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Genome-wide alterations in CpG island methylation induced by arsenite

Alterazioni genomiche nella metilazione delle isole CpG indotte dall'arsenite

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Summary

Aberrant methylation of tumour suppressor CpG islands, resulting in epigenetic gene silencing, is common in human cancer. However, the mechanism responsible for inducing gene silencing remains unclear. Epidemiologic data have confirmed that the human carcinogen arsenic is associated with epigenetic silencing in several tumour-types. In this study, we set out to investigate the role exposure to arsenic plays in inducing CpG island methylation. To test the effects of the USA Environmental Protection Agency's drinking water arsenic limits (10 ppb), two clonal populations of immortalized human keratinocytes were treated with this level of arsenic, and methylation at CpG islands was compared to pooled, untreated keratinocytes using a 12K genome-wide CpG microarray. Islands with at least a 1.5-fold increase or decrease in methylation were examined. Comparing arrays from duplicate treated, clonal populations, a global decrease in methylation was seen, that involved more than 3000 CpG island loci, with a concomitant increase in methylation at over 500 CpG loci. Between arrays, the specific loci that exceeded the methylation change cutoff had > 99% concordance, demonstrating both reproducibility and specificity. Consistent with alterations from human tumours, this pattern of globally decreased CpG island methylation and local CpG island hypermethylation suggests that arsenic contributes to epigenetic alterations in human cancer. Eur. J. Oncol., 12 (1), 9-13, 2007

Key words: **DNA, CpG islands, methylation, arsenic, epigenetic silencing, keratinocytes**

Riassunto

La metilazione anomala delle isole CpG degli oncosoppressori, che determina un disattivazione epigenetica dei geni, è comune nei tumori umani. Tuttavia, il meccanismo responsabile della disattivazione genica rimane da chiarire. Dati epidemiologici hanno confermato che l'arsenico, cancerogeno per l'uomo, comporta una disattivazione epigenetica in diversi tipi tumorali. In questo studio ci siamo proposti di valutare il ruolo che gioca l'esposizione ad arsenico nell'indurre la metilazione delle isole CpG. Per determinare gli effetti dei limiti per l'arsenico nell'acqua da bere (10 ppb) fissati dall'Environmental Protection Agency degli USA, due popolazioni clonali di cheratinociti umani immortalizzati sono state trattate con questo livello di arsenico, e la metilazione delle isole CpG è stata confrontata con un *pool* di cheratinociti non trattati, usando *microarray* genomici 12K per le isole CpG. Sono state esaminate le isole con un aumento o una diminuzione della metilazione di almeno 1,5 volte. Confrontando i dati degli *arrays*, ottenuti in duplicato, per le popolazioni di cloni trattate, è stata osservata una riduzione globale della metilazione, che interessava più di 3000 loci di isole CpG, con un contemporaneo aumento della metilazione di oltre 500 loci CpG. Confrontando gli *arrays*, i loci specifici che superavano il *cutoff* di alterazione della metilazione avevano una concordanza di > 99%, dimostrando così riproducibilità e specificità. In accordo con le alterazioni dei tumori umani, questo *pattern* di metilazione delle isole CpG complessivamente diminuita e di locale ipermetilazione di isole CpG suggerisce che l'arsenico contribuisce alle alterazioni epigenetiche nei tumori umani. Eur. J. Oncol., 12 (1), 9-13, 2007

Parole chiave: **DNA, isole CpG, metilazione, arsenico, disattivazione epigenetica, cheratinociti**

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Introduction

Epigenetic gene inactivation, associated with DNA methylation, occurs in virtually all types of human cancer. Methylation occurs via addition of a methyl-group to cytosine in a CpG dinucleotide and can result in silencing of transcription when occurring in the context of a CpG island in the promoter region of a gene[1]. Approximately half of all human genes contain a CpG island that, when hypermethylated, may silence gene expression. Aberrant CpG island methylation is associated with the recruitment of methyl-binding domain proteins and subsequently, histone deacetylases. The deacetylated histones alter the chromatin conformation to its heterochromatic form that is not permissible to transcription. However, the mechanisms that target specific CpG islands for hypo- or hypermethylation are not understood. Interestingly, although gene-specific CpG island hypermethylation is common in cancer, global hypomethylation is far more prevalent[2]. The mechanisms contributing to *de novo* methylation losses and gains in cancer are unknown, despite well-studied associations between exposures and methylation alterations. Nonetheless, methylation-induced silencing has been demonstrated to be related to carcinogen exposure in lung cancer and various other solid tumour types[3-6].

