Original article

P-glycoprotein expression is associated with sestamibi washout in primary hyperparathyroidism

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Background: **The detection of parathyroid adenomas by ^{99m}Tc-labelled hexakis 2-methoxyisobutyl isonitrile (sestamibi) scintigraphy is influenced by several factors, including tumour size and serum level of parathyroid hormone (PTH). This study examined the relationship between sestamibi accumulation and multidrug resistance (MDR)-related P-glycoprotein (P-gp) expression in a large series of surgically excised parathyroid tumours.**

Methods: **Seventy-eight patients underwent dual-phase sestamibi imaging before parathyroidectomy. Expression of P-gp within tumour cells was assessed by immunohistochemistry. Tumour size was measured and the ellipsoid volume calculated. Scan results were analysed in relation to preoperative serum levels of calcium and PTH, P-gp expression and tumour volume.**

Results: **Sixty-four of the 78 sestamibi scans were positive and 14 negative. Smaller adenomas (less than 0·5 cm^3) were more likely to be sestamibi negative than larger lesions ($P = 0·006$). Ten of 14 adenomas with negative imaging showed strong P-gp membrane positivity and 45 of 64 lesions with a positive scan did not show P-gp membrane expression, indicating a significant association between high P-gp membrane immunoreactivity and negative sestamibi result ($P = 0·006$).**

Conclusion: **These data suggest an association between P-gp membrane expression and false-negative sestamibi scan result. Inhibition of the P-gp transmembrane pump using MDR modulators may therefore improve the sensitivity of sestamibi scintigraphy.**

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Introduction

The development of minimally invasive parathyroidectomy[1] has been made possible by improvements in imaging techniques for accurate localization of the parathyroid glands. ^{99m}Tc-labelled hexakis 2-methoxyisobutyl isonitrile (sestamibi) scintigraphy has become the method of choice for the preoperative localization of parathyroid adenomas[2], with a sensitivity in localizing abnormal parathyroid glands ranging from 75 to 91 per cent[3–6]. Although superior to other imaging techniques, this still represents significant variation in sensitivity, which can be attributed partially to differences in imaging protocols. There is also evidence that single-photon emission computed tomography and subtraction techniques may offer improved sensitivity, albeit marginal, over the simpler dual-phase technique[7]. The best reported sensitivity of 91 per cent[6] still leaves a substantial false-negative rate, which may influence the surgical approach and cure rate following surgery.

Previous studies have investigated sestamibi accumulation in abnormal parathyroid glands and reasons for false-negative scan results. One important factor is gland size. It has been suggested that the large size of parathyroid tumours, coupled with a corresponding increase in blood flow, is responsible for sestamibi uptake in abnormal glands[6]. However, false-negative results have been reported for large glands and very small adenomas may be detectable, indicating that size is not the only important factor[8,9]. Other factors said to influence sestamibi

uptake include serum levels of calcium, parathyroid hormone (PTH) and phosphorus, and the predominant cell type (for example oxyphil content)[9,10].

Several studies have explored the relationship between sestamibi imaging and multidrug resistance (MDR)-related P-glycoprotein (P-gp). P-gp is a 170 000-Da transmembrane lipoprotein encoded by the *MDR* gene. Expression in normal tissues is variable, with high levels in epithelial cells, leading to the suggestion that P-gp protects against toxic substances[11]. P-gp expression facilitates the efflux of chemotherapeutic drugs from cancer cells, rendering tumours resistant to chemotherapy[8]. The mechanism is thought to involve diffusion of substrates into the cell, which bind reversibly to P-gp on the cytoplasmic side of the cell membrane. The P-gp efflux pump then transports these substrates out of the cell by an adenosine 5′-triphosphate-dependent process.

Sestamibi shares physical and chemical characteristics with substances known to be transported by P-gp. Both *in vitro* and *in vivo* studies have confirmed that it is a transport substrate for P-gp[12,13]. There is evidence that abnormal parathyroid glands have low levels of P-gp expression compared with normal parathyroids. Mitchell and colleagues[11] reported that 18 of 19 excised normal, but only one of 18 adenomatous, parathyroid glands were positive for P-gp expression. The same study found that abnormal parathyroid tissue had a higher retention of sestamibi per gram than normal glands on γ count.

