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Increased upstream methylation has no influence on the overexpression of the parathyroid hormone-related protein gene in squamous cell carcinoma of the lung

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Abstract

Humoral hypercalcaemia of malignancy (HHM) commonly results from the excessive production of a parathyroid hormone-related protein (PTHrP) by tumours. We have previously shown malignancy is associated with increased DNA methylation in the 5′ region of the *PTHrP* gene. In a series of patients with lung carcinoma and relatively high serum calcium levels, 3 patients showed substantially increased *PTHrP* gene methylation while 5 patients showed no change in methylation status in this region. Patients showed marked tumour-specific expression of *PTHrP* through the P1 and P3 promoters with more general tumour and non-tumour expression through the P2 promoter. The lack of potential key regulatory CpG sites in the P1 promoter and the complete demethylation in the P2 and P3 promoters suggests methylation does not influence tumour-specific expression of *PTHrP*. Although demethylation may be a prerequisite for P2 and P3 expression, the overexpression of the *PTHrP* gene in cancer cells must be mediated through mechanisms other than DNA methylation.

Keywords: Parathyroid hormone-related protein; DNA methylation; Promoter regions; Regulation of gene expression; Squamous cell carcinoma

1. Introduction

Hypercalcaemia is a common complication of cancer and is estimated to occur in at least 10% of patients with advanced malignancies [1]. This 'humoral hypercalcaemia of malignancy' (HHM) commonly results from the production of a parathyroid hormone-like protein (PTHrP) by the tumour [2]. PTHrP is structurally distinct from parathyroid hormone (PTH), but at the N-terminus 8 out of 13 amino acids are identical [3–5] and PTHrP mimics the hypercalcaemic action of PTH by binding to the same receptor [6].

The *PTHrP* gene is a complex transcriptional unit composed of eight exons and spanning 15 kb (Fig. 1). Three isoforms spanning 139, 173 and 141 amino acids are encoded from transcripts generated by 3′ alternative splicing [7–9]. The gene appears to be under the control of three distinct promoters: promoters 1 and 3 have classical TATA box elements [7,9–11], whereas promoter 2 is GC-rich [12]. The unusual use of both TATA and GC-rich promoters suggests regulation of the *PTHrP* gene is complex. There are only a limited number of functional studies on the basal promoter activity or the regulation of transcription and the physiological relevance of 5′ alternative splicing and the differential promoter usage is still unclear. However, subtle changes in the regulation of *PTHrP* expression are likely to be causative in the development of HHM.

Methylation of cytosine residues within CpG dinucleotide sequences of the DNA is an important mechanism by which gene activity is modulated [13]. An early and consistent molecular change in neoplasia is an alteration in cytosine methylation in the DNA. Neoplastic cells simultaneously harbour widespread genomic hypomethylation, regional areas of hypermethylation and increased DNA-methyltransferase activity (reviewed in [14]). Our studies have sought to examine whether there is a change in methylation status of the *PTHrP* promoter associated with the overexpression of the gene found in tumorigenesis. Our previous work [15] has

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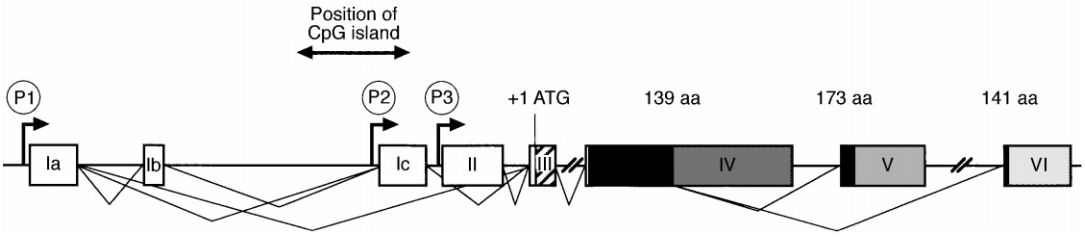

Fig. 1. Genomic organisation of the human parathyroid hormone-related protein (*PTHrP*) gene. Exons are shown as follows: 5′-non-coding exons, □; preproregion, ▨; mature coding region, ■; 3′ untranslated regions, ■. Splicing patterns are indicated by fine lines connection exons. aa, amino acids.

