



Tumour nuclear oestrogen receptor beta 1 correlates inversely with parathyroid tumour weight

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Abstract

Primary hyperparathyroidism (PHPT) is a common endocrinopathy, frequently caused by a parathyroid adenoma, rarely by a parathyroid carcinoma that lacks effective oncological treatment. As the majority of cases are present in postmenopausal women, oestrogen signalling has been implicated in the tumourigenesis. Oestrogen receptor beta 1 (ERB1) and ERB2 have been recently identified in parathyroid adenomas, the former inducing genes coupled to tumour apoptosis. We applied immunohistochemistry and slide digitalisation to quantify nuclear ERB1 and ERB2 in 172 parathyroid adenomas, atypical adenomas and carcinomas, and ten normal parathyroid glands. All the normal parathyroid glands expressed ERB1 and ERB2. The majority of tumours expressed ERB1 (70.6%) at varying intensities, and ERB2 (96.5%) at strong intensities. Parathyroid carcinomas expressed ERB1 in three out of six cases and ERB2 in five out of six cases. The intensity of tumour nuclear ERB1 staining significantly correlated inversely with tumour weight ($P=0.011$), and patients whose tumours were classified as ERB1-negative had significantly greater tumour weight as well as higher serum calcium ($P=0.002$) and parathyroid hormone levels ($P=0.003$). Additionally, tumour nuclear ERB1 was not expressed differentially with respect to sex or age of the patient. Levels of tumour nuclear ERB2 did not correlate with clinical characteristics. In conclusion, decreased ERB1 immunoreactivity is associated with increased tumour weight in parathyroid adenomas. Given the previously reported correlation with tumour-suppressive signalling, selective oestrogen receptor modulation (SERMs) may play a role in the treatment of parathyroid carcinomas. Future studies of SERMs and oestrogen treatment in PHPT should consider tumour weight as a potential factor in pharmacological responsiveness.

Key Words

- ▶ parathyroid adenoma
- ▶ parathyroid carcinoma
- ▶ primary hyperparathyroidism
- ▶ oestrogen receptor beta
- ▶ oestrogen
- ▶ selective oestrogen receptor modulators
- ▶ Visiopharm

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Introduction

Primary hyperparathyroidism (PHPT) is defined as a parathyroid tumour producing excessive amounts of parathyroid hormone (PTH), which in turn leads to

hypercalcaemia. Most parathyroid tumours are adenomas but are nonetheless associated with an increased risk of cardiovascular and cerebrovascular diseases as well as



cancer (1, 2, 3). Parathyroid carcinomas are very rare (<1% of parathyroid tumours), but today's oncological treatment regimens have only a limited effect (4). According to World Health Organization (WHO) criteria, parathyroid tumours are classified as atypical adenomas if they show histopathological signs of malignancy (i.e. capsular engagement, trabecular growth, marked nuclear pleomorphism, fibrous bands and increased MIB-1 proliferation index) but lack distant metastasis, local invasion of surrounding organs, vascular/neural invasion and evidence of recurrence after parathyroidectomy (5). This group of tumours is believed to include both parathyroid carcinomas and histologically adverse adenomas without malignant potential. Several diagnostic markers have been proposed for distinguishing between the two (6).

Recurrent genetic aberrations in parathyroid tumours have been identified during previous studies, e.g. inactivating mutations of *MEN1* in parathyroid adenomas or cell division cycle 73 (*CDC73*) in parathyroid carcinomas (7, 8, 9). Recent whole-exome sequencing studies of parathyroid adenomas have overall revealed few non-synonymous mutations, including ones in a limited number of reoccurring genes (10, 11, 12). Additional underlying genomic events are yet to be identified and the current understanding of parathyroid tumour genetics cannot fully explain the aetiology of the common parathyroid adenoma. As parathyroid adenomas are frequently found in postmenopausal women, a causality coupled to female hormones or their receptors was proposed at an early stage.

Oestrogens are a group of steroid hormones with important reproductive functions in fertile women, but low levels of oestrogens are also found in men as well as in postmenopausal and prepubertal women. Oestrogens function by binding to oestrogen receptor α (ERA), encoded by the ER gene 1 (*ESR1*), or to ERB1, encoded by the *ESR2* (13, 14, 15). ERA and ERB1 both act as DNA-binding transcription factors upon ligand activation. Several splice variants of ERB exist; the well-characterised variant is ERB2 (also known as ERBcx), which lacks a ligand-binding domain and is believed to inhibit ERA function (16, 17). A membranous G protein-coupled ER (GPER/GPR30) with non-genomic effects has also been described (18). ER signalling has been found to be associated with tumorigenesis of other tissues, most notably that of breast, where loss of ERB1 is thought to be associated with a poorer prognosis (19, 20).

Results from several studies have indicated a functional association between oestrogen signalling and

parathyroid functionality *in vitro*, with effects on PTH secretion, DNA synthesis and gene transcription (21, 22, 23, 24).

Oestrogen signalling was suspected to affect parathyroid tumour development but initial results indicated that parathyroid glands in both normal and tumorous states lacked ERA expression (25). Recently, however, two ERB isoforms (ERB1 and ERB2) were identified in parathyroid adenomas and the adjacent normal parathyroid tissue; moreover, in several cases, nuclear expression of ERB1 was found to be weaker in the tumour than in the corresponding normal rim. Additionally, an inverse relationship was observed between ERB1 nuclear expression and tumour weight. Selective activation of ERB1 by agonist treatment *in vitro* was found to functionally alter parathyroid tumour gene transcription in a tumour-suppressive manner (24).

In this study, we sought to assess the findings outlined above by investigating the expression of ERB1 and ERB2 in a large panel of parathyroid specimens, including a set of prospectively collected parathyroid adenomas, retrospectively collected extremely large parathyroid adenomas, atypical adenomas, parathyroid carcinomas and normal parathyroid glands.

