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Allelic imbalance in sporadic parathyroid carcinoma and evidence for its *de novo* origins

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Abstract

Purpose—Parathyroid cancer is a rare, clinically aggressive cause of primary hyperparathyroidism, and whether these malignancies generally evolve from preexisting benign adenomas or arise *de novo* is unclear. Further, while inactivation of the *CDC73* (*HRPT2*) tumor suppressor gene, encoding parafibromin, is a major contributor, other genes essential to parathyroid carcinogenesis remain unknown. We sought to identify genomic regions potentially harboring such oncogenes or tumor suppressor genes, and to gain insight into the origins and molecular relationship of malignant vs benign parathyroid tumors.

Methods—We performed genome-wide copy-number and loss of heterozygosity (LOH) analysis using Affymetrix 50K SNP Mapping Arrays and/or comparative genomic hybridization (CGH) on 16 primary parathyroid carcinomas, local recurrences or distant metastases, and matched normal controls, from 10 individuals.

Results—Recurrent regions of allelic loss were observed on chromosomes 1p, 3, and 13q suggesting that key parathyroid tumor suppressor genes are located in these chromosomal locations. Recurrent allelic gains were seen on chromosomes 1q and 16, suggesting the presence of parathyroid oncogenes on these chromosomes. Importantly, the most common alteration in benign parathyroid adenomas, loss of 11q, was not found as a recurrent change in the malignant parathyroid tissues. Molecular allelotyping using highly polymorphic microsatellite markers provided further confirmation that the prevalence of 11q loss is markedly and significantly lower in carcinomas as compared with adenomas.

Conclusions—Our observations provide molecular support for the concept that sporadic parathyroid cancer usually arises *de novo*, rather than evolving from a preexisting typical benign adenoma. Further, these results help direct future investigation to ultimately determine which of the candidate genes in these chromosomal locations make significant contributions to the molecular pathogenesis of parathyroid cancer.

Keywords

primary hyperparathyroidism; parathyroid carcinoma; allelic imbalance; DNA microarray

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Primary hyperparathyroidism is a common endocrine disorder characterized by excessive secretion of parathyroid hormone (PTH) leading to hypercalcemia. Benign, sporadic, non-familial parathyroid adenomas are the most frequent cause of primary hyperparathyroidism, and in developed nations their clinical manifestations are generally mild. Parathyroid carcinoma, in contrast, is an uncommon cause of hyperparathyroidism often associated with severe clinical manifestations and significant mortality. Distinguishing between parathyroid carcinoma and adenoma is notoriously difficult on histopathologic grounds, and a definitive diagnosis of carcinoma depends upon the presence of distant metastases or local invasion of surrounding structures [1–3].

The molecular pathogenesis of parathyroid carcinoma is poorly understood. In fact, it remains controversial whether parathyroid cancers typically arise from preexisting benign adenomas through further accumulation of genetic abnormalities, akin to the colorectal cancer model [4] or *de novo*, without a clinically or histologically detectable intermediate [2]. Two genes have been solidly established as contributors to the molecular pathogenesis of benign parathyroid adenoma [5], the *CCND1/PRAD1* oncogene [6–8] and the *MEN1* tumor suppressor gene [9]. The involvement of the cyclin dependent kinase inhibitor gene *CDKN1B*, encoding p27, in sporadic parathyroid tumorigenesis has also been shown through rare germline and somatic mutations in parathyroid adenomas [10], complemented by evidence from a p27-mutant rodent [11], and by germline involvement in a subset of human MEN1-like cases [11,12]. A few additional candidate genes, including *CTNNA1* - catenin [13–15], *EZH2* [16] and *POT1* [17], have been identified which exhibit rare somatic mutations in sporadic parathyroid adenomas but their ability to function as primary drivers of parathyroid tumorigenesis in an experimental model system has yet to be established. None of these genes are known to contribute commonly to malignant parathyroid tumorigenesis on the basis of clonal mutation, although this issue has not been thoroughly assessed. In contrast, the *HRPT2* (*CDC73*) tumor suppressor gene, encoding parafibromin, is clearly an important contributor to parathyroid cancer, with mutations having been identified in a large percentage of sporadic malignant parathyroid tumors [18–20] but virtually never in sporadic, benign adenomas [21]. Further, individuals with the hyperparathyroidism jaw tumor syndrome (HPT-JT), a rare autosomal dominant condition caused by germline *HRPT2* mutation, are at markedly increased risk for parathyroid carcinoma as well as benign parathyroid tumors, fibro-osseous jaw lesions, uterine tumors and a variety of kidney lesions with variable penetrance [22,23].