shown that in the lung, new methylation events upstream of the P2 promoter mark neoplastic change. These findings were surprising since such increased methylation is usually associated with a decreased expression in normal cells [16]. A more detailed examination with cultured lung cells [17] showed that partial methylation in the genes' 'CpG island' did not inhibit expression of the downstream (P2 and P3) promoters. Either, methylation of the 5′ non-coding region of the *PTHrP* gene did not play a role in the regulation of adjacent promoters, or maintenance of the demethylated state in the 170 bp at the 3′ end of the CpG island was fundamental for use of the *PTHrP* promoters. The cell line studies also indicated that the GC-rich promoter might play a 'housekeeping' role in between the TATA P1 and P3 promoters. Cell lines in culture are known to exhibit unusual patterns of methylation [18,19] and care must be taken in interpreting results in a clinical context. Thus, the aim of this study was to clarify the relationship between *PTHrP* gene methylation and expression, making a comprehensive study of the methylation status of all three *PTHrP* promoters and determining physiological relevance by extending our analysis to normal and tumour lung tissue.

2. Materials and methods

2.1. Isolation of tissue DNA and RNA

Humoral hypercalcaemia and PTHrP production are commonly associated with squamous cell carcinoma of the lung [20] and so lung tissue has been the focus of this study. For cell line studies, primary cultures of human lung fibroblasts represented 'normal' tissue and the epidermoid bronchial carcinoma-derived BEN cell line [21] represented tumour cells. Jurkat T-lymphocyte cells served as a negative control for *PTHrP* expression (as described in [22]). Analysis of clinical samples was confined to patients with poorly to moderately differentiated squamous cell carcinoma of the lung, where subsequent pathology revealed resection margins clear from tumour. The sample group was made up of 7 males and 1 female covering an age range of 57–74 years. Patients had serum Ca^{2+} levels at the top end of the reference range but with the exception of one, patients were not considered hypercalcaemic.

Lung fibroblasts and BEN cells were maintained in Dulbecco's modified Eagles medium and Jurkat T-cells in Roswell Park Memorial Institute (RPMI) 1640 medium. Growth media were supplemented with 10% heat-inactivated fetal calf serum containing 100 μg/μl penicillin and 100 μg/ml streptomycin. Genomic DNA was isolated from cultured cells using a Wizard Genomic DNA purification kit (Promega, Southampton, UK). RNA was isolated using a Total RNA Isolation System (Promega).

Samples of tumour and a portion of the resection margin were collected and processed within 3 h of surgery. Genomic DNA and total RNA were simultaneously isolated using a TwinPrep DNA/RNA isolation kit (InViSorb TwinPrep DNA/RNA kit, Bioline Ltd, London, UK). Briefly, tissue was homogenised in a 'lysis' buffer containing chaotrophic compounds that inactivate endogenous RNases. Genomic DNA was then removed by incubation with a mineral carrier. The resulting total RNA containing supernatant was extracted with phenol/chloroform, and the RNA precipitated with isopropanol. RNA samples were stored, resuspended in ethanol at −70°C. The carrier-bound genomic DNA was washed and eluted from the carrier material in low salt buffer. DNA concentrates were stored at 4°C.

2.2. Analysis of methylated CpG dinucleotides in genomic DNA

The methylation status of the DNA was analysed using a chemical modification and sequencing method. 2 μg of genomic DNA was modified using sodium bisulphite as described by Frommer and colleagues [23]), but with the dialysis stage replaced by extraction of DNA using glass milk (Geneclean II Kit, Qbiogene, Middlesex, UK) [24]. Modified DNA was redissolved in a volume of 100 μl and 5 μl (≈100 ng genomic DNA) used in the amplification reaction. The study determined the methylation status of the CpG dinucleotides in the 5′ region −3530 bp to −18 bp (where +1 is the translation

