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# Molecular analysis of the HPJ-JT syndrome and sporadic parathyroid carcinogenesis

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*Chapter 6*

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**Original Paper**

# Differential expression of the calcium sensing receptor and combined loss of chromosomes 1q and 11q in parathyroid carcinoma

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## Abstract

**Malignant transformation of parathyroid tumours is rare. Nevertheless, this small subset of malignant tumours often creates diagnostic and therapeutic problems. In this work, the morphological characteristics of 26 primary parathyroid carcinomas and seven metastases have been studied. Furthermore, immunohistochemical expression profiles for the calcium sensing receptor (CASR), cyclin D1 (CCND1), and Ki-67 were determined for parathyroid carcinomas and compared with adenomas and hyperplasias using a tissue microarray. Loss of heterozygosity (LOH) of the chromosome 1q region containing the *HRPT2* gene and chromosome 11q (*MEN1*) was determined in the carcinomas. In contrast to the adenomas and hyperplasias, 31% of carcinomas demonstrated down-regulation of CASR. A significant correlation was found between CASR expression and the Ki-67 proliferation index. Chromosome 1q and chromosome 11q LOH were found in 12 of 22 (55%) and 11 of 22 (50%) carcinomas tested, respectively. Combined 1q and 11q LOH was seen in 8 of 22 (36%) carcinomas, in contrast to the low percentage of LOH reported in both regions in adenomas. In conclusion, this study demonstrates that combined 1q and 11q LOH in parathyroid tumours is suggestive of malignant behaviour. Strong down-regulation of the CASR protein is seen in a proportion of parathyroid carcinomas with a high proliferation index.** **Copyright © 2003 John Wiley & Sons, Ltd.**

**Keywords: parathyroid; parathyroid carcinoma; parathyroid adenoma; immunohistochemistry; tissue micro-array; LOH; CASR**

## Introduction

Parathyroid hyperfunction concomitant with parathyroid tumourigenesis is one of the most common endocrinopathies, with a prevalence of about 1 per 1000 individuals. Although parathyroid carcinoma is rare (less than 1%) in patients with hyperparathyroidism, it creates diagnostic and therapeutic problems, partly due to its heterogeneous morphological appearance. Classically, the presence of fibrous bands and mitotic activity are considered to be histological features of malignancy. However, a definitive diagnosis of parathyroid carcinoma can sometimes not be established, based on morphology alone, in the absence of invasion of adjacent structures, vaso-invasive growth, and/or metastasis. Prognosis is variable, and early diagnosis and radical surgery seem to be favourable prognostic factors.

Little is known about the pathways involved in parathyroid tumourigenesis. The multiple endocrine neoplasia tumour suppressor gene (*MEN1*) is mutated in 30% of sporadic parathyroid tumours (mostly adenomas). Furthermore, overexpression of the cyclin D1 oncogene (CCND1) seems to be involved in parathyroid tumourigenesis. Analysis of RB and P53 showed varying results or ruled out their importance [1].

An inherited form of parathyroid carcinoma occurs as part of a rare syndrome, the so-called hyperparathyroidism–jaw tumour syndrome (HPT–JT: OMIM; 145 001), a syndrome characterized by primary hyperparathyroidism due to neoplastic transformation of parathyroid tissue in combination with rare tumours such as ossifying fibromas of the maxilla or mandible, bilateral renal cysts, hamartomas, and Wilms' tumours [2–8]. In HPT–JT, 10% of all parathyroid tumours are or become malignant [4–16]. Mutations in the *HRPT2* gene at chromosome1q25–32 are responsible for HPT–JT and are also found in a selection of patients with familial isolated hyperparathyroidism (FIHP) [6,17,18]. *HRPT2* mutations are found in a limited subset of sporadic parathyroid adenomas [18]. This gene is considered to be a tumour suppressor, according to LOH studies, although chromosome 1q21–32 LOH was found in only 17% (in two of the ten families) of the adenomas. In LOH studies performed on parathyroid carcinomas from proven *HRPT2*-linked patients [4,6,7,16,19], however, all of the tumours (5) showed chromosome 1q21–32 LOH.

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The latter observation might indicate that chromosome 1q loss, probably in combination with mutation, plays an important role in parathyroid carcinoma formation.

As the diagnosis of parathyroid carcinoma is important but difficult, we tried to find specific features that characterize parathyroid malignancy and as such, can possibly serve as diagnostic markers of these tumours. We therefore studied the morphological characteristics of a series of parathyroid carcinomas in combination with immunohistochemistry (IHC) for the calcium sensing receptor (CASR), cyclin D1, and Ki-67 using a tissue micro-array (TMA). Comparison with a TMA from parathyroid adenomas and hyperplasias was performed. Furthermore, LOH analysis of the chromosome 1q region containing the *HRPT2* gene and of chromosome 11q (*MEN1*) was performed on the parathyroid carcinomas.