To identify new locations of pathogenetically important oncogenes or tumor suppressor genes, and to potentially shed light upon the molecular evolution of these tumors, we performed genome-wide copy-number analysis using SNP Mapping Arrays and comparative genomic hybridization (CGH) on a series of clinically and pathologically well-characterized parathyroid carcinomas.

MATERIALS AND METHODS

Patients and Tumor Specimens

Sixteen primary, recurrent or metastatic parathyroid carcinomas and paired peripheral blood or other non-tumor control samples were obtained from 10 patients who underwent surgery for primary hyperparathyroidism. Patients were diagnosed with parathyroid carcinoma according to stringent clinico-pathological criteria [24,2]. All patients given the diagnosis of carcinoma had evidence of either gross invasion or distant metastasis. No patient had undergone irradiation of the neck or had clinical manifestations or family history of multiple endocrine neoplasia. Three patients were later found to be positive for germline mutation of *HRPT2*, but had presented as sporadic cases with no family history or clinical manifestations of HPT-JT.

At initial parathyroidectomy, the 10 patients with parathyroid carcinoma ranged in age from 20 to 68 years; six were men, and four were women. All patients had hypercalcemia and elevated serum levels of parathyroid hormone. No patients were treated with radiation therapy or chemotherapy.

An additional 56 patients with primary hyperparathyroidism due to a single, typical parathyroid adenoma, with no features suggestive of malignancy or multi-gland disease, were also included in this study.

Genomic DNA was extracted from each sample using either proteinase K digestion for surgical samples or sucrose gradient centrifugation for blood samples, followed by phenol-chloroform extraction and ethanol precipitation.

Affymetrix 50K SNP Mapping Array Analysis

High molecular weight DNA from twelve primary or metastatic parathyroid carcinomas from 9 patients and non-tumor control DNA from the same individuals was examined by Affymetrix 50K SNP Mapping Array Analysis. Briefly, genomic DNA was digested with Xba I, adapter sequences were ligated on to the resulting sticky ends and subjected to PCR (polymerase chain reaction) amplification using a single, proprietary primer. Resulting PCR fragments were purified and quantified prior to fragmentation and end-labeling. Labeled fragments were hybridized overnight to Affymetrix 50K Xba SNP Mapping Arrays (Affymetrix, Santa Clara, CA), then washed stained and scanned, all under conditions as recommended by the manufacturer. Data analysis was performed using Gene Chip Operating Software, Gene Chip DNA Analysis Software (Affymetrix) and Copy Number Analyzer for Gene Chip (CNAG) [25]. Two tumors (tumors 1 and 2a) were compared to non-self reference due to low signal-noise ratio.

Comparative Genomic Hybridization (CGH)