initiation codon), encompassing all three promoters and a CpG island. DNA was analysed in small fragments (< 440 bp), using overlapping primers to cover 3.5 kb of DNA. Oligonucleotide sense and antisense primers recognising bisulphite modified DNA are listed in Table 1. Primers were sited at positions where there were no CpG dinucleotides, so as to ensure no bias in the assessment of sequence methylation. The amplification reaction was set up in a volume of 50 μl, containing 1× reaction buffer, 12.5 pmol of each primer, 0.2 mM solutions of each deoxynucleotide triphosphate (dNTP) and 2.5 U Taq DNA polymerase (Promega). Magnesium chloride concentration was optimised for each separate reaction (Table 1). The reactions were set up using a 'hot start' with a paraffin wax interphase separating the DNA template from the primers. Amplifications were performed on a DNA thermal cycler (Omnigene, Hybaid, Ashford, Kent, UK) with denaturation at 94°C for 30 s, optimised annealing temperature (50–61°C, see Table 1) for 1 min, extension at 72°C for 1 min and varying cycle number (see Table 1). Polymerase chain reaction (PCR) products were purified using DNA affinity columns (Wizard PCR Preps DNA Purification System, Promega) and fragments sequenced using end-labelled ([γ-^{33}P] ATP) primers in a cycle DNA sequencing reaction (Thermosequenase Cycle Sequencing Kit, Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK). The extent of methylation at each CpG dinucleotide was assessed visually from an autoradiograph of the sequencing reaction, comparing the density of the band in the 'methylated' track with that in the 'unmethylated' track. Methylation was assessed as approximately 100% (complete), 90%, 75%, 50%, 25%, 10% (trace) or 0% (unmethylated). The level of methylation represents the 'average' for all of the cells derived from the tissue sample and assessments were only made for one of the strands of DNA.

2.3. Analysis of promoter usage

The differential use of promoters in the *PTHrP* gene was investigated by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA isolated using the TwinPrep DNA/RNA isolation kit was digested using RNase-Free DNase (RQ1, Promega) to remove any residual genomic DNA. Approximately 10 μg of total RNA was reverse-transcribed from 0.5 μg of oligo-dT primer using 25 U avian myeloblastosis virus reverse transcriptase (AMVRT) in 1× RT buffer with 1 mM each dNTP, 5 mM $MgCl_2$ and 20 U RNasin ribonuclease inhibitor (all components of a Reverse Transcription System Kit, Promega). 20 μl of reaction mixture was incubated for 30 min at room temperature, 1 h at 42°C and 5 min at 90°C.

Published primer sets were used for the detection of PCR products derived from the activity of the *PTHrP* promoters (Table 2). A *β_2-microglobulin* internal standard and the P1 and P3 *PTHrP* promoters were detected using the primers described by Campos and coworkers [25]. P2 activity was detected using the primers described

Table 1
Details of PCR primers and the specific amplification conditions used in the analysis of methylated DNA[a]

Fragment	Primer sequence (5′ to 3′)	Nucleotide location	Annealing temperature (°C)	Mg^{2+} conc (mM)	No. of cycles
1	AAGGTGTATTTATATGATAG	−3530 to −3511	52	1.5	60 (using Bio-X-Act)
	AATACCTCTTCCAAACTACC	−3223 to −3204			
2	GAAGAGTAGAGAGAAGATTG	−3056 to −3037	55	1.5	55
	TAAAATTTCCTCTCCTCCAC	−2936 to −2917			
3	GTAAGTATTAAATGGTTTAT	−2580 to −2561	50	1.5	60
	CCACAACTTCCCTTAAATAT	−2353 to −2334			
4	ATATTTAAGGGAAGTTGTGG	−2353 to −2334	55	1.5	55
	CTCCTACCTTCCTCAATTCA	−2218 to −2199			
5	GTTGTAGTGAATTAGTGGGA	−2143 to −2124	55	2.5	35
	CAATCCTACTAATAAAATTC	−1970 to −1951			
6	TTAGTAGGATTGTTTAGAAG	−1962 to −1943	55	2	35
	CTCAAAAATCCCAAATTACA	−1679 to −1660			
7	TAGGGTATTAGAGAAAGGAT	−1641 to −1622	55	1.75	35
	ATACATAAAAACCTTCCTCC	−1364 to −1345			
8	GGAATGGGTTAGGGAGGAAG	−1376 to −1357	61	2.5	35
	ACAACAAAACTCCCCCCTCC	−972 to −953			
9	GGTAGAGTTTTTGATAGTTT	−1009 to −990	56	1.4	35
	CACACACCCTAAAAACAAAT	−588 to −569			
10	ATTTGTTTTTAGGGTGTGTG	−588 to −569	55	1.5	45
	TATACTTTCTCCAAACCACA	−231 to −212			
11	TGTGGTTTGGAGAAAGTATA	−231 to −212	55	1.5	45
	TCCACTACTAAACCAATCTC	+9 to +28			

PCR, polymerase chain reaction.
[a] The translation initiation codon, ATG, in exon III is designated nucleotide 1 and used as the frame of reference.

