Cancer Society estimates that 20,180 new cases of ovarian cancer and 15,310 consequent deaths will occur in the United States in 2006 [2]. The high mortality rate in ovarian cancer can be attributed to diverse factors, including the fact that women with ovarian cancer often present minimal symptoms, and that accurate diagnostic tools for ovarian cancer are virtually non-existent [3].

Gene specific hypermethylation is known to cause silencing of genes involved in multiple pathways. In the case of ovarian cancer, hypermethylation has been detected in genes such as BRAC1, ARHI, DAPK and hMLH1 (reviewed in [3]). In contrast, gene specific hypomethylation can also result in an increase in gene expression in cancer, as shown for BRCA2 and Synuclein γ in ovarian cancer [11, 12].

Global hypomethylation has been reported to affect 25% of the genome in ovarian cancer [4]. Very little is known about the specific sequences that are affected by loss of methylation other than generally centromeric and juxtracentromeric satellite DNA seem to be consistently

hypomethylated [5, 6]. Around 43% of our genome is made up retroelements, and most of these elements are silenced through methylation in non-malignant cells [7, 8]. Alterations in the methylation levels of retroelements may result in detrimental effects for the cancer cells like gene mutations caused by new insertions, altered gene expression of neighboring genes, and chromosomal rearrangement, driven by ectopic recombination between homologous retrotransposons (reviewed in [8]).

In Chapter 2, the methylation levels of two families of retrotransposons, LINE1 (non-LTR long interspersed retroelements) and HERV-W, (human endogenous retrovirus) were studied. In both cases, ovarian adenocarcinoma had reduced methylation levels when compared to benign adenomas. For the samples for which RNA was available, lower methylation levels correlated with an increase of expression. These results are consistent with the hypothesis that changes in methylation during cancer development may lead to retroelement activation, and raise some interesting questions about the nature of hypomethylation. For example, is hypomethylation random within families of elements or are some elements specifically affected by it? It also leads to more detailed studies concerning the precise location of the hypomethylated elements within the genome, and the effects this hypomethylation has on neighboring genes, since hypomethylation of retroelements could lead to the expression of nearby oncogenes normally silenced due to the more compacted chromatin structure of methylated DNA.

In Chapter 3, the methylation level of *HLA-G* was studied in ovarian cancer. *HLA-G* is a member of the Human Leukocyte Antigen (HLA) family and its expression has been reported to be upregulated in different types of cancers, including ovarian cancer [13]. The methylation status of the promoter region of *HLA-G* has been studied in cancer cell lines [14] and an inverse

correlation between expression and methylation has been observed for a CpG enriched region of ~450 bp upstream of the transcription start site [15]. In our study, the methylation status of *HLA-G* was determined in the ovarian cancer cell line BG-1. This cell line has no detectable expression of HLA-G and showed high methylation in the 450 bp CpG rich promoter region. Treatment with the demethylating agent 5-aza-dC caused an increase of expression as well as a decrease of methylation, suggesting a correlation between expression and methylation levels in this ovarian cancer cell line. In this dissertation I provide the first comparison between expression and methylation levels of this specific 450 bp promoter region of *HLA-G* in cancer and non-cancer ovarian tissue samples, by comparing 8 benign adenomas, 8 adenocarcinomas, and 4 surface epithelium samples from normal ovaries. Expression levels of *HLA-G* were very low or non-existent in seven out of eight benign and in six out of eight cancer samples. Expression from normal cells obtained from brushings of normal ovaries was intermediate to expression from adenoma and adenocarcinoma cells obtained through LCM. No correlation between expression and methylation levels was found for our set of samples, and results suggest that in ovarian cancer expression is not correlated with *HLA-G* methylation levels within the CpG rich in the *HLA-G* promoter. This is in contrast to what was observed in the ovarian cancer cell line and suggests that methylation is not responsible for changes in *HLA-G* expression in ovarian cancer, or if it is, it is regulated by regions other than the CpG rich region located in the *HLA-G* promoter. Examination of a second region in Exon1 also showed no correlation between expression and methylation. Our results suggest that DNA methylation might not have any control over *HLA-G* regulation of expression in ovarian tissue, even though expression and methylation are correlated in the ovarian cancer cell line BG-1.

Regardless of this lack of inverse correlation in tissue, we found a significant higher level of methylation of the promoter region of adenomas and adenocarcinomas than in normal epithelium samples, as well as site-specific differences at 10 CpG dinucleotides. These results suggest that HLA-G is undergoing a process of hypermethylation during the development of ovarian cancer although methylation levels had no correlation with expression of HLA-G.

