The aim of the study was to analyze the gene expression of JUN and CCND1 in a group of parathyroid tissues obtained from patients with primary hyperparathyroidism in comparison to hyperplastic parathyroid and normal/atrophic parathyroid tissues by real-time quantitative PCR. Our goal was to validate the conclusion of Forsberg et al (2005) who reported overexpression of JUN in parathyroid adenomas by a joint microarray and QPCR study.

Material and methods. The analysis of JUN, CCND1 was carried out by QPCR in 14 parathyroid adenomas, 8 hyperplasia cases and 50 normal/atrophic parathyroid samples taken intraoperatively. Expression of the examined genes was normalized to the reference index (geometric mean of reference genes expression: EIF3S10, UBE2D2, ATP6V1E).

Results. We observed a decrease of JUN expression in parathyroid adenomas in comparison to both normal/atrophic and hyperplastic parathyroids. The fold change value was 0.71 in comparison of adenomas to normal/atrophic samples (p = 0.044) and 0.75 to hyperplastic glands (p = 0.003). For CCND1 we observed one case of parathyroid adenoma with a very clearly increased expression, while 3 adenomas (21.4% of all adenomas) exhibited the increase over the highest value seen in normal parathyroids (fold change = 3.52).

Conclusions. In parathyroid adenomas we were not able to confirm any overexpression of JUN gene, as suggested by the previous study. On the contrary, we observed a distinct inhibition of JUN RNA expression in comparison to non-neoplastic parathyroids. For CCND1 gene overexpression in parathyroid adenomas, we report the frequency of 21.4%.

Key words: parathyroid adenoma, JUN gene, CCND1 gene, QPCR

Primary hyperparathyroidism (PHPT) is a relatively frequent endocrinopathy, however, its molecular etiology remains poorly understood. PHPT is characterized by calcium-insensitive hypersecretion of parathyroid hormone following the increased parathyroid cell proliferation. The main cause of autonomous parathyroid function is a single benign adenoma (80% of PHPT cases). In 15-20% of cases PHPT is due to hyperplasia of parathyroid
glands, while parathyroid carcinomas are very rare (less than 1%) (1). Majority of tumors are sporadic, however, about 5% of them are associated with MEN1 or 2A (Multiple Endocrine Neoplasia types 1 and 2A), Hyperparathyroidism-Jaw Tumor Syndrome or Familial Isolated Hyperparathyroidism.

In the parathyroid tumorigenesis several genes and chromosomal changes have been analyzed. Among them loss of menin (MEN1), the gene located at chromosome 11q13, and gain/overexpression of cyclin D1 (CCND1), also located at chromosome 11q13, are the best known (2). Somatic deletion of chromosome 11 is the most frequent genetic event in sporadic parathyroid adenoma (3, 4), seen in about 30% of cases either by CGH or LOH. Loss of neighbouring genes at 11q13 locus has been confirmed by their decreased expression in a microarray based study (2). The same study reported for the first time the overexpression of JUN gene in benign parathyroid adenomas in comparison to normal parathyroid glands, suggesting its role in their molecular pathogenesis. The expression of JUN was increased more than twofold in 6/17 of the parathyroid adenomas investigated (2).

The JUN gene is a protooncogene located in chromosomal region 1p32. Its protein product is a member of the AP-1 family of proteins (5), which act as transcriptional activators (6). JUN is required for a variety of biological processes that influence oncogenic transformation including cell differentiation, proliferation, and apoptosis (7). One of suggested function is to antagonize the proapoptotic activity of p53 and p21 (6, 8). Overexpression of this gene is common in human tumors (e.g. lung, osteosarcoma and liposarcoma, prostate cancer) (9-12) and promotes cellular proliferation and DNA synthesis (5).

