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ORIGINAL ARTICLE

The relationship between proliferation activity and parathyroid hormone levels in parathyroid tumors

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Summary

Purpose: *This article examines as to whether the Ki-67 index may be useful as a marker for cell proliferation, as well as to whether Ki-67 immunohistochemical expression and parathyroid hormone (PTH) levels are useful in distinguishing between parathyroid carcinoma (PC) and adenoma.*

Methods: *A retrospective analysis of 50 patients (10 with PC and 40 with adenoma) who had been previously diagnosed with primary hyperparathyroidism (PHPT) was conducted. Normal parathyroid glands served as the control group. Immunostaining of Ki-67 was estimated through image analysis and the results were statistically analyzed.*

Results: *Ki-67 was higher in PC patients (median 785.15) compared to adenoma patients (median 297.41; Mann–Whitney U-test p<0.001). ROC analysis confirmed that Ki-67 has a positive predictive marker in diagnosing cancer. Mann-Whitney U-test confirmed a highly statistically significant difference in the preoperative PTH levels between the PC and adenoma group (p <0.001). The PTH serum preoperative level was higher in PC patients (median 1721) than in those with adenoma (median 189.5). A highly significant correlation was also found between Ki-67 and preoperative PTH levels (p <0.001).*

Conclusion: *A higher rate of cellular proliferation was noted in malignant tumors as compared to benign tumors. Moreover, the expression profile of Ki-67 and high PTH levels in this study indicates a role for them as potential markers of malignancy.*

Key words: *hyperparathyroidism, Ki-67, parathyroid carcinoma, parathyroid hormone*

Introduction

Hyperparathyroidism is a common disorder characterized by hypercalcemia due to increased PTH secretion. In most cases, a single benign adenomatous gland (adenoma) is found. Most of the parathyroid adenomas studied have been found to be monoclonal neoplasms supporting a pathogenic role of oncogenes or tumor suppressor genes [1,2].

PC is an uncommon finding, accounting for only 1-2% of patients that suffer from PHPT while a higher incidence (approximately 5%) has been reported in Italy and Japan [3-5].

The diagnosis of PC is often difficult to establish since its clinical features are similar to those of benign primary hyperparathyroidism. Fine needle aspiration is not useful in establishing a diagnosis, which is finalized only after confirming infiltration beyond the tumor capsule [6].

Ki-67 is a useful antigen which determines the proliferation activity in adenoma, hyperpla-

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sia, and carcinoma of the parathyroid glands. In recent studies, the Ki-67 expression has been reported as being between 1.36% and 3.3% in parathyroid adenomas [7].

The aim of this study was to measure the expression of the Ki-67 labelling index in parathyroid tumors and to correlate this index with PTH levels.

Methods

Between 2001 and 2012, 50 patients with PHPT were treated for PC (N=10) and for adenoma (N=40). All patients underwent initial parathyroid surgery at the Center for Endocrine Surgery, Clinical Centre of Serbia. Of the patients 17 were male and 43 female, with a mean age 52.65 years (range 15-77). The control group consisted of 10 cases of normal parathyroid gland tissue. Their clinical diagnosis was histologically confirmed. Most of the lesions were located in the neck. One patient had an ectopic retrosternal tumor.

The study protocol was approved by the local Ethics Committee and all participants provided oral consent.

Immunohistochemical examination

Representative 3μm tissue sections were taken, which were then heated at 55° C in order to dissolve the paraffin used in the process, and were thereafter deparaffinized in xylene (3x5 min) to be rehydrated through immersion in graded ethanols. Antigen retrieval was enhanced by autoclaving the slides in sodium citrate buffer (pH 6.0) for 30 min. Endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide-methanol buffer for 25 min. The rabbit monoclonal Ki-67 antibodies utilized (Abcam, Cambridge, UK; 1:100) were incubated at 4° C for 10 hrs. Immunostaining was performed by the avidin-biotin peroxidase complex (ABC) method (Vectastain ABC-*Elite* kit, Vector Laboratories, Burlingame, CA). Staining was visualized using 3.3 diaminobenzidine tetrachloride (DAB). The slides were counterstained with Mayer hematoxylin and mounted in Canada balsam. Negative controls were done by replacing the primary antibody with phosphate buffered solution (PBS).

