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Original Paper

Patterns of DNA Methylation of the Parathyroid Hormone-related Protein Gene in Human Lung Carcinoma

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Humoral hypercalcaemia of malignancy often results from production of parathyroid hormone-related protein (PTHrP) by the tumour. We have investigated whether malignancy is associated with epigenetic changes in the *PTHrP* gene in lung. In normal and tumour tissue, there was a general background of nonmethylation in the *PTHrP* gene. In the 5′ region, there appeared to be increased methylation of sites upstream of the promoter, P2. The extent of methylation increased from germ line to normal tissue to tumour tissue to tumour cell line, indicating that new methylation events in this region mark neoplastic change in lung cells.

Key words: parathyroid hormone-related protein, DNA methylation, lung carcinoma
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INTRODUCTION

HUMORAL HYPERCALCAEMIA of malignancy (HHM) often results from production of a parathyroid hormone-like protein by the tumour [1]. The parathyroid hormone-related protein (PTHrP) and parathyroid hormone are structurally distinct [2], but, as 8 of the first 13 amino acids are identical, they bind to the same receptor and have equivalent hypercalcaemic action [3]. Why PTHrP is overproduced by certain tumours is unclear; although a number of agents influence PTHrP production (reviewed in [4]), the main mechanisms of *PTHrP* gene regulation have not been clarified.

One mechanism by which gene activity is modulated is through methylation of cytosine residues within CpG dinucleotide sequences in the DNA [5]. The 5′ region of the *PTHrP* gene contains a high proportion of these dinucleotides in a "CpG island" [6] upstream of exon Ic, but it is unknown whether changes in the methylation status of the region are associated with gene expression. An early and consistent molecular change in neoplasia is an alteration in cytosine methylation in DNA (reviewed in [7]); consequently, this study examined the methylation status of the 5′ region of the *PTHrP* gene in relation to tumorigenesis.

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MATERIALS AND METHODS

Humoral hypercalcaemia and PTHrP production are commonly associated with squamous cell carcinoma of the lung [8]. Hence, lung tissue was chosen as a model system, using normal (post-mortem) lung, resected lung carcinoma and an established cell line BEN, derived from epidermoid bronchial carcinoma [9]. BEN cells are known to express the *PTHrP* gene [10].

The methylation status of the *PTHrP* gene was first investigated by restriction enzyme analysis, using a pair of isoschizomeric enzymes differentially sensitive to methylation. *Msp*I will cut whether or not the central C of the CCGG restriction site is methylated, whereas *Hpa*II will only cut if this C is unmethylated.

Genomic DNA was prepared by the method of Herrmann and Frischauf [11] or Gill and associates [12]. DNA was predigested with *Hind*III (5U/μg DNA) overnight at 37°C, extracted, then similarly digested with *Msp*I or *Hpa*II. 15 μg of DNA was fractionated by agarose electrophoresis and alkali blotted on to Hybond N+ (Amersham). Blots were prehybridised, then hybridised with a radioactive probe specific to the 5′ region of the *PTHrP* gene (Figure 1). Blots were washed and autoradiographed using standard procedures [14].

Enzyme analysis could only elucidate the methylation status of a small proportion of the CpG dinucleotides. A detailed analysis of the methylation status was determined by sequencing two fragments, one within, and one remote from the CpG island. Sequencing was used to detect methylation changes after chemical modification of the DNA [15]. Sodium bisulphite converts cytosine residues to uracils where 5-methylcytosines remain unreactive. Following amplification by PCR, the uracils are amplified as thymines, while 5-methylcytosine residues are