Table 2
Details of PCR primers and the specific amplification conditions used in the analysis of promoter usage

Fragment	Primer sequence (5′ to 3′)	Product size (bp)	Annealing temperature (°C)	Number of cycles	DNA polymerase + specific conditions
β²-microglobin	ACCCCCACTGAAAAAGATGA ATCTTCAAACCTCCATGATG	123	56	30	Taq
P1	AGGTACCTGCTTTCTAATAA AACCGAGCGGAGGAATGTTC	320	56	45	Bio-X-act
P2	TTCTCCGGCAGGTTTG TGCGATCAGATGGTGAAGGA	468	57	60	Bio-X-act + 5% dimethyl sulphoxide (DMSO)
P3	AGCTGACTTCAGAGGGGGAA TCTCCGCTCGCGCTCGGGAC	287	63	40	Taq

by Southby and associates [26]. The PCR conditions described by these authors were further optimised to facilitate ready visualisation on ethidium bromide gels. One-twenty fifth of the reverse transcription reaction was used per PCR in a 50 μl reaction mixture, containing 1× reaction buffer, 12.5 pmol of each primer, 0.2 mM solutions of each dNTP, 1.5 mM $MgCl_2$ and 4U DNA polymerase (Taq DNA polymerase, Promega; Bio-X-Act DNA polymerase, Bioline). Each reaction required the specific modifications summarised in Table 2. 'Hot start' PCR amplifications were performed with denaturing at 94°C for 30 s, optimised annealing temperature (56–63°C, Table 2) for 1 min and extension at 72°C for 1 min. Cycle number was individually optimised to ensure visualisation on ethidium bromide-stained gels (Table 2).

2.4. Statistical analysis

The statistical effect of the type of tissue (normal or tumour) and the patient's age on the per cent methylation was evaluated within the region of the *PTHrP* gene that showed methylation of CpG dinucleotides (nucleotide position −3424 through to −1510). Stepwise analysis and a Kruskal–Wallis test were performed using Statistical Package for the Social Sciences (SPSS) software.

3. Results

3.1. Cell line analysis

The methylation of CpG dinucleotides in the *PTHrP* gene was analysed in a 3.5 kb region upstream of the transcription initiation site (Fig. 2). This region encompassed all three of the *PTHrP* gene promoters. Around the P1 promoter (−3500 and −3000 bp), CpG dinucleotides are rare and in all of the cell lines most were methylated to some degree, but there appeared little pattern to levels of methylation. Further downstream, as CpG dinucleotides became more frequent, patterns of methylation emerged. In the lung fibroblasts, CpG dinucleotides were maximally methylated at −2500 bp. Then downstream CpG methylation diminished to a value of zero at approximately −1500. In the BEN cells, CpG dinucleotides were maximally methylated at approximately −2000 to −1500 and a relativethe the lung fibroblasts higher proportion of the sites remained methylated. Methylation diminished to zero at approximately −900 bp. In the Jurkat cell line, methylation followed a similar pattern to the BEN cells with a nadir at approximately −750 bp. However, between −750 and 0 bp variable, but substantial, methylation could be detected at CpG sites in these cells.

Earlier RT-PCR analysis [17] had shown that only in BEN cells were *PTHrP* transcripts produced from the P1 promoter. Expression from the P2 promoter appeared to act like a 'housekeeping' gene with all three cell lines utilising this promoter. P3 activity could be detected in the lung fibroblasts and in the BEN cells (Fig. 3).

3.2. Analysis of the resected tumour samples

Analysis of eight resected tumour samples measuring the methylation status in non-tumour and tumour tissue showed some common patterns of methylation in the *PTHrP* gene. Methylation in the region −3500 to −3000 bp showed substantial, but variable, levels of methylation. Methylation of the CpG sites from −3000 bp to approximately −1200 bp gradually decreased to zero, remaining at that level up to the transcription start site. Although all the samples followed a similar pattern of loss of CpG methylation through the 5′ region, there were quite specific differences in the methylation patterns with individual patients. In 3 of the 8 (38%) patients, tumour samples were substantially more methylated than non-tumour tissue taken from the resection margin (Fig. 4). For 5 out of 8 patients, there appeared little difference in the levels of methylation between the tumour and non-tumour tissue. The methylation patterns of the non-tumour samples were similar to those of a *post mortem* lung sample with no history of lung pathology (data not shown). It was therefore deemed that although this tissue was physically close to the tumour, the resection margin declared clear of malignancy could be used as a 'normal' lung control.