Subsequent research has proved inconclusive. Some studies show no relationship[9,14] and others a definite correlation[15] between sestamibi parathyroid imaging and P-gp expression. In the largest study of 47 patients with parathyroid adenomas, all eight tumours positive for P-gp expression were negative on sestamibi scanning, whereas the 39 P-gp-negative tumours were positive[16]. The aim of the present study was to examine further the role of P-gp expression and other factors in parathyroid sestamibi scintigraphy in a large series of 78 surgically excised parathyroid tumours.

Methods

The study included 78 patients with single-gland disease, all of whom had parathyroidectomy for primary hyperparathyroidism at the Department of Endocrine Surgery, Addenbrooke's Hospital, Cambridge during the years 2000–2004. Patients had confirmed biochemical hyperparathyroidism, with documented indices for serum calcium (available for 69 patients) and PTH (available for 71 patients). All had preoperative sestamibi scintigraphy, followed by ultrasonography if scintigraphy was inconclusive.

Dual-phase parathyroid imaging was performed 5 min and 2 h after intravenous administration of 600 MBq sestamibi using a large field-of-view single-headed γ camera fitted with a parallel-view collimator to focus on the neck and mediastinum, and a pinhole collimator to view the thyroid. Data were processed by dedicated computers and results interpreted by experienced nuclear medicine physicians. Results were reported as positive, negative or indeterminate for parathyroid adenoma. Negative and indeterminate cases were grouped together for further analysis.

A focused parathyroidectomy technique[17] was used when preoperative imaging suggested unifocal disease. Bilateral neck exploration was used when imaging was negative, discordant or showed multigland disease. Following gland excision, intraoperative serum PTH measurement was used to confirm biochemical cure. Parathyroid tissue was immediately placed in formalin and sent for histological examination.

The tumour dimensions were measured at the time of receipt in the histopathology laboratory. Tumour volume was calculated according to the formula for ellipsoid volume (V)[9,10], where $V = \pi/6 \times \text{width} \times \text{length} \times \text{depth}$. Tumours smaller than 0·5 cm^3 were considered to be small. All others were considered large.

Immunohistochemistry

Formalin-fixed paraffin sections (4 μm) were cut and deparaffinized in an oven for 5 min, and then rehydrated through graded alcohols to water. Antigen retrieval was performed using a microwave technique (800 W), in 500 ml low-antigen retrieval solution (pH 6·0; Dako, Ely, UK) for 15 min. The sections were then washed for 5 min in running water. Endogenous peroxidase was blocked by incubating with 0·1 per cent hydrogen peroxide in methanol for 15 min at room temperature, followed by a further 5-min wash in running water. The sections were incubated overnight in a moist chamber at 4°C with primary antibody (NCL-JSB-1; Novacastra Laboratories, Newcastle upon Tyne, UK) at a one in 25 dilution. After washing in Tris-buffered saline (pH 7·6), secondary detection was performed with EnVision™ (Dako) in accordance with the manufacturer's instructions. Appropriate positive and negative controls were used, and a negative control was included specifically for each sample.

Following development of an appropriate scoring system by examination of a series of sections by two pathologists, the assessment of P-gp expression was undertaken by one of them. Difficult or borderline cases were re-examined by the two pathologists on a conference multiheaded microscope, and a final score agreed. Both pathologists were blinded

to the clinical outcome and sestamibi scan results. The intensity of membrane staining was scored as absent, weak, moderate or strong, and the proportion of tumour cells showing expression assessed semiquantitatively as less than 10, 11–33, 34–66 or more than 66 per cent. The same scoring system was used for cytoplasmic immunoreactivity. For further analysis, samples with staining intensity characterized as absent or weak were compared with those showing moderate or strong immunoreactivity. In addition, lesions with 33 per cent or fewer tumour cells showing membrane immunopositivity were compared with those showing more than 33 per cent. *Fig. 1* shows examples of histological P-gp expression.

Statistical analysis

Statistical analysis, including χ^2 test and Fisher's exact test where appropriate, was carried out using StatView® version 5.0.1 software (SAS Institute, Cary, North Carolina, USA). $P < 0{\cdot}050$ was considered to be significant.