## Materials and methods

### Parathyroid carcinomas

Formalin-fixed, paraffin wax-embedded tumour tissue from 26 primary parathyroid carcinomas, six regional lymph node metastases, and one lung metastasis from 30 patients was obtained from different laboratories in The Netherlands using PALGA (Dutch National Information System for Pathology, Utrecht, The Netherlands). One parathyroid carcinoma (No 30) came from a documented HPT–JT family [16]. Thirty samples had undoubted features of carcinoma, namely the presence of vascular invasion and/or metastasis [20] based on evaluation of representative haematoxylin and eosin-stained slides from each tumour by a pathologist (HM) and the initial pathology report. Three cases were diagnosed as carcinomas based on their clinical presentation; definitive vascular invasion was not found in these cases. Each tumour was scored for the overall histological pattern (gross architecture), vascular invasion, cysts, and fibrous bands. Furthermore, variation in nuclear size, the presence of nucleoli, cytoplasmic characteristics, mitotic activity, haemosiderin deposition, and the presence or absence of inflammation were evaluated.

Available data from the pathology reports regarding tumour size, weight, and gross appearance were incomplete and have not been included except for additional information regarding the presence of cysts or vascular invasion. There were 15 males (age range 32–76 years, mean age 56.8 years) and 15 females (age range 30–83 years, mean age 59 years).

### Parathyroid adenoma/hyperplasia

Lesions from 109 patients were used to construct a TMA (see below). These comprised 93 primary parathyroid adenomas (87 sporadic and five MEN1) and 26 hyperplasias [12 primary hyperparathyroidism (HPT), three secondary HPT, three tertiary HPT, and eight MEN1]. Of the 109 patients, 39 were males (age range 18–76 years, mean 41.4 years) and 70 females (age range 12–81 years, mean 56.5 years).

### Tissue micro-array (TMA)

Whenever possible, triplicate tissue cores with a diameter of 0.6 mm, as selected by a pathologist (HM), were taken from each specimen (carcinoma, adenoma or hyperplasia) (Beecher Instruments, Silver Springs, MD, USA) and arrayed on a recipient paraffin block, using standard procedures [21]. Nine cores of nine normal parathyroid tissues were added to serve as internal controls.

### Loss of heterozygosity (LOH) analysis

Eighteen primary parathyroid carcinomas, five metastases to regional lymph nodes, and one lung metastasis from 22 patients from whom both tumour and normal tissue were available were screened for LOH. Genomic DNA was isolated from the paraffin wax-embedded material using standard methods. Seven microsatellite markers located in the *HRPT2* region were selected: D1S428, D1S492, D1S384, D1S081*, D1S556*, D1S173*, D1S422 (markers with an asterisk were obtained through the HPT–JT linkage consortium). These markers covered a 10 Mb area at 1q21–1q41 (source UDB: <URL> http://bioinformatics.weizmann.ac.il/udb/).

D11S4940, D11S4946, and PYGM were the markers used for analysis of the *MEN1* region. Polymerase chain reactions (PCRs) were performed according to standard procedures. Fluorescence-labelled PCR products were electrophoresed using an ABI 310 automated sequencer (Applied Biosystems, Foster City, USA) and the results were analysed using the ABI prism GeneScan 3.1 program. As we used paraffin wax tissue blocks obtained from different hospitals and years, not all the markers gave interpretable results, even after repeated experiments.

The threshold for LOH, comparing normal and tumour DNA, was defined as 40% reduction of one allele, equating to a ratio of $\geq 1.7$ or $\leq 0.59$ [22]. The density of the tumour cells was greater than 80%. The threshold for retention ranged from 0.76 to 1.3. For so-called grey areas with ratios of 0.60–0.75 and 1.31–1.69, no definite decision was made. For HPT–JT case 30, LOH was concluded using HRPT2 mutation specific primers and therefore not counted.