Materials and methods

Patient samples and ethical statements

All parathyroid adenomas were obtained from patients who underwent surgery at Karolinska University Hospital after informed consent and local ethical approval were obtained. Both the prospective cohort of 146 adenomas, including extensive clinical biochemistry, and the retrospective set of 15 adenomas with large glandular weight have been described previously (26, 27, 28). Parathyroid carcinomas and atypical adenomas were collected on a worldwide basis: eight have previously been described as part of a study of historical material (29). Additionally, three cases of parathyroid carcinomas were obtained from Kyiv City Teaching Endocrinological Centre (Kiev, Ukraine) with informed consent and local ethical approval. In total, 172 tumours were included in this study. All specimens were classified according to the WHO criteria. Clinical data are summarised in Table 1.

Normal parathyroid glands were obtained from normocalcaemic patients undergoing thyroid surgery, where normal parathyroid glands were identified en passant in the histopathological material. Cases of invasive ductal breast carcinoma and normal breast tissue

Table 1 Tumour and clinical characteristics.

Tumours	172
Adenomas	161
Atypical adenomas	5
Carcinomas	6
S-calcium (mmol/l)	
Min	1.31
Mean	1.46
Median	1.43
Max	1.89
Tumour weight (mg)	
Min	52
Mean	1720
Median	494
Max	27 800
P-PTH (ng/l)	
Min	54
Mean	161
Median	117
Max	2420

served as positive controls. Cases of breast carcinoma and normal tissues were anonymised in accordance with Swedish Biobank law.

Immunohistochemistry

Slide deparaffinisation, rehydration and high pH antigen retrieval were performed using a PT link system (Dako, Glostrup, Denmark). Immunostaining was performed using an IntelliPATH FLX automated stainer (HistoLab, Gothenburg, Sweden) with primary antibodies targeting human ERB1 diluted to 1:200 (rabbit monoclonal, EPR3778; Abcam, Cambridge, UK) and human ERB2 diluted to 1:300 (mouse monoclonal, clone 57/3; AbD Serotec, Oxford, UK), for 45 min at room temperature. Primary antibodies were visualised using a HRP-coupled polymer incorporating 3,3'-diaminobenzidine (DAB) chromogen. Before polymer incorporation, anti-ERB2 was treated with an anti-mouse probe, yielding additional signal amplification. Haematoxylin was used for counterstaining.

Experimental optimisation of ERB1 was determined using anonymised slides of breast cancer and parathyroid specimens, evaluating antigen retrieval solution (low- and high-pH) as well as antibody concentrations and incubation time.

To validate antibody specificity, anti-ERB1 blocking experiments were conducted as described later in this study. For ERB2, experiments were conducted as described previously (24). A single slide of multiple parathyroid specimens was run in each experiment for comparison to exclude batch effects. Slides were selected randomly for batch runs, with no entire clinical subgroup run in a single batch.

Scoring of immunohistochemistry

All slides were scanned using a Hamamatsu digital scanner (Hamamatsu, Shizuoka, Japan) for manual and digital scoring. The authors (F Haglund, C C Juhlin and A Höög) performed manual scoring cooperatively, reaching consensus in all cases. ERB1 and ERB2 nuclear staining (positive, weak or negative) was evaluated in tumourous and adjacent normal parathyroid tissue (normal rim). Tumours and normal rim with either weak or positive nuclear staining showing a significant proportion (>25%) of negative cells (as either an overall mixture of positive and negative nuclei or a negative subpopulation within the slide) were scored as heterogeneous ('mix').

Quantification of nuclear staining intensity was performed as described previously for all slides (24). In short, digitalised images were analysed using the Visiopharm Tissuemorph Digital Pathology (Visiopharm, Hoersholm, Denmark) image analysis software. An algorithm for nuclei-specific signal selection was manually designed for each case. Staining intensity in the nuclei was estimated by measuring the unmodified signal intensity in a DAB-specific layer (signal filter provided by Visiopharm Tissuemorph Digital Pathology). DAB intensity was measured using the standard Red-Green-Blue (RGB) integer value scale, ranging from 0 (no signal) to 255 (fully saturated signal).

Immunoblotting

Western blotting was performed to validate the specificity of the anti-ERB1 (EPR3778) antibody, which has not, to our knowledge, previously been described. Sample protein was extracted by cutting fresh frozen breast or parathyroid tissue, followed by incubation in RIPA buffer and treatment with proteinase inhibitors for 30 min on ice. Samples were centrifuged at 13 000 *g* for 30 min in 4 °C to remove residual cellular debris. Protein concentration was measured by A280 absorption using a Nanodrop instrument (Thermo Fisher Scientific, Inc., Waltham, MA, USA); 5–12% BisTris gels were loaded with 75 µg protein/sample. Magic Mark XP Western and Novex Sharp protein standards were used to identify protein size and migration. Protein migration was performed for 50 min at 200 V in a wet chamber using MOPS buffer with reducing conditions and proteins were transferred onto a nitrocellulose membrane (all reagents from Life Technologies). Membranes were blocked in 5% non-fat milk and incubated in anti-ERB1 (EPR3778, 1:2000 in 5% milk) overnight at 4 °C followed by a 1-h room

temperature incubation with an HRP-coupled secondary anti-rabbit antibody (1:10 000 in 5% milk). Membranes were developed with ECL (Amersham, GE Healthcare, Little Chalfont, UK) reagent, digitally scanned and edited in Adobe Photoshop. No additional bands or features were removed or enhanced in the digitalising process. The antibody revealed a single band at approximately 58 kDa, close to the predicted size of 59.2 kDa for ERB1, in normal breast tissue and parathyroid adenomas (Supplementary Figure 1, see section on supplementary data given at the end of this article).