Fifteen primary or metastatic parathyroid carcinomas from 10 patients were analyzed by CGH, using previously described methods [26]. Briefly, tumor DNA, serving as probe DNA, and normal reference DNA were differentially labeled using standard nick translation with fluorescein 12-deoxyuridine 5-triphosphate and Texas Red 5-deoxyuridine 5-triphosphate (NEN-Dupont, Boston, MA), respectively. Equal amounts of tumor and normal reference DNA were co-precipitated with unlabeled human Cot-1 DNA (Gibco-BRL, Gaithersburg, MD). Probe DNA and reference DNA were hybridized to normal human metaphase chromosomes prepared by phytohemagglutinin-stimulated peripheral blood lymphocyte culture. Hybridization was performed for 48 hours prior to washing and counterstaining with 4,6-diamino-2-phenylindole (DAPI) for the identification of the chromosomes. The green and red fluorescence intensities of the hybridization signals and DAPI staining patterns were captured with a cooled charge-coupled device camera (Photometrics, Tucson, AZ) attached to a Nikon Microphot-SA microscope. Fluorescence ratio profiles for each chromosome were calculated using the Quantitative Image Processing System (QUIPS, Vysis Inc., Downers Grove, IL). For each hybridization, the data from 12–14 representations of each chromosome were combined to obtain the mean and 95% confidence interval for that ratio, plotted next to the ideogram for that chromosome. Gains or losses of entire chromosomes or chromosomal regions were detected on the basis of the ratio profiles deviating from the green to red balance value of 1.0. The upper and lower threshold limits for defining chromosomal gains and losses were set to 1.20 and 0.80, respectively. These threshold values were determined by CGH experiments using two differentially labeled normal genomic DNA samples. In these negative control experiments, the mean green to red ratio was well within 1.20-0.80 over the entire length of all chromosomes, thus providing robust and highly stringent criteria for the determination of

gains and losses in tumor samples. Metaphase spreads, with uniform high intensity fluorescence in both green and red colors on both homologous chromosomes and with no background spots, were selected for evaluations. The centromeric and heterochromatic regions, p arm of acrocentric chromosomes and telomeric regions were not included in the interpretation of gains and losses.

Molecular Allelotyping

Loss of heterozygosity (LOH) on chromosome arm 11q was specifically assessed by molecular allelotyping of highly polymorphic microsatellite markers. D11S987, D11S4191 and D11S787 were PCR amplified using fluorescently labeled primers (Sigma-Aldrich, St. Louis, MO) specific for each microsatellite. Calculations were as previously described [27], with preferential loss of at least 50% of signal from one allele being scored as LOH.

RESULTS

Affymetrix 50K SNP Mapping Array Analysis

Twelve parathyroid carcinomas from 10 patients were analyzed by SNP array analysis, with complete results shown in Table 1. Ten tumors (83%) showed clonal chromosomal imbalances that included gains and losses of partial or entire chromosome lengths; all tumors that demonstrated chromosomal abnormalities had at least 4 distinct chromosomal changes. One tumor (tumor 4) showed a complex pattern that included multiple gains and losses on the same chromosomes. Copy number neutral LOH events, designated here as uniparental disomy (UPD), were identified in three tumors (tumor 4, 8a and 8b). Highly recurrent regions of loss were identified on chromosomes 1p (6 of 12 tumors, or 50%), 3 (6 of 12 tumors, or 50%), 13q (7 of 12 tumors, or 58%), and 14 (5 of 12 tumors, or 42%) and highly recurrent gains were seen on chromosomes 1q (5 of 12 tumors, or 42%) and 16 (5 of 12 tumors, or 42%).

CGH

Fifteen carcinomas from 10 patients were analyzed by CGH, with detailed results shown in Table 1 and Figure 1. Eleven tumors (73%) showed clonal chromosomal imbalances that included gains and losses of partial or entire chromosome lengths. Among the 15 tumors, seven had 2 or more chromosomal changes. Six abnormalities were found recurrently: loss of chromosome 1p (3 of 15 tumors, or 20%), gain of chromosome 1q (4 of 15 tumors, or 27%), loss of 3 (5 of 15 tumors, or 33%), loss of 13q (5 of 15 tumors, or 33%), gain of 16 (5 of 15 tumors, or 33%) and gain of 20 (3 of 15 tumors, or 20%).

Molecular Allelotyping

Fifteen carcinomas from 10 patients and 56 adenomas were assessed for LOH on chromosome 11q. All samples included in the study were informative for at least one of the three markers. 11q LOH was present in only one of the 15 carcinomas examined (6.7%). In stark contrast, LOH was found in 20 of the 56 adenomas examined (35.7%). The frequencies of LOH in these two groups were statistically significant (p-value less than 0.03 using the Fisher's Exact test).