### Immunohistochemical analysis of cyclin D1, CASR, and Ki-67

Immunohistochemical staining was performed on 5 μm sections of the tissue array, using a paraffin sectioning aid system (Instrumedics Inc, Hackensack, NJ, USA). Sections were deparaffinized, treated with 0.3% $H_2O_2$ in methanol, and submitted to antigen retrieval by microwave oven treatment for 10 min in 10 mM citrate buffer (pH 6) for cyclin D1, CASR,

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and Ki-67. Tissue sections were incubated overnight at room temperature with monoclonal mouse anti-human CASR (ADD antibody) [23] (1 : 16 000; NPS Pharmaceuticals, Inc, Salt lake City, UT, USA), cyclin D1 (1 : 500, clone DCS-6; Neomarkers, Fremont, CA, USA), and Ki-67 (1 : 300, clone MIB-1 [10,11]; DAKO, Denmark). The sections were then washed (3 × 5 min in PBS) and incubated (30 min) with biotinylated secondary antibody in 1% BSA in PBS and washed (3 × 5 min in PBS) and incubated (30 min) with HRP/streptavidin complex (SABC). Diaminobenzidine tetrahydrochloride (DAB) was used as a chromogen, followed by counterstaining with haematoxylin. As a negative control, the primary antibody was omitted. Tonsil served as a positive control for cyclin D1 and Ki-67, and kidney tissue as a positive control for CASR. Expression was scored by light microscopy.

For cyclin D1, both the staining intensity (0, no staining; 1, weak; 2, moderate; 3, strong intensity, as related to a positive internal control) and the percentage of positive tumour cells (0 = 0%; 1 = <10%; 2 = 10–30%; 3 = 30–50%; 4 = 50–80%; 5 = >80%) were evaluated and scores of the intensity and the percentage of positive cells were added. If 30% or more of the sample cells showed nuclear immunopositivity for cyclin D1 (and score >3), the sample was considered to be positive [24]. In cases classified as negative, the proportion of the stained cells never exceeded 10%. The calcium sensing receptor was evaluated for the presence or absence of clear and regular membranous staining [25]. Ki-67 staining was quantified by counting all the cells in a punch (>600), ensuring a total of at least 1000 cells as the punches were present on the tissue array in triplicate. The percentage of positive nuclei was calculated.

### Statistical analysis

Summary statistics were obtained utilizing established methods: chi-square analysis [26] was undertaken using the SPSS statistical package (version 9.0.0) to determine any association between categorical variables (as shown in Table 1).

## Results

### Histological parameters of parathyroid carcinomas

We scored the histological pattern (partly shown in Table 1) at low microscopic magnification of 26 primary parathyroid carcinomas and seven metastases (the primary tumour was also analysed in three of these cases). One sample was not scored for some of the features, as it was only a small lymph node metastasis (No 19). There were two common patterns. The majority of the parathyroid carcinoma foci (primary tumours and metastases) had a sheet-like architecture (19/32, 59%), 6/32 (19%) had a nodular pattern, and the remaining 7/32 (22%) had other types of architecture. At higher magnification, 6/32 (19%) tumour foci showed a region with a trabecular tumour cell arrangement. There was no mitotic activity in 10/33 (30%) tumour foci, sporadic mitotic activity in 13/33 (39%), and >1/10 HPF in 10/33 (30%). In 23/33 (70%) of the tumour foci analysed, a region with fibrous bands was seen.

Cystic features were seen in 10/33 (30%) of the tumour foci. No variation in nuclear size was seen in 3/33 (9%); 13 (39%) showed little nuclear anisomorphism, 11/33 (33%) moderate differences, and 6/33 (18%) had very pleomorphic nuclei. In 9/33 foci (27%), we did not see nucleoli in nuclei; in 17/33 (52%) of foci, we found less than 50% of the nuclei with nucleoli; and in 7/33 (21%), we saw nucleoli in more than 50% of the nuclei. The majority (19/33, 61%) of tumour foci showed a granular cytoplasm reminiscent of chief cells; 4/33 (12%) consisted of oxyphilic cells; 2/33 (6%) of so-called water-clear cells; 5/33 (15%) had a mixture of water-clear and granular cytoplasm (transitional water-clear cells); 2/33 (6%) foci a mixture of oxyphilic and water-clear cells; and 1/33 (3%) a mixture of granular and oxyphilic cells (so-called transitional oxyphilic cells).

In 2/33 (6%) foci, we found signs of inflammation, and deposition of haemosiderin was found in 10/32 (31%) tumour foci.

### Immunohistochemical analysis

Overexpression of cyclin D1 was identified in 17/30 (57%) parathyroid carcinomas (Table 2), 13/90 (14%) adenomas, and 10/23 (43%) hyperplasias. Nine/29 (31%) parathyroid carcinomas showed an irregular or absent staining pattern for CASR. In the 'adenoma/hyperplasia' TMA, only 1/104 of the interpretable tumours (an adenoma) showed such irregular or absent calcium sensing receptor staining. Seventeen of 30 (57%) carcinomas had Ki-67 nuclear positivity, a marker of proliferative activity, in 0–5% of cells; 11/30 (37%) had Ki-67 nuclear positivity in 5–20% of cells; and 2/30 (7%) of the samples had Ki-67 positivity in more than 20% of the cells. In the aforementioned adenoma/hyperplasia TMA, all of the 117 interpretable cases showed Ki-67 positivity in 0–5% of cells.