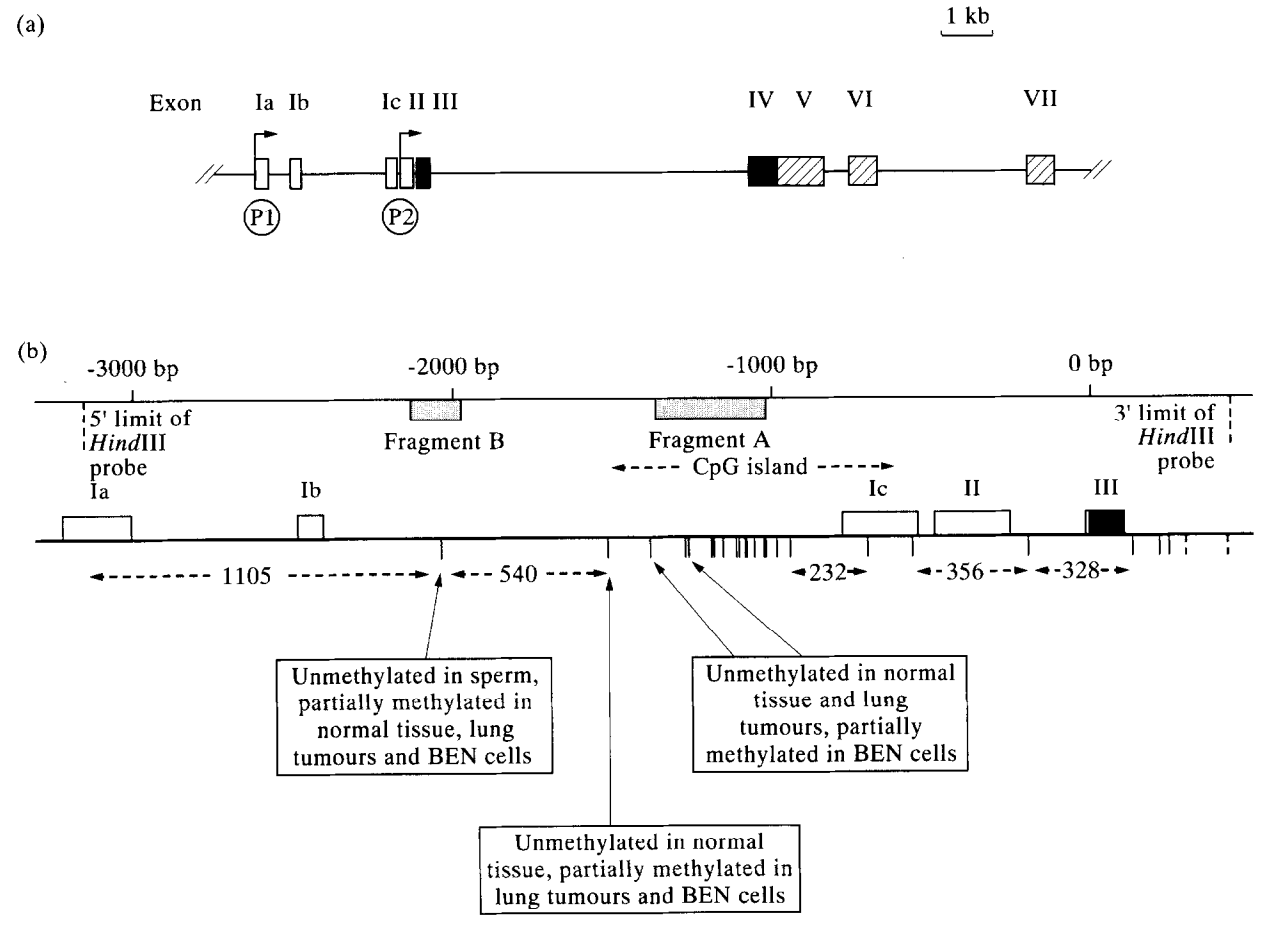

Figure 1. (a) Organisation of the human *PTHrP* gene. Exons are indicated by boxes designated by a Roman numeral. Open boxes are 5'-noncoding regions, solid boxes are coding regions and hatched boxes are 3'-noncoding regions. P1 and P2 designate the upstream and downstream promoters, respectively. Other transcription initiation sites lying between P1 and P2 may also be used. (b) Detailed structure of the 5' region of the *PTHrP* gene. Limits of the restriction enzyme probe and position of the amplified fragments are marked. The frequency of *MspI*/*HpaII* (CCGG) sites are illustrated by short vertical bars below the gene. Dashed lines indicate possible CCGG site, as determined by Suva and associates [13] or from sequence data (not shown).

still read as cytosines. 2 μg genomic DNA was modified using sodium bisulphite as described by Frommer and associates [15]. Following amplification, fragments were sequenced using end-labelled ([γ-^{33}P]ATP) primers in a cycle DNA sequencing reaction (Promega).

RESULTS

Restriction enzyme analysis

In a number of normal tissues (Figure 2a), three *Msp*I fragments of 350 bp, 540 bp and 1.1 kb could be localised to the upstream region of the *PTHrP* gene. From the published sequence, it appears that the two larger fragments covered the upstream end of this region from -3155 to -2050 and -2050 to -1510, respectively, whilst two 350 bp fragments could be localised to exons II and III (see Figure 1b). In sperm, digestion with *Hpa*II gave similar patterns to *Msp*I indicating that the CCGG sites detected are unmethylated. However, in lung, an extra 1.7 kb band was seen with *Hpa*II digestion indicating at least one methylated site within the 5' region. If this 1.7 kb band is the product of the 540 bp and 1.1 kb band, then this suggests partial methylation of the CCGG site of -2050.

