DNA methylation in the promoter region of the p16 (CDKN2/MTS-1/INK4A) gene in human breast tumours

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Summary The p16 (CDKN2/MTS-1/INK4A) gene is one of several tumour-suppressor genes that have been shown to be inactivated by DNA methylation in various human cancers including breast tumours. We have used bisulphite genomic sequencing to examine the detailed sequence specificity of DNA methylation in the CpG island promoter/exon 1 region in the p16 gene in DNA from a series of human breast cancer specimens and normal human breast tissue (from reductive mammaplasty). The p16 region examined was unmethylated in the four normal human breast specimens and in four out of nine breast tumours. In the other five independent breast tumour specimens, a uniform pattern of DNA methylation was observed. Of the nine major sites of DNA methylation in the amplified region from these tumour DNAs, four were in non-CG sequences. This unusual concentration of non-CG methylation sites was not a general phenomenon present throughout the genome of these tumour cells because the methylated CpG island regions of interspersed L1 repeats had a pattern of (almost exclusively) CG methylation similar to that found in normal breast tissue DNA and in DNA from tumours with unmethylated p16 genes. These data suggest that DNA methylation of the p16 gene in some breast tumours could be the result of an active process that generates a discrete methylation pattern and, hence, could ultimately be amenable to therapeutic manipulation.

Keywords: breast tumour; p16; CpG island; DNA methylation; genomic sequencing; SP1 sites

Alterations in the function of multiple genes are required for the full development of oncogenic phenotype in humans and other mammals (Knudson, 1971; Vogelstein et al, 1987). In the case of tumour-suppressor genes, it is now clear that loss of function can not only occur through allelic loss or mutation, but also through loss of function mediated by DNA methylation (Baylin, 1992; Szyf, 1994; Merlo et al, 1995). The p16 gene (CDKN2/MTS-1/INK4A) is one such gene. This gene acts to inactivate CDK4 and CDK6 cyclin-dependent kinases and, hence, controls the entry of cells from G1 to S-phase (Kamb, 1995; Hara et al, 1996). As such, it is a prime candidate as a tumour-suppressor gene. However, whereas this gene was found to be subject to frequent homozygous deletion in tumours, point mutations were not commonly detected. Consequently, the role of p16 as a tumour suppressor was questioned until it was shown that the p16 gene was also frequently inactivated through a process involving DNA methylation without point mutation (Merlo et al, 1995; Herman et al, 1996a).

In human breast tumours, it has been reported that > 30% of primary tumours and many tumour-derived cell lines have inactive methylated copies of the p16 gene (as assayed at only a few sites using methylation-sensitive restriction endonucleases) (Merlo et al, 1995). Herman et al (1996b) have further shown by bisulphite genomic sequencing that in a tumour cell line (H157) a region of the p16 gene becomes completely methylated at all CG dinucleotides. Extrapolating from this finding, they developed an assay that will detect a very small proportion of tumour cells containing methylation at all CG sites as a sensitive method of detecting minimal residual disease. This method uses polymerase chain reaction (PCR) primers that amplify specifically from the fully CG-methylated form of the gene after bisulphite conversion of unmethylated cytosines to uracils (Frommer et al, 1992). Using this assay, they have demonstrated the presence in a series of human tumour-derived cell lines and in some human tumours of apparently fully CG-methylated forms of p16 and other genes (Herman et al, 1996b).

Here, we report the results of genomic sequencing from DNA from a series of human breast samples, both normal and tumour, and demonstrate a common specific pattern of tumour-related DNA methylation in a number of independent human breast tumours. Rather than a non-specific methylation at CG dinucleotides in this gene, this methylation has characteristics that could better be explained as the product of a gene-specific alteration in the local secondary structure of the promoter/exon 1 region that transforms it into a highly efficient substrate for asymmetric de novo methylation.

MATERIALS AND METHODS

Human tissue specimens were portions of samples provided for routine pathology assessment that were in excess of the requirements for that purpose and were obtained with the approval of the relevant ethics committees. All samples were stored at −70°C until DNA was extracted by the guanidine isothiocyanate/caesium chloride method for the isolation of both RNA and DNA (Chirgwin et al, 1979), and further purified by extraction with phenol/chloroform. For genomic sequencing of sites of DNA methylation, 5 µg of genomic DNA was modified with metabisulphite after alkali denaturation using five or six cycles of 94°C for 3 min, 55°C for 57 min and subsequently desulphonated as described previously.
RESULTS

In the bisulphite method for the genomic sequencing of sites of DNA methylation, unmethylated cytosines are converted to uracils and, through subsequent PCR amplification, to thymines (Frommer et al., 1992). After this process, the two strands of any DNA sequence after bisulphite modification.