The statistical correlation between the determined parameters was determined using the chi-square test. A significant correlation was found between CASR expression and the proliferation (Ki-67) index ($p < 0.05$). A Ki-67 index ≥5% was present in eight of the nine samples that showed down-regulation of CASR. This observation is illustrated in Figure 1 with three tumours, one with normal CASR (case 18) and two with altered CASR staining (cases 4 and 23).

Significant correlations were found between cyclin D1 and Ki-67 ($p < 0.05$). In 12/18 (67%) carcinomas with overexpression of cyclin D1, the Ki-67 index was ≥5%, whereas only 3/12 (27%) parathyroid carcinomas with normal cyclin D1 expression had a Ki-67 index ≥5%.

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**Table 1.** Overview of the histological, immunohistochemical, and genetic (LOH) data of 30 parathyroid carcinoma cases (33 tumour foci)

| Patient | Sex | Diagnosis | Cyst | Fibrotic bands | Mitosis | Vaso-invasive | Age (years) | Pattern | Variation in nuclear size | Cytoplasm | Trabecular | Ki-67 | CASR | Cyclin D1 | LOH 1q | LOH 11q |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | F | Prim | Yes | Yes | Sporadic | Yes | 30 | Sheet | Little | Gran | Yes | 5 | 0 | 5 | No | No |
| 2 | F | Prim | No | No | Sporadic | Yes | 83 | Nod | Little | Gran | No | 5.8 | I | 4 | No | Yes |
| 3 | F | Prim | No | Yes | >1/10 HPF | Yes | 74 | Other | Large | Oxy | No | 10 | I | 3 | Yes | Yes |
| 4 | M | Prim | No | Yes | >1/10 HPF | Yes | 55 | Other | Moderate | Gran/eos | No | 27.5 | 0 | 4 | Yes | Yes |
| 5 | F | Prim | Yes | Yes | >1/10 HPF | Yes | 77 | Sheet | Large | Gran | No | 1 | I | 0 | Yes | No |
| 6 | M | Prim | Yes | Yes | Sporadic | Yes | 72 | Other | Moderate | Gran | No | 9.6 | 0 | 0 | Yes | Yes |
| 7 | F | Prim | Yes | No | No | Yes | 78 | Sheet | No | clear | No | 0.1 | I | 4 | No | No |
| 8 | M | Prim | No | Yes | Sporadic | Yes | 32 | Sheet | Little | Gran | No | 10 | 0 | 4 | Yes | No |
| 9 | M | Prim | No | Yes | >1/10 HPF | No | 71 | Sheet | Moderate | Gran | No | 1.9 | I | 5 | nd | nd |
| 10 | F | Prim | No | No | No | Yes | 49 | Sheet | No | Gran/cle | No | 1.3 | I | 6 | No | No |
| 11 | M | Prim | No | Yes | Sporadic | No | 58 | Nod | Little | Gran | No | 2 | I | 0 | No | No |
| 12 | M | Prim | No | Yes | Sporadic | Yes | 49 | Sheet | Moderate | Gran/cle | No | 1.4 | I | 0 | Yes | No |
| 13 | M | Prim | No | Yes | No | Yes | 32 | Sheet | Moderate | Gran/cle | No | 5.6 | I | 4 | nd | nd |
| 14 | M | Prim | No | Yes | No | Yes | 76 | Sheet | Moderate | Gran | No | 0.6 | I | 0 | nd | nd |
| 15 | F | Prim | No | Yes | Sporadic | Yes | 74 | Sheet | Little | Gran | No | 10 | I | 0 | Yes | Yes |
| 15 | F | Reg LN | No | Yes | Sporadic | Yes | 75 | Nod | Little | Gran | No | 10 | I | 0 | Yes | Yes |
| 16 | F | Prim | Yes | Yes | No | Yes | 66 | Other | Large | Oxy/cle | No | 2.7 | I | 0 | Yes | Yes |
| 16 | F | Reg LN | Yes | No | No | Yes | 73 | Sheet | Large | Oxy | No | 2.8 | I | 0 | Yes | Yes |
| 16 | F | Reg LN | Yes | No | Sporadic | Yes | 73 | Sheet | Moderate | Oxy | No | 0.6 | I | 0 | Yes | Yes |
| 17 | M | Lung MT | No | Yes | >1/10 HPF | Yes | 42 | Sheet | Little | Gran | No | 6.3 | I | 5 | No | Yes |
| 18 | F | Prim | No | Yes | Sporadic | Yes | 51 | Sheet | Little | Gran/cle | Yes | 3 | I | 0 | Yes | Yes |
| 19 | F | Reg LN | No | No | >1/10 HPF | Yes | 30 | nd | Moderate | Clear | nd | 35 | 0 | 7 | nd | nd |
| 20 | F | Prim | No | Yes | >1/10 HPF | Yes | 52 | Sheet | Moderate | Gran | Yes | 3.3 | I | 4 | No | Yes |
| 21 | M | Prim | Yes | No | No | Yes | 54 | Other | Large | Oxy/cle | Yes | 5.5 | I | 4 | Yes | Yes |
| 22 | F | Prim | No | No | >1/10 HPF | Yes | 37 | Sheet | Moderate | Gran | No | 0.6 | I | 4 | nd | nd |
| 23 | M | Reg LN | No | No | >1/10 HPF | Yes | 41 | Nod | Little | Gran | No | 9.8 | 0 | 4 | No | No |
| 24 | F | Prim | No | Yes | No | Yes | 62 | Nod | Little | Gran | No | 2.4 | 0 | 0 | No | No |
| 25 | M | Prim | No | Yes | No | No | 39 | Sheet | Little | Gran | Yes | 1.5 | I | 4 | Yes | Yes |
| 26 | F | Prim | Yes | Yes | Sporadic | Yes | 80 | Other | Large | Gran | No | 5 | nd | 4 | nd | nd |
| 27 | M | Reg LN | No | Yes | >1/10 HPF | Yes | 55 | Sheet | Little | Gran | No | 13.6 | 0 | 0 | Yes | No |
| 28 | M | Prim | Yes | No | Sporadic | Yes | 65 | Sheet | Little | Gran | No | 0.3 | I | 0 | nd | nd |
| 29 | M | Prim | No | Yes | No | Yes | 63 | Nod | No | Gran/cle | No | 0.2 | I | 0 | nd | nd |
| 30 | F | Prim | No | Yes | Sporadic | Yes | 36 | Other | Moderate | Oxy | Yes | 15 | 0 | 2 | No | No |