Analysis of the levels of β_2-*microglobulin* showed approximately equivalent amounts of RNA in each sample. RT-PCR of Na^+K^+ATP-ase, a much larger housekeeping protein of 650 bp, resulted in broadly similar amounts of amplicon in each patient sample (data not shown). This indicated that, even though there was an unavoidable time delay between removal of tissue and extraction of RNA, the RNA remained of

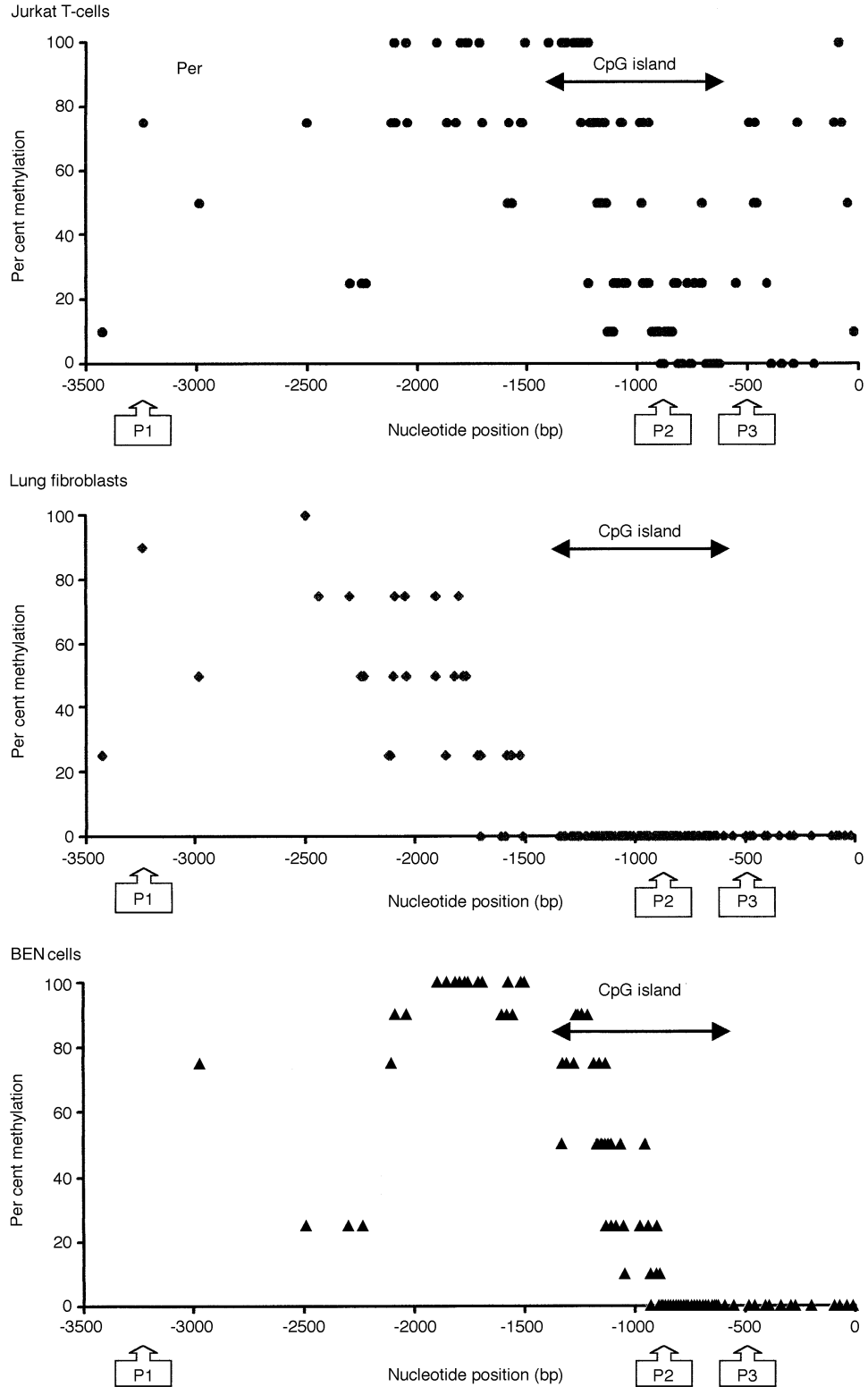

Fig. 2. DNA methylation status of the 5′ region of the *PTHrP* gene in Jurkat T-cells, lung fibroblasts and the BEN cell line. The positions of the gene's three promotors and 'CpG island' are marked.