Results

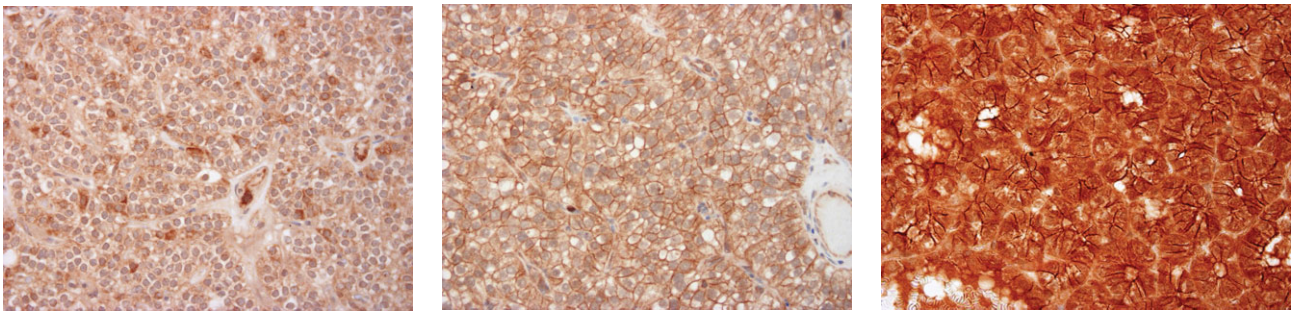
There were 78 patients (20 men, 58 women) with a mean age of 62 (range 25–90) years. The mean serum calcium concentration was 2·77 mmol/l (normal range 2·12–2·65 mmol/l) and the mean serum PTH level was 155·68 ng/l (normal range 10–65 ng/l).

Two patients had parathyroid carcinoma, two were considered suspicious for malignancy and a single parathyroid adenoma was excised in the remaining 74 patients. Sestamibi scintigrams were obtained in all patients. Of these, 64 were successful in locating an abnormal parathyroid gland and 14 did not identify an abnormality (seven negative, seven indeterminate). Sensitivity was 82 per cent. Of the malignant and suspicious lesions, one of the latter was not detected by sestamibi scanning.

Immunohistochemistry was performed on all 78 specimens. Ten of 14 patients with a negative sestamibi scan had lesions with a high proportion of P-gp membrane staining (more than 33 per cent), whereas 45 of 64 patients with a positive scan had adenomas with a low proportion of P-gp membrane staining (33 per cent or less). There was a significant relationship between a high proportion of membrane P-gp expression and a negative sestamibi scan result ($P = 0{\cdot}006$). There was no association between a positive sestamibi result and either P-gp membrane intensity ($P = 0{\cdot}251$) or P-gp cytoplasmic intensity ($P = 0{\cdot}404$). Of the four non-benign tumours, one of the two malignant lesions and one of the two suspicious lesions had a high proportion of P-gp membrane staining.

Parathyroid volume was calculated for 67 of the specimens. The ellipsoid volume ranged from 0·15 to 10·47 (mean 1·55) cm^3. Seven of 18 small adenomas had a negative sestamibi result, whereas 45 of 49 large adenomas had a positive finding. There was a significant association between large adenoma size and positive sestamibi result ($P = 0{\cdot}006$). Of the four large adenomas that were negative on sestamibi scan, two had a high level of membrane P-gp expression.

There was no relationship between preoperative serum PTH level (analysed in two categories: either less than, or equal to and greater than, the median of 110·5 ng/l) and sestamibi result ($P = 0{\cdot}547$) or with proportion of P-gp membrane expression ($P = 0{\cdot}625$) (*Table 1*). There was no association between preoperative serum calcium level (also analysed in two categories: less than, or equal to or greater than, the median of 2·74 mmol/l) and sestamibi result ($P = 0{\cdot}073$) or proportion of P-gp membrane expression ($P = 0{\cdot}218$) (*Table 1*).



a Weak membrane and cytoplasmic staining

b Moderate membrane and weak cytoplasmic staining

c Strong membrane and cytoplasmic staining

Fig. 1 Patterns of P-glycoprotein expression: **a** weak membrane and cytoplasmic staining; **b** moderate membrane and weak cytoplasmic staining; **c** strong membrane and cytoplasmic staining

Table 1 Sestamibi scan findings in relation to P-glycoprotein expression, parathyroid volume, and preoperative serum parathyroid hormone and calcium levels

	Sestamibi scan result		
	Negative or indeterminate	Positive	*P**
P-glycoprotein membrane staining (% of cells)			0·006
≤ 33	4	45	
> 33	10	19	
Parathyroid volume (cm^3)			0·006
< 0·5	7	11	
≥ 0·5	4	45	
Preoperative serum PTH (ng/l)			0·547
< 110·5	6	33	
≥ 110·5	7	25	
Preoperative serum calcium (mmol/dl)			0·073
< 2·74	10	27	
≥ 2·74	3	29	

PTH, parathyroid hormone. *Fisher's exact test.