nd = no data; prim = primary tumour; lung MT = lung metastasis; reg LN = regional lymph node metastasis; nod = nodular; gran = granular; cle = clear; oxy = oxyphilic. Ki-67: percentage of positive nuclei. CASR: I = normal staining; 0 = absent or faint staining. Cyclin D1: sum of score of intensity and percentage of positive cells (see the materials and methods section).

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**Table 2.** Percentage of parathyroid hyperplasias, adenomas, and carcinomas that showed overexpression (cyclin D1, Ki-67) or loss of expression/altered staining (CASR). Significance: *p* value of chi-square test comparing the different groups

| Marker | Hyperplasian $n = 26$ | Adenoma $n = 93$ | Carcinoma $n = 30$ | Significance |
|---|---|---|---|---|
| CASR | 0 | 1 | 31 | $p < 0.05$ |
| Cyclin D1 | 43 | 14 | 57 | $p < 0.05$ |
| Ki-67 | 0 | 0 | 44 | $p < 0.05$ |

As expected, the absence of mitosis was correlated with a low Ki-67 index (<5%) in 8/10 (80%) samples.

### LOH analysis at the MEN1/HRPT2 loci

LOH of chromosome 1q was found in 12/22 (55%) cases analysed and 11/22 (50%) showed loss of chromosome 11q (Figure 2). The LOH pattern identified was identical in different regions of primary tumours (eg cases 10, 20, and 25) or in comparison between primary tumours and metastases (cases 15 and 16, Figure 2). Eight of 22 (36%) cases showed LOH of both chromosome 1q and 11q, whereas LOH of either chromosome 1q or 11q occurred in four (16%) and three samples (12%), respectively. Seven out of 22 (32%) cases tested showed LOH at neither chromosome 11q nor chromosome 1q using the markers investigated.

No chromosomal boundary of LOH could be identified with the markers used.

Four of the six parathyroid carcinomas with cystic features in our series in which LOH status was determined showed LOH of chromosome 1q (67%) and three of these four showed both chromosome 1q and chromosome 11q loss. In tumours without cystic features, we found LOH at chromosome 1q in 8/16 and in five of these eight, loss of both chromosome 1q and chromosome 11q.