Antibody blocking

The EPR3778 immunising peptide sequence was kindly provided by Abcam. A corresponding peptide with >75% purity was commercially produced (GeneScript, Piscataway, NJ, USA). Antibody immunoreactivity was confirmed using a dot-blot protocol (results not shown). The antibody was then incubated with the blocking peptide (diluted to >100-fold excess molarity) for 1 h at room temperature followed by 1 h at 4 °C. An unblocked antibody dilution was run in parallel to the blocking experiments using otherwise the same conditions (Supplementary Figure 2, see section on supplementary data given at the end of this article).

Statistical analysis

All data were analysed using SPSS version 22.0 (IBM, Inc., Chicago, IL, USA). All statistical analyses assumed a non-normal distribution and two-tailed tests when applicable. Bivariate analyses of covariance were performed using Spearman's ranked correlation. Categorical variable distribution was analysed using Fisher's exact test. The Mann–Whitney *U* tests for unpaired data or the Wilcoxon tests for paired data were performed for comparisons between groups. For categorical variables with more than two levels, Kruskal–Wallis one-way ANOVA was employed. $P < 0.05$ was considered statistically significant.

Tumour weight quartiles were defined for statistical comparison: the first weight quartile ranged from 52 to 254 mg, the second and third quartiles from 262 to 1020 mg and the fourth quartile from 1035 to 27 800 mg.

Box plots were created using SPSS or R version 3.1.2. Box whiskers represent the lowest value still within 1.5 interquartile range (IQR) of the lower quartile, and the highest value still within 1.5 IQR of the upper quartile.

Results

Validation of digitally measured intensity by comparison with manual scoring

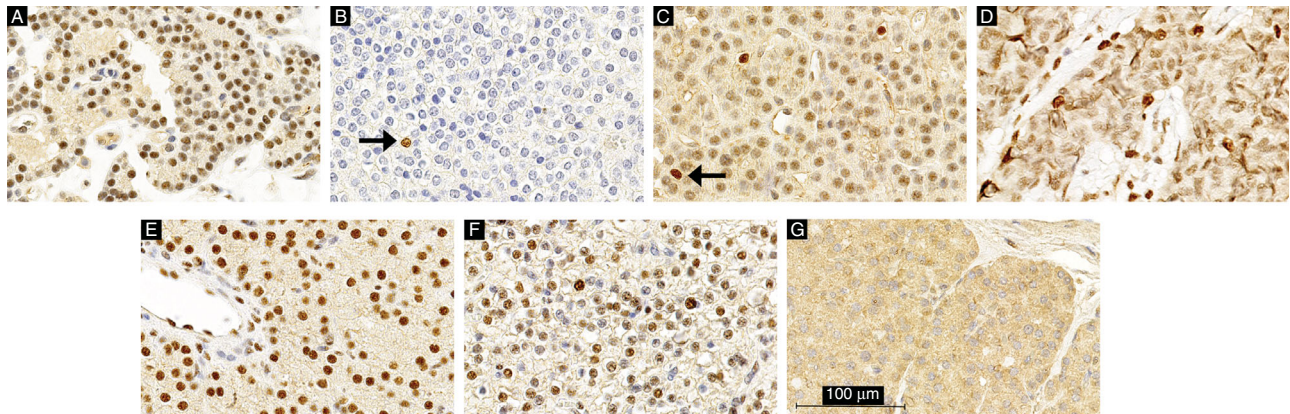
The overall association between manual and digital scoring results was strong: ERB1 nuclear intensity as measured by Visiopharm Tissuemorph was significantly associated with the results from manual scoring (ERB1 visualised in Supplementary Figure 3, see section on supplementary data given at the end of this article, Spearman's ranked $r = 0.642$, $P < 0.0005$). The intensity of individual cases did not exclusively overlap the manual measurement (e.g. tumour nuclear staining heterogeneity was not included in this model). Statistical correlation between manual and digital scoring was not performed for ERB2 on account of few samples being available with weak (=1) or negative (=3) ERB2 staining.

Heterogeneous expression of ERB1 in parathyroid tumours

We observed varying levels of ERB1 nuclear expression in all normal parathyroid glands (10/10) and the majority of parathyroid tumours (120/170 = 70.6%); about half of the tumours exhibited a heterogeneous mix of negative and positive cells (52/120 = 43%) (Fig. 1A, B and C, Supplementary Figure 4A, B and C, see section on supplementary data given at the end of this article). Specifically, nuclear immunoreactivity was scored as weak or positive in three out of six (50%) parathyroid carcinomas (Supplementary Figure 5), one out of seven (14%) atypical adenomas and 116 out of 157 (74%) regular adenomas. Tumour ERB1 nuclear intensity did not differ significantly between tumours with different histopathological diagnoses or tumour-dominant cell type (chief-, oxyphil or mixed cell types).

Tumour nuclear ERB1 expression correlates inversely with tumour weight. Both digital and manual scores of ERB1 levels correlated inversely with tumour weight (Spearman's rank correlation, $r = -0.20$, $P = 0.011$, Fig. 2A). Nuclear ERB1 intensity was also significantly lower in tumours of the highest (fourth) versus the lowest (first) weight quartiles (Kruskal–Wallis all quartiles: $P = 0.024$ and Mann–Whitney *U* tests: $P = 0.007$, Fig. 2B).

