The Epigenetics of Oral Cancer

Richard Shaw FDS FRCS

Regional Maxillofacial Unit

University Hospital Aintree

Liverpool L9 7AL, UK

Contact: tel : 0151 529 5290

Fax:0151 529 5288

Email: freedna@gmail.com

The epigenetics of oral cancer

1. Epigenetics in normal cells

The term epigenetics defines those heritable changes in gene expression that are not coded in the DNA sequence. To clarify this definition, it is important to understand that chemical modifications to DNA and its associated proteins can alter gene expression without altering the DNA sequence. Whereas genetic abberations change expression by altering the sequence of adenine (A)-thymine (T) and cytosine (C)-guanine (G) base pairs, epigenetic changes do not affect the underlying base pair sequence. The forms of epigenetic modification occurring in human cells are known as DNA methylation and histone deacetylation. DNA methylation is a modification of the DNA molecule itself in which methyl groups are added to cytosine nucleotides in specific areas of the gene by the enzyme DNA methyltransferase. Methylation directly switches off gene expression by preventing the binding of transcription factors as illustrated in Figure 1

DNA does not exist as a naked molecule, but in association with proteins called histones to form a complex substance known as chromatin. Changes to the structure of the chromatin also have a profound influence on gene expression. If chromatin is a condensed, tight-knit structure, the factors involved in gene expression cannot get access to the DNA, and consequently the gene will be switched off. Conversely, if the chromatin is chemically changed to a loose, more open structure, the genes can be, in effect, switched on (Figure 2).

These epigenetic events are important in the physiology of normal cells. During embryonic development, hypermethylation silences a proportion of genes which dictates the path of differentiation. Even adult human cells have enormous potential for growth, such that if unhindered, a single cell can reach a mass of 1kg in 40 days by mitotic division[46]. Epigenetic changes govern a series of cellular "checks and balances" restraining this growth. These include tumour suppressor genes that are active in normal cells but become silenced in cancer.

2. Epigenetic changes in cancer

2.1.1. Genetics versus epigenetics?

Changes in methylation of DNA in cancer were first recognised by Feinberg in 1983[19], however it was originally thought that these were linked to a general disruption of the cell cycle, perhaps an effect and not the cause, of malignancy. The intervening two decades have seen molecular research principally directed towards genetic changes as the basis for cancer. Techniques such as loss of heterozygosity in chromosome regions thought to contain tumour suppressor genes[21], microsatellite instability[22,41] or identification of individual gene mutations[2] have been widely reported in the field of oral cancer. Whilst giving an insight into the processes underlying cancer, the explosion of genetic information has yet to translate into clinical benefit in oral cancer, as with many other sites. The discovery that tumour suppressor genes often fail to be expressed in the absence of a detectable genetic change, along with significant technological advances have recently led to greater research emphasis on cancer

epigenetics[16]. Figure 3 illustrates the exponential increase in published research in the epigenetics of human cancer.

Silencing of tumour suppressor genes is central to the development of cancer. It was originally thought that both alleles of a tumour suppressor gene had to be altered by mutation or deletion (Knudsen's "two hit" hypothesis) for it to become inactivated. Gene silencing is now also recognised in the absence of any genetic change, suggesting a new model of bi-allelic inactivation[46] involving hypermethylation (Figure 4), whereby one or both alleles might be affected by aberrant methylation at the gene promoter. The aging process[32] and auto-immune diseases, as well as cancer, may be mediated by gradual accumulation of epigenetic changes[51].

2.2. Epigenetic changes seen in cancer

2.2.1. Global hypomethylation

Feinberg[17-19] first described the overall pattern of hypomethylation in human cancer, specifically a 10% reduction in genomic 5-methylcytosine content in pre-malignant and malignant colonic polyps. This presumably represents an overall increase in gene expression and cellular synthetic activity. Hypomethylation has however subsequently received less attention than the finding of hypermethylation in certain areas of the genome also seen in cancer.

2.2.2. Hypermethylation in tumour suppressor genes

Hypermethylation occurs in certain regions of tumour suppressor genes known as promoters. These are characterised by a high density of cytosine nucleotides known as CpG islands. It is assumed that during evolution, CpG (Cytosine – Guanine nucleotide pairings) sites were evenly distributed throughout the genome. However, methylation renders cytosine more prone to mutations, and thus the frequency of cytosine has gradually reduced, except in gene promoter regions, which were spared, leaving CpG "islands". The mechanism for this retention of cytosine in CpG islands is unknown, but it is interesting to speculate that it may have been due to their function as gene promoters. These CpG islands are approximately 500 base pairs in length, within which CpG form more than 55% of the nucleotides, and they are found in the promoter regions of 40% of mammalian genes. There are thought to be around 45,000 CpG islands distributed around the human genome. Physiological methylation of CpG islands causes long term gene silencing and, in a similar way, aberrant DNA methylation of the promoter region of certain genes is now thought to be a key mechanism for carcinogenesis. The attachment of 5-methylcytosine binding protein to methylated cytosine bases interferes with the binding of transcriptional proteins to gene promoters, halting the expression of that gene. Genes commonly found to be hypermethylated in cancer include tumour suppressors, metastasis related genes, DNA repair genes, hormone receptor genes and those inhibiting angiogenesis. In normal cells, the pattern of DNA methylation in any particular

cell type is conserved following replication by a maintenance DNA methylase. The mechanism by which aberrant DNA methylation occurs is, however, unclear.

The pattern of hypermethylation is specific to the tumour type[14,20], for example the DNA repair gene BRCA1 is hypermethylated in breast and ovarian cancer[15], but not other sites[14]. The term “methylotype” to signify the pattern of promoter hypermethylation has been used in an analogous way to the genetic term “genotype”[13]. Much of the publish research concentrates on 15-20 genes, the function of which is known in the context of cancer. Other studies have attempted to gain an overall picture of the global pattern of methylation. A study examining 97 tumour specimens from various sites demonstrated an average of 600 CpG islands were aberrantly methylated in various cancers[7]. Clearly our understanding of these profound epigenetic changes across the genome is, as yet, incomplete.

2.2.3. Histone modification

The basic unit of chromatin is the nucleosome which comprises 146 base pairs of DNA surrounding a histone octomer (Figure 2). De-acetylation of histones gives them a positive charge and which interacts with the negative charge of DNA producing a closed structure, repressive for transcription and hence gene silencing. Histone de-acetylase (HDAC) thus mediates gene silencing. HDAC inhibitors can reverse gene silencing in certain instances, but not in genes which contain hypermethylated CpG islands. Methylation of lysine in histones is also implicated in gene regulation. The combination of both histone modification and

acetylation is known as the “histone code”[16] and significant cross talk occurs between DNA methylation and the histone code which together mediate gene silencing. The balance of evidence from microarray studies with 5AZA and HDAC inhibitors is that DNA hypermethylation is the dominant event but that these factors may occur in concert[16].