Quantification of the immunohistochemical staining

In order to evaluate the expression of Ki-67, only the stained nuclei were assessed. To determine the density of Ki-67 positive cells per mm2 the multipurpose test system M42 by Weibel (University of Wiskonsin, Madison, USA) was used. The objective micrometer (Reichert, Wien 2mm/200) test system was used to calibrate an Olympus BH-2 microscope to x400 magnification (10x40 eyepiece lens), according to the defined field of measurement as to test the density at 0.016 mm^2. In order to test the density of Ki-67 positive cells/mm^2, 10 "hot spots" were numbered successively. The absolute value of the density of positive cells in these were stereometrically assessed [8]. The arithmetic mean of the values obtained was defined as the finite number of the Ki-67 positive cells per mm3 of the case. After determination of the median of the Ki-67 positive cells, the patients with primary hyperparathyroidism were divided into two groups: those with low expression (values ≤ to the median) and those of high expression (values > than the median). The proliferation index was thereby derived from the absolute values of the determined Ki-67 in comparison to those with deviation from the median.

The expression of Ki-67 was evaluated by two independent pathologists (Figures 1 and 2).

Statistics

SPSS 15.0 software was used for the statistical analyses of the data collected. Any difference was considered significant when the p value was <0.05. The sta-

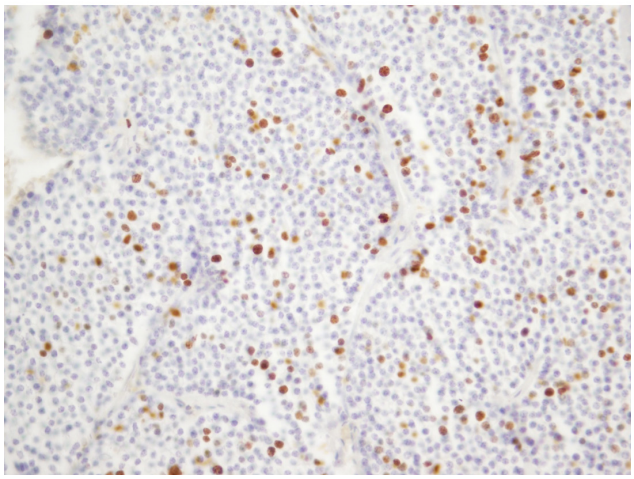

Figure 1. Ki-67 immunostaining in parathyroid carcinoma. Numerous Ki-67 positive cells are evident (H&E x20).

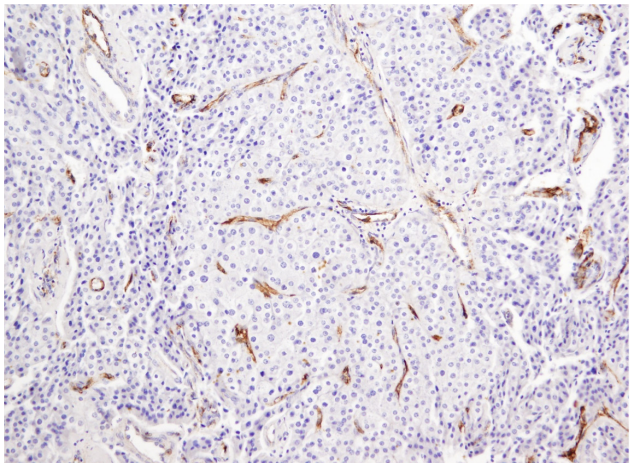

Figure 2. Ki-67 immunostaining in parathyroid adenoma. A smaller number of Ki-67 positive cells are evident (H&E x20).

Table 1. Preoperative and postoperative values of PTH in parathyroid cancer and adenoma patients

		PTH preoperative	*PTH postoperative*
Parathyroid cancer	N	10	10
	$\bar{x}$	1824.02	105.84
	SD	861.47	99.02
	Median	1721.00	78.20
	Min	400.00	5.00
	Max	3000.00	362.87
Adenoma	N	40	40
	$\bar{x}$	266.04	24.34
	SD	235.75	20.18
	Median	189.50	19.50
	Min	83.00	.45
	Max	1311.00	87.00

PTH: parathyroid hormone, SD: standard deviation

tistical methods used were the Mann-Whitney U-test and ROC curve analysis.

Results

Four females (40%) and 6 males (60%), mean age 53.00±14.17 years formed the PC group.

