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# The profile of *ErbB*/*Her* family genes copy number assessed by real-time PCR in parathyroid adenoma and hyperplasia associated with sporadic primary hyperparathyroidism

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**Hyperparathyroidism (pHPT) is a relatively frequent endocrinopathy, however, the molecular mechanisms of its etiology remain poorly understood. This disorder is mainly associated with benign tumours (adenoma) and hyperplasia of the parathyroid, hence, the focus is directed also to genes that are likely to be involved in carcinogenesis. Among such genes are *ErbB*/*Her* family genes already used in diagnosis of other tumours (e.g., breast carcinoma) and reported also to play a role in development of endocrine lesions. So far, ErbB-1/Her-1/EGFR expression has been detected in pHPT-associated adenomas and hyperplasia as opposed to no expression in normal parathyroid tissue. Moreover, losses or gains of the fragments of chromosomes where *ErbB*/*Her* genes are located have been reported. In this study, the gene dosage of *ErbB*/*Her* family genes were determined for the first time in parathyroid adenomas, hyperplasia and morphologically unchanged tissue in order to establish their putative role in the development of the disease. Genomic DNA was isolated from 33 patients with sporadic hyperparathyroidism and the gene copy numbers were assessed using real-time PCR. The *ErbB*/*Her* genes' profile was unaltered in most of the examined samples. Two low-level amplifications of *ErbB-1*/*Her-1*/*EGFR* gene, two deletions of *ErbB-2*/*Her-2*, and six deletions of *ErbB-4*/*Her-4* were found. The *ErbB-3*/*Her-3* gene remained unaffected. No correlation with clinical parameters was found for any gene. Both the low number of alterations and a lack of their associations with clinical parameters exclude the prognostic value of the *ErbB*/*Her* genes family in parathyroid tumourigenesis. Nevertheless, the *ErbB-4*/*Her-4* deletions seem to be interesting for further investigations, especially in the context of PTH secretion.**

**Keywords:** hyperparathyroidism, parathyroid adenoma, parathyroid hyperplasia, quantitative real-time PCR, *ErbB*/*Her*

## INTRODUCTION

Sporadic primary hyperparathyroidism (pHPT) is one of the most frequent endocrine disorders (Adami *et al*., 2002). Its prevalence is estimated as 1:500 to 1:1000. pHPT is characterized by increased secretion of parathyroid hormone (PTH), which in consequence leads to hypercalcemia connected with renal complications, hypertension and dysregulation of metabolism of the whole organism (reviewed in Carling, 2001; Imanishi, 2002; Miedlich *et al*., 2003; Younes *et al*., 2005). The molecular ba-

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**Abbreviations:** A, amplification; AGP, parathyroid adenoma; CGH, comparative genomic hybridization; D, deletion; FISH, fluorescent *in situ* hybridization; HypGP, hyperplasia; N, normal gene copy number; pHPT, sporadic primary hyperparathyroidism; PTH, parathormon.

sis of this disorder remains unclear. The variety of described abnormalities suggests different genetic defects leading to changes and/or dysfunctions of parathyroid cells (Kameyama *et al.*, 2000). pHPT in most cases is associated with parathyroid adenoma (AGP, *adenoma glandulae parathyroideae,* 70–90% of all cases), hyperplasia (HypGP, *hyperplasia glandulae parathyroideae,* 15–20%) and only in very few cases with parathyroid carcinoma (Monson, 2000; Marx, 2002). Hence, very often the interest is directed to well-known genes involved also in tumourigenesis of other organs, such as *CCND1*/*PRAD1*, *MEN1*, *RET*, *p53*, *RAS*, *pRb*. Nevertheless, no significant association has been shown to date between any genetic factor and parathyroid tumourigenesis or pHPT, apart from two genes: *CCND1*/*PRAD1* (cyclin D1 gene) (Hemmer *et al.*, 2001) and *MEN1* (multiple endocrine neoplasia 1 gene) (reviewed in Carling, 2001; Imanishi, 2002; Miedlich *et al.*, 2003).