2.3. Gene imprinting

Imprinting is an epigenetic change that occurs on only one parental allele of a gene and is traditionally associated with genes mapping to the X chromosome where its function is to prevent gene dosage differences between males and females. Loss of imprinting (LOI) may conceivably lead to, for example, predisposition to malignancy, should this involve a tumour related gene. The critical difference is that in most human cancers, the tumour demonstrates hypermethylation at certain loci, but that normal cells within the same subject are not methylated in the same way. Recent evidence[9] in colorectal cancer has shown certain individuals who have LOI at a growth factor gene (human insulin-like growth factor II gene:IGF2), have much higher rates of colorectal cancer. However these patients have LOI in all cells and studies have clearly demonstrated that the high cancer risk is independent of known environmental risk factors. Those treating oral and oro-pharyngeal cancer will be aware of young non-smoking, non-drinking patients who due to “bad luck” or “bad genes” develop tumours, and it is now believed that LOI may be one mechanism responsible for this phenomenon. The concept of inherited epigenetic

susceptibility to tobacco related cancers[80] is certainly of interest. Identification of these individuals might allow more focused, and hence more cost effective, prevention strategies in OSCC.

3. Hypermethylation in oral cancer

In recent years, there has been a rapid increase of interest in hypermethylation in human cancers (Figure 3). A summary of studies investigating hypermethylation in oral and other head and neck sites is given in Table 1

3.1. Correlation of hypermethylated gene promoters with prognosis in oral cancer

There may be potential for therapeutic advantage if specific epigenetic aberrations could be shown to correlate with tumour behaviour. Some of the studies listed in Table 1 have attempted to correlate the clinicopathological staging of the tumour with promoter hypermethylation in the gene studied. Additionally, a number of the studies also attempt to demonstrate loss of expression of the gene by techniques such as immunohistochemistry. Unfortunately, some studies bulk all head and neck sites together, so separate interpretation of the oral cancer data can be difficult.

3.1.1. E-Cadherin

Cell adhesion molecules maintain stable tissue structure and loss of expression correlates with tumour invasiveness and metastasis[70]. *E-cadherin* is a trans-membrane glycoprotein responsible for cell-cell adhesion, the reduced expression of which is highly correlated with regional metastasis in OSCC. Mutations (i.e. genetic not epigenetic) of *E-cadherin* gene are seen at some tumour sites e.g. breast, stomach[38,69]. However, in the head and neck, promoter hypermethylation is more significant and has been demonstrated in 46% of specimens investigated. Several studies relate *E-cadherin* hypermethylation to adverse histological grade[49,56] and poor survival[3]. Yeh et al. [82] did not find this association although they also found *E-cadherin* expression did not correlate with methylation opening up the possibility that other , genetic, changes were present in this Taiwanese study.

3.1.2. DAP-kinase

Reduced expression of the enzyme *DAP-kinase* (Death Associated Protein) is associated with loss of apoptosis, cell immortality and their relationship to metastasis. Reduced expression has been correlated with metastasis in lung cancer[34]. *DAP-kinase* promoter hypermethylation has been shown in 27% of H&N specimens investigated, however only in one study has been significantly correlated with nodal stage[57]. Other studies failed to find any correlation in oral[35] or nasopharyngeal[77] specimens.

3.1.3. p16, p15, p14

These cell cycle regulatory genes have been extensively studies and promoter hypermethylation is common in OSCC (*p15*:30%, *p16*:76%) however, no significant correlation with clinicopathological characteristics or prognosis has been observed[12,33,35,57,75]. Further, these epigenetic aberrations have also been shown in "normal" and dysplastic oral lesions[4], and consequently, may be involved in the early stages of carcinogenesis and related to exposure to alcohol and tobacco[4,35,79]. *p14* hypermethylation, perhaps surprisingly, has been related to good prognosis in one study[50].

3.1.4. DCC

Ogi et al[50]. demonstrated that DNA methylation in the promoter region of *DCC* (Deleted in Colorectal Cancer) was significantly correlated (P=0.036) with mandibular invasion in oral cancer, which in turn is now recognised as a negative prognostic indicator[61].

3.1.5. "MINT" family CpG islands

These CpG islands are associated with tumours at several sites[71,72], however their functions are uncertain as they are not located near any known genes. Ogi et al. demonstrated significant correlation with poor survival in oral cancers where hypermethylation was found at *MINT 1* & *MINT 31*, but not *MINT 2* & *MINT27*[50].

4. Epigenetic changes in circulating DNA

A most significant development in the history of epigenetics was the development of methylation-specific polymerase chain reaction (MSP) using bisulphate modification of DNA by Herman at the Johns Hopkins, Baltimore, USA in 1996[30]. This allowed precise mapping of DNA methylation patterns in CpG islands across the entire genome which has stimulated huge interest in this field. This method is also highly sensitive, allowing reliable detection of only 0.1% methylated alleles of a given CpG island. Crucially though, very small quantities of DNA are required to perform MSP and the process can be partially automated by the introduction of technical improvements such as pyrosequencing[5,74]. Tumour DNA is known to be present in a variety of body compartments in cancer sufferers[37,67], however it has previously been technically difficult to reliably identify specific genetic changes known to correlate with the primary tumour[8,65]. The significance of this is, perhaps not that molecular changes in the primary tumour can be identified by examination of circulating DNA, but that the elimination or persistance of tumour following treatment might be inferred. This concept of circulating DNA as a tumour marker has been explored in a number of tumour subsites[63].

4.1. Sources of free DNA

4.1.1. Serum / Plasma Free DNA.

Small fragments of extra-cellular DNA are known to circulate in the blood of patients with diseases such as inflammatory bowel disease and rheumatoid arthritis[36,60,73], as well as many malignant conditions[83]. The first report regarding hypermethylation status of serum DNA samples and their correlation to the primary head and neck tumour originated from the Johns Hopkins in 2000[57]. In this study, pre-operative blood specimens were taken and anaylsed using MSP in a series of 50 patients. A pattern of CpG island hypermethylation at one or more gene promoter (*p16*, *MGMT* and/or *DAP-kinase*) was observed in 21/50 (42%) samples and corresponded to that observed in the primary tumour. 5 of the 21 “serum-positive” patients developed distant metastases (24%), while only 1 of 29 “serum-negative” patients did so (3%). The authors comment that pre-treatment tumour DNA seems to correlate with tumour load and would make a good tumour marker. Hibi et al.[31] performed a similar study using *p16* promoter hypermethylation alone in oesophageal cancer in 2001. 23% of patients were “serum positive” on a pre-operative sample but the study found no correlation with clinical outcome. Another study from Hong Kong[78] demonstrated the presence of *DAP-kinase* promoter methylation in the peripheral blood of 8 of 24 (33%) nasopharyngeal cancer patients.