reasonable integrity with no differential loss of larger amplicons (such as P2). The utilisation of the *PTHrP* promoters is illustrated in Fig. 3. P1 expression was detected in five tumour samples, for P2, expression was measured in six tumour samples and two non-tumour samples and for P3, expression was detected in five tumour samples. As care must be taken not to 'over-interpret' the RT-PCR results, we attempted to verify the promoter expression data by the use of primer extension studies. However, this technique proved insufficiently sensitive given the small amounts of tissue at our disposal. RT-PCR reactions were carefully optimised to ensure all reactions were in a linear phase and the appearance of PCR product genuinely reflected promoter expression (data not shown).

As the two different patterns of methylation in the patient samples could not be linked to *PTHrP* expression, we investigated whether other covariates might be involved. Ageing has been shown to be a major contributor to the hypermethylation of gene CpG islands in

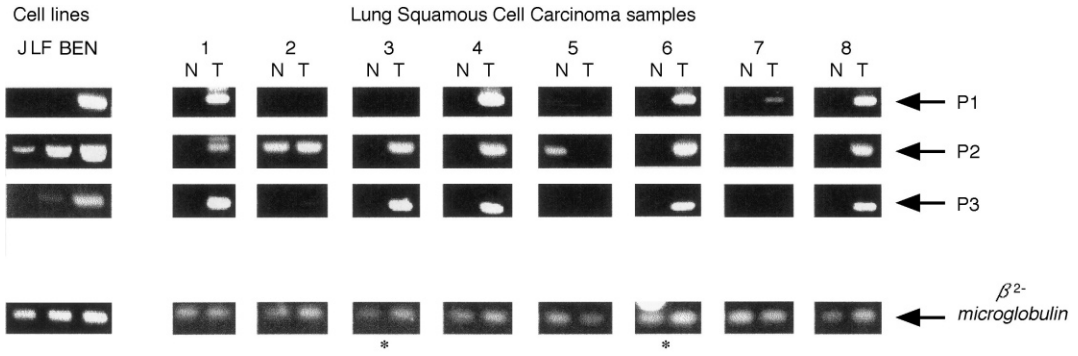

Fig. 3. *PTHrP* promotor usage in cell lines and resected lung samples. J, Jurkat T-cells; LF, lung fibroblasts; BEN, BEN cells. Patients have been designated numbers: N, 'normal tissue'; T, tumour tissue. *indicates patients illustrated in Fig. 4.