One of the genes which have already been examined in parathyroid benign tumours is that encoding ErbB-1/Her-1/EGFR. This protein is a member of the ErbB/Her family of tyrosine kinase receptors and among others is involved in cellular signal transduction (reviewed in Zaczek *et al.*, 2005). Altered expression of ErbB/Her receptors might lead to impaired cell proliferation. *ErbB*/*Her* genes are often mutated or have altered expression in cancer and precancerous cells, which is often a sign of progression and development of disease (Vogt *et al.*, 1998; Xu *et al.*, 2002; Awaya *et al.*, 2005; Saxby *et al.*, 2005; Sassen *et al.*, 2008). *ErbB*/*Her* aberrations have also been reported in some types of endocrine lesions, e.g., of thyroid, pheochromocytoma and pancreas (Sworczak e*t al.*, 2002; Freudenberg *et al.*, 2005; Saxby *et al.*, 2005; Lee *et al.*, 2007). *ErbB-1*/*Her-1*/*EGFR* expression has been found in pHPT-related adenomas and hyperplasia (Sadler *et al.*, 1996; Gülkesen *et al.*, 2001) and, interestingly, recent data have documented an involvement of its activation in the initiation of parathyroid hyperplasia as a consequence of secondary hyperparathyroidism (Cozzolino *et al.*, 2005).

Additionally, comparative genomic hybridization (CGH) and fluorescent *in situ* hybridization (FISH) data document some gains and losses of chromosome fragments in a pool of pHPT-associated AGPs (Agarwal *et al.*, 1998; Palanisamy *et al.*, 1998; Kytölä *et al.*, 2000; Garcia *et al.,* 2002; Forsberg *et al.,* 2005). The observed chromosomal changes concern also chromosomes 2, 7, 12 and 17 where the *ErbB*/*Her* family genes are localized.

Hence, the aim of our study was to examine variations of the copy number of *ErbB*/*Her* genes in pHPT-related adenoma and hyperplasia as well as in morphologically unchanged parathyroid tissue. Furthermore, the possible impact of chromosomal aberrations on the disease status was analyzed.

## MATERIAL AND METHODS

**Surgical specimens.** Human parathyroid tissue samples were collected from 33 patients from the Northern part of Poland, affected with sporadic pHPT. All patients had no history of familial hyperparathyroidism or a multiple endocrine neoplasia syndrome. The patients were diagnosed and treated in the Department of Endocrinology and Internal Medicine at the Medical University of Gdańsk (Poland) in years 2001–2006. After surgical removal tissue specimens were frozen and stored at –80°C. Material was classified by pathologist. A selected part of the material was used for DNA extraction. Ten out of 33 cases (30.3%) were classified as adenoma (AGP) and 23 (69.7%) as hyperplasia (HypGP). There were 24 females and 9 males among patients. The mean age at the time of surgery was 59.26 year (range: 29–77 year). Additional demographic and clinical information was available for 33 patients. The mean size of the maximal dimension of excised AGP and HypGP was 20.67 mm (range: 8–50 mm). All patients had increased levels of serum parathormon (PTH), calcium (Ca), creatinine and alkaline phosphatase and a decreased phosphate (P) serum concentration. Seven samples characterized as morphologically unchanged parathyroid obtained during thyroidectomy from patient not affected with hyperparathyroidism were taken as a control.

**DNA isolation.** DNA was isolated from 20 mg of selected parathyroid tissue with the usage of QIAamp® DNA Mini Kit (Qiagen, Germany) as described by the manufacturer. Control DNA was isolated in the same way from SKBR-3 (ATCC HTB-30) and MDA-MB-468 (ATCC HTB-132) cell lines. The purity of DNA was verified by the ratio $A_{260}/A_{280}$ = 1.70–1.95. For further procedures DNA was prepared at concentration of 10 ng/µl and stored at –20°C.