4.1.2. Saliva / oral rinse.

Lopez et al.[40] reported on the value of gene promoter Hypermethylation, as demonstrated, using MSP, in oral rinses in 2003. Methylation of *p16*, *p14* and *MGMT* was observed in 44%, 12% and 56% of the oral samples respectively. DNA hypermethylation was more frequent in patients with previous OSCC. The study concludes that this technique was non-invasive and highly sensitive and could be used to monitor patients with pre-malignant and malignant oral lesions. Whether the DNA was intra or extra-cellular is difficult to prove. A similar principle is currently being applied in the Liverpool Lung Project[23] where sputum samples are being monitored in a large study investigating the possibility of early detection of malignancy using hypermethylation.

4.1.3. Urine.

Su et al.[68] report finding small fragments of tumour DNA in the urine of colorectal cancer patients. Remarkably, these are filtered unchanged through the glomerular membrane and are readily detected. There are no reports as yet of the detection of tumour DNA in urine from oral cancer patients, or on the use of hypermethylation as a specific method of detection.

4.2. Hypermethylation of circulating DNA as a tumour marker in oral cancer.

The application of Methylation Specific PCR (MSP) in early diagnosis, tumour surveillance and prescription of neo-adjuvant therapy has been suggested[64]. Promoter hypermethylation is well suited to use as a tumour marker for a number of reasons. Hypermethylation occurs with high frequency in oral cancer, such that if several well chosen CpG islands are used, a "signature" should be available for any tumour ("informativity"). Also the sensitivity and specificity are high and can be used in a non-invasive manner, e.g. on blood specimens. However, heterogenicity within a tumour or between the primary tumour and its metastases however may present difficulties. Metastatic oral cancers methylate a greater proportion of CpG islands than do the primary tumours, and do so at different subsets of loci. Smiraglia et al.[66] studied 1300 CpG islands amongst HNSCC patients, utilising tissue from matched primary and metastatic tumour. They found that many loci methylated in a patient's primary tumour were no longer methylated in the metastatic tumour, an unexpected finding. The two possible explanations were epigenetic heterogeneity within the primary tumour and plasticity, i.e. that the tumour develops the ability to silence and re-activate genes in a dynamic way in order to gain survival advantage. This work challenges previous hypotheses that tumours spread by gradual accumulation of genetic changes.

The potential for clinical application of circulating DNA been demonstrated in a study that followed the incidence of the *BamHI-W* fragment of the Epstein Barr virus genome in recurrent nasopharyngeal cancer[76]. Cell-free EBV DNA was detected in 61% of patients with recurrence, and its quantity postoperatively reflected whether salvage surgery achieved a negative surgical margin. The most convincing epigenetic study is, however, from outside the head and neck field. Ryan et al.[55] followed a series of colorectal cancer patients with regular blood sampling for hypermethylation of the *KRAS2* gene promoter, which had occurred in 60/94 studied primary tumours. 16 of the 60 showed persistent KRAS2 promoter hypermethylated in the serum DNA. Ten of these (63%) developed a recurrence compared with only 1/44 (2%) patients who remained serum negative (p=0.0000). The authors concluded that longitudinal monitoring of postoperative blood for serum mutant KRAS2 was more prognostic for recurrence than Dukes' stage.

5. Reversibility of epigenetic events as a therapeutic target.

Since epigenetic modification plays such an important role in cancer, novel therapeutic strategies are being developed that are based on the reversal of DNA methylation and the inhibition of histone deacetylation.

5.1.1. Gene promoter methylation

Hypermethylation is reversible by agents such as 5-Azacytidine (5 AC), and this has been shown promise in early clinical studies in haematological[47] and lung[45] as well as head and neck[6] malignancy. The cell cycle regulator *p15*, which is methylated in high grade myelodysplastic syndrome and predicts for malignant transormation has been targeted with this approach. Treatment with 5AC was effective both clinically and biologically and was correlated with a decrease in *p15* hypermethylation[10]. The ability of 5AC to reactivate p16 in a HNSCC cell line has been demonstrated, as has its consequent modification of histone H3 configuration[6]. Unfortunatetely, the available inhibitors of DNA methylltransferase (MTI) are not specific for a particular gene leading to problems of toxicity[26] Theoretically,these drugs may also reverse physiologically methylated genes and produce unwanted expression or even new malignancy of a differing kind, although this has not yet been reported with MTIs[26].

5.1.2. Histone acetylation

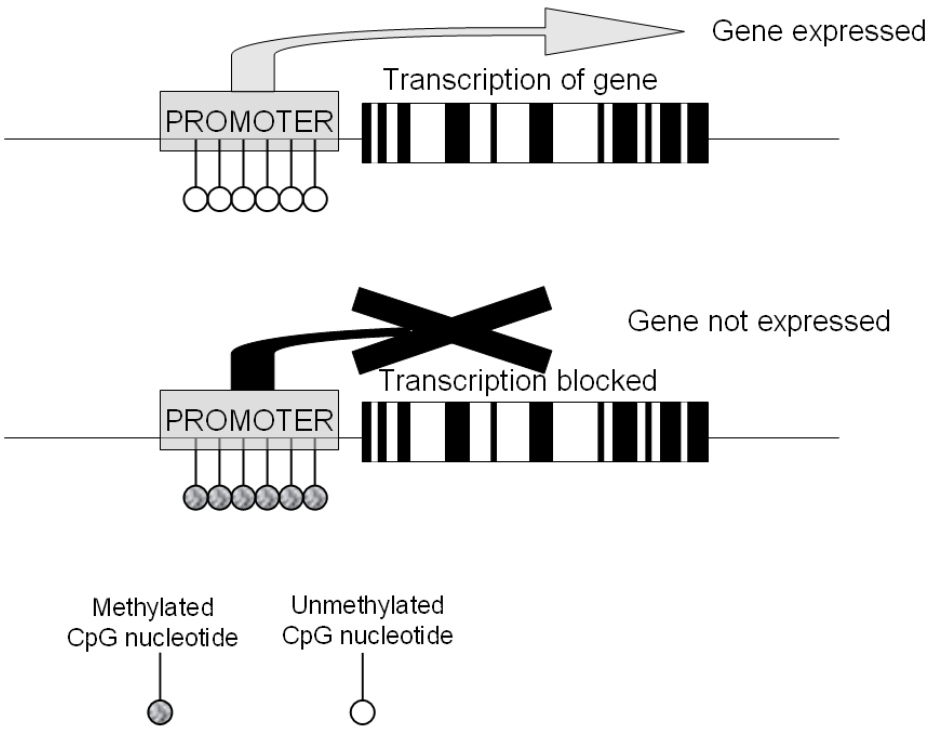
In a similar way to the above, histone deacetylase inhibitors (HDAC) have shown promise in trials. There are a number of classes of HDAC, which have been used in haematological conditions, but some of which have also demonstrated efficacy in early clinical trials in solid tumours.[42,58] As mentioned above, histone modification in a HNSCC cell line has been seen after treatment with 5AC[6]. The synergic effect of 5AC and HDAC inhibitors used together is also currently showing promise[59] in the laboratory.