In Chapter 4, a study of global gene hypermethylation was performed using the OVCAR3 ovarian cancer cell line. Wei et al. [9] analyzed the methylation status of 7776 CpG islands in ovarian cancer, and found 956 of them to be hypermethylated in high grade tumors compared to normal samples. In our study the expression profile of OVCAR3 was monitored before and after demethylation treatment (5 μ m 5-aza-deoxycytidine), and 831 genes were found to be differentially expressed after the treatment. Five genes were randomly selected for detailed analysis of their methylation status. Results showed a decrease of methylation in four of the five selected genes, suggesting that genes upregulated after 5-aza-dC treatment are candidates for being transcriptionally silenced through hypermethylation. Since results in cell lines do not always reflect methylation patterns in tissue [10], our list of upregulated genes after 5-aza-dC treatment was compared to a list of genes found to be downregulated in ovarian carcinoma. This comparison provided a total of 102 genes candidates for hypermethylation and subsequent silencing during ovarian cancer development.

References

1. Jones PA, Baylin SB: The fundamental role of epigenetic events in cancer. *Nat Rev Genet* 2002, 3(6):415-428.
2. Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C, Thun MJ: Cancer statistics, 2006. *CA Cancer J Clin* 2006, 56(2):106-130.

3. Balch C, Huang TH, Brown R, Nephew KP: The epigenetics of ovarian cancer drug resistance and resensitization. *Am J Obstet Gynecol* 2004, 191(5):1552-1572.
4. Cheng P, Schmutte C, Cofer KF, Felix JC, Yu MC, Dubeau L: Alterations in DNA methylation are early, but not initial, events in ovarian tumorigenesis. *Br J Cancer* 1997, 75(3):396-402.
5. Ahluwalia A, Yan P, Hurteau JA, Bigsby RM, Jung SH, Huang TH, Nephew KP: DNA methylation and ovarian cancer. I. Analysis of CpG island hypermethylation in human ovarian cancer using differential methylation hybridization. *Gynecol Oncol* 2001, 82(2):261-268.
6. Widschwendter M, Jiang G, Woods C, Muller HM, Fiegl H, Goebel G, Marth C, Muller-Holzner E, Zeimet AG, Laird PW *et al*: DNA hypomethylation and ovarian cancer biology. *Cancer Res* 2004, 64(13):4472-4480.
7. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W *et al*: Initial sequencing and analysis of the human genome. *Nature* 2001, 409(6822):860-921.
8. Kazazian HH, Jr.: Mobile elements: drivers of genome evolution. *Science* 2004, 303(5664):1626-1632.
9. Wei SH, Chen CM, Strathdee G, Harnsomburana J, Shyu CR, Rahmatpanah F, Shi H, Ng SW, Yan PS, Nephew KP *et al*: Methylation microarray analysis of late-stage ovarian carcinomas distinguishes progression-free survival in patients and identifies candidate epigenetic markers. *Clin Cancer Res* 2002, 8(7):2246-2252.
10. Ehrlich M: Expression of various genes is controlled by DNA methylation during mammalian development. *J Cell Biochem* 2003, 88(5):899-910.
11. Chan KY, Ozcelik H, Cheung AN, Ngan HY, Khoo US: Epigenetic factors controlling the BRCA1 and BRCA2 genes in sporadic ovarian cancer. *Cancer Res* 2002, 62(14):4151-4156.
12. Gupta A, Godwin AK, Vanderveer L, Lu A, Liu J: Hypomethylation of the synuclein gamma gene CpG island promotes its aberrant expression in breast carcinoma and ovarian carcinoma. *Cancer Res* 2003, 63(3):664-673.
13. Singer G, Rebmann V, Chen YC, Liu HT, Ali SZ, Reinsberg J, McMaster MT, Pfeiffer K, Chan DW, Wardelmann E *et al*: HLA-G is a potential tumor marker in malignant ascites. *Clin Cancer Res* 2003, 9(12):4460-4464.
14. Mouillot G, Marcou C, Rousseau P, Rouas-Freiss N, Carosella ED, Moreau P: HLA-G gene activation in tumor cells involves cis-acting epigenetic changes. *Int J Cancer* 2005, 113(6):928-936.

15. Moreau P, Mouillot G, Rousseau P, Marcou C, Dausset J, Carosella ED: HLA-G gene repression is reversed by demethylation. *Proc Natl Acad Sci U S A* 2003, 100(3):1191-1196.