**Measurement of gene copy number by real-time PCR.** A set of primers was designed for real-time PCR (sequences 5′→3′) as follows: *ErbB-1*/*Her-1*/*EGFR* forward (F): TCTGCATTCCTGCCGAGTTC, *ErbB-1*/*Her-1*/*EGFR* reverse (R): GCAGTCTCCACTCCATGCTCA, *ErbB-2*/*Her-2* F: CTGCTGGTCGTGGTCTTGG, *ErbB-2*/*Her-2* R: CTGCAGCAGTCTCCGCATC, *ErbB-3*/*Her-3* F: GCTCATCCTGGCCAACAC, *ErbB-3*/*Her-3* R: CATACCCGATCAGCACCAGTG, *ErbB-4*/*Her-4* F: TGCTGCT GCTCACTCATTGG, *ErbB-4*/*Her-4* R: CTGCGCGTCTGGAGTTGTTG, 3p F: CTCATAGGCGAAGGCACCAG, 3p R: GGTCAAGTTCCGCACACACC. Real-time PCR was performed on a LightCycler ver. 2.0 (Roche, Germany). PCR reactions were prepared in a total volume of

10 μl using LC FastStart DNA Master SYBR Green I (Roche, Germany) as described by the manufacturer, with $MgCl_2$ at a final concentration of 2.5 μM and primers at a final concentration of 0.5 μM each. The DNA template was added at 10 ng per sample. The following temperature profile was used: denaturation — 10 min at 95°C; quantification (40 cycles) — 10 s at 95°C and 10 s at 60°C with single fluorescence acquisition; melting curve — 0 s at 95°C, 15 s at 65°C and 0 s at 95°C with continuous fluorescence acquisition (ramping rate 0.1°C/s); cooling — 30 s at 40°C. The ramping rate — if not mentioned otherwise — was set at 20°C/s. The Cp values and melting curves of all samples were obtained and analyzed. For every sample the mean of three Cp values for each gene was calculated into its concentration according to a standard curve prepared for each gene separately. Then, the ratio between the concentration of the gene of interest and a reference gene was counted and normalized to the related ratio of normal parathyroid tissue for the same gene. The gene dosage was compared to the cut-off values and defined as amplification (A), deletion (D) or normal gene copy number (N).

**Statistics.** Non-parametric Kruskal–Wallis test was performed to compare the results with the clinical data. The level of significance was set to 0.05 for a significant correlation.

## RESULTS

In order to analyse the *ErbB/Her* family genes' dosages in pHPT the quantitative real-time PCR was established with the usage of region on chromosome 3p as a reference. A standard curve was obtained for each tested gene and the efficiency of reaction was checked. The Cp values of each gene from all samples were counted into dosage according to the appropriate standard curve. The cut-off values were determined as follows: below 0.5 as a deletion (D), 0.5–2.0 as a normal gene copy number (N) and above 2.0 as an amplification (A). According to these values, 10 parathyroid adenomas, 23 hyperplasia and 7 normal parathyroid tissues were examined for copy number of the *ErbB/Her* genes.

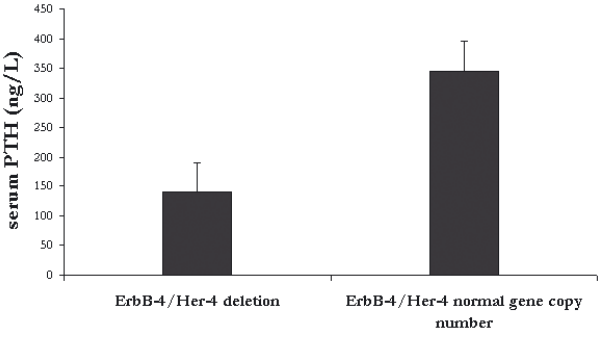


**Figure 1. Parathormon concentration in serum of patients affected with sporadic hyperparathyroidism and bearing deletion or normal gene copy number of *ErbB-4/Her-4* gene.**

The control normal parathyroid tissue not affected with pHPT revealed normal copy numbers of all tested genes with one exception where, interestingly, an *ErbB-4/Her-4* deletion appeared. The assessment of gene copy numbers for two cell lines with known amplified *ErbB-1/Her-1/EGFR* or *ErbB-2/Her-2* gene (respectively, MDA468 and SKBR322 cell line) confirmed the method's accuracy and sensitivity.