Ki-67 was higher in PC patients (median=785.15) as compared to adenoma patients (median=297.41) with Mann–Whitney U-test, U=10.50; Z=4.598; p<0.001; Figure 3).

The area under the curve (AUC) for ROC curve for the Ki-67 was highly significant (0.979; p<0.001), indicating that PC patients were 97.9% more likely to have higher values of Ki-67 than adenoma patients. For the threshold of Ki-67=511.54 the sensitivity was 100% with 86% specificity. These results showed that a Ki-67 value >511.54 was highly indicative of cancer as a probable diagnosis.

The Man-Whitney U-test confirmed a highly significant difference in the preoperative PTH levels between the PC and adenoma patients (U=9.00, Z=4.361, p<0.001). The preoperative PTH serum level was higher in the PC group (median =1721) than in the adenoma group (median =189.5) (1).

The AUC for PTH in the ROC curve was highly significant (0.978; p<0.001), indicating that PC patients were 97.9% more likely to have higher values of PTH than adenoma patients.

For a threshold of PTH=768, the sensitivity

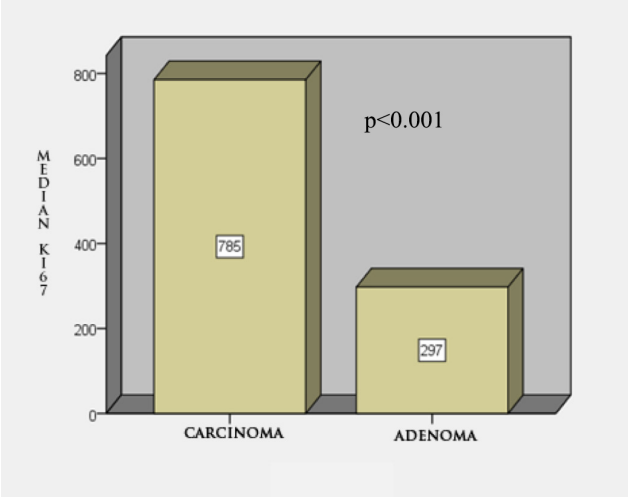

Figure 3. Ki-67 values in carcinoma and adenoma.

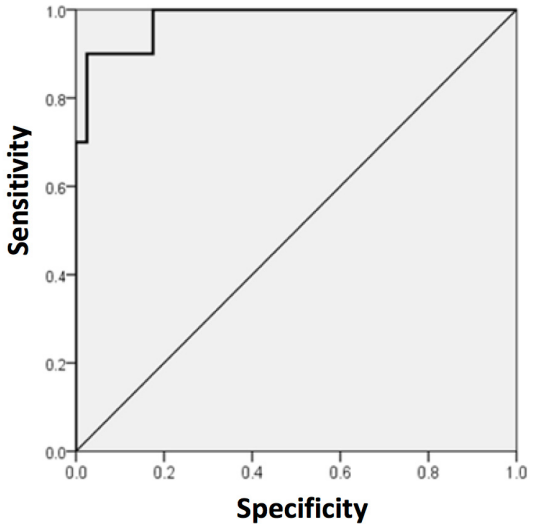

Figure 4. ROC for parathyroid hormone preoperative levels. PTH>768, sensitivity=90.0 (55.5-98.3), specificity=97.5 (86.6-99.6).

was 80% and specificity 100% (Figure 4). If the threshold was >768 in the ROC curve, this was highly indicative of cancer as a probable diagnosis.

A highly significant correlation was found between Ki-67 and preoperative PTH levels (r=0.562, p<0.001). The correlation was positive, meaning that patients found to have higher values of Ki-67 also have higher values in their PTH levels.

Since the Ki-67 based on the ROC analysis has been found to be a good predictor for the diagnosis of PC, it can be concluded, on the basis of high preoperative PTH values, that the values of Ki-67 will also be high, which may also indicate a PC diagnosis.

Discussion

PC presents a range of clinical and biochemical characteristics, but is generally considered to have an indolent evolution, characterized by slow growth and culminating in death from the metabolic complications of hypercalcaemia if untreated [9].

The diagnosis of PC is difficult and is based on morphological features that are not totally reliable. Several molecular markers have proved useful in the evaluation of PC, but their sensitivity, specificity, or both prove to be rather low [10].