The methylation pattern of the *PTHrP* gene from BEN cells was very different from normal tissue. The 540 bp and 1.1 kb *Msp*I fragments were replaced by a cluster of bands between 1.6 and 2.0 kb, suggesting methylation of CCGG sites between -2050 and -1300 as well as the -2050 site. *PTHrP* mRNA could readily be detected, indicating transcriptional activity in the *PTHrP* gene of these BEN cells (Figure 2a).

Digestion of lung tumour samples produced similar fragment patterns to normal lung. However, in five out of six squamous cell carcinomas and one adenocarcinoma there was an extra band of 1.8 kb in the *Hpa*II digest. Since the 1.7 kb fragment in normal lung spanned -3155 to -1510 and position -3155 represents the probe 5' boundary, the 1.8 kb band must be due to further methylation of the -1510 CCGG site.

5-Methylcytosine sequencing analysis

The CpG methylation status of two fragments, one within, and one remote from the CpG island, were analysed by sequencing. Fragment B was amplified from the upstream region, bases -2123 to -1971 (Figure 2b). In sperm DNA, the six CpG dinucleotides within this region remained completely unmethylated. In normal lung and lung tumour, these sites were partially methylated, but with a trend to increased methylation in the tumour samples. In the BEN cells, the six CpG dinucleotides were completely methylated.

In the CpG island fragment, bases -1356 to -1010 (fragment A), the results were very different (Figure 2c). In germ line,