6. Conclusions

Progress in the field of molecular oncology is rapid and it is difficult for the clinician treating cancer to keep abreast of important new developments. The rather obscure terminology used in epigenetic research, as in other molecular fields, can be confusing. This review aims to demystify epigenetics and explain its potential clinical relevance to oral cancer in diagnosis, staging, surveillance, and its potential in offering novel therapeutic targets. The role of promoter hypermethylation affecting individual tumour suppressor genes, or the genomic "methylotype", has been discussed in relation to the aetiology and prognosis of oral cancer. Epigenetic changes offer new therapeutic targets, which have yet to be explored in oral squamous cell carcinoma, but have shown promise in other tumour sites. Finally, the ability to detemine the pattern of hypermethylation with great sensitivity and specificity, in both tumour tissue and in circulating free DNA, has potential clinical application in early diagnosis, non-invasive testing and tumour surveillance. Translational research attempting to exploit this, particularly in oral cancer and other head neck sites is currently progressing within a number of centres and may offer an interesting avenue for accurate molecular staging.

The contribution of Dr Janet Risk (Lecturer in Genetics, Molecular Oncology Group, University of Liverpool) & Mr James Brown, (Consultant Oral & Maxillofacial Surgeon, University Hospital Aintree) in the development of this manuscript are gratefully acknowledged.

Figure 1: Methylation of CpG islands in gene promoter region prevents transcription



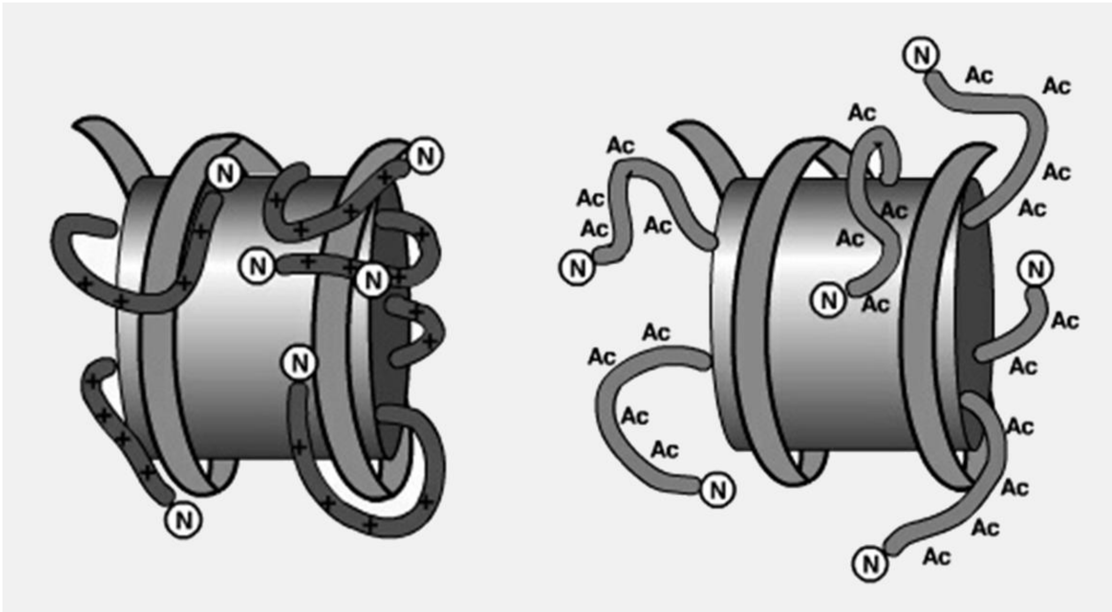
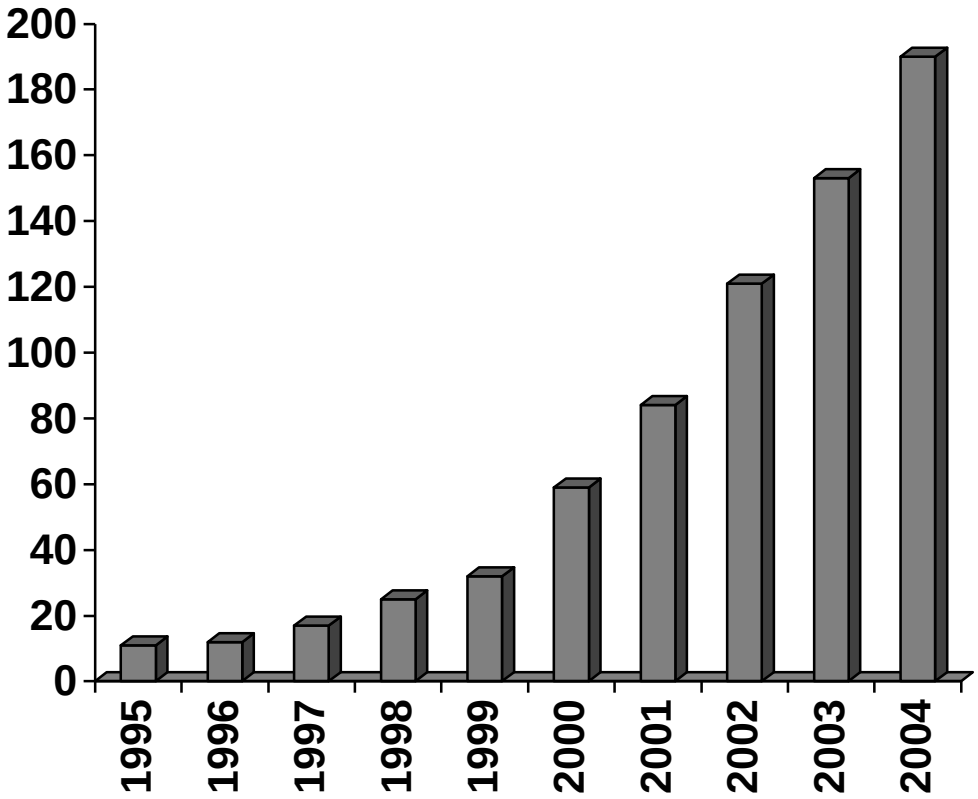
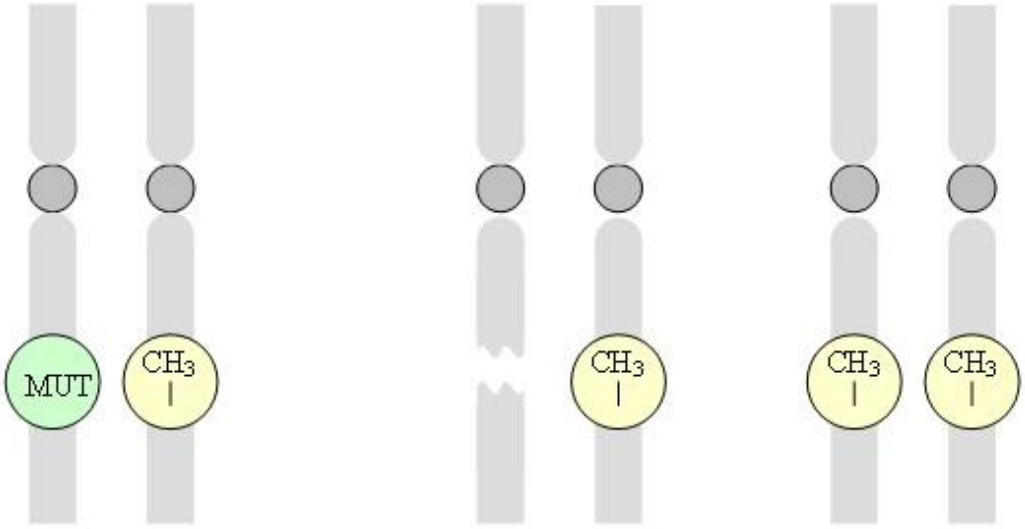