Tumours with negative nuclear ERB1 staining (compared with weak or positive staining) had significantly higher levels of plasma intact PTH (Mann–Whitney *U* tests: $P = 0.027$ and $P = 0.003$, Fig. 2C) and serum ionised calcium (Mann–Whitney *U* tests: $P < 0.0005$

**Figure 1**

Representative immunohistochemistry slides showing ERB1 nuclear staining in (A) positive parathyroid adenoma, (B) negative parathyroid adenoma, (C) weak parathyroid adenoma and (D) positive ductal mammary carcinoma; ERB2 nuclear staining in (E) positive parathyroid adenoma,

(F) mixed negative and positive staining pattern in parathyroid adenoma and (G) negative parathyroid adenoma. Arrows in (B) and (C) indicate single cells with strong ERB1 immunoreactivity subject to manual scoring.

and $P=0.002$, Fig. 2D). ERB1 nuclear staining intensity showed a weakly significant inverse correlation with PTH levels but not with calcium levels (Spearman's ranked $r=-0.17$, $P=0.036$ and $r=-0.16$, $P=0.055$ respectively). Tumour ERB1 nuclear intensity did not remain a statistically significant predictor of PTH after correction for tumour weight (PTH as a dependent variable in linear regression, crude B, -0.246 , $P=0.002$; adjusted B -0.087 , $P=0.219$).

Indications that loss of ERB1 is a tumour-specific phenomenon

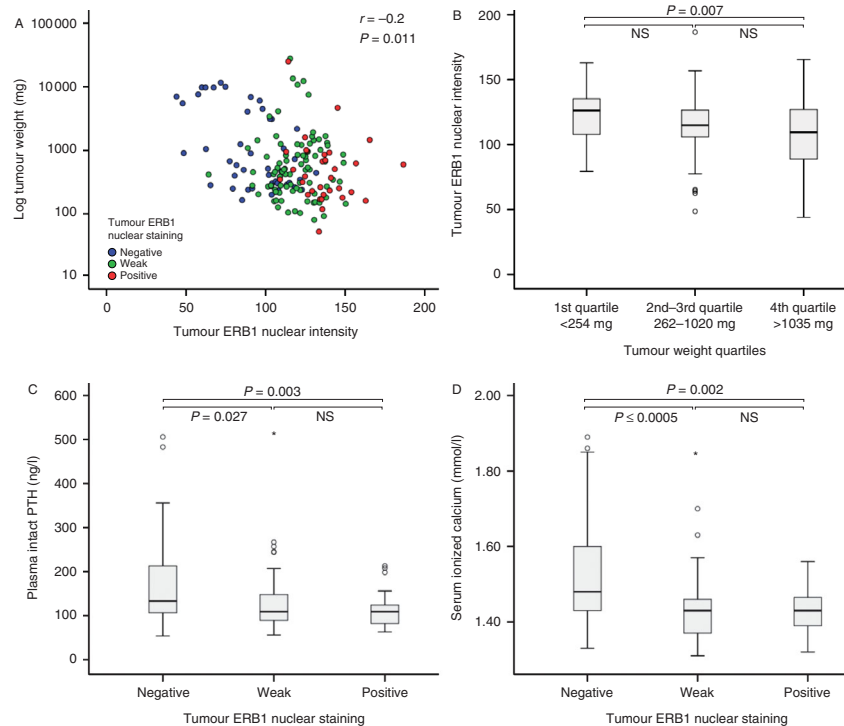
A comparison between tumour and normal rim ERB1 signal intensity was performed on an individual-case basis (normal rim was present in 60 samples, all adenomas). For comparative purposes, an arbitrary difference of ± 10 integer values (RGB range 0–255) was chosen to classify the cases as having stronger, equivalent or weaker tumour staining compared with normal rim (illustrated in Supplementary Figure 6A, see section on supplementary data given at the end of this article); 21 out of 60 cases were classified as having weaker intensity (tumour), 28 out of 60 as having equivalent intensity and 11 out of 60 as having stronger intensity (tumour). Overall, tumours had a significantly lower ERB1 nuclear intensity than the corresponding normal rim (Wilcoxon, $P=0.027$), which in turn was similar to that of normal parathyroid glands. Additionally, no significant differences were observed when the intensities of normal rims from different (tumour) weight

quartiles were compared with each other or with normal parathyroid glands (data not shown). This supports the notion that normal parathyroid cells express ERB1 with limited variation, and that the extracellular environment in PHPT (e.g. increased serum calcium and PTH) does not strongly regulate ERB1 levels in normal parathyroid cells.

As tumours with a lower ERB1 nuclear intensity were found to weigh more, we investigated the overall tumour versus normal classification in association with tumour weight (Supplementary Figure 6B). We observed that the majority of large tumours were classified as having a decreased tumour ERB1 intensity compared with the normal rim. Results of comparisons of the ERB1 nuclear staining intensity of tumour and normal rim samples classified according to tumour weight quartiles of the samples ($n=60$) indicate that a significant decrease in tumour intensity was limited to tumours of the largest (fourth) weight quartile (Wilcoxon, $P=0.004$, Fig. 3).

Sex of the patient is not associated with tumour nuclear ERB1 levels

ERB1 levels (both manual and digital scores) in tumours from males did not differ significantly from those for tumours obtained from females. To investigate the potential association of menopause with tumour levels of ERB1, female patients were grouped according to age: <40 ($n=8$), 41–55 ($n=41$) or >55 ($n=83$) years. Using these criteria, no significant difference in tumour nuclear ERB1 was observed between these groups.

**Figure 2**

Association of clinical characteristics with nuclear ERB1 immunoreactivity in parathyroid tumours. (A) Scatter plot of tumour ERB1 nuclear intensity (digital scoring) and tumour weight (log₁₀-scale), showing a significant inverse correlation (Spearman's rank correlation: $r = -0.20$, $P = 0.011$). Colours of circles indicate tumour ERB1 nuclear staining (manual scoring): blue, negative; green, weak and red, positive. (B) Box plots of tumour ERB1 nuclear intensity (digital scoring) by tumour weight quartiles, showing a significant difference (Mann–Whitney U test: $P = 0.007$) between the lowest

(first) and the highest (fourth) weight quartiles; significantly higher levels of (C) plasma intact parathyroid hormone (Mann–Whitney U test: $P = 0.027$ and 0.003); and (D) serum ionised calcium (Mann–Whitney U test: $P \leq 0.0005$ and 0.002) in patients with parathyroid tumours classified as nuclear ERB1-negative (manual scoring) compared with weak or positive respectively. Open circles represent outlier values and asterisks represent extreme values (outside $1.5\times$ and $3\times$ interquartile range, respectively).