One example of a ubiquitous human carcinogen to which humans are exposed is the nongenotoxic carcinogen arsenic. Humans are exposed to arsenic through drinking water and it is known to accumulate in skin and hair with chronic exposure[7]. Although arsenic exposure has been associated with cancer of the lung, bladder, and skin, the mechanism by which it contributes to carcinogenesis remains unclear[8, 9]. However, since oxidative methylation of arsenic is required for its metabolism *in vivo,* arsenic exposure may alter the methyl-donor pool enough to cause aberrant changes in CpG island methylation. In fact, *in vitro* inorganic arsenic exposure has been recently associated with both hypermethylation of CpG sites as well as global hypomethylation[10-12]. In addition, a significant relationship between arsenic exposure and tumour suppressor hypermethylation has been described in bladder cancer[13].

In order to further assess whether arsenic contributes to aberrant methylation losses and gains we treated primary human keratinocytes with the United States Environmental Protection Agency's (US EPA) current drinking water arsenic limit of 10 ppb, and then characterized the genome-wide pattern of CpG island methylation in arsenite treated *versus* untreated cells.

Materials and methods

Cell culture

Primary human keratinocytes immortalized by transfection with hTERT (courtesy J. Rheinwald, Harvard Medical School) were maintained in keratinocyte serum-free medium from GIBCO (Invitrogen, Carlsbad, CA, USA) supplemented with 25 μg/ml bovine pituitary extract, 0.2 ng/ml epidermal growth factor, 0.4 mM $CaCl_2$ and antibiotics. Clonally derived cells were expanded in the presence of 10 ppb sodium arsenite with a treatment period of 14 days. Both treated and untreated cells were harvested, pelleted and subjected to DNA extraction with a DNeasy tissue kit (Qiagen, Valencia, CA, USA).

DNA preparation

Primary human keratinocyte DNA was digested with *MseI* for fragmentation. Linker primers H12 5'-TAATCCCTCGGA-3' and H24 5'-AGGCAACTGTGCTATCCGAGGGAT-3' were ligated to the fragmented DNA and a test PCR was conducted with H24 primer comparing ligated and unligated DNA to verify H12 and H24 linkage. Following ligation DNA samples were cleaned with a Qiagen MinElute PCR purification kit (Qiagen, Valencia, CA, USA). Samples were then digested with methyl-sensitive CpG cutting enzyme *BstUI* which will not cut at CpG sites if methylated. This digestion was followed by alternate CpG site cutting enzyme, *HpaII*. Digest products were amplified by PCR with H24 primer followed by amino-acyl dUTP incorporation. PCR products were then labelled with fluorescent dyes Alexa555 for untreated samples and Alexa647 for sodium arsenite-treated samples.

CpG island microarray

Human CpG island 12K arrays with CpG islands associated with approximately 7,000 unique genetic loci were purchased from Microarray Centre (Toronto, Canada), for CpG island differential methylation hybridization analysis. Fluorescently labelled DNAs were mixed and incubated with 100 μl DIG Easy hybridization solution (Roche, Indianapolis, IN, USA), 5 μl of 10 mg/ml yeast tRNA (Invitrogen), and 5 μl of 10 mg/ml calf thymus DNA (Sigma-Aldrich) for 2 minutes at 65°C and then transferred onto the array. CpG island arrays were washed, dried and scanned with a GenePix 4000B slide scanner and analyzed with GenePix Pro 6.0 software. Array results from multiple treated keratinocyte clones were independently normalized to internal alien DNA

control features. Cutoff values corresponding to either a 1.5-fold increase or 1.5-fold decrease in methylation were derived from the ratio of median dye intensities following independent normalization of arrays.