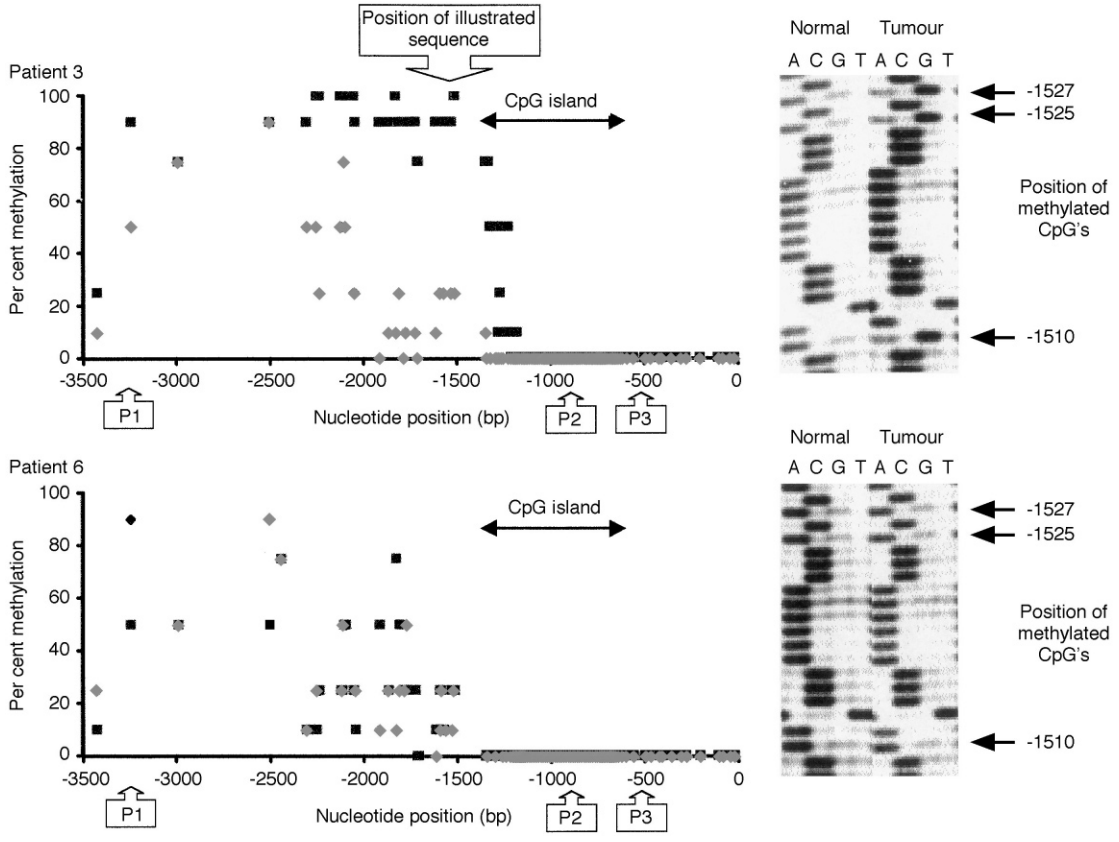

Fig. 4. DNA methylation status of the 5′ region of the *PTHrP* gene in 2 patients with lung carcinoma. ◆, normal lung; ■, tumour tissue. Autoradiographs illustrate methylated CpGs in DNA at −1528 to −1508 bp. Methylated cytosines are seen in the 'G' track through complementary strand sequencing.

carcinogenesis [27,28] and so the statistical effect of the type of tissue (normal or tumour) and the patient's age on the per cent methylation was evaluated. Stepwise analysis and a Kruskal–Wallis test showed in this study that the age of patients did not have a significant effect on the level of methylation, whereas the tissue type was clearly significant.

4. Discussion

Our previous studies have shown that increased methylation in the 5′ region of *PTHrP* marks neoplastic changes in lung tissue [15]. As increased methylation is usually associated with decreased expression in normal cells [16], our work has centred on whether methylation influences the regulation of the *PTHrP* gene effecting the overexpression of the gene seen in squamous cell carcinoma. As such, this detailed study has sought to investigate whether location or extent of increased methylation was related to promoter usage and whether there was any link in gene methylation status and promoter usage with patient serum calcium levels.

Around the P1 promoter CpG dinucleotides are relatively rare. In all of the cell lines, normal and tumour tissue, levels of methylation of these sites was considerable (up to 90%) but variable (Figs. 2 and 4). However, expression off the P1 promoter was confined to the tumour cell line, BEN and 5/8 of the resected tumour samples. In promoter regions without CpG islands it is thought methylation of individual sites near, or directly within, transcription factor-binding motifs affects binding, thereby modulating gene expression [14]. There are few CpG sites close to the P1 TATA box or the c-adenosine monophosphate (cAMP) response element [29] and levels of methylation at these sites were disparate. As *PTHrP* expression was highly tumour-specific, it seems likely methylation plays no role in *PTHrP* expression through this promoter.

CpG dinucleotides at the 3′ end of the CpG island, around the P2 promoter, were almost universally free from methylation (Figs. 2 and 4). Expression through the P2 promoter was seen in all of the cell line samples to varying degrees and in 2/8 normal and 6/8 tumour lung samples (Fig. 3). The more general expression from the P2 promoter corroborates the earlier cell line studies that expression from the P2 promoter shows the characteristics of a housekeeping gene, with an unmethylated CpG island and more ubiquitous gene expression. Recent work using BEN cells has shown calcitonin and other agonists cause an increase in transcription initiated from both P1 and P3 promoters with no observed effect on the P2 promoter [29]. Thus, other work also suggests that the nature of P2 regulation is different from that of P1 and P3 regulation. If P2 does behave like a housekeeping gene it would be expected that the CpG island should remain unmethylated regardless of the expression levels, with DNA methylation having no role in the control of gene expression [13].