...methylation of most DNA from that sample. In this study, PCR primers for the p16 gene were designed so that they would be able to prime amplification from both modified and unmodified (native) forms of the target sequence, irrespective of methylation status of any or all cytosines in the primer sequences (Woodcock et al., 1997, 1998). This was achieved by choosing a site for the forward primer that was rich in guanines and contained the minimum number of cytosines (and also lacked simple sequence tracts). Any cytosine in the PCR forward primers was synthesized as a degenerate site (C or T), irrespective of dinucleotide context.

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was not observed in any of the clones from the other modified DNAs. In these two distinct methylation patterns observed in the ts34 clones, base 244 was the only methylated site common to both patterns (Figure 2). In the remaining sets of tumour-derived p16 clones that did not exhibit discrete methylation patterns, there was a slightly higher proportion of (apparently random) methylated bases present overall than in clones from normal breast DNAs. However, methylation levels were somewhat lower overall than in the tumours with discrete methylation.

In the tumour-derived clones exhibiting discrete methylation patterns, a high proportion of residual cytosines (methylation sites) were on non-CG contexts (Figures 1 and 2). In the predominant tumour methylation pattern, five out of nine sites in the region amplified were in m CG dinucleotides, but three out of nine were in m CTG contexts and one in what would have been m CCTTCG. In the secondary methylation pattern found in some clones from ts34, the original sequence contexts would have been m CACG, m CTG and m CACCG. The few random residual cytosines that were observed in clones from normal breast DNAs were almost exclusively in the context of CG dinucleotides (not illustrated).

To test whether this unusually high proportion of non-CG methylation observed in the p16 gene from these tumour DNAs was not observed in any of the clones from the other modified DNAs. In these two distinct methylation patterns observed in the ts34 clones, base 244 was the only methylated site common to both patterns (Figure 2). In the remaining sets of tumour-derived p16 clones that did not exhibit discrete methylation patterns, there was a slightly higher proportion of (apparently random) methylated bases present overall than in clones from normal breast DNAs. However, methylation levels were somewhat lower overall than in the tumours with discrete methylation.

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might reflect some widespread deregulation (or misregulation) of the methylation processes, DNA sequences from the methylated CpG island region of human L1 dispersed repeat elements (Woodcock et al., 1997) were amplified, cloned and sequenced. This allowed the sampling from multiple dispersed sites of methylation through the genome rather than from just a single site as would be the case with a unique protein coding sequence. In clones from these sequences amplified from bisulphite-modified DNAs from normal breast samples, a total of 37 consistently methylated sites were observed in a 460-bp region with an average 55.5% residual cytosines at these sites. (These are observed frequencies of cytosines only with no correction having been made for sequence variations within the repeat sequence family.)

L1 clones from two tumour DNAs with basically unmethylated p16 genes (ts21 and ts30) had 34.0% residual cytosines present on average at these sites, whereas the average for the clones from the tumours with methylated p16 was 42.5% residual cytosines. The methylation levels in dispersed repeats are likely to reflect average methylation levels in total genomic DNA, and are consistent with previous observations that tumours frequently have lower total genomic methylation levels which may be accompanied by increased methylation in some normally unmethylated CpG island regions (such as the p16 promoter) (reviewed by Szyf, 1994). The relative levels of methylated cytosines at each of these consistently methylated sites in L1 elements from DNAs from normal and tumour-derived DNAs (with and without p16 methylation) are shown in Figure 3. All 37 of the consistently methylated sites in clones from the L1 repeats from both normal and tumour DNAs were in CG dinucleotides. However, there were some methylated non-CG sites observed. The occurrence of sites of non-CG methylation, however, was suggestive of individual polymorphisms in the control of epigenetic modification. For example, seven out of nine clones from one normal breast sample (nb1) had a 5′-C-CA sequence. This site was also methylated in four out of nine and one out of ten clones from two other sets of normal breast-derived clones (nb2 and nb5 respectively), but absent from clones from the fourth normal breast sample and also from all tumour-derived clones. Also, the tumour DNA ts30 in which the p16 gene was unmethylated had two 5′-CAG sites, one 5′-CTT and one 5′-CATTAG site in up to one-third of clones (not illustrated). In addition, at site no. 15 in ts30 clones, 0 out of 12 clones had a residual cytosine at this CG dinucleotide. Rather, five of these clones had a residual cytosine as the prior base (i.e. originally 5′-CCG rather than C-CCG).