Results

Following clonal expansion of keratinocytes either in medium with 10 ppb sodium arsenite or without, cells were harvested, DNA was extracted, digested, and then annealed to linker primers. Following a test PCR reaction, samples were subjected to digestion with two methylation sensitive enzymes and amplified to incorporate an additional linker, and then a fluorescent dye to indicate treatment status. Finally, samples were hybridized to the 12K CpG island array, which is spotted with CpG islands associated with approximately 7,000 unique gene loci. Two arrays were hybridized with independent, clonally-expanded treated keratinocyte DNA and pooled untreated keratinocyte DNA (fig. 1). Arrays were then scanned, and subjected to independent normalization. Following signal normalization, cutoff values corresponding to either a 1.5-fold increase or 1.5-fold decrease in methylation were derived from the ratio of median dye intensities. We found that the same number of CpG island loci, 3058, had a 1.5-fold or more decrease in methylation in both arrays, comparing sodium arsenite treated keratinocytes to controls. Furthermore, 503 CpG island loci had a 1.5-fold or more increase in methylation in both arrays comparing sodium arsenite-treated keratinocytes to controls. These results indicate a pattern of widespread CpG island hypomethylation, with a simultaneous and far less extensive CpG island hypermethylation.

When comparing the list of CpG island loci that exceed the cutoff for increased methylation between the two arrays, the lists are nearly identical, having 99% similarity. Additionally, the list of CpG island loci that exceed the cutoff for decreased methylation between the two arrays had 99% similarity, indicating that sodium arsenite treatment gives highly reproducible changes in methylation levels at specific CpG islands. Although the specific loci were very similar, the rank order of loci on these lists between arrays was less similar, but this can be explained by the inherent variability in hybridization between arrays that cannot be entirely accounted for with statistical normalization techniques. Among CpG island loci with increased methylation, a number of tumour suppressor and putative tumour suppressor loci were observed, a selected list of which is presented in Table 1. Among CpG island loci with decreased methylation, many oncogenes and putative oncogenes were included and a selected list is presented in Table 2.

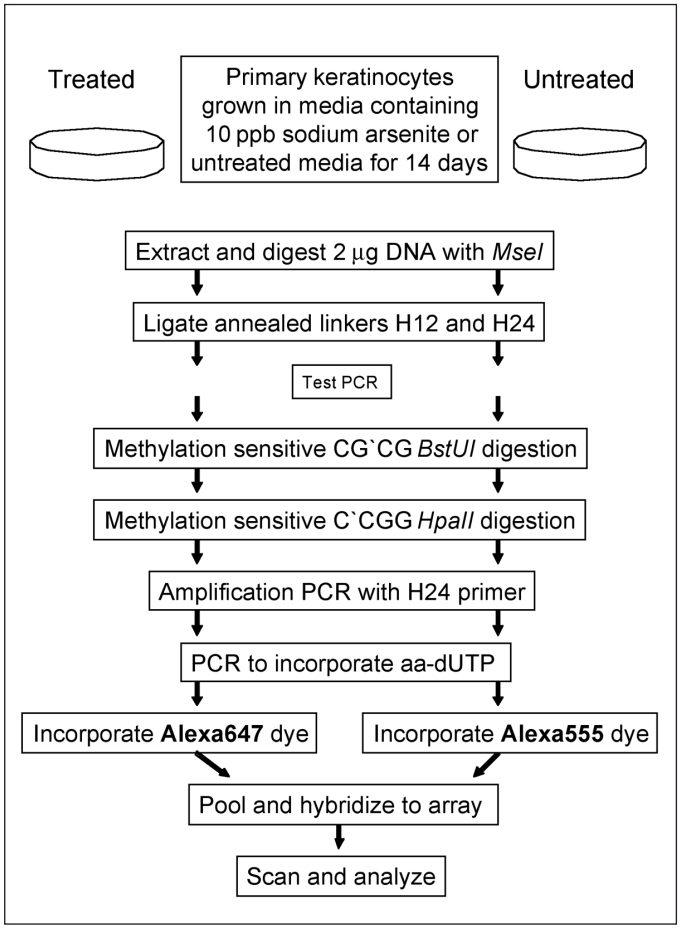

Fig. 1. Array-based differential methylation hybridization assay design

Table 1 - Selected tumour suppressor and putative tumour suppressor loci with increased CpG island methylation in sodium arsenite-treated keratinocytes

