Molecular-cytogenetic characterization of head and neck cancer: Identification of novel prognostic factors and gene targets for therapy [double dissertation 2]
Wreesmann, V.B.; Singh, B.

Citation for published version (APA):
Chapter 16

Screening for Genetic Aberrations in Papillary Thyroid Cancer by Using Comparative Genomic Hybridization

Bhuvanesh Singh, Dennis Lim, Juan C. Cigudosa, Ronald Ghossein, Ashok R. Shaha, Ashok Poluri, Volkert B. Wreesmann, Michael Tuttle, Jatin P. Shah, Pulivarthi H. Rao

Screening Papillary Thyroid Cancer

Abstract

**Background.** Determination of the genetic composition of papillary thyroid cancers may help explain differences in observed clinical behavior. Comparative genomic hybridization (CGH) is a novel molecular cytogenetic assay that allows simultaneous detection of gains, losses, and amplification of genetic information, making it an ideal screening tool. The aim of this study was to identify genetic aberrations occurring in papillary thyroid cancers by using CGH analysis.

**Methods.** CGH analysis was performed on 21 individual cases of papillary thyroid cancers. Nonparametric statistical comparisons were performed with the Fisher exact test.

**Results.** Genetic abnormalities were identified by CGH in 10 of 21 cases (48%). A recurrent pattern of aberrations was seen in cases where genetic changes were detected, involving losses at chromosome arms 1p and 9q and chromosomes 17, 19, and 22, and gains at chromosome 4 and chromosome arms 5q, 6q, 9q, and 13q. The loss of chromosome 22 was unique to younger patients \((P=.05)\) and was associated with a higher rate of regional lymphatic metastasis \((19\% \text{ vs } 80\%, P=.02)\).

**Conclusions.** Two genetically unique groups of patients were identified by using CGH analysis. One group had no detectable aberrations; the other had a recurrent pattern of aberrations, localizing to the identical chromosomal loci. This pattern of aberrations suggests that the involved loci may contain genes important in thyroid carcinogenesis. The clinical significance of the presence of copy number changes detected by CGH needs to be determined. In addition, molecular cloning of involved genes in each of the aberrations is warranted.

Although they originate from the same progenitor cell, thyroid cancers represent a heterogenous group of neoplasms that vary in their clinical behavior from relatively innocuous well-differentiated tumors to the universally fatal anaplastic types. Papillary thyroid cancers (PTC) are the most common type of thyroid neoplasms, which in themselves manifest significant variability in clinical behavior.[1] The 20-year statistics show a 99% survival rate for the majority of patients with low-risk PTC, but patients with high-risk cancers, poorly differentiated subtypes, or both, fare significantly worse.[7] Although several authors have attempted to explain variability in clinical behavior by analysis of the genetic complement of the tumor, these attempts have been constrained by the sparse knowledge pertaining to the genetic basis for PTC.

Cytogenetic studies identified abnormalities in 27% of cases, with the most common recurrent changes including inv(10)(q22.2q21.2) in 7%, t(10;17)(q11.2;q23) in 3%, and chromosome 1 aberrations in 3% of cases.[2,3] Fewer than 30 cases of PTC with aberrations have been successfully karyotyped, reflecting the difficulty in culturing these tumors. Loss of heterozygosity studies have also yielded a low rate of detection of aberrations (23%) without any identifiable predilection to chromosomal loci.[3] On the molecular level, unlike most human cancers, p53 mutations are rare in well-differentiated thyroid cancers, but may be more common in radiation-associated PTC.[4,5] Interestingly, Jennings et al [6] found elevated MDM2 levels in 33% of PTC, suggesting an alternate mechanism for p53 inactivation in these tumors. Alterations of the RET
gene, which is mapped to 10q, have been identified in up to 50% of cases, especially those associated with radiation exposure, making it the most common aberration in these tumors.[4,7] However, none of the molecular aberrations are convincingly linked with clinical outcome. It may be concluded that many of the consequential aberrations in PTC remain unidentified.