Tumour nuclear ERB1 does not correlate with other clinical features

The tumour nuclear ERB1 expression was not related to coexistence of patient diabetes, hypertension, osteoporosis or increased markers of bone metabolism (serum alkaline phosphatase, carboxy-terminal collagen or amino-terminal pro-peptide of type 1 collagen).

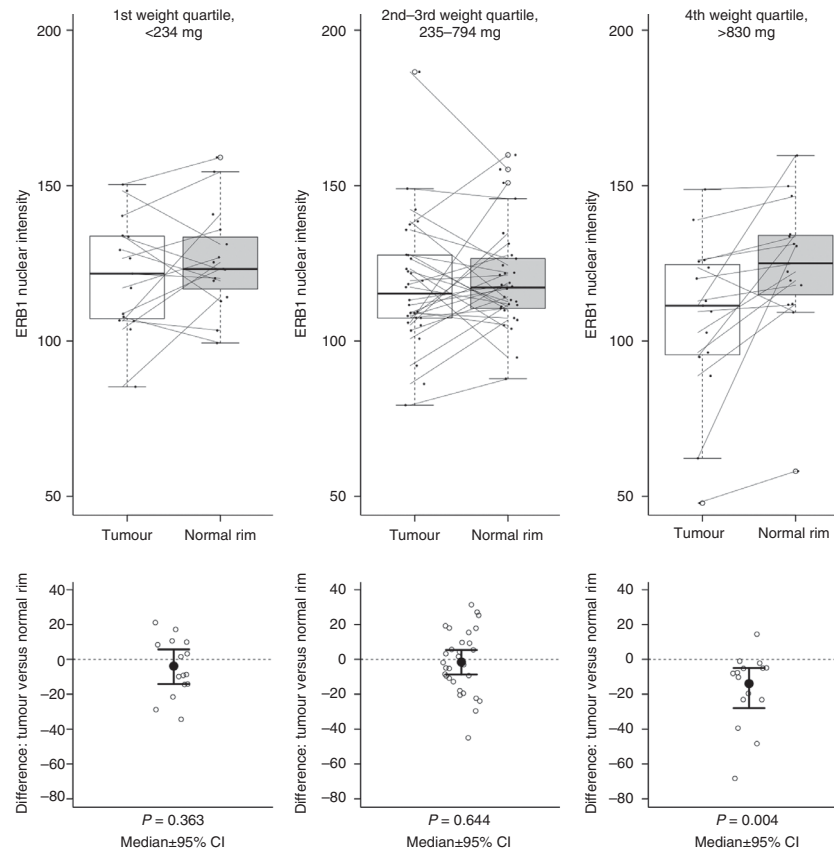
Identification of single cells with strong ERB1 nuclear immunoreactivity in parathyroid tissue

We initially observed single cells with strong positive ERB1 expression in parathyroid tumours with otherwise weak ERB1 expression, varying in density between specimens (Fig. 1B and C, indicated by black arrows). These single cells were manually counted in five high-power fields (HPFs) for each tumour: 34 out of 151 (22.5%) had ≤ 1 cell/HPF (range: min–max 0–126, median 19, mean 25), but as the number of cells did not correlate with any

clinical or tumour characteristic, their possible role or function remains ambiguous. While these cells exhibited a tumour cell-like morphology, no further characterisation of them was performed. These cells were sometimes observed in normal parathyroid tissue but the small size of normal parathyroid glands and parathyroid rims did not permit manual counting in HPF.

ERB2 is widely expressed in parathyroid tumours and normal glands

All the normal parathyroid glands (10/10) and the vast majority of the parathyroid tumours (165/171 = 96.5%) expressed strong nuclear ERB2 immunoreactivity (Fig. 1E, F and G, Supplementary Figure 4D, E and F). Tumour ERB2 nuclear staining intensity was similar in tumours from males and females and did not correlate with age, serum ionised calcium or plasma PTH of patients. No correlation was found with tumour weight or histopathological

**Figure 3**

(Upper) Box plots of nuclear ERB1 staining intensity in parathyroid tumours (white) and normal rim (grey) classified according to tumour weight quartiles. The intensities for individual tumours and the corresponding normal rims are plotted as filled circles and joined by lines for paired comparisons. (Lower) Median ($\pm 95\%$ CI) difference in ERB1 nuclear

staining intensity between tumour and corresponding normal rim classified according to tumour weight quartiles. Individual cases are plotted as open circles. A significant decrease in tumour nuclear ERB1 was observed in tumours of the largest (fourth) weight quartile (Wilcoxon's signed rank test: $P=0.004$).

diagnosis. ERB2 expression did not differ significantly between tumours, normal rims and normal parathyroid glands.

Discussion

It has been suggested previously that oestrogen signalling has a role in parathyroid tumour development (21, 22, 23, 24). To investigate this notion, we studied the expression of ERB1 and ERB2 in a large set of parathyroid tumours.

A comparison of the intensity of nuclear ERB1 immunoreactivity showed that the levels were similar in tumours, normal rims and normal parathyroid glands. As we observed a wider distribution in the tumours, we proposed the hypothesis that ERB1 levels could be altered in the tumourous tissue. Indeed, the overall nuclear intensity of ERB1 was significantly lower in the tumours compared with the corresponding normal rims. The

tumour nuclear immunoreactivity of ERB1 correlated inversely with tumour weight, by both digital and manual scoring methods. This prompted us to further investigate the potential relationship between a decrease in tumour nuclear ERB1 levels and an increased tumour weight.