The majority of pHPT-affected patients had a completely unchanged profile of the *ErbB/Her* genes. Six patients with pHPT (18.2%) revealed abnormalities of at least one examined gene.

Two low-level amplifications of *ErbB-1/Her-1/EGFR* were found (6.1%), two (6.2%) deletions of *ErbB-2/Her-2* and five (15.1%) deletions of *ErbB-4/Her-4*. There were no amplifications of *ErbB-2/Her-2* and no aberrations of *ErbB-3/Her-3*. Simultaneous aberrations of two genes were found in three patients (9.1%). There were two cases with co-deletions of *ErbB-2/Her-2* and *ErbB-4/Her-4* and one case with amplification of *ErbB-1/Her-1/EGFR* and deletion of *ErbB-4/Her-4*. Alterations in more than two genes were found. The range and the mean of

**Table 1. The gene dosages of *ErbB/Her* family in 33 parathyroid tissues of the patients affected with sporadic primary hyperparathyroidism.**

Note that 32 samples were tested for *ErbB–2/Her-2* gene dosage.

| Gene | Gene dosage | | Gene status | Frequency | |
|---|---|---|---|---|---|
| | Range | Mean | | No. of cases | % |
| | – | – | deletion | – | – |
| *ErbB-1/Her-1/EGFR* | 0.65–1.98 | 1.03 | normal | 31 | 94.90 |
| | 2.30–2.86 | 2.58 | amplification | 2 | 5.10 |
| | 0.38–0.43 | 0.41 | deletion | 2 | 6.25 |
| *ErbB-2/Her-2* | 0.72–1.31 | 0.98 | normal | 30 | 93.75 |
| | – | – | amplification | – | – |
| | – | – | deletion | – | – |
| *ErbB-3/Her-3* | 0.50–1.44 | 1.01 | normal | 33 | 100 |
| | – | – | amplification | – | – |
| | 0.20–0.46 | 0.31 | deletion | 5 | 15.15 |
| *ErbB-4/Her-4* | 0.68–1.12 | 0.92 | normal | 28 | 84.85 |
| | – | – | amplification | – | – |

**Table 2. Characteristics of patients with sporadic primary hyperparathyroidism and altered profile of ErbB/Her genes**

| Patient code | *ErbB-1/Her-1/ EGFR* | *ErbB-2/ Her-2* | *ErbB-3/ Her-3* | *ErbB-4/ Her-4* | Sex | Age | Histology | Diameter (mm) | PTH (ng/L) |
|---|---|---|---|---|---|---|---|---|---|
| Pth37 | n | n | n | D | m | 46 | AGP | 35 | 156 |
| Pth39 | A | n | n | D | f | 77 | AGP | 12 | 222 |
| Pth38 | n | n | n | D | f | 52 | HypGP | 18 | 101 |
| Pth3 | n | D | n | D | m | 45 | HypGP | 30 | 133 |
| Pth0 | n | D | n | D | m | 76 | HypGP | 30 | 147 |
| Pth21 | A | n | n | n | f | 69 | HypGP | 20 | 302 |

n, normal gene copy number; A, amplification; D, deletion; f, female; m, male; AGP, parathyroid adenoma; HypGP, parathyroid hyperplasia; PTH, parathormon.

the assessed gene dosages as well as the frequency of amplifications, deletions and normal gene copy numbers found for each gene in all examined samples are presented in Table 1. The clinical data of selected parathyroid samples with the *Erb*/*Her* alterations are presented in Table 2. No significant correlations were found between any gene's copy number and clinical parameters like patients' age, type of changes, size and localization, PTH, Ca, P and alkaline phosphatase level. However, an interesting tendency was observed in samples bearing an *ErbB-4*/*Her-4* deletion. The concentration of serum PTH was decreased in those samples in comparison with samples with a normal *ErbB-4*/*Her-4* gene copy number ($P>0.05$) (Fig. 1).