**DISCUSSION**

Using genomic sequencing of DNAs from human clinical specimens, we have observed a common pattern of DNA methylation in the p16 gene from a series of independent breast tumour samples. This methylation pattern is of unusual sequence specificity and contains a high proportion of methylation at asymmetric sites. We consider that this unusual methylation pattern is not an experimental artefact for the following reasons:

1. The PCR product from modifications of human genomic DNAs were not used unless clones from the control plasmid were effectively modified (<0.2% residual cytosines excluding sites of endogenous E. coli dcm methylation) (Woodcock et al., 1997).

2. In clones from modified DNAs from four normal breast samples and from four of the breast tumours, the p16 gene was essentially unmethylated (in a total of 72 independent clones comprising 33 from normal breast samples and 39 from four tumour samples). Thus, methylation at the specific set of sites observed in these five breast tumours is specific to DNA from these tumours and cannot represent some region that is intrinsically resistant to bisulphite modification.

Hence, it appears that the residual cytosines present in clones from modified DNAs from some human breast tumours represent a characteristic tumour-specific epigenetic modification to the DNA. Although clones from one of the tumour specimens showed evidence of a second pattern of DNA methylation in the p16 gene, clones with the common predominant pattern of methylation were also present. (It is conceivable that these two methylation patterns represent two independent lines of oncogenic evolution in this particular tumour.)

Herman et al (1996b) have shown that, in some human tumour-derived cell lines and some clinical specimens, the p16 gene can be present with complete methylation of CG dinucleotides. We cannot say from these data that the breast tumour DNAs used in this study do not contain some p16 sequences with complete methylation at all of the CG dinucleotides. If copies of this gene with complete CG methylation were present at, for example, <10% of the frequency of sequences with the methylation pattern we have observed, we would have a low probability of recovering them, considering also that these clinical samples contain a mixture of normal cells. However, we have shown previously that

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**Figure 3** Proportion of clones with residual cytosines in each of the consistently methylated sites from the methylated CpG island region from human L1 dispersed repeats. Values are given as percentage of the total clones with cytosines averaged over all the clones from normal breast DNAs ( ), the clones from tumours with methylated p16 gene ( ), and the clones from tumour DNAs where p16 exhibited low levels of random methylation (as in normal breast) ( ). The x-axis represents the numbering from 5′ to 3′ of the consistently methylated CG sites. Sets of full sequence data relating site number to base number are available on request.
the PCR amplification and cloning methodology employed here does not bias the data through the selective recovery of clones from less methylated forms of the target sequence (Woodcock et al, 1997). It has also been shown that, during adaptation of cells to in vitro culture, many normally unmethylated sites in CpG island sequences can become methylated (Antequera et al, 1990). The observation of complete methylation in all CG dinucleotides in the p16 gene from a human tumour-derived cell line is more likely to represent a later stage in the evolution of epigenetic modification of this CpG island rather than an artefact of in vitro culture. The ability to amplify from primary human tumours using PCR primers specific for the bisulphite-modified form of fully CG methylated p16 gene argues strongly for the presence of some cells within primary tumours with this pattern of methylation (Herman et al, 1996b). The absence of clones with such a methylation pattern in this study could be due to such cells being in relatively low abundance. This would not be inconsistent with total methylation at CG dinucleotides representing an important and characteristic stage of tumour evolution, however an assessment of the minimal residual disease using an assay that identifies cells with total methylation of p16 would fail to detect tumour cells with other patterns of methylation.