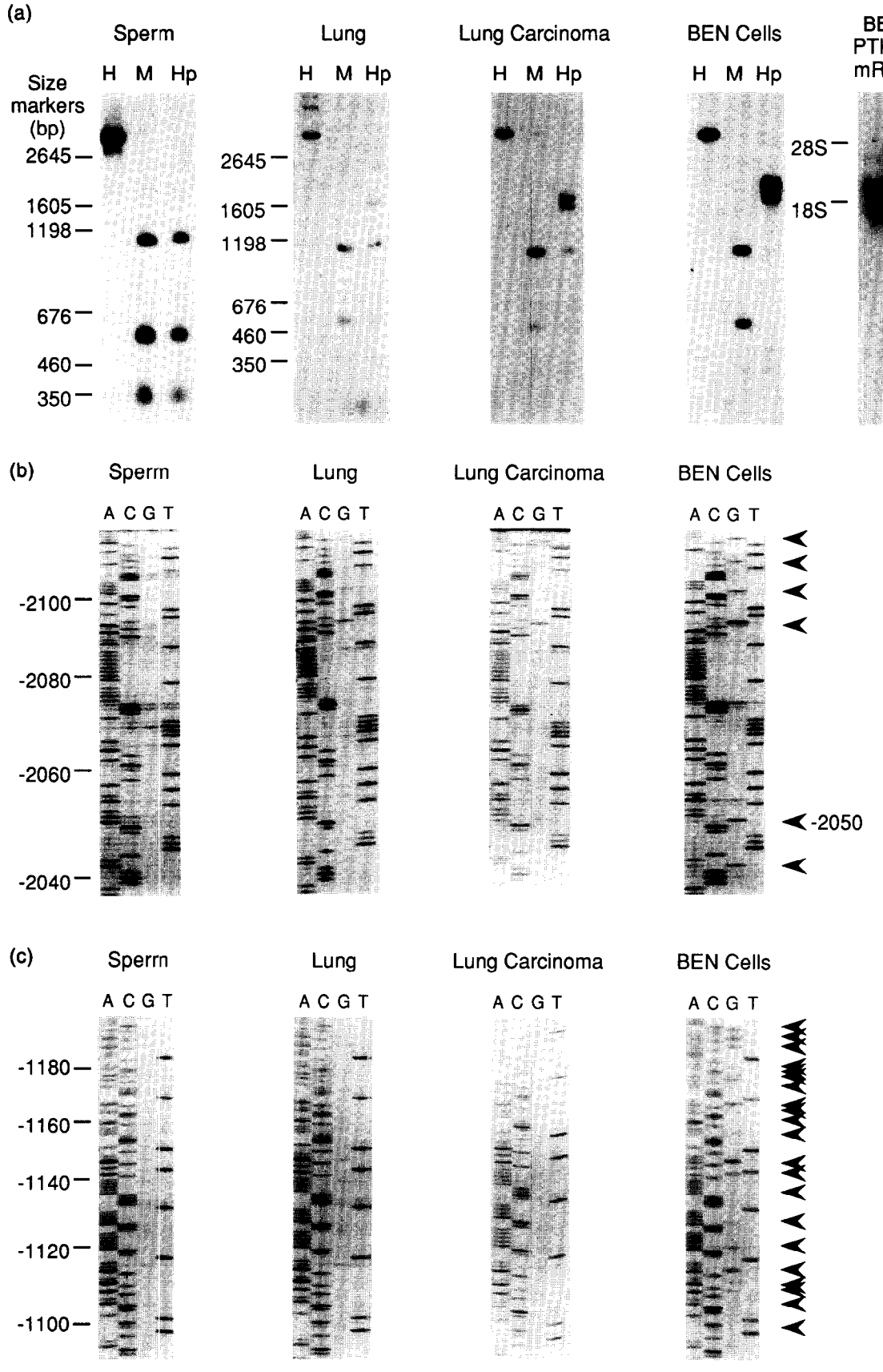

Figure 2. Methylation status of the 5′ region of the *PTHrP* gene in sperm, normal lung, lung carcinoma and the BEN cell line. (a) DNA methylation was investigated by restriction enzyme fragment analysis. H, *Hind*III, M, *Msp*I, Hp, *Hpa*II. Using pGEM markers, the fragment sizes (mean ± S.D.) were calculated: *Msp*I digest: 1104 ± 56 (average number of samples = 8), 538 ± 29 (8), 343 ± 17 (8); *Hpa*II digest: 1825 ± 50 (4), 1680 ± 76 (5), 1120 ± 45 (5), 532 ± 19 (5), 342 ± 8 (5). BEN cell poly(A) + RNA was electrophoresed on a denaturing agarose gel, Northern blotted and hybridised with a full length gene probe. (b) 5-methylcytosine sequencing of the *PTHrP* gene upstream region (fragment B). Numbers on the left indicate the upstream nucleotide position. Arrows on the right mark the position of methylatable cytosines in CpG dinucleotide sequences. Sequencing of the complementary strands means methylated cytosines are seen in the "G" track. (c) 5-methylcytosine sequencing of *PTHrP* gene CpG island (fragment A). Fragment A was sequenced exactly as above.

normal lung and lung tumour no methylation was apparent in any of the 44 sites analysed. However, in the BEN cell line, all these sites showed full or the partial presence of 5-methylcytosine.

DISCUSSION

Within the 5′ region of the *PTHrP* gene, there is methylation of the *MspI* site of -2050 in normal tissue, lung tumour and BEN cells, but not in germ line tissue (sperm). A downstream *MspI* site of -1510 is partially methylated in lung tumour and BEN cell DNA, and further methylation of positions -1378 and -1267 occurs in BEN cells. DNA sequencing of the upstream region of nucleotides -2123 to -1971 showed a trend of increased methylation associated with the neoplastic state. Thus, upstream of the P2 promoter, there is an apparent gradient of methylation during cellular development and progression to the neoplastic state, from germ line → tissue → tumour → tumour cell line.

Sequencing of a fragment of the CpG island showed that, in all tissue samples studied, the region was completely unmethylated. Thus, it appears that the *PTHrP* gene is similar to other housekeeping and tissue-specific genes in having an essentially unmethylated CpG island. In tissue-specific genes, there is an inverse correlation between DNA methylation and gene expression (reviewed in [16]) and methylation-free status of the promoter sequence is thought to be important for active transcription [5]. In contrast, although unmethylated in tissue samples, the CpG island in the BEN cell DNA was heavily methylated; moreover, this methylation did not interfere with gene expression as *PTHrP* mRNA was readily detected. Complete demethylation is, therefore, not essential for expression of the *PTHrP* gene.

In many established cell lines, CpG island methylation inactivates non-essential genes, causing loss of cell type-specific function [17]. In BEN cells, the *PTHrP* gene would be expected to be non-essential and it is heavily methylated; surprisingly, this methylation is not accompanied by loss of expression.

Cancer cells show a redistribution of methylation patterns which include *de novo* methylation of normally unmethylated areas, such as CpG islands (reviewed in [7]). For example, altered chromosomal methylation patterns accompany transformation of human bronchial epithelial cells [18]. Our data also suggest that new methylation events in the *PTHrP* gene mark neoplastic change. It is thought that CpG island methylation could interfere with normal expression of growth-suppressing or differentiation-inducing genes [7], but rather than reducing gene activity, our findings indicate upstream methylation is linked to increased *PTHrP* gene expression. It might, therefore, be considered whether *de novo* methylation events are associated with a switch to a different promoter, as occurs with promoter-specific imprinting in the human *IGF-II* gene [19]. To obtain a full understanding of the effect of methylation, it will be necessary to determine the CpG status of the whole region and to link it to examination of gene expression in different tissues.

In summary, this work has identified methylation events in the P2 promoter/CpG-rich region of the *PTHrP* gene. Methylation does not inactivate the gene and new methylation in this region may mark neoplastic change in lung cells. Increased methylation is found in tissues where a higher level of PTHrP expression would be expected. Further work is needed to investigate whether malignancy-associated epigenetic change in the *PTHrP* gene can be directly linked to overexpression of the protein.

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