DISCUSSION

Here we present results of genome-wide copy-number analysis using Affymetrix 50K SNP Mapping Arrays and comparative genomic hybridization (CGH) performed on a series of clinically and pathologically well-characterized parathyroid carcinomas. Parathyroid carcinoma, while rare, is an aggressive disease that is clinically distinct from benign parathyroid neoplasia. Severe, symptomatic hypercalcemia is often seen at initial

presentation, in contrast with the mild and minimally symptomatic hypercalcemia typical of patients with benign tumors. While there are a few reports of carcinoma occurring within (and apparently evolving from) an adenoma or a hyperplastic parathyroid gland [28–32], the disproportionately overwhelming prevalence of typical sporadic parathyroid adenoma compared with carcinoma implies that progressive transformation from typical adenoma to carcinoma must be extremely rare. On the other hand, patients with germline *HRPT2* mutations do indeed appear to develop parathyroid carcinomas that evolve from preexisting benign or atypical adenomas, and might explain those rare reports of apparent progression. Substantial evidence for a progression model has been demonstrated in colon cancer and other solid tumors, with normal tissue advancing through hyperplastic/dysplastic and benign neoplasia stages, via incremental accumulation of acquired genetic abnormalities, before becoming malignant.

In a progression model, genetic alterations already present in early/benign disease are found at equal or greater frequencies in advanced/malignant disease, and additional alterations (that were important for progression) are present selectively in the malignant tumors. For this progression model to be generally true for parathyroid cancer, the same genetic alterations already present in parathyroid adenomas should be at least equally well represented in parathyroid carcinoma, with additional acquired genomic changes found in carcinomas. While few somatically mutated genes have been identified in either parathyroid adenoma or carcinoma, many recurrent regions of clonal allelic imbalance have been found in both tumor types. The most common (and most informative) alteration in benign parathyroid tumors, loss of 11q, occurs in at least 35% of parathyroid adenomas [33–37,16,17] and, quite strikingly, was not identified as a recurrent change in our series of malignant parathyroid tumors. Further, when 11q LOH was directly assessed using microsatellite markers, we found a strong, statistically significant difference in the rate of 11q LOH in adenomas versus carcinomas. Additionally, a review of previous studies by other groups [33] [35] [38] also shows a statistically significant difference (p-value less than 0.004 using the Fisher's exact test) between adenomas and carcinomas when the most stringent definition of carcinoma is used: 39% (14 of 36) of adenomas show losses on 11q, while losses on 11q are seen in only 7% (2/28) of unequivocal carcinomas. Since a progression model would predict that 11q loss would be found in at least 35% of carcinomas, our observations suggest that parathyroid cancer generally arises *de novo*, rather than evolving from a preexisting typical benign adenoma. Clinically, this conclusion can provide added reassurance to physicians who may opt to defer surgery for a presumptive parathyroid adenoma until and unless the patient's symptoms progress [39].

While loss of 11q does not appear to contribute to the pathogenesis of parathyroid cancer, this study identified several genomic regions of recurrent allelic imbalance likely to contain genes important to the development of this devastating tumor. Highly recurrent regions of loss, suggesting the presence of a key tumor suppressor gene(s), were identified on chromosomes 1p, 3, 13q and 14. Highly recurrent gains, suggesting the presence of a driver oncogene(s) were seen on chromosomes 1q and 16. While recurrent changes on 1p, 3, 13q and 16 have been described in prior studies using comparative genomic hybridization and molecular allelotyping [33,35,36,38], our results narrow the target regions on 1p and 13q, where a tumor suppressor gene (or genes) important to the pathogenesis of parathyroid cancer is likely to be located. Recurrent allelic loss on chromosome 14 in parathyroid cancer is a novel finding identified by our study. Additionally, this is the first study to describe copy number neutral LOH events in parathyroid cancer.