We found that tumours in the largest (fourth) weight quartile had a significantly lower immunoreactivity than the corresponding rim, in contrast to tumours in the other weight quartiles. As the ERB1 levels of the normal rims were equivalent to those of normal parathyroid glands, we concluded that this could represent a tumour-specific reduction in ERB1 nuclear immunoreactivity, which in turn is associated with higher tumour weight. The coupling of ERB1 function to tumour suppression in parathyroid tumour cells *in vitro* also supports this role (24). The difference in nuclear ERB1 immunoreactivity could reflect the relative tumour sensitivity to ER modulation. Thus, future pharmacological studies of

SERMs in PHPT should consider tumour weight as a potential factor in patient/tumour responsiveness.

To investigate the potential difference in tumour nuclear ERB1 levels in pre- and postmenopausal female patients, we divided these patients into subgroups. No statistically significant difference between the groups was detected. While no direct measurement of oestrogen levels was performed, this indicates that tumour ERB1 levels are not strongly dependent on the menopausal status of female patients. Without a reliable medical history, however, one cannot rule out a causal linkage to oestrogen levels or hormone replacement therapy. Results from epidemiological studies were indicative of an inverse association between PHPT incidence and oestrogen replacement therapy in Rochester, MN, as well as an increased risk of parathyroid adenoma with high parity (30, 31, 32). The potential effects of oestrogen and oestrogen compounds on parathyroid tumourigenesis deserve further attention.

Results of functional studies have indicated a causal linkage between PTH secretion and oestrogen treatment in parathyroid cell cultures. We observed a significant inverse relationship between patient PTH levels and tumour nuclear ERB1 levels, but it ceased to be significant after correction for tumour weight. Further *in vitro* analyses of PTH secretion in parathyroid tumour cells with a focus on ER signalling would be of interest.

We observed tumours with either an increased or a decreased nuclear ERB1 staining intensity compared with normal rim. Cases classified as having a stronger tumour intensity (18%) had a mean increase of 19.8 (16–24; 95% CI integer value), while tumours with a weaker intensity (35%) had a mean decrease of 26.1 (19.6–33; 95% CI integer value). The smaller difference observed in tumours with an increased intensity compared with the normal rim might explain the lack of association with clinical variables in this group.

The nuclear immunoreactivity of ERB1 in the six parathyroid carcinomas was not apparently different from that in the parathyroid adenomas. Considering the inverse correlation among tumour weight, nuclear ERB1 intensity and the apparent larger size of these tumours, the nuclear ERB1 levels were higher than expected. This is remarkable as atypical adenomas have been reported to exhibit lower levels of nuclear ERB1 compared with adenomas. Similarly, we found low levels of ERB1 in atypical adenomas (six out of seven were classified as ERB1-negative). While the number of analysed cases is small, this may be a reflection of the different tumour aetiologies of entities. As our set

contained only a limited number of atypical adenomas and carcinomas, the presented correlations between tumour ERB1 levels and tumour size as well as patient biochemistry can with certainty only be applied to parathyroid adenomas.

We found an overall strong and homogenous expression of ERB2 in parathyroid tumours (96.5%) and normal parathyroid glands (100%). While the biological function of ERB2 in parathyroid tissue is unknown, the retention of expression of this splice variant signifies that the loss of ERB1 expression is limited to that isoform. Furthermore, the structural properties of ERB2 determine that the receptor cannot mediate oestrogen signalling by itself (16, 17). As parathyroid tissue effectively lacks ERA, the down-regulation of ERB1 would effectively silence the ligand-mediated oestrogen signalling in parathyroid tumours. As ERB2 was found to be widely expressed across our panel, this might indicate a vital housekeeping function for this receptor in parathyroid tumours, albeit not directly influencing the clinical parameters analysed in this study. Future insights into the function of ERB2 would help to elucidate the complex context of ER signalling.

Parathyroid carcinomas are a rare entity but additional treatment options are still warranted. Studies of SERMs in PHPT have focused on pharmacological effects on bone metabolism, serum calcium and tumour PTH secretion. While these are important outcomes in patients with parathyroid adenomas, the potential tumour-suppressive effects may be of equal importance in patients with parathyroid carcinomas. If the effects of ERB1 activation in carcinomas are similar to those in *ex vivo* cultured parathyroid adenoma cells, SERMs and oestrogens may have a role to play in the treatment of malignant lesions. Parathyroid carcinomas are so rare that randomised double-blinded studies are ruled out. Case reports on individual patients with metastasising carcinomas and simultaneous treatment with SERMs may provide anecdotal evidence of the potential tumour-suppressive activity. Goepfert and colleagues (33) reported treatment of a single parathyroid carcinoma with hexestrol, a synthetic ER agonist. After 4 weeks, they found a significant decrease in serum calcium levels and significant tumour regression as determined by radiology. To our knowledge, there are only a few other reports of treatment with oestrogen compounds or SERMs in patients with metastatic parathyroid carcinomas and more are needed.

We identified ERB1 at an overall lower frequency in parathyroid tumours than described previously. In the current study, we classified 29% as ERB1-negative; the

remaining tumours exhibited a mix of negative and positive cells at a frequency of 43%. In the previously published study from our institution (24) ERB1 expression was observed in all the investigated cases and a mosaic pattern of ERB1-positive and -negative tumour cells was identified in 19 out of 35 tumours (54%); however, the distribution of the variation in tumour ERB1 nuclear immunostaining was similar to that in this study. We believe that the difference could be attributed to the sensitivity of the primary antibodies.