## Discussion

In sporadic parathyroid adenomas, both mutations in the *HRPT2* gene [18] and LOH of chromosome 1q are uncommon [27–32], being present in 2–4% and 0–9% of cases, respectively. In HPT–JT-related tumours, the percentage of LOH of chromosome 1q in adenomas is relatively low (17%) compared with that in carcinomas (100%). The incidence of carcinoma in HPT–JT syndrome is approximately 10%, whereas it is less than 1% in sporadic primary HPT. Taken together, this suggests an important role for the *HRPT2* gene in parathyroid carcinoma tumourigenesis. The high percentage of LOH at 1q that we found in our set of sporadic parathyroid carcinomas seems to support this hypothesis and might underscore the important role of (LOH of) *HRPT2* in the development of sporadic parathyroid carcinoma. To our knowledge, no LOH data are available for the 1q region in parathyroid carcinoma and the reported percentage of LOH of chromosome 1q in adenomas is low (8%) [28].

LOH of chromosome 11q13 in 50% of the parathyroid carcinomas is higher than reported for parathyroid adenomas (with LOH of chromosome 11q13 in 30% of cases) [25,29,32]. This finding might also suggest involvement of the *MEN1* gene in the tumourigenesis of some parathyroid carcinomas.

A striking feature of our cohort of parathyroid carcinomas is the combination of chromosome 1q21–32 and 11q13 loss, both of which were lost in 36% of cases. To our knowledge, this is the first time that this combination of loss has been described in parathyroid carcinoma, whereas in parathyroid adenoma such an event appears to be rare [27,32]. Therefore it might be concluded that the combined 1q/11q loss can be used as a supportive criterion in the diagnosis of parathyroid carcinoma. The findings also seem to suggest that inactivation of *HRPT2* on chromosome 1q21–32 may function either independently or in concert with *MEN1* gene inactivation to promote parathyroid carcinogenesis.

Immunohistochemistry for CASR showed decreased or absent expression in parathyroid carcinomas (31%), whereas expression was decreased in only one adenoma. So far, no mutations in the coding sequence of the *CASR* gene have been described [33–35]. In parathyroid adenomas, however, a decrease in the CASR mRNA level of 24–98% has been detected [36,37].

At the protein level, a reduction in the intensity of staining for the CASR protein (14–60%) has also been described [34,38]. Our results in parathyroid adenomas appear to be different from the results of the latter studies. Using expression arrays (Haven *et al*, manuscript in preparation), we clearly see that expression of CASR is greatly diminished in parathyroid carcinomas and to a lesser extent in adenomas, supporting our immunohistochemical data. The differences between these observations might also be explained by the use of different antibodies.

In our study, the Ki-67 proliferation index in parathyroid carcinomas is, as described previously, significantly higher in parathyroid carcinomas (>5%) than in adenomas/hyperplasias (<1%) [39–42].

It is striking that the altered expression of the CASR protein in 31% of the parathyroid carcinomas seems to occur concomitantly with a high Ki-67 proliferation index in these tumours. In contrast, in 119 parathyroid adenomas/hyperplasias in our TMA, a very low proliferation index (mean 0.4%) in combination with positive staining for the CASR receptor was seen.

The association between the proliferation index and a decrease in the expression of CASR has been previously reported for secondary hyperparathyroidism [43–45]. Ritter *et al* [43] showed in uraemic rats that parathyroid cell proliferation triggers a cascade of effects that leads (directly or indirectly) to down-regulation of CASR.