The major concentration of methylated sites in the p16 promoter/exon 1 region examined in this study is in a region containing two 9-bp direct repeats separated by 9 bp (Figure 1 and Figure 2). Overlapping with this central region and the 3′ repeat is a SP1 core consensus site (Minth and Dixon 1990; Thiesen and Bach, 1990; Merchant et al, 1991). Methylation in CG dinucleotides has been shown not to affect SP1 binding (Harrington et al, 1988). The presence of SP1 sites has also been shown to protect against de novo methylation and to induce demethylation adjacent to the SP1 site (Brandeis et al, 1994; Macleod et al, 1994; Silke et al, 1995). There are also two other SP1 consensus sites 70–100 bp upstream in two 24-bp tandem repeats with the more proximal repeat containing the p16 ATG initiation codon plus the first of the tumour-specific methylation sites. In this instance, this concentration of SP1 binding sites has not prevented tumour-specific methylation in this p16 CpG island region.

Methylation, particularly the high concentration of non-CG methylation observed in the p16 gene region, was not part of some general non-specific epigenetic modification of the tumour genomes. When we sampled the sequence specificity of methylation in multiple regions of the normal and tumour breast DNAs using the CpG island region from the 5′ end of human L1 repeats, methylation was consistently found at more sites than we observed previously in L1 elements from non-transformed human embryonic fibroblasts (Woodcock et al, 1997). In this L1 region, 29 consistently methylated sites were observed in embryonic fibroblast DNA as opposed to the 37 observed in this study with DNA from normal human breast. Also, for the L1 elements from embryonic fibroblasts, 4 out of 29 consistently methylated positions were in non-CG sites, whereas, in this study, the consistently methylated sites were all in CG dinucleotides. However, some specific sites of non-CG methylation were present but these seemed to be specific to the individual, and may represent polymorphisms in genetic factors that determine epigenetic events. However, overall, there is no evidence for a general increase in non-CG methylation in the genome of those tumours that have a high proportion of non-CG methylation in their p16 genes.

There is now evidence that mice (and presumably all other mammals) have more than one DNA methyltransferase gene. ES cells from complete knockouts of the known DNA methyltransferase gene retain residual DNA methylation as well as the ability to methylate sequences de novo (Lei et al, 1996; Tucker et al, 1996). This residual DNA methyltransferase gene has been suggested to represent the activity responsible for the wave of de novo methylation that occurs in the genome of the preimplantation embryo (Monk, 1990). However, the discrete methylation patterns observed in p16 gene from tumour DNAs with their high proportion of methylation at non-CG sites is very different from the specificity of methylation in methyltransferase-knockout ES cells. This methylation is apparently totally CG-specific and it appears to be randomly positioned within the set of sites that are methylated in the normal adult mouse genome (Woodcock et al, 1998). Rather, the high proportion of non-CG methylation in the tumour p16 genes is more likely to be related to the process whereby sites of CNG methylation in transfected plasmid DNAs can be maintained over many cell generations in mouse cells in culture (Clark et al, 1995).

For a distinct methylation pattern to be present, there must be some mechanism that efficiently restores this methylation in the daughter strands after each round of replication. Otherwise, such a methylation pattern would rapidly be lost. Maintenance of DNA methylation at any site in mammalian genomes in non-CG sites is conceptually inherently more difficult than that in CG dinucleotides in which there is the symmetrically placed +CG template in the parental strand (Holliday and Pugh, 1975; Riggs, 1975). One possible exception is methylation in CNG sequences (as in plant DNAs) in which a parental template would be displaced by only one base, although this is not a documented function of the mammalian DNA methyltransferase. However, it has also been shown that distortions of the DNA duplex that promote the extra-helical extrusion of any cytosine base will render such a cytosine an efficient substrate for de novo methylation (Smith et al, 1992; Klimasauskas and Roberts, 1995; Laayoun and Smith, 1995). We suggest that one possible explanation for the discrete pattern of DNA methylation observed in the p16 gene in breast tumour DNAs is that it is the result of sequence-specific protein binding that induces the DNA duplex in this region to be distorted, rendering certain specific bases efficient substrates for asymmetric de novo methylation. If this were the case, inactivation of some tumour-suppressor genes through DNA methylation would, thus, be an active process in the tumour rather than through random methylation at CG dinucleotides and, hence, be more amenable to ultimate therapeutic manipulation.

ACKNOWLEDGEMENTS

We would like to thank Dr Michael Henderson for his help both in obtaining tumour samples and in organizing such collections from other sources. This study was partially financed by a grant to DMW from the National Health and Medical Research Council of Australia.

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