There is a little knowledge about the putative contribution of JUN to the pathogenesis of parathyroid tumors. For validation of the conclusions drawn by Forsberg et al, we decided to analyze the gene expression of JUN in an independent set of PHPT parathyroid intraoperative samples – adenomas and hyperplastic glands – in comparison to normal/atrophic parathyroid tissues. We widened our study by the evaluation of CCND1 expression, also reported to be overexpressed by the same RNA-based study.

CCND1 is a proto-oncogene located at chromosome 11q13 and its protein product, cyclin D1, is a key regulator of the cell cycle. Cyclin D1 binds to Cdk4 and Cdk6 to form pRB kinase. Upon phosphorylation, pRB loses its repressive activity for the E2F transcription factor which then activates transcription of several genes required for the transition from the G1- to S-phase and for DNA replication (13, 14). The CCND1 gene is rearranged and overexpressed in many human tumors (e.g. breast, hepatocellular or oesophageal carcinomas) (15, 16), which is believed to play a critical role in tumor development and in maintenance of the malignant phenotype. CCND1 overexpression may be caused by chromosomal rearrangement due to a pericentric inversion with break points at 11q13 and 11p15, which has been suggested to bring the CCND1 under the 5´ regulatory sequences of the PTH gene (17). In other malignant neoplasms, including lung, breast, sarcoma, and colon cancer, CCND1 overexpression results from induction by oncogenic signals rather than a clonal somatic mutation or rearrangement in the CCND1 gene (18).

MATERIAL AND METHODS

The study was approved by the Ethic Committee of M. Skłodowska-Curie MSC Cancer Center and Institute of Oncology in Gliwice.

Patients

The analysis of gene expression was carried out in 23 patients, diagnosed with PHPT and referred to parathyroid surgery. There were 21 women and 2 men, aged 31-76 years (median 50 years). All but two patients with positive MEN1 family history were diagnosed with sporadic PHPT. Excision of adenoma with the intraoperative identification of all parathyroids or subtotal parathyreoidectomy were performed respectively. Intraoperatively, fragments of each of the respective normal/atrophic parathyroid gland (excision intended for pathological identification) and the fragments of the excised adenomas and hyperplastic glands were taken and stored in RNAlater at 4°C. In total, for the qPCR analysis 14 adenomas confirmed by postoperative histopathological evaluation (PA), 8 hyperplastic parathyroids (PH) and 50 fragments of normal/atrophic parathyroids (NP) were included.
Isolation of RNA

Total RNA was extracted from homogenised frozen tissue using Mini Kits (Qiagen), which included a DNase step. RNA quantity was measured by NanoDrop ND-1000 minispectrophotometer and quality was estimated by Agilent 2100 using RNA 6000 Nano Assay (Agilent Technologies). RNA integrity, assessed by RIN index (Agilent 2100), was within the 4.1-9.1 range.

cDNA synthesis

cDNA was synthetised from 500 ng of total RNA by Omniscript Kit (Qiagen), with mixture of oligo-dT and random nonamers primers (Sigma) and 10U RNase inhibitor (Sigma). The reaction was done in 37°C for 1 hour.

Quantitative real-time reverse transcription-PCR (QPCR)

Analysis of gene expression was performed by real-time quantitative PCR with the use of fluorescent probes (Universal Probe Library, Roche). Amplicons were designed using a Web-based application (www.roche-applied-science.com/sis/rtpcr/upl) (tab.1).

QPCR was carried out in a 96-well optical reaction plate using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Five microliters of template cDNA (equivalent to 500 ng of total RNA) were