Interestingly, tumors from the same individual (designated by a/b in Table 1) did not always show the exact same patterns of copy number abnormalities. In patients with germline *HRPT2* mutation (example patient 6 shown in Table 1), these distinct chromosomal

abnormality patterns are likely indicative of two independent primary tumors, as opposed to a primary tumor and its recurrence or metastasis. Seemingly sporadic parathyroid carcinoma patients with germline *HRPT2* mutations are thought to represent phenotypic variants of the hyperparathyroidism-jaw tumor syndrome (HPT-JT) [18], an autosomal dominant disorder involving primary hyperparathyroidism, ossifying fibroma of the maxilla or mandible and renal abnormalities. Parathyroid tumors in these patients often occur asynchronously and with an increased likelihood of malignancy [40]. While this patient showed no family history of HPT-JT nor other clinical manifestations of the syndrome, our finding of distinct chromosomal alterations in two tumors from one patient with a detectable germline *HRPT2* mutation lends support to the hypothesis that a subset of patients presenting with seemingly sporadic parathyroid carcinoma may indeed represent phenotypic variants of HPT-JT [18]. These patients and their families may be at increased risk of developing additional parathyroid, jaw or renal tumors. Germline testing for *HRPT2* mutations must be considered in the small subset of patients in which primary hyperparathyroidism is due to parathyroid carcinoma; mutation positive patients and their families should be carefully monitored [41].

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Figure 1.

DNA copy number changes in 15 primary or metastatic parathyroid carcinomas from 10 patients. Summary of all gains and losses detected by CGH. The red bars on the left side of the chromosome ideograms indicate losses and the green bars on the right side indicate gains of the corresponding chromosomal region for each individual tumor.

Table 1

Allelic imbalance identified in parathyroid carcinoma

Tumor ⁺	SNP Array Result	CGH Result
1 [*]	-3, +5, +16, -13	-3, +5, -13q, +16, +22
2a [*]	-1p35.1-pter, +1q22-qter, -3, -13, -14, +20, +21	-1p33-36.3, +1q22-44, -3, -13, -14, +20, -21
2b	-1p35.1-pter, +1q23.1-qter, -3, -13, -14, +16, +20	-1p33-36.3, +1q22-44, -3, -13, -14, +16, +20
3a	not done	-2q34-37
3b	not done	-2q34-37, -13q11-14
3c	not done	no changes
3d	-2q36.1-qter, -13q12.13-q21.1, -14q24.2-q24.3	no changes
4	1 ^{**} , UPD 3, 5 ^{**} , +16, +21	+1q, -3, +5q23-35, -6, +12, +16
5 [‡]	no changes	no changes
6a [‡]	no changes	no changes
6b [‡]	+12, -13, +16, -18, +20	+12p
7 [‡]	not done	-13q13-34
8a	UPD 1p13.1-pter, -1p13.1-p11.2, -3, -4, -8, -9, -11, -12, -14, -15, -17, -18, -21, UPD 22	-1p231-36.3, +16p, +x
8b	-1p11.2-pter, -2, +3, UPD 4, +5, -6, -8p22.1-qter, -9p23-p21.3, -9p21.2-pter, -10, +11, -12, -13, UPD 14, -15, -16p11.2-pter, -17, -18q12.1-qter, -21q21.1-qter, UPD 22	not done
9	-1p31.2-pter, +1q21.1-qter, +11p15.1-15.2, +12p11.1-pter, +11q13.3-q21.1, -16q12.1	+x
10	+1q21.1-qter, -3, -6, +12p11.1-pter, -13, -15, +16, -18, +20	+1q, -3, +8, -15q, +16p, -18, +20

⁺Tumors with the same number followed by a letter indicate independent tumors obtained from the same patient

[‡]patients with germline *HRPT2* mutations

^{*}compared to non-self reference (use of self-reference yielded high signal to noise ratio)

^{**}multiple abnormalities