## DISCUSSION

pHPT is a frequently diagnosed endocrinopathy with severe consequences for the whole organism. The majority of diagnosed cases are associated with benign parathyroid tumours. Hence, genes known to be involved in tumourigenesis of other organs are tested as factors potentially related to the status of illness and possibly indicating the prognosis for patients. In this study, genes of the *ErbB*/*Her* family — known to play a role in tumourigenesis of other organs — were investigated for the first time in pHPT-related adenoma and hyperplasia as well as in morphologically unchanged parathyroid tissue.

The reference locus (on chromosome 3p) was chosen by analysis of the available CGH and FISH literature data on sporadic parathyroid adenomas and carcinomas. It is believed to be the least frequently altered region in the Caucasian race (Agarwal *et al*., 1998; Kytölä *et al*., 2000; Garcia *et al*., 2002). Those studies also show that regions where genes of the *ErbB*/*Her* family are located (chromosomes 2, 7, 12 and 17) reveal some gains (particularly chromosome 17) or losses. Nevertheless, these changes did not occur very often (Agarwal *et al*., 1998; Palanisamy *et al*., 1998; Garcia *et al*., 2002). In general, *ErbB*/*Her* aberrations are detected mainly in patients affected with more advanced types of tumour. In the case of benign tumours, abnormalities are not observed (Brandt *et al*., 1995; Schwartz *et al*., 1998; Xu *et al*., 2002) or appear only very rarely (Vogt *et al*., 1998; Awaya *et al*., 2005; Dimova *et al*., 2006), but then they are correlated with the later stages and/or progression of the disease (Awaya *et al*., 2005). In our experiments most of the samples had unchanged gene profiles. The only detected amplifications were those of the *ErbB-1*/*Her-1*/*EGFR* gene. The hypothesis about *ErbB-1*/*Her-1*/*EGFR* involvement in parathyroid tumourigenesis has been suggested by other authors who performed *in situ* hybridization or immunohistochemistry (Sadler *et al*., 1996; Gülkesen *et al*., 2001), and recently also in secondary hyperparathyroidism as an initial event for hyperplasia (Cozzolino *et al*., 2005). Gülkesen *et al*. (2001) noticed also that *ErbB-1*/*Her-1*/*EGFR* overexpression might be related to young age of patients. In contrast, in our analysis both patients with the *ErbB-1*/*Her-1*/*EGFR* amplification were of the age of around 70. We did not find any relationship between these amplifications and other clinical parameters. The deletions of the *ErbB-2*/*Her-2* gene found in two cases are not common in any type of tumours where mainly amplifications play an important role in carcinogenesis and are a prognostic factor (Sworczak *et al*., 2002; Xu *et al*., 2002; Awaya *et al*., 2005; Freundenberg *et al*., 2005).

The relatively high prevalence of deletions of the *ErbB-4*/*Her-4* gene in our study is interesting. To the best of our knowledge, *ErbB-4*/*Her-4* deletions have been detected very rarely in tumours (Zaczek *et al*., 2008). The reason of this apparent discrepancy might be the type of method used in previous studies. Real-time PCR used in our analysis is a very sensitive and precise tool enabling exact determination of gene dosage which was not possible with other methods. However, the losses on chromosome 2q (where *ErbB-4*/*Her-4* is located) in parathyroid tissue reported in the literature might also support our results (Palanisamy *et al*., 1998; Kytölä *et al*., 2000).

The possible relationship between *ErbB-4*/*Her-4* status and serum PTH should be investigated on

a larger group of patients. The present results can only signal a tendency which seems to be worthy of further interest in the context of hypertension related to pHPT and rising knowledge about the biological role of ErbB-4/Her-4.

## CONCLUSIONS

In the present study no relationship between the histological type of changes or other clinical data and *Erb/Her* genes' copy number alterations was found in patients affected with pHPT. Thus, no member of the ErbB/Her family can serve as prognostic factor in the studied disorder. Nevertheless, the presence of relatively numerous ErbB-4/Her-4 abnormalities among the examined patients suggests their possible role in pHPT.

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