| Table 1. Amplicons used for quantitative real-time PCR measurement of analyzed genes |
|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| Gene | Symbol | Gene ID | Primer sequence | Probe number and sequence (Universal Probe Library, Roche) |
| Geny badane / investigated genes: |
| Proto-oncogene c-jun | JUN | NM_002228.3 | F 5’-CCAAAGGATAGTG CGGATGTTT-3’ R 5’-CTGCTCCCTCTCC ACTGCAAC-3’ | 19 GGCTGGAG |
| Cyclin D1 G1/S-specific cyclin-D | CCND1 | NM_053056.2 | F 5’-GAAGATCGTCGGGCACCTG-3’ R 5’-GACCTCCTCCTGCACTTCT-3’ | 67 TGCTGGAG |
| Housekeeping genes: |
| Eukaryotic translation initiation factor 3, subunit 10 theta, 150/170kDa | EIF3S10 | NM_003750.1 | F 5’-AGTAGAGCGCTGTACCATGA-3’ R 5’-GGGTATATGAGGCGAGAAAT-3’ | 61 TTGCCCAG |
| Ubiquitin-conjugating enzyme E2D 2 (UBC4/5 homolog, yeast) | UBE2D2 | NM_003339.2 | F 5’-AATGCGAGCATTTGCTCCTGA-3’ R 5’-CACAAACAGAGACAGATGGGAC-3’ | 67 CTCCAGCA |
| ATPase, H+ transporting, lysosomal 31kDa, V1 subunit E isoform 1 | ATP6V1E | NM_001696.2 | F 5’-AAGCGGCTGCTGGATCTCAT-3’ R 5’-GCATTGACCACAAACAGG-3’ | 03 CCCAGCAG |
| β-glucuronidase | GUSB | NM_000181.1 | F 5’-CGCCCTGCTGCTCTGTATTCC-3’ R 5’-TCCCACAGGAGATGTGAGA-3’ | 57 CGGGCG |
| Hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/acyl-Coenzyme A hydratase (trifunctional protein), alpha subunit | HADHA | NM_000182.3 | F 5’-GCTTGGCGCCCTGATGOT-3’ R 5’-TCCCACAGGAGATGTGAGA-3’ | 65 TCCTCCAG |
| β-actin | ACTB | NM_001101.2 | F 5’-ATGCGCATGGAAGGTTTC-3’ R 5’-GGATGCCACAGGACTCCAT-3’ | 11 CTCCAGCAG |
added to 15 µl of PCR reaction mix containing 10 µl TaqMan Universal PCR Master Mix (Applied Biosystems), 1 µl forward and reverse primers (200 nM), 1 µl probe (100 nM) and water. Thermal cycling condition were as follows: 50°C for 2 min (incubation and activation AmpErase UNG), 95°C for 10 min (activation AmpliTag Polymerase DNA), 95°C for 15 sec (dennuration) and 60°C for 1 min (annealing and extension). Every sample was examined in duplications. The standard curve, used in experiments, was prepared from serial dilutions of human reference RNA (Stratagene). Expression of the examined genes was normalized to the reference index, obtained by calculation of geometric mean of reference genes expression: \( eIF3S10 \), \( ATP6V1E \), \( UBE2D2 \). The genes were selected by geNorm software. We analyzed expression of six housekeeping genes: \( GUSB \), \( ACTB \), \( HADHA \), \( ATP6V1E \), \( eIF3S10 \) and \( UBE2D2 \) in parathyroid adenomas and normal/atrophic parathyroid samples. Expression of these genes was used to obtain normalization factor. The most stable housekeeping genes were \( EIF3S10 \) and \( UBE2D2 \), the less stable gene was \( GUSB \) (fig. 1A).

The analysis of pairwise variation showed that the normalization index based on expression of 2 housekeeping genes fulfilled criteria given by Vandesompele et al. (19) for acceptable pairwise variation between the genes in the reference index (less than 0.15). We decided to use the normalization factor based on expression of 3 genes because the pairwise variation for this factor was significantly better than factor based on 2 genes and was slightly worse than factor based on 4 genes (fig. 1B). None of the 3 genes selected exhibited a statistically significant difference in gene expression between parathyroid adenomas, parathyroid hyperplasias and normal/atrophic parathyroid samples.

Statistical analysis

To determine between-group differences, we used Mann-Whitney nonparametric test. Differences were considered significant at a p
Gene expression is decreased in parathyroid adenoma

The fold change was calculated by dividing values medians of expression.