Figure 2 Deacetylated nuclseosome with tight structure (left) and acertylated nucleosome with loose structure (right)

Figure 3 Number of PubMed entries by year for epigenetics or hypermethylation in relation to all cancer sites. (2004 data is extrapolated from 142 entries by Sep 1st 2004)





MUTATION & METHYLATION **DELETION (LOH) & METHYLATION** **BI-ALLELIC METHYLATION**

Figure 4 **Model of bi-allelic inactivation involving both genetic (mutation or deletion) and epigenetic aberrations (hypermethylation)** [46]

Table 1 Hypermethylated genes in HNSCC

Hallmark [28]	Gene			Ref.	Total H&N Patients	Hyper-meth. shown	% Hyper-meth.
	Name	Locus	Function				
1. Insensitivity to antigrowth signals	p16	9p21	Cell cycle – cyclin kinase inhibitor induces differentiation	12,14,27,29,31,33,35,43,44,48,50,52,57,75,78,79,81	956	728	76%
	p15	9p21	TGF beta-mediated cell cycle arrest	4,14,50,75,78,79	340	103	30%
	RARbeta	3p24	Regulatory protein & apoptosis	43	51	26	26%
	Sigma 14-3-3	22q12	Glucocorticoid signaling	25	92	32	35%
2. Self sufficiency to growth signals	RASSF1A	3p21	RAS pathway regulation	11,29,43,78	218	20	9%
3. Evading apoptosis	p14	9p21	Pro-apoptosis	43,50,62	204	38	19%
	DAP-kinase	19q34	Pro-apoptosis (p53-dependent apoptotic checkpoint)	29,35,43,50,54,57,77,78	577	157	27%
4. Sustained angiogenesis	VHL	3p26-25	Von Hippel-Lindau suppressor gene	81	48	0	0%
	p73	1p36	Angiogenesis & apoptosis	43	51	1	2%
5. Tissue invasion & metastasis	E-Cadherin	16q22	Cell-cell adhesion	3,29,43,49,56,75,82	433	201	46%
	ABO	9q34	Blood group – glycosylation relates to tumour cell motility	24	30	10	33%
	DCC	18q21	Cell-cell adhesion "Deleted in Colorectal Cancer"	50	96	16/96	17%
6. Genome instability	hMLH1	3p21	DNA mismatch repair	39,50,75,78	271	37	14%
	MGMT	10q26	DNA repair for alkylated guanine	35,43,53,57,75,84	545	183	34%
	p53	17p13	DNA repair	81	48	2	4%
Caricnogen detoxification	ATM	11q22	Ataxia-telangectasia mutated gene: Genotoxic stress & radiotherapy	1	100	25	25%
	GSTP1	11q13	Glutathione transferase	14,57	201	0	0%
Unknown	MINT 1, 2, 27,31		Mostly unknown but associated with malignancy[71,72]	50	96	22,8,15,14	23,8, 16,15

References

1. AI L, VO QN, ZUO C, LI L, LING W, SUEN JY, HANNA E, BROWN KD, & FAN CY. Ataxia-telangiectasia-mutated (ATM) gene in head and neck squamous cell carcinoma: promoter hypermethylation with clinical correlation in 100 cases. Cancer Epidemiol.Biomarkers Prev. 2004: **13:** 150-156.

2. BOYLE JO, HAKIM J, KOCH W, VAN DER RP, HRUBAN RH, ROA RA, CORREO R, EBY YJ, RUPPERT JM, & SIDRANSKY D. The incidence of p53 mutations increases with progression of head and neck cancer. Cancer Res. 1993: **53:** 4477-4480.

3. CHANG HW, CHOW V, LAM KY, WEI WI, & YUEN A. Loss of E-cadherin expression resulting from promoter hypermethylation in oral tongue carcinoma and its prognostic significance. Cancer 2002: **94:** 386-392.

4. CHANG HW, LING GS, WEI WI, & YUEN AP. Smoking and drinking can induce p15 methylation in the upper aerodigestive tract of healthy individuals and patients with head and neck squamous cell carcinoma. Cancer 2004: **101:** 125-132.

5. COLELLA S, SHEN L, BAGGERLY KA, ISSA JP, & KRAHE R. Sensitive and quantitative universal Pyrosequencing methylation analysis of CpG sites. Biotechniques 2003: **35:** 146-150.

6. COOMBES MM, BRIGGS KL, BONE JR, CLAYMAN GL, EL NAGGAR AK, & DENT SY. Resetting the histone code at CDKN2A in HNSCC by inhibition of DNA methylation. Oncogene 2003: **22:** 8902-8911.

7. COSTELLO JF, FRUHWALD MC, SMIRAGLIA DJ, RUSH LJ, ROBERTSON GP, GAO X, WRIGHT FA, FERAMISCO JD, PELTOMAKI P, LANG JC, SCHULLER DE, YU L, BLOOMFIELD CD, CALIGIURI MA, YATES A, NISHIKAWA R, SU HH, PETRELLI NJ, ZHANG X, O'DORISIO MS, HELD WA, CAVENEE WK, & PLASS C. Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. Nat.Genet. 2000: **24:** 132-138.

8. COULET F, BLONS H, CABELGUENNE A, LECOMTE T, LACOURREYE O, BRASNU D, BEAUNE P, ZUCMAN J, & LAURENT-PUIG P. Detection of plasma tumor DNA in head and neck squamous cell carcinoma by microsatellite typing and p53 mutation analysis. Cancer Res. 2000: **60:** 707-711.