Gene	Description	Ratio of medians treated / untreated
TP53BP1	Tumour protein p53 binding protein 1	3.67
NKAP	Nuclear NF-kappaB activating protein	2.38
PCAF	p300/CBP-associated factor	2.26
TNRSF10B	Tumour necrosis factor receptor superfamily, member 10b	2.05
HOXD10	Homeo box D10	1.75
TP53	Tumour protein p53	1.72
CDON	Cell adhesion downregulated by oncogenes	1.60
MDC1	Mediator of DNA damage checkpoint 1	1.53

Table 2 - Selected oncogene and putative oncogene loci with decreased CpG island methylation in sodium arsenite-treated keratinocytes

Gene	Description	Ratio of medians treated / untreated
RAB21	Member of *RAS* oncogene family	2.78
ETS1	*v-ets* avian erythroblastosis virus E26 oncogene homolog 1	2.63
RAB6A	Member of *RAS* oncogene family	1.89
ABL1	*v-abl* Ableson murine leukaemia viral oncogene family	1.89
BC068522	*v-jun* avian sarcoma virus 17 oncogene homolog	1.82
RAB38	Member of *RAS* oncogene family	1.79
M13930	*c-myc* oncogene	1.75
ERBB4	*v-erb-a* erythroblastic leukaemia viral oncogenes homolog 4	1.75

Discussion

Our results demonstrate a pattern of CpG island methylation that is consistent with general pattern from human tumours; where both global hypomethylation, and site-specific CpG island hypermethylation occur simultaneously. Since these results are taken from independent sodium arsenite-treated keratinocyte clones, which were compared to pooled untreated keratinocytes, and the concordance among normalized loci exceeding cutoff values is extremely high, our results are both reproducible and very specific.

Although the genome-wide CpG island array is a powerful tool, it has limitations. It is well established that CpG island hypermethylation can silence gene expression, and that hypomethylation can induce gene expression; however, the resolution of the CpG island microarray does not allow for conclusions regarding changes in expression. Individual loci known to be hypermethylated on the array can be subjected to methylation specific polymerase chain reaction to investigate gene silencing, or to reverse transcription polymerase chain reaction to investigate gene expression changes in future experiments. Furthermore, an array-array approach combining the CpG island with an expression microarray experiment could also address this issue. The specific limitation of inferring expression changes from these data can be illustrated as follows: the *TP53* locus was hypermethylated, however it is known that this critical tumour suppressor gene cannot be silenced by hypermethylation. One caveat is that partial CpG island methylation can reduce gene expression without complete silencing, yet this finding prompts the question of whether the definition of a CpG island must include the ability to completely silence gene expression. Nonetheless, the genome-wide CpG island microarray is a powerful tool that has allowed us to observe that arsenic exposure alters CpG island methylation levels in a pattern that recapitulates alterations observed in human tumours.

The mechanism by which arsenic exposure contributes to the observed changes in methylation is not known. However, the widespread hypomethylation in arsenic treated keratinocytes may be related to a reduction in the available methyl-donor pool from metabolism of arsenic. Less well understood is how exposure to arsenic results in hypermethylation of some CpG island loci. Also, the CpG island loci with altered methylation levels were highly similar between the two independent arrays, indicating that some loci may be specifically targeted, or more susceptible to changes in methylation; however, the mechanism by which this occurs remains obscure.

The results presented in this study agree with the previously mentioned *in vitro* studies of arsenic and DNA methylation[10-12]. However, our work, using an array-based approach, investigates CpG island methylation levels on a genome-wide scale, as well as using a low treatment level of arsenic that matches the new US EPA limit for drinking water arsenic of 10 ppb. Importantly, this level of arsenic treatment results in global hypomethylation, as well as more localized CpG island hypermethylation, a pattern that matches what is observed in tumour cells. Therefore, our work adds to evidence suggesting arsenic participates in epigenetic events that contribute to carcinogenesis even with a low-dose, short-duration treatment.

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