The geNorm application software for Microsoft Excel was additionally used to identify the most stable reference gene under the described conditions, and to determine the optimal number of reference genes required for reliable normalization of QPCR data.

RESULTS

Among the 14 adenomas and 8 cases of parathyroid hyperplasia investigated, there was no single case of JUN expression higher than the range observed in normal samples (fig. 2A). On the contrary, the median JUN expression in parathyroid adenomas (PA) was significantly lower in comparison to normal/atrophic parathyroid glands (NP) (p=0.044) and even in comparison to hyperplastic glands (PH) (p=0.003) (tab. 2). For the one case of adenoma and one case of hyperplasia diagnosed within the MEN1 syndrome there were no differences in comparison to other cases. For the all JUN data gained by our QPCR study, the fold change value was 0.8 in comparison of parathyroid adenomas/hyperplasias to normal/atrophic samples. Median expression level was: 1.903 (IQR = 1.600) in normal/atrophic parathyroids, 1.348 (IQR = 0.442) in parathyroid adenomas and 1.807 (IQR = 1.274) in parathyroid hyperplasia, with no difference between the groups of PH and NP (p=0.49). The range of JUN gene expression in non-neoplastic parathyroids was rather wide, with the highest value 10.7-fold higher than the lowest one.

For CCND1, 3 adenomas (21.4% of all adenoma samples) exhibited the increase over the highest value seen in normal parathyroids (fold change = 3.52) and in 6 adenomas (42.9% of total adenoma samples) the expression level exceeded the 75 percentile of the normal/atrophic glands. In parathyroid hyperplasia, there were no cases of distinctly increased CCND1 expression. The inspection of all values obtained indicated that there was one case of parathyroid adenoma with a very clearly increased expression (fold change value = 21.76) (fig. 2B). However, the median values for CCND1 were similar in all compared groups: 0.219 (IQR = 0.158) in normal/atrophic parathyroids, 0.224 (IQR = 0.291) in parathyroid adenomas and 0.269 (IQR = 0.172) in parathyroid hyperplasia (tab. 2). The fold change between median values in parathyroid adenomas and normal/atrophic samples was 1.02. In the subgroup of 3 parathyroid adenomas with the increased CCND1 expression the median level of JUN was 1.335, as compared to 1.476 in the remaining adenomas. This difference was statistically not significant.

There was no relation between JUN and CCND1 expression, in the PA and NP sub-

![Fig. 2. Scatter plot of gene expression in parathyroid adenomas and normal/atrophic parathyroid tissues. A) JUN gene expression, B) CCND1 gene expression. The cases of hyperplasia are marked with Δ, the two patients with MEN1 related PHPT are marked with coloured dots](image-url)
groups by Spearman rank correlation, while we observed a correlation between \textit{JUn} and \textit{CCND1} expression in parathyroid hyperplasia (PH) \((R = 0.857; p = 0.007)\) (fig. 3).

**DISCUSSION**

Our study was intended as an independent confirmation for the results obtained by Forsberg et al who reported overexpression of \textit{JUn} in 6/17 (35\%) parathyroid adenomas. Such reanalysis is always necessary for validation of microarray based conclusions. We collected a slightly smaller number \((n = 14)\) of parathyroid adenomas and a significantly larger group of normal/atrophic glands \((n = 50)\) and widened the study by inclusion of parathyroid hyperplasia.

The conclusion from our analysis is contrary to the results obtained by Forsberg et al: not only there was no increase in \textit{JUn} expression in parathyroid adenomas, but its expression levels were significantly lower. The down-regulation of \textit{JUn} gene expression was characteristic for benign parathyroid neoplasia and was not observed in hyperplastic glands. In the literature data there is only one report considering the expression of \textit{JUn} in parathyroid adenomas. As already mentioned above, Forsberg et al found clear overexpression of \textit{JUn} in the microarray-based analysis of 8 parathyroid adenomas (the two probesets for \textit{JUn} were the top overexpressed genes in comparison to two normal tissues) and they verified it including 9 additional adenomas (in total, the confirming of QPCR was done in 17 adenomas). However, they did not widen the normal control group which consisted of only 2 normal parathyroids. Our study was done on a much larger set of sample parathyroid tissues, which showed a very wide range of \textit{JUn} expression. Additionally, we included also a group of PHPT-related hyperplasia where no change in \textit{JUn} expression was observed in comparison to the control group.