Cell proliferation is one of the most important biological mechanisms in oncogenesis.

Ki-67 has been widely used to evaluate cell proliferation in various types of tumors and other lesions.

Ki-67 is a nuclear antigen expressed in all phases of the cell cycle (G1, S, and G2) except G0. It is used as a proliferation marker. Therefore, Ki-67 levels reflect cell division activity. Ki-67 antigen is a bimolecular complex of 345 kDa and 395 kDa proteins. Ki-67 reactivity is now widely accepted as a marker of proliferative activity and correlates well to other cell kinetic measurements. It has proven useful in evaluating the proliferative activity of parathyroid adenoma and hyperplasia [11,12]. In recent years, several investigators have reported on the use of Ki-67 labelling index for differential diagnosis between adenoma and PC [11,13,14].

Few reports, however, have focused on the use of Ki-67 for prognostication. A higher tumor proliferating fraction detected by Ki-67 immunostaining is associated with aggressive neoplasms. Wang et al. [15] have found that all parathyroid adenomas were immunoreactive to Ki-67, while only 2 out of 15 (13.3%) demonstrated Ki-67 positivity in the residual rim of normal parathyroid tissue. Abbona et al. [16] demonstrated that PCs have a higher proliferating fraction than parathyroid adenomas and hyperplasia. Iihara et al. [17] found that the Ki-67 labelling index was much higher in PC than in adenoma patients, as well as that the same model demonstrated an increased Ki-67 expression which was associated with significantly shorter disease-free survival (p=0.005) in PC compared with adenoma patients.

A significant relationship was found between the Ki-67 and serum PTH levels in a study carried out by Gozu et al. [7]. Therein, the Ki-67 expression in PC was significantly higher than in adenoma. Ki-67 was found to be 2% in adenoma and 25% in carcinoma in the Gozu's study. This also shows that Ki-67 can be helpful in lesions which prove difficult to differentiate between adenoma and carcinoma [18,19]. PC showing a Ki-67 expression ≥ 5% is likely to recur, which may be particularly more common in the early postoperative period for cases with a Ki-67 expression ≥ 10% [17].

Observing a higher Ki-67 expression in adenoma than in normal parathyroid tissue indicates that the adenoma is of clonal proliferation [1]. Clonal analyses have shown that parathyroid adenomas are monoclonal [1,20,21]. All adenomas have been determined to be monoclonal and normal parathyroid tissue to be polyclonal in the study of Larian et al. [20].

In this paper, it has been found that Ki-67 was higher in PC patients compared to adenoma patients (Figures 1,2), as well as that Ki-67 may prove of assistance in lesions that are difficult to differentiate between adenoma and carcinoma.

PTH has also been examined as a potential factor in distinguishing PC from benign lesions of PHPT. Cavalier et al. confirmed the utility of the inverted 3rd/2nd-generation PTH ratio as a marker of PC (sensitivity: 81.8%; specificity: 97.3%) [22].

In our study we found that the sensitivity was 80% for a PTH threshold of 768, which had a specificity of 100% (Figure 4). Based on these results, it can be concluded that the value of PTH was greater than 768, highly indicative of cancer as a probable diagnosis. This result is supported by a previous study that PTH serum levels are useful for the prediction of PC [23].

The results of this study suggest that serum level of PTH may be helpful in predicting PC in patients with PHPT before the primary operation. We conclude that the expression of Ki-67 proliferation index is much more pronounced in parathyroid carcinoma than in adenoma, helping therefore the discrimination between these two tumor types. What is more, this study has shown that Ki-67 and PTH are good tools in the diagnosis of carcinoma and adenoma -as a statistically significant correlation exists between Ki-67 and PTH.

Authors' contributions

Conceived and designed the study: ZI. Performed operations: IP. Pathology: SJ, ST, DD. Data analysis: ZI, KT, II. Wrote the first draft of the manuscript: ZI. Contributed to the writing of the manuscript: ZI. Agree with manuscript results and conclusions: ZI, MI, SJ, IP, ST, KT, VZ, MZ, II, DD. Jointly developed the structure and arguments for the paper: ZI, MI, SJ, IP, ST, KT, VZ, MZ, II, DD. Made critical revisions and approved final ver¬sion: ZI. All authors reviewed and approved the final manuscript.

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