Discussion

Membrane expression of P-gp in parathyroid tumours is associated with a negative result on sestamibi scintigraphy, in keeping with the findings of smaller studies[15,16,18]. Of note is the present observation that only membrane, and not cytoplasmic, P-gp expression is related to sestamibi findings. Although other studies have examined expression of P-gp in both the cytoplasm and cell membrane[9,15,19], none specified which was used in the analysis. The putative mechanism of P-gp as a membrane efflux pump for sestamibi is consistent with the pattern of membrane staining described in this study. The role of cytoplasmic P-gp expression remains unclear.

Although the main focus of this study was the relationship between sestamibi scanning and P-gp, technical factors that may influence the results of sestamibi scintigraphy should be considered. The dual-phase planar scintigraphy protocol employed in this study yielded a sensitivity of 82 per cent, somewhat lower than the best reported sensitivity of 91 per cent in a study using the same technique[6].

One way of eliminating the effect of different imaging techniques on sensitivity is to examine γ counts of excised parathyroid tissue, rather than scintigraphy results. Ugur and colleagues[9] failed to find a correlation between sestamibi scanning and P-gp expression in a study of 31 patients with primary hyperparathyroidism, although they did note that *ex vivo* parathyroid γ counts were higher in P-gp-negative lesions. O'Doherty and Kettle[2] suggested that subtraction imaging may be better suited to detection of P-gp-positive lesions than the dual-phase protocol, as it could identify early uptake of sestamibi before the P-gp efflux pump removed it from the gland. One hypothesis is that dual-phase scintigraphy may miss some smaller adenomas that are associated with early washout. This is supported in the present study where sestamibi scanning was more likely to detect larger lesions, as well as other studies that have found correlations between sestamibi uptake and parathyroid volume or weight[9,14,15]. This relationship can probably be explained by the increased blood flow into a larger gland with consequent increased uptake of sestamibi, as well as the detection advantage of a larger object in relation to system resolution.

The relationship between tumour size and sestamibi scintigraphy cannot, however, fully explain the imaging results. Some large adenomas escape detection, yet some small ones exhibit substantial sestamibi uptake. Yamaguchi *et al.*[15] compared ^{201}Tl with ^{99m}Tc-labelled sestamibi scintigraphy and, although both agents have the same uptake mechanism, the amount of sestamibi retained per gram of adenoma was much higher, leading to the conclusion that sestamibi uptake could not be explained by blood flow alone.

Two of the four large adenomas that were negative on sestamibi scan in the present study had high levels of P-gp expression, suggesting that P-gp influences the effectiveness of sestamibi scintigraphy. The impact of size on sestamibi uptake might, however, more often outweigh the effect of P-gp-related efflux, so that large adenomas may still be detected by sestamibi scan even if they express high levels of membrane P-gp. Of the 19 lesions that were both sestamibi positive and showed high percentage expression of membrane P-gp, 12 were larger than 0·5 cm^3, and this group also included very large lesions of 2·51 and 3·14 cm^3. The present results support the hypothesis that gland size and P-gp expression are two of the principal factors in sestamibi accumulation and retention. Further work is required to elucidate the relative importance of size on the uptake and P-gp on the efflux of sestamibi.

There was no association between sestamibi result or P-gp expression and preoperative serum PTH or calcium concentration. Previous evidence is inconclusive, as some studies noted an association between sestamibi uptake and high serum level of PTH[9] and calcium[20], whereas others found no relationship[10,19]. There is little consensus on the explanation for this weak association, although it has been suggested that calcium may play a role in modifying sestamibi kinetics by altering the membrane potential[21]. It is possible that an increase in serum calcium is simply a

consequence of more active hyperparathyroidism, and that this is responsible for amplified sestamibi uptake.

Most research on P-gp expression has focused on its role in MDR and how this can be modified to improve the sensitivity of cancers to chemotherapy. The first generation of MDR modulators included verapamil and cyclosporin, but low potency and side-effects at the concentrations required to inhibit MDR function precluded clinical use[22]. Second- and third-generation modulators are more potent and specific with lower side-effect profiles. One of the most promising of the third-generation agents is the anthranilamide derivative XR9576 (tariquidar), a potent and specific inhibitor of P-gp[23]. The use of such a P-gp inhibitor may therefore increase the sensitivity of preoperative sestamibi scintigraphy.

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