9. CRUZ-CORREA M, CUI H, GIARDIELLO FM, POWE NR, HYLIND L, ROBINSON A, HUTCHEON DF, KAFONEK DR, BRANDENBURG S, WU Y, HE X, & FEINBERG AP. Loss of imprinting of insulin growth factor II gene: a potential heritable biomarker for colon neoplasia predisposition. Gastroenterology 2004: **126:** 964-970.

10. DASKALAKIS M, NGUYEN TT, NGUYEN C, GULDBERG P, KOHLER G, WIJERMANS P, JONES PA, & LUBBERT M. Demethylation of a hypermethylated P15/INK4B gene in patients with myelodysplastic syndrome by 5-Aza-2'-deoxycytidine (decitabine) treatment. Blood 2002: **100:** 2957-2964.

11. DONG SM, SUN DI, BENOIT NE, KUZMIN I, LERMAN MI, & SIDRANSKY D. Epigenetic inactivation of RASSF1A in head and neck cancer. Clin.Cancer Res. 2003: **9:** 3635-3640.

12. EL NAGGAR AK, LAI S, CLAYMAN G, LEE JK, LUNA MA, GOEPFERT H, & BATSAKIS JG. Methylation, a major mechanism of p16/CDKN2 gene inactivation in head and neck squamous carcinoma. Am.J.Pathol. 1997: **151:** 1767-1774.

13. ESTELLER M. Cancer epigenetics: DNA methylation and chromatin alterations in human cancer. Adv.Exp.Med.Biol. 2003: **532:** 39-49.

14. ESTELLER M, CORN PG, BAYLIN SB, & HERMAN JG. A gene hypermethylation profile of human cancer. Cancer Res. 2001: **61:** 3225-3229.

15. ESTELLER M, FRAGA MF, GUO M, GARCIA-FONCILLAS J, HEDENFALK I, GODWIN AK, TROJAN J, VAURS-BARRIERE C, BIGNON YJ, RAMUS S, BENITEZ J, CALDES T, AKIYAMA Y, YUASA Y, LAUNONEN V, CANAL MJ, RODRIGUEZ R, CAPELLA G, PEINADO MA, BORG A, AALTONEN LA, PONDER BA, BAYLIN SB, & HERMAN JG. DNA methylation patterns in hereditary human cancers mimic sporadic tumorigenesis. Hum.Mol.Genet. 2001: **10:** 3001-3007.

16. FEINBERG AP. Cancer epigenetics takes center stage. Proc.Natl.Acad.Sci.U.S.A 2001: **98:** 392-394.

17. FEINBERG AP, GEHRKE CW, KUO KC, & EHRLICH M. Reduced genomic 5-methylcytosine content in human colonic neoplasia. Cancer Res. 1988: **48:** 1159-1161.

18. FEINBERG AP & VOGELSTEIN B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature 1983: **301:** 89-92.

19. FEINBERG AP & VOGELSTEIN B. Hypomethylation of ras oncogenes in primary human cancers. Biochem.Biophys.Res.Commun. 1983: **111:** 47-54.

20. FELTUS FA, LEE EK, COSTELLO JF, PLASS C, & VERTINO PM. Predicting aberrant CpG island methylation. Proc.Natl.Acad.Sci.U.S.A 2003: **100:** 12253-12258.

21. FIELD JK. Genomic instability in squamous cell carcinoma of the head and neck. Anticancer Res. 1996: **16:** 2421-2431.

22. FIELD JK, KIARIS H, HOWARD P, VAUGHAN ED, SPANDIDOS DA, & JONES AS. Microsatellite instability in squamous cell carcinoma of the head and neck. Br.J.Cancer 1995: **71:** 1065-1069.

23. FIELD JK & YOUNGSON JH. The Liverpool Lung Project: a molecular epidemiological study of early lung cancer detection. Eur.Respir.J. 2002: **20:** 464-479.

24. GAO S, WORM J, GULDBERG P, EIBERG H, KROGDAHL A, LIU CJ, REIBEL J, & DABELSTEEN E. Genetic and epigenetic alterations of the blood group ABO gene in oral squamous cell carcinoma. Int.J.Cancer 2004: **109:** 230-237.

25. GASCO M, BELL AK, HEATH V, SULLIVAN A, SMITH P, HILLER L, YULUG I, NUMICO G, MERLANO M, FARRELL PJ, TAVASSOLI M, GUSTERSON B, & CROOK T. Epigenetic inactivation of 14-3-3 sigma in oral carcinoma: association with p16(INK4a) silencing and human papillomavirus negativity. Cancer Res. 2002: **62:** 2072-2076.

26. GILBERT J, GORE SD, HERMAN JG, & CARDUCCI MA. The clinical application of targeting cancer through histone acetylation and hypomethylation. Clin.Cancer Res. 2004: **10:** 4589-4596.

27. GONZALEZ MV, PELLO MF, LOPEZ-LARREA C, SUAREZ C, MENENDEZ MJ, & COTO E. Deletion and methylation of the tumour suppressor gene p16/CDKN2 in primary head and neck squamous cell carcinoma. J.Clin.Pathol. 1997: **50:** 509-512.

28. HANAHAN D & WEINBERG RA. The hallmarks of cancer. Cell 2000: **100:** 57-70.

29. HASEGAWA M, NELSON HH, PETERS E, RINGSTROM E, POSNER M, & KELSEY KT. Patterns of gene promoter methylation in squamous cell cancer of the head and neck. Oncogene 2002: **21:** 4231-4236.

30. 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.

31. HIBI K, TAGUCHI M, NAKAYAMA H, TAKASE T, KASAI Y, ITO K, AKIYAMA S, & NAKAO A. Molecular detection of p16 promoter methylation in the serum of patients with esophageal squamous cell carcinoma. Clin.Cancer Res. 2001: **7:** 3135-3138.

32. HOLLIDAY R. Is DNA methylation of X chromosome genes stable during aging? Somat.Cell Mol.Genet. 1991: **17:** 101-103.

33. HUANG MJ, YEH KT, SHIH HC, WANG YF, LIN TH, CHANG JY, SHIH MC, & CHANG JG. The correlation between CpG methylation and protein expression of P16 in oral squamous cell carcinomas. Int.J.Mol.Med. 2002: **10:** 551-554.

34. INBAL B, COHEN O, POLAK-CHARCON S, KOPOLOVIC J, VADAI E, EISENBACH L, & KIMCHI A. DAP kinase links the control of apoptosis to metastasis. Nature 1997: **390:** 180-184.

35. KULKARNI V & SARANATH D. Concurrent hypermethylation of multiple regulatory genes in chewing tobacco associated oral squamous cell carcinomas and adjacent normal tissues. Oral Oncol. 2004: **40:** 145-153.

36. LEON SA, EHRLICH GE, SHAPIRO B, & LABBATE VA. Free DNA in the serum of rheumatoid arthritis patients. J.Rheumatol. 1977: **4:** 139-143.