Analysis of the methylation status of CpG sites towards and through the P3 promoter showed that, with the exception of Jurkat T-cells, the whole region from P2 to the transcription start site was unmethylated. P3 expression showed tight tumour specificity (Fig. 3) and it is, therefore, clear methylation does not play a direct role in the tumour-specific regulation of the *PTHrP* gene through the P3 promoter. The substantially methylated CpG sites in the Jurkat T-cells may contribute to the lack of P3 expression in these cells and so it seems likely demethylation is a prerequisite for transcription factor access.

RT-PCR analysis revealed marked tumour specific expression of *PTHrP*. This confirmed the work of others [30], that high levels of expression in this type of tumour can be seen even in the absence of hypercalcaemia. It seems likely that in these cases, tumours synthesise large amounts of PTHrP, but secrete the peptide at levels insufficient to cause hypercalcaemia. However, there appears to be secretion of sufficient PTHrP to induce mild effects on calcium metabolism, pushing serum levels in to the upper part of the normal reference range (Table 3).

Analysis of the methylation status of the CpG dinucleotides in the 5′ region of the *PTHrP* gene in patients with squamous cell carcinoma of the lung revealed two patterns of methylation. In some patients, tumour DNA was significantly more methylated than the normal tissue at the resection margin (Fig. 4). These results were in line with an earlier analysis [15]. However, in 5 of the patients there appeared to be little difference in the level of methylation between the non-tumour and tumour tissue. The variable methylation patterns did not show any particular relationship with the pattern of promoter usage or Ca^{2+} levels and so there appeared to be no consistent link between *PTHrP* gene methylation and gene expression in this tumour tissue. In an earlier study in renal cell carcinoma cell lines, analysis of four CpG dinucleotides 5′ to the CpG island (at nucleotides −2030, −1890, −1510 and −1500) showed increased methylation to be correlated with an absence of *PTHrP* expression [31]. In our lung tumour samples, all these sites were methylated to some degree, while the samples showed increased gene activity with respect to the normal tissue. Thus, as in our earlier study [17] we were unable to link increased DNA methylation with reduced *PTHrP* expression in lung tissue.

In conclusion, patients with squamous cell carcinoma of the lung show differing levels of methylation in the *PTHrP* gene. In this study, 5 (63%) of patients showed similar patterns of methylation in normal and tumour tissue. However, in a number of patients (3, 38%), tumour samples were significantly more methylated. 5

Table 3
Patient clinical information and summary of *PTHrP* gene methylation status and promoter usage[a]

Patient	Age (years)	Sex	Preoperative Ca^{2+} level (mmol/l)	Methylation status upstream of the −1510 site			Promotor usage					
							P1		P2		P3	
				t > n	t = n	t < n	n	t	n	t	n	t
1	74	M	2.5	24	3	2		++		++		++
2	57	M	2.49	20	3	0			++	++		
3	70	M	2.36	29	2	0				++		++
4	62	F	2.61	10	9	6		++		++		++
5	57	M	2.31	4	12	3			++			
6	58	M	n/a	9	13	6		++		++		++
7	68	M	2.44	13	7	8		+				
8	70	M	2.32	7	17	4		++		++		++

n/a, not available; M, male; F, female; n, normal tissue; t, tumour.

[a] Preoperative calcium levels represent the last blood analysis done prior to surgery and the values have been corrected to account for serum albumin levels. The reference range for serum calcium is 2.15–2.60 mmol/l. The level of methylation was assessed in each CpG site by logging whether methylation was greater in the tumour sample compared with the normal (t > n), the same (t = n), or less in the tumour sample compared with the normal (t < n). This analysis was only carried out in CpG sites upstream of that at −1510 where there was a substantial amount of methylation. Promoter usage is indicated by a '+' as illustrated in Fig. 3.

patients showed marked tumour-specific expression of *PTHrP* through its P1 and P3 promoter whereas there was a more general tumour and non-tumour expression of the P2 promoter in 6 patients. The lack of potential key regulatory CpG sites in the P1 promoter and the complete demethylation in the P2 and P3 promoter suggests methylation does not influence tumour-specific expression. Although demethylation may well be a prerequisite for P2 and P3 expression, the overexpression of the *PTHrP* gene in cancer cells must be mediated through mechanisms other than DNA methylation.

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