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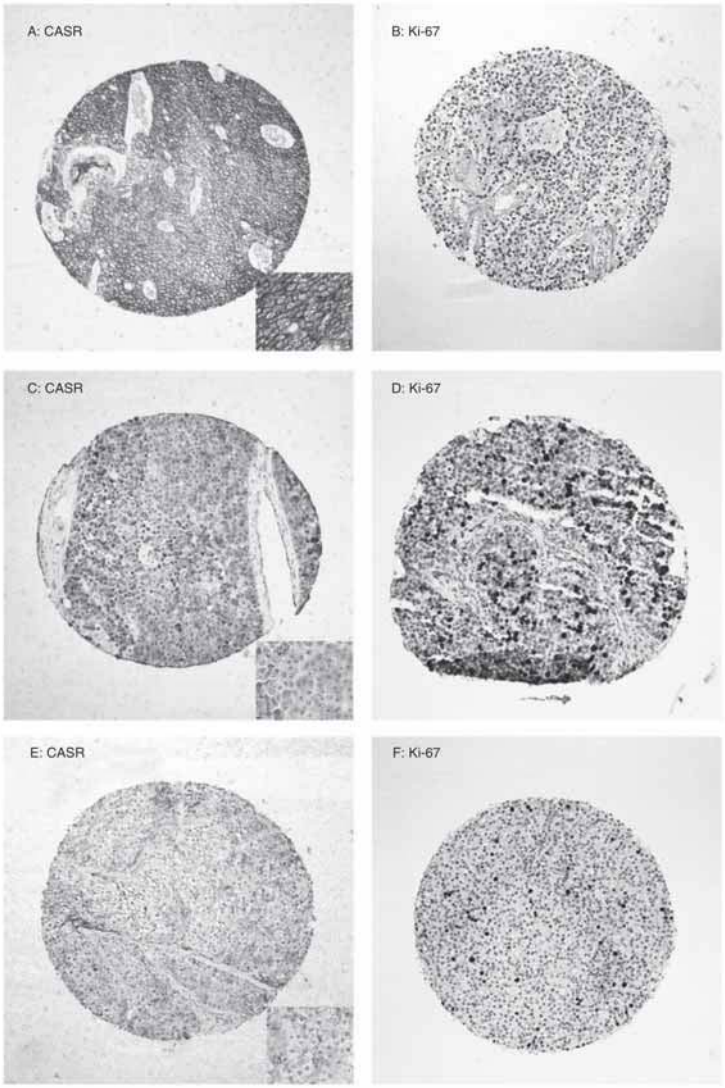


**Figure 1.** Immunohistochemical staining of a 5 μm section from a tissue micro-array showing the immunophenotype of three different parathyroid carcinomas: one (A, B) shows membranous staining from CASR (A) and no Ki-67 positivity (B); the second (C, D) shows no staining for CASR and strong Ki-67 positivity, whereas the third (E, F) shows diminished, but not absent, CASR staining, concomitant with moderate overexpression (≥5%) of Ki-67 (magnification ×100; inserts ×200)

The finding that cyclin D1 overexpression is more common in parathyroid carcinoma (55%) and hyperplasia (43%) than in adenomas (14%) is in agreement with previous studies [46–48], but contrasts with the results of Stojadinovic *et al*, who reported a lower percentage of overexpression in carcinomas than in adenomas [42]. The latter observation may be due to the different antibodies used or the small number of

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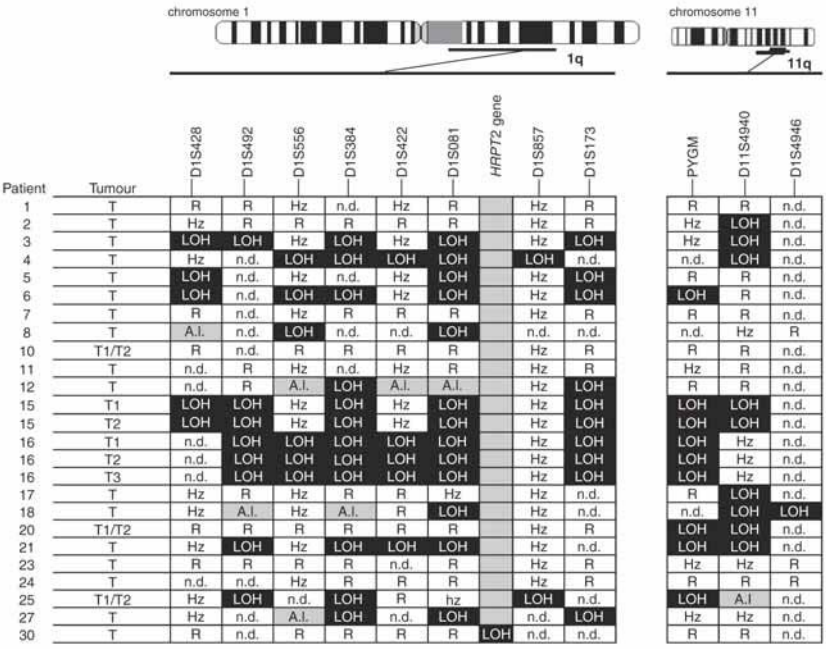