\textit{JUn} (c-Jun) is a member of \textit{JUn} transcription factors family, where \textit{JUND} also belongs. \textit{JUND}-mediated transcription is inhibited by MEN1 gene product and, as this last gene is often lost in parathyroid tumors, this is expected to be important for parathyroid neoplasia (2).

In the case of \textit{CCND1}, we observed the very strong overexpression of the gene only in one sporadic adenoma (7.1\%) with no change in the median gene expression level in this subgroup. However, \textit{CCND1} expression exceeded the upper range of the values observed in normal/atrophic parathyroid glands in 3/11 adeno-

<table>
<thead>
<tr>
<th>Gene</th>
<th>Parathyroid adenoma (PA)</th>
<th>Parathyroid hyperplasia (PH)</th>
<th>Normal/atrophic glands (NP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JUn</td>
<td>Median</td>
<td>1,348</td>
<td>1,807</td>
</tr>
<tr>
<td></td>
<td>Fold change</td>
<td>PA vs PH 0.75</td>
<td>PH vs NP 0.95</td>
</tr>
<tr>
<td></td>
<td>p value</td>
<td>p= 0.003</td>
<td>ns</td>
</tr>
<tr>
<td>CCND1</td>
<td>Median</td>
<td>0.224</td>
<td>0.269</td>
</tr>
<tr>
<td></td>
<td>Fold change</td>
<td>PA vs PH 0.83</td>
<td>PH vs NP 1.23</td>
</tr>
<tr>
<td></td>
<td>p value</td>
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Test U Mann-Whitney’as / U Mann-Whitney test

![Fig. 3. Correlation plot for JUn and CCND1 in hyperplasias. 75 percentil in normal/atrophic parathyroid samples is marked with lines for JUn and CCND1 (R = 0.857; p = 0.007).](image-url)
gene expression is decreased in parathyroid adenoma

Our own results indicate that parathyroid adenomas are heterogenous in respect to \textit{CCND1} amplification/overexpression and this mechanism should not be considered as mandatory in the pathogenesis of parathyroid neoplasia.

Experimental studies have shown that in human tumoral parathyroid cells, cyclin D1 expression is regulated by \textit{CaSR} and is dependent on the level of \textit{FGF}, \textit{EGF} and \textit{TGF beta} signaling (25). Interestingly, in murine PHPT transgenic model, \textit{CCND1} is down regulated by the transient over-expression of parafibromin (26, 27). In this context it is important to state that \textit{HRPT2} gene expression was present in all adenomas investigated by us (data not shown) but did not correlate with the expression of either \textit{JUN} or \textit{CCND1}.

The comparison of gene expression in parathyroid adenomas was related to parathyroid fragments taken from the same PHPT patients. It may be assumed that the remaining parathyroids were suppressed by the autonomous function of the adenoma and this could influence their gene expression profile. However, for both genes we would rather expect an overestimation of the gene overexpression in this context, while the contrary result was obtained for \textit{JUN}. Simultaneously, by inclusion of over 50 normal parathyroids our judgement on non-neoplastic expression of both genes was much more detailed than in other studies done on the RNA level.

CONCLUSIONS

In parathyroid adenomas we were not able to confirm any overexpression of \textit{JUN} gene, as suggested by previous study. On the contrary, we observed a distinct inhibition of \textit{JUN} expression in comparison to non-neoplastic parathyroids. For \textit{CCND1} gene overexpression in parathyroid adenomas, we report the frequency of 21.4%.

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