The advent of comparative genomic hybridization (CGH), a powerful molecular cytogenetic method, has significantly enhanced the capacity to screen solid tumors for genetic aberrations.[8] CGH can be performed without prior knowledge of potential aberrations, as is required for loss of heterozygosity and molecular analysis, and is independent of the need for cell culture, as is required for cytogenetic assessment. In the 2 studies analyzing PTC by CGH in the literature, the results have been quite variable in both the rate of detection of genetic aberrations and the specific sites of aberrations detected.[9,10] Nonetheless, several novel copy number changes were identified, albeit at a low prevalence, including gains at chromosome arms 1q, 5q, and 21q, and at chromosome 7 and a loss at 16q12-q13.[9,10] Hemmer et al [10] correlated the presence of these aberrations with older age and the presence of cervical nodal metastasis. These studies have been confounded by the inclusion of several different pathologic and prognostic groups. In the current study, a prognostically uniform group of cases of pathologically confirmed well-differentiated PTC were subjected to genome wide screening by CGH analysis to identify the patterns of genetic aberrations occurring in these tumors.

Figure 1. Chromosomal aberrations detected by comparative genomic hybridization in 21 papillary thyroid carcinomas. Bars to the right of the chromosome represent gains in genetic information and bars to the left represent losses. The chromosomal loci for gains and losses is represented by the location and extent of each bar.
Methods

Study population

The study population included patients with pathologically confirmed PTC, American Joint Commission for Cancer stage I or II by the 5th edition of the staging system, and who had stored frozen tumor tissue. Cases with evidence of distant metastasis or extensive extrathyroidal invasion were excluded. The tissue specimens were confirmed to contain more than 70% tumor by analysis of corresponding histologic sections.

Comparative genomic hybridization

Comparative genomic hybridization (CGH) was performed as described in detail elsewhere.[8] In brief, DNA was extracted by cutting and washing approximately 1 cm [3] of tissue in ice-cold phosphate-buffered saline solution followed by suspension in digestion buffer (10 mmol/L TRIS-hydrogen chloride, 10 mmol/L EDTA (pH 8), 0.5% sodium dodecylsulfate, and 0.1 mg/mL proteinase K). The suspension was incubated for 12 hours at 50°C and extraction was performed once with phenol, twice with 25:24:1 phenol/chloroform/isoamyl alcohol, and once with pure chloroform. The DNA was precipitated and collected by using a glass hook. After washing in 70% alcohol, the DNA was resuspended in 100 mL TE buffer (pH 8). Quantification of the DNA was performed by using electrophoresis in a 0.8% agarose gel and by using Eagle Eye II analytic software (Stratagene).

CGH analysis (Figure) was performed by using 2 g of DNA from cell lines and placental DNA confirmed to have a normal karyotype. The DNA from each cell line was labeled by using nick translation (Gibco-BRL) with flourescein-12-dUTP and from normal placentas with Texas red-5-uUTP (NEN Dupont). Nick translation was performed according to manufacturer's protocol (Gibco-BRL) with minor modification. The reac-

Table 1. Comparison of population characteristics by the presence of genetic aberrations by CGH