37. LEON SA, SHAPIRO B, SKLAROFF DM, & YAROS MJ. Free DNA in the serum of cancer patients and the effect of therapy. Cancer Res. 1977: **37:** 646-650.

38. LIM SC, ZHANG S, ISHII G, ENDOH Y, KODAMA K, MIYAMOTO S, HAYASHI R, EBIHARA S, CHO JS, & OCHIAI A. Predictive markers for late cervical metastasis in stage I and II invasive squamous cell carcinoma of the oral tongue. Clin.Cancer Res. 2004: **10:** 166-172.

39. LIU K, HUANG H, MUKUNYADZI P, SUEN JY, HANNA E, & FAN CY. Promoter hypermethylation: an important epigenetic mechanism for hMLH1 gene inactivation in head and neck squamous cell carcinoma. Otolaryngol.Head Neck Surg. 2002: **126:** 548-553.

40. LOPEZ M, AGUIRRE JM, CUEVAS N, ANZOLA M, VIDEGAIN J, AGUIRREGAVIRIA J, & MARTINEZ DP. Gene promoter hypermethylation in oral rinses of leukoplakia patients--a diagnostic and/or prognostic tool? Eur.J.Cancer 2003: **39:** 2306-2309.

41. MAO L, LEE DJ, TOCKMAN MS, EROZAN YS, ASKIN F, & SIDRANSKY D. Microsatellite alterations as clonal markers for the detection of human cancer. Proc.Natl.Acad.Sci.U.S.A 1994: **91:** 9871-9875.

42. MARSHALL JL, RIZVI N, KAUH J, DAHUT W, FIGUERA M, KANG MH, FIGG WD, WAINER I, CHAISSANG C, LI MZ, & HAWKINS MJ. A phase I trial of depsipeptide (FR901228) in patients with advanced cancer. J.Exp.Ther.Oncol. 2002: **2:** 325-332.

43. MARUYA S, ISSA JP, WEBER RS, ROSENTHAL DI, HAVILAND JC, LOTAN R, & EL NAGGAR AK. Differential methylation status of tumor-associated genes in head and neck squamous carcinoma: incidence and potential implications. Clin.Cancer Res. 2004: **10:** 3825-3830.

44. MERLO A, HERMAN JG, MAO L, LEE DJ, GABRIELSON E, BURGER PC, BAYLIN SB, & SIDRANSKY D. 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. Nat.Med. 1995: **1:** 686-692.

45. MOMPARLER RL & AYOUB J. Potential of 5-aza-2'-deoxycytidine (Decitabine) a potent inhibitor of DNA methylation for therapy of advanced non-small cell lung cancer. Lung Cancer 2001: **34 Suppl 4:** S111-S115.

46. MOMPARLER RL & BOVENZI V. DNA methylation and cancer. J.Cell Physiol 2000: **183:** 145-154.

47. MOMPARLER RL, RIVARD GE, & GYGER M. Clinical trial on 5-aza-2'-deoxycytidine in patients with acute leukemia. Pharmacol.Ther. 1985: **30:** 277-286.

48. NAKAHARA Y, SHINTANI S, MIHARA M, UEYAMA Y, & MATSUMURA T. High frequency of homozygous deletion and methylation of p16(INK4A) gene in oral squamous cell carcinomas. Cancer Lett. 2001: **163:** 221-228.

49. NAKAYAMA S, SASAKI A, MESE H, ALCALDE RE, TSUJI T, & MATSUMURA T. The E-cadherin gene is silenced by CpG methylation in human oral squamous cell carcinomas. Int.J.Cancer 2001: **93:** 667-673.

50. OGI K, TOYOTA M, OHE-TOYOTA M, TANAKA N, NOGUCHI M, SONODA T, KOHAMA G, & TOKINO T. Aberrant methylation of multiple genes and clinicopathological features in oral squamous cell carcinoma. Clin.Cancer Res. 2002: **8:** 3164-3171.

51. RICHARDSON BC. Role of DNA methylation in the regulation of cell function: autoimmunity, aging and cancer. J.Nutr. 2002: **132:** 2401S-2405S.

52. ROSAS SL, KOCH W, COSTA CARVALHO MG, WU L, CALIFANO J, WESTRA W, JEN J, & SIDRANSKY D. Promoter hypermethylation patterns of p16, O6-methylguanine-DNA-methyltransferase, and death-associated protein kinase in tumors and saliva of head and neck cancer patients. Cancer Res. 2001: **61:** 939-942.

53. ROSAS SL, KOCH W, COSTA CARVALHO MG, WU L, CALIFANO J, WESTRA W, JEN J, & SIDRANSKY D. Promoter hypermethylation patterns of p16, O6-methylguanine-DNA-methyltransferase, and death-associated protein kinase in tumors and saliva of head and neck cancer patients. Cancer Res. 2001: **61:** 939-942.

54. ROSAS SL, KOCH W, COSTA CARVALHO MG, WU L, CALIFANO J, WESTRA W, JEN J, & SIDRANSKY D. Promoter hypermethylation patterns of p16, O6-methylguanine-DNA-methyltransferase, and death-associated protein kinase in tumors and saliva of head and neck cancer patients. Cancer Res. 2001: **61:** 939-942.

55. RYAN BM, LEFORT F, MCMANUS R, DALY J, KEELING PW, WEIR DG, & KELLEHER D. A prospective study of circulating mutant KRAS2 in the serum of patients with colorectal neoplasia: strong prognostic indicator in postoperative follow up. Gut 2003: **52:** 101-108.

56. SAITO Y, TAKAZAWA H, UZAWA K, TANZAWA H, & SATO K. Reduced expression of E-cadherin in oral squamous cell carcinoma: relationship with DNA methylation of 5' CpG island. Int.J.Oncol. 1998: **12:** 293-298.

57. SANCHEZ-CESPEDES M, ESTELLER M, WU L, NAWROZ-DANISH H, YOO GH, KOCH WM, JEN J, HERMAN JG, & SIDRANSKY D. Gene promoter hypermethylation in tumors and serum of head and neck cancer patients. Cancer Res. 2000: **60:** 892-895.

58. SANDOR V, BAKKE S, ROBEY RW, KANG MH, BLAGOSKLONNY MV, BENDER J, BROOKS R, PIEKARZ RL, TUCKER E, FIGG WD, CHAN KK, GOLDSPIEL B, FOJO AT, BALCERZAK SP, & BATES SE. Phase I trial of the histone deacetylase inhibitor, depsipeptide (FR901228, NSC 630176), in patients with refractory neoplasms. Clin.Cancer Res. 2002: **8:** 718-728.

59. SHAKER S, BERNSTEIN M, MOMPARLER LF, & MOMPARLER RL. Preclinical evaluation of antineoplastic activity of inhibitors of DNA methylation (5-aza-2'-deoxycytidine) and histone deacetylation (trichostatin A, depsipeptide) in combination against myeloid leukemic cells. Leuk.Res. 2003: **27:** 437-444.