| Patient | Tumour | D1S428 | D1S492 | D1S556 | D1S384 | D1S422 | D1S081 | *HRPT2* gene | D1S857 | D1S173 | PYGM | D11S4940 | D1S4946 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | T | R | R | Hz | n.d. | Hz | R | | Hz | R | R | R | n.d. |
| 2 | T | Hz | R | R | R | R | R | | Hz | R | Hz | LOH | n.d. |
| 3 | T | LOH | LOH | Hz | LOH | Hz | LOH | | Hz | LOH | Hz | LOH | n.d. |
| 4 | T | Hz | n.d. | LOH | LOH | LOH | LOH | | LOH | n.d. | n.d. | LOH | n.d. |
| 5 | T | LOH | n.d. | Hz | n.d. | Hz | LOH | | Hz | LOH | R | R | n.d. |
| 6 | T | LOH | n.d. | LOH | LOH | Hz | LOH | | Hz | LOH | LOH | R | n.d. |
| 7 | T | R | n.d. | Hz | R | R | R | | Hz | R | R | R | n.d. |
| 8 | T | A.I. | n.d. | LOH | n.d. | n.d. | LOH | | n.d. | n.d. | n.d. | Hz | R |
| 10 | T1/T2 | R | n.d. | R | R | R | R | | Hz | R | R | R | n.d. |
| 11 | T | n.d. | R | Hz | n.d. | Hz | R | | Hz | R | Hz | R | n.d. |
| 12 | T | n.d. | R | A.I. | LOH | A.I. | A.I. | | Hz | LOH | R | R | n.d. |
| 15 | T1 | LOH | LOH | Hz | LOH | Hz | LOH | | Hz | LOH | LOH | LOH | n.d. |
| 15 | T2 | LOH | LOH | Hz | LOH | Hz | LOH | | Hz | LOH | LOH | LOH | n.d. |
| 16 | T1 | n.d. | LOH | LOH | LOH | LOH | LOH | | Hz | LOH | LOH | Hz | n.d. |
| 16 | T2 | n.d. | LOH | LOH | LOH | LOH | LOH | | Hz | LOH | LOH | Hz | n.d. |
| 16 | T3 | n.d. | LOH | LOH | LOH | LOH | LOH | | Hz | LOH | LOH | Hz | n.d. |
| 17 | T | Hz | R | Hz | R | R | Hz | | Hz | n.d. | R | LOH | n.d. |
| 18 | T | Hz | A.I. | Hz | A.I. | R | LOH | | Hz | n.d. | n.d. | LOH | LOH |
| 20 | T1/T2 | R | R | R | R | R | R | | Hz | R | LOH | LOH | n.d. |
| 21 | T | Hz | LOH | Hz | LOH | LOH | LOH | | Hz | n.d. | LOH | LOH | n.d. |
| 23 | T | R | R | R | R | n.d. | R | | Hz | R | Hz | Hz | R |
| 24 | T | n.d. | n.d. | Hz | R | R | R | | Hz | R | R | R | R |
| 25 | T1/T2 | Hz | LOH | n.d. | LOH | R | hz | | LOH | n.d. | LOH | A.I | n.d. |
| 27 | T | Hz | n.d. | A.I. | LOH | n.d. | LOH | | n.d. | LOH | Hz | Hz | n.d. |
| 30 | T | R | n.d. | R | R | R | R | LOH | n.d. | n.d. | R | R | n.d. |

**Figure 2.** Details of LOH analysis. LOH (shaded black): allele ratio $\geq 1.7$ or $\leq 0.59$. Retention (R): allele ratio between 0.76 and 1.3. Grey area (A.I.): allele ratio between 0.60–0.75 and 1.31–1.69. n.d. = no data; Hz = homozygous

samples included in the study ($n = 8$ in the study of Stojadinovic *et al*). Using expression arrays (Haven *et al*, manuscript in preparation), we clearly see the same trend that we now describe using immunohistochemistry.

No relationship could be found between the histological parameters and the genetic or immunohistochemical features of the parathyroid carcinomas, other than the recently described relationship between cystic appearance and chromosome 1q LOH. Cystic morphology is linked to HPT–JT tumours and chromosome 1q LOH. In sporadic cystic parathyroid adenomas, chromosome 1q LOH is present in 20% of the samples as opposed to 0–9% in tumours without cystic changes [49]. We saw the same trend in the parathyroid carcinomas, with 67% of the cystic parathyroid carcinomas showing chromosome 1q LOH compared with 50% of the parathyroid carcinomas without cysts.

In conclusion, our study suggests that the combined loss of chromosomes 1q and 11q in parathyroid tumours might be an indication of malignant behaviour. Loss of CASR protein expression in combination with an increased proliferation rate seems, in our hands, an almost typical feature of parathyroid carcinoma. Furthermore, TMA is a very powerful tool for the analysis of a large number of different tumours at once, the equal staining conditions making the comparison easy in such heterogeneous and cell-rich tumours as those from the parathyroid.

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