The heterogeneity of tumour nuclear ERB1 could reflect an increase in cellular proliferation, which is indicated by the tumour-specific association between ERB1 levels and tumour weight. Generally, parathyroid tumours exhibit a low MIB-1 proliferative index, making comparisons difficult. A further investigation of this association by regular immunohistochemistry would require a more sensitive marker of proliferation. Additionally, the staining heterogeneity could reflect actual tumour ERB1 signalling. Upon ligand activation, ERB1 translocates to the nuclei, where it acts as a transcription factor. Hence, negative tumour nuclei may be unresponsive to oestrogen signalling due to altered receptor turnover or inhibitory mechanisms.

In conclusion, we have found widespread expression of ERB1 and ERB2 in a large set of parathyroid tumours. While the variations in ERB2 expression were small, the nuclear immunoreactivity of ERB1 was significantly decreased in larger parathyroid tumours, indicating the potential tumour-suppressive function of ERB1 in parathyroid tumourigenesis.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EC-14-0109>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

F Haglund, G Rosin, I-L Nilsson, C C Juhlin, C Larsson, J Hartman and A Höög contributed to study concept and design: F Haglund, G Rosin, C C Juhlin, S Norenstedt and A Dinets performed acquisition of data. F Haglund, I-L Nilsson, C C Juhlin, Y Pernow, J Hartman and A Höög interpreted the data: F Haglund, C C Juhlin, C Larsson, J Hartman and A Höög drafted and revised the manuscript. F Haglund, G Rosin, I-L Nilsson, C C Juhlin, Y Pernow, S Norenstedt, A Dinets, C Larsson, J Hartman and A Höög critically revised the manuscript.

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References

- Hedbäck GM & Odén AS. Cardiovascular disease, hypertension and renal function in primary hyperparathyroidism. *Journal of Internal Medicine* 2002 **251** 476–483. (doi:10.1046/j.1365-2796.2002.00984.x)
- Nilsson IL, Yin L, Lundgren E, Rastad J & Ekblom A. Clinical presentation of primary hyperparathyroidism in Europe – nationwide cohort analysis on mortality from nonmalignant causes. *Journal of Bone and Mineral Research* 2002 **17** N68–N74.
- Nilsson IL, Zedenius J, Yin L & Ekblom A. The association between primary hyperparathyroidism and malignancy: nationwide cohort analysis on cancer incidence after parathyroidectomy. *Endocrine-Related Cancer* 2007 **14** 135–140. (doi:10.1677/erc.1.01261)
- Al-Kurd A, Mekel M & Mazeh H. Parathyroid carcinoma. *Surgical Oncology* 2014 **23** 107–114. (doi:10.1016/j.suronc.2014.03.005)
- DeLellis RA, Lloyd RV, Heitz PU & Eng C. Tumours of endocrine organs. In *World Health Organization Classification of Tumour Pathology and Genetics*, Eds RA DeLellis, RV Lloyd, PU Heitz & C Eng. Lyon: IARC Press, 2004.
- Juhlin CC & Höög A. Parafibromin as a diagnostic instrument for parathyroid carcinoma – lone ranger or part of the posse? *International Journal of Endocrinology* 2010 **2010** 324964. (doi:10.1155/2010/324964)
- Chandrasekharappa SC, Guru SC, Manickam P, Olufemi SE, Collins FS, Emmert-Buck MR, DeBelenko LV, Zhuang Z, Lubensky IA, Liotta LA *et al.* Positional cloning of the gene for multiple endocrine neoplasia-type 1. *Science* 1997 **276** 404–407. (doi:10.1126/science.276.5311.404)
- Lemmens I, Van de Ven W, Kas J, Zhang CX, Giraud S, Wautot V, Buisson N, De Witte K, Salandre J, Lenoir G *et al.* Identification of the multiple endocrine neoplasia type 1 (MEN1) gene. The European Consortium on MEN1. *Human Molecular Genetics* 1997 **6** 1177–1183. (doi:10.1093/hmg/6.7.1177)
- Carpten JD, Robbins CM, Villablanca A, Forsberg L, Prescittini S, Bailey-Wilson J, Simonds WF, Gillanders EM, Kennedy AM, Chen JD *et al.* HPR2, encoding parafibromin, is mutated in hyperparathyroidism–jaw tumor syndrome. *Nature Genetics* 2002 **32** 676–680. (doi:10.1038/ng1048)
- Cromer MK, Starker LF, Choi M, Udelsman R, Nelson-Williams C, Lifton RP & Carling T. Identification of somatic mutations in parathyroid tumors using whole-exome sequencing. *Journal of Clinical Endocrinology and Metabolism* 2012 **97** E1774–E1781. (doi:10.1210/jc.2012-1743)
- Newey PJ, Nesbit MA, Rimmer AJ, Attar M, Head RT, Christie PT, Gorvin CM, Stechman M, Gregory L, Mihai R *et al.* Whole-exome sequencing studies of nonhereditary (sporadic) parathyroid adenomas. *Journal of Clinical Endocrinology and Metabolism* 2012 **97** E1995–E2005. (doi:10.1210/jc.2012-2303)
- Doiung CP & Arnold A. Recurrent ZFX mutations in human sporadic parathyroid adenomas. *Oncoscience* 2014 **1** 360–366.