60. SHAPIRO B, CHAKRABARTY M, COHN EM, & LEON SA. Determination of circulating DNA levels in patients with benign or malignant gastrointestinal disease. Cancer 1983: **51:** 2116-2120.

61. SHAW RJ, BROWN JS, WOOLGAR JA, LOWE D, ROGERS SN, & VAUGHAN ED. The influence of the pattern of mandibular invasion on recurrence and survival in oral squamous cell carcinoma. Head Neck 2004: **26:** 861.

62. SHINTANI S, NAKAHARA Y, MIHARA M, UEYAMA Y, & MATSUMURA T. Inactivation of the p14(ARF), p15(INK4B) and p16(INK4A) genes is a frequent event in human oral squamous cell carcinomas. Oral Oncol. 2001: **37:** 498-504.

63. SIDRANSKY D. Circulating DNA. What we know and what we need to learn. Ann.N.Y.Acad.Sci. 2000: **906:** 1-4.

64. SIDRANSKY D. Circulating DNA. What we know and what we need to learn. Ann.N.Y.Acad.Sci. 2000: **906:** 1-4.

65. SILVA JM & BONILLA F. Correspondence re: F. Coulet et al., Detection of plasma tumor DNA in head and neck squamous cell carcinoma by microsatellite typing and p53 mutation analysis. Cancer Res., 60: 707-709, 2000. Cancer Res. 2001: **61:** 8595-8596.

66. SMIRAGLIA DJ, SMITH LT, LANG JC, RUSH LJ, DAI Z, SCHULLER DE, & PLASS C. Differential targets of CpG island hypermethylation in primary and metastatic head and neck squamous cell carcinoma (HNSCC). J.Med.Genet. 2003: **40:** 25-33.

67. STROUN M, ANKER P, LYAUTEY J, LEDERREY C, & MAURICE PA. Isolation and characterization of DNA from the plasma of cancer patients. Eur.J.Cancer Clin.Oncol. 1987: **23:** 707-712.

68. SU YH, WANG M, BRENNER DE, NG A, MELKONYAN H, UMANSKY S, SYNGAL S, & BLOCK TM. Human urine contains small, 150 to 250 nucleotide-sized, soluble DNA derived from the circulation and may be useful in the detection of colorectal cancer. J.Mol.Diagn. 2004: **6:** 101-107.

69. TANAKA N, ODAJIMA T, OGI K, IKEDA T, & SATOH M. Expression of E-cadherin, alpha-catenin, and beta-catenin in the process of lymph node metastasis in oral squamous cell carcinoma. Br.J.Cancer 2003: **89:** 557-563.

70. THOMAS GJ & SPEIGHT PM. Cell adhesion molecules and oral cancer. Crit Rev.Oral Biol.Med. 2001: **12:** 479-498.

71. TOYOTA M, AHUJA N, SUZUKI H, ITOH F, OHE-TOYOTA M, IMAI K, BAYLIN SB, & ISSA JP. Aberrant methylation in gastric cancer associated with the CpG island methylator phenotype. Cancer Res. 1999: **59:** 5438-5442.

72. TOYOTA M, HO C, AHUJA N, JAIR KW, LI Q, OHE-TOYOTA M, BAYLIN SB, & ISSA JP. Identification of differentially methylated sequences in colorectal cancer by methylated CpG island amplification. Cancer Res. 1999: **59:** 2307-2312.

73. TOYOTA M, ITOH F, KIKUCHI T, SATOH A, OBATA T, SUZUKI H, ISHII S, ENDO T, TOKINO T, & IMAI K. DNA methylation changes in gastrointestinal disease. J.Gastroenterol. 2002: **37 Suppl 14:** 97-101.

74. UHLMANN K, BRINCKMANN A, TOLIAT MR, RITTER H, & NURNBERG P. Evaluation of a potential epigenetic biomarker by quantitative methyl-single nucleotide polymorphism analysis. Electrophoresis 2002: **23:** 4072-4079.

75. VISWANATHAN M, TSUCHIDA N, & SHANMUGAM G. Promoter hypermethylation profile of tumor-associated genes p16, p15, hMLH1, MGMT and E-cadherin in oral squamous cell carcinoma. Int.J.Cancer 2003: **105:** 41-46.

76. WEI WI, YUEN AP, NG RW, HO WK, KWONG DL, & SHAM JS. Quantitative analysis of plasma cell-free Epstein-Barr virus DNA in nasopharyngeal carcinoma after salvage nasopharyngectomy: a prospective study. Head Neck 2004: **26:** 878-883.

77. WONG TS, CHANG HW, TANG KC, WEI WI, KWONG DL, SHAM JS, YUEN AP, & KWONG YL. High frequency of promoter hypermethylation of the death-associated protein-kinase gene in nasopharyngeal carcinoma and its detection in the peripheral blood of patients. Clin.Cancer Res. 2002: **8:** 433-437.

78. WONG TS, KWONG DL, SHAM JS, WEI WI, KWONG YL, & YUEN AP. Quantitative plasma hypermethylated DNA markers of undifferentiated nasopharyngeal carcinoma. Clin.Cancer Res. 2004: **10:** 2401-2406.

79. WONG TS, MAN MW, LAM AK, WEI WI, KWONG YL, & YUEN AP. The study of p16 and p15 gene methylation in head and neck squamous cell carcinoma and their quantitative evaluation in plasma by real-time PCR. Eur.J.Cancer 2003: **39:** 1881-1887.

80. WU X, ZHAO H, SUK R, & CHRISTIANI DC. Genetic susceptibility to tobacco-related cancer. Oncogene 2004: **23:** 6500-6523.

81. YEH KT, CHANG JG, LIN TH, WANG YF, TIEN N, CHANG JY, CHEN JC, & SHIH MC. Epigenetic changes of tumor suppressor genes, P15, P16, VHL and P53 in oral cancer. Oncol.Rep. 2003: **10:** 659-663.

82. YEH KT, SHIH MC, LIN TH, CHEN JC, CHANG JY, KAO CF, LIN KL, & CHANG JG. The correlation between CpG methylation on promoter and protein expression of E-cadherin in oral squamous cell carcinoma. Anticancer Res. 2002: **22:** 3971-3975.

83. ZIEGLER A, ZANGEMEISTER-WITTKE U, & STAHEL RA. Circulating DNA: a new diagnostic gold mine? Cancer Treat.Rev. 2002: **28:** 255-271.

84. ZUO C, AI L, RATLIFF P, SUEN JY, HANNA E, BRENT TP, & FAN CY. O6-methylguanine-DNA methyltransferase gene: epigenetic silencing and prognostic value in head and neck squamous cell carcinoma. Cancer Epidemiol.Biomarkers Prev. 2004: **13:** 967-975.