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Aberrations present</th>
<th>Aberrations absent</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age</td>
<td>40 years</td>
<td>43 years</td>
<td>NS</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 45 y</td>
<td>6 (60%)</td>
<td>7 (64%)</td>
<td>NS</td>
</tr>
<tr>
<td>&gt; 50 y</td>
<td>4 (40%)</td>
<td>4 (36%)</td>
<td></td>
</tr>
<tr>
<td>Female sex, n (%)</td>
<td>6 (60)</td>
<td>7 (64)</td>
<td>NS</td>
</tr>
<tr>
<td>Median size, cm</td>
<td>3.1</td>
<td>2.5</td>
<td>NS</td>
</tr>
<tr>
<td>Lymphatic metastasis, n (%)</td>
<td>4 (40)</td>
<td>3 (27)</td>
<td>NS</td>
</tr>
<tr>
<td>Median TNM stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>6 (60%)</td>
<td>7 (64%)</td>
<td>NS</td>
</tr>
<tr>
<td>II</td>
<td>4 (40%)</td>
<td>4 (36%)</td>
<td>NS</td>
</tr>
<tr>
<td>Extrathyroidal extension, n (%)</td>
<td>1 (10)</td>
<td>1 (9)</td>
<td>NS</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td>0</td>
<td>0</td>
<td>NS</td>
</tr>
<tr>
<td>Coexistent Hashimoto's thyroiditis</td>
<td>1 (10%)</td>
<td>0</td>
<td>NS</td>
</tr>
<tr>
<td>Total thyroidectomy, n (%)</td>
<td>6 (60)</td>
<td>9 (82)</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS, Not significant. Statistical comparisons performed by using the Fisher exact test.

277
tion was performed at 15°C and terminated when the product size was 500 to 2000 base pair in size. A Sephadex G-50 column was then used to separate the labeled DNAs and 200 ng of normal and cell line DNA was coprecipitated with 15 g of cot-1 DNA (Gibco/BRL) and suspended in a hybridization mix (Oncor). The suspension was hybridized for 3 days at 37°C to normal metaphase chromosome spreads prepared from phytohemagglutinin (PHA) stimulated lymphocytes from healthy people. Upon completion of hybridization, the slides were washed and the chromosomes counterstained with 4',6'-diamidino-2 phenylindole (DAPI) to allow their identification.

**Image analysis**

Ten individual metaphases were captured for each case with a cooled-charge coupled device camera attached to a Nikon Microphot-SA microscope. Only metaphases with strong and uniform hybridization were selected for analysis. Each metaphase was processed by using the Quantitative Image Processing System (QUIPS, Applied Imaging). The chromosomes were identified by DAPI banding analysis, segmented, local background subtracted, and the median axis identified. Individual chromosomes with poor hybridization, overlap, or other features limiting accurate analysis were excluded. Red, green, and blue fluorescence was analyzed for all metaphase spreads, normalized to a standard length, and statistically combined to show the red:green signal ratio and 95% confidence intervals for the entire chromosome. Copy number changes were detected based on the variance of the red:green ratio profile from the standard of 1. Ratio values of 1.2 and 2.0 were defined as thresholds for gains and amplifications, respectively. Losses were defined as ratios of 0.8 or lower. The centromeric regions of chromosomes 1, 9, 16, and Y and the p-arms of acrocentric chromosomes were not analyzed because of uniformly variable hybridization resulting from the presence of heterochromatic DNA content. Chromosomes X and Y were also excluded because of gender conflict between the healthy female placenta and tumor specimens from men.

**Clinical data**

Clinical information was collected for each case from available hospital and physi-

---

**Table 2. Comparison of population characteristics by age**

<table>
<thead>
<tr>
<th></th>
<th>Age &lt; 45 y</th>
<th>Age &gt; 45 y</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female sex, n (%)</td>
<td>6 (46)</td>
<td>7 (88)</td>
<td>NS</td>
</tr>
<tr>
<td>Median size, cm</td>
<td>2.9</td>
<td>2.6</td>
<td>NS</td>
</tr>
<tr>
<td>Lymphatic metastasis, n (%)</td>
<td>7 (54)</td>
<td>0</td>
<td>.01</td>
</tr>
<tr>
<td>Extrathyroidal extension, n (%)</td>
<td>2 (15)</td>
<td>0</td>
<td>NS</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td>0</td>
<td>0</td>
<td>NS</td>
</tr>
<tr>
<td>Coexistent Hashimoto's thyroiditis</td>
<td>1 (8%)</td>
<td>0</td>
<td>NS</td>
</tr>
<tr>
<td>Total thyroidectomy, n (%)</td>
<td>9 (69)</td>
<td>6 (75)</td>
<td>NS</td>
</tr>
<tr>
<td>Presence of a chromosomal aberration</td>
<td>6 (46%)</td>
<td>4 (50%)</td>
<td>NS</td>
</tr>
<tr>
<td>Chromosome 22 loss</td>
<td>5 (38%)</td>
<td>0</td>
<td>.05</td>
</tr>
</tbody>
</table>

NS, Not significant. Statistical comparisons performed by using the Fisher exact test.
Screening Papillary Thyroid Cancer

cian records. Demographic (age and gender), tumor (size, presence of lymphatic metastasis), and pathologic data (presence of extrathyroidal extension and differentiation) were collected for all cases. Descriptive statistics were used to summarize study data. The Fisher exact test was used for nonparametric, qualitative comparisons. Statistical significance was accepted at a 2-tailed $P$ value of less than or equal to .05.

**Results**

**Study population**

The study population consisted of 8 men and 13 women ranging in age from 13 to 77 years, with a median of 42 years. One 13-year-old patient had a history of radiation exposure from the Chernobyl accident. The median tumor size was 2.8 cm, ranging from 1.1 to 6.5 cm. Two patients under the age of 45 had focal extrathyroidal extension, but none of the patients displayed extensive invasion of local structures. Lymph node metastases were identified in 7 patients at presentation. No cases were associated with distant metastasis. Surgical management consisted of total thyroidectomy in 15 cases (71%) and less than total thyroidectomy in 6 cases (29%).

Genetic analysis and clinical correlation. Genetic aberrations were identified in 10 of 21 cases (48%). In cases with aberrations, the median number of changes (gains and losses) was 6.5, with a range of 1 to 12. An outlier case in our series with 12 aberrations was from the 13-year-old who had been exposed to radiation as a child in the Chernobyl accident. The most common sites of chromosomal gains were 4q, 5q, 6q, and 13q, and losses at 1p36, 9qter, 17, 19, and 22 (Figure). The presence of aberrations was not influenced by the presenting clinical characteristics, stage, or treatment (Table 1). Analysis of aberrations by age group showed some significant differences. Although the presence of an aberration as not different by age at presentation, analysis of individual chromosomal aberrations shows that loss of genetic information at chromosome 22 was unique to patients under the age of 45 (Table 2; $P=.05$). In addition, chromosome 22 loss was associated with a higher rate of regional lymph node metastasis (19% vs 80%; $P=.02$).

**Discussion**

The use of CGH for the genetic screening of solid tumors has yielded significant novel genetic information and has lead to the discovery of novel prognostic markers and oncogenes in several tumor systems, including thyroid cancers.[9,10] Using this technique, Chen et al [9] identified a recurrent amplification at 2p21 and found over-expression and mutation of protein kinase C-epsilon, suggesting its role as an oncogene in thyroid tumors.

In our analysis, genetic aberrations were detected in 10 of 21 cases (48%) of PTC. This success rate for detecting genetic aberrations by CGH is greater than the 27% rate reported for conventional cytogenetic analysis and 23% for loss of heterozygosity studies.[2-3] In contrast, Hemmer et al detected aberrations in only 12% of 26 cases of PTC analyzed by CGH analysis, but Chen et al found, at minimum, a 50% prevalence of aberrations among 16 cases of PTC.[9,10] Significantly higher rates of aberrations have been
identified in follicular carcinoma by CGH, ranging from 54% to 80% of cases.[9-12] A recent report analyzing 8 cell lines from anaplastic thyroid carcinomas detected aberrations in all but 1 case (95%).[14] Interestingly, benign adenomas of the thyroid also have CGH-detectable aberrations in more than 50% of cases.[9-12]

In our study population, the median rate of aberrations per case was 6.5, which is in sharp contrast to Hemmer's findings of a median of 1 aberration per case.[10] Of note, the single patient with PTC in our series who was exposed to radiation as a child from the Chernobyl accident had 12 aberrations, far outnumbering the remainder of the study population. The median number of aberrations reported in the literature for follicular adenomas is 3.5 in 18 cases with detectable aberrations. [11,12] The median number of aberrations