- 13 Jensen EV & Jacobsen HI. Fate of steroid estrogens in target tissues. In *Biological Activities of Steroids in Relation to Cancer*, pp 161–174. Eds G Pincus & EP Vollmer. New York: Academic Press, 1960.
- 14 Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S & Gustafsson JA. Cloning of a novel receptor expressed in rat prostate and ovary. *PNAS* 1996 **93** 5925–5930. (doi:10.1073/pnas.93.12.5925)
- 15 Petersen DN, Tkalcic GT, Koza-Taylor PH, Turi TG & Brown TA. Identification of estrogen receptor β_2 , a functional variant of estrogen receptor β expressed in normal rat tissues. *Endocrinology* 1998 **139** 1082–1092. (doi:10.1210/endo.139.3.5840)
- 16 Maruyama K, Endoh H, Sasaki-Iwaoka H, Kanou H, Shimaya E, Hashimoto S, Kato S & Kawashima H. A novel isoform of rat estrogen receptor beta with 18 amino acid insertion in the ligand binding domain as a putative dominant negative regulator of estrogen action. *Biochemical and Biophysical Research Communications* 1998 **246** 142–147. (doi:10.1006/bbrc.1998.8590)
- 17 Zhao C, Dahlman-Wright K & Gustafsson JA. Estrogen signalling via estrogen receptor β . *Journal of Biological Chemistry* 2010 **285** 39575–39579. (doi:10.1074/jbc.R110.180109)
- 18 Hammes SR & Levin ER. Extranuclear steroid receptors: nature and actions. *Endocrine Reviews* 2007 **28** 726–741. (doi:10.1210/er.2007-0022)
- 19 Heldring N, Pike A, Andersson S, Matthews J, Cheng G, Hartman J, Tujague M, Ström A, Treuter E, Warner M *et al.* Estrogen receptors: how do they signal and what are their targets. *Physiological Reviews* 2007 **87** 905–931. (doi:10.1152/physrev.00026.2006)
- 20 Nilsson S, Koehler KF & Gustafsson J. Development of subtype-selective oestrogen receptor-based therapeutics. *Nature Reviews. Drug Discovery* 2011 **10** 778–792. (doi:10.1038/nrd3551)
- 21 Greenberg C, Kukreja SC, Bowser EN, Hargis GK, Henderson WJ & Williams GA. Parathyroid hormone secretion: effect of estradiol and progesterone. *Metabolism* 1987 **36** 151–154. (doi:10.1016/0026-0495(87)90009-6)
- 22 Duarte B, Hargis GK & Kukreja SC. Effects of estradiol and progesterone on parathyroid hormone secretion from human parathyroid tissue. *Journal of Clinical Endocrinology and Metabolism* 1988 **66** 584–587. (doi:10.1210/jcem-66-3-584)
- 23 Boucher A, D'Amour P, Hamel L, Fugère P, Gascon-Barré M, Lepage R & Ste-Marie LG. Estrogen replacement decreases the set point of parathyroid hormone stimulation by calcium in normal postmenopausal women. *Journal of Clinical Endocrinology and Metabolism* 1989 **68** 831–836. (doi:10.1210/jcem-68-4-831)
- 24 Haglund F, Ma R, Huss M, Sulaiman L, Lu M, Nilsson IL, Höög A, Juhlin CC, Hartman J & Larsson C. Evidence of a functional estrogen receptor in parathyroid adenomas. *Journal of Clinical Endocrinology and Metabolism* 2012 **97** 4631–4639. (doi:10.1210/jc.2012-2484)
- 25 Prince RL, MacLaughlin DT, Gaz RD & Neer RM. Lack of evidence for estrogen receptors in human and bovine parathyroid tissue. *Journal of Clinical Endocrinology and Metabolism* 1991 **72** 1226–1228. (doi:10.1210/jcem-72-6-1226)
- 26 Sulaiman L, Nilsson I-L, Juhlin CC, Haglund F, Höög A, Larsson C & Hashemi J. Genetic characterization of large parathyroid adenomas. *Endocrine-Related Cancer* 2012 **19** 389–407. (doi:10.1530/ERC-11-0140)
- 27 Norenstedt S, Pernow Y, Brismar K, Säaf M, Ekip A, Granath F, Zedenius J & Nilsson IL. Primary hyperparathyroidism and metabolic risk factors, impact of parathyroidectomy and vitamin D supplementation, and results of a randomized double-blinded study. *European Journal of Endocrinology* 2013 **6** 795–804. (doi:10.1530/EJE-13-0547)
- 28 Norenstedt S, Pernow Y, Zedenius J, Nordenström J, Säaf M, Granath F & Nilsson IL. Vitamin D supplementation after parathyroidectomy: effects on bone mineral density – a randomized double-blind study. *Journal of Bone and Mineral Research* 2014 **4** 960–967. (doi:10.1002/jbmr.2102)
- 29 Juhlin CC, Villablanca A, Sandelin K, Haglund F, Nordenström J, Forsberg L, Bränström R, Obara T, Arnold A, Larsson C *et al.* Parafibromin immunoreactivity: its use as an additional diagnostic marker for parathyroid tumor classification. *Endocrine-Related Cancer* 2007 **14** 501–512. (doi:10.1677/ERC-07-0021)
- 30 Wermers RA, Khosla S, Atkinson EJ, Hodgson SF, O'Fallon WM & Melton LJ. The rise and fall of primary hyperparathyroidism: a population-based study in Rochester, Minnesota 1965–1992. *Annals of Internal Medicine* 1997 **126** 433–440. (doi:10.7326/0003-4819-126-6-199703150-00003)
- 31 Wermers RA, Khosla S, Atkinson EJ, Achenbach SJ, Oberg AL, Grant CS & Melton LJ. Incidence of primary hyperparathyroidism in Rochester, Minnesota, 1993–2001: an update on the changing epidemiology of the disease. *Journal of Bone and Mineral Research* 2006 **21** 171–177. (doi:10.1359/JBMR.050910)
- 32 Rastad J, Ekeboom A, Hultin H, Wu J, Lundgren E, Hsieh CC & Lambe M. Childbearing and the risk of parathyroid adenoma – a dominant cause for primary hyperparathyroidism. *Journal of Internal Medicine* 2001 **250** 43–49. (doi:10.1046/j.1365-2796.2001.00849.x)
- 33 Goepfert H, Smart CR & Rochlin DB. Metastatic parathyroid carcinoma and hormonal chemotherapy. Case report and response to hexestrol. *Annals of Surgery* 1966 **164** 917–920. (doi:10.1097/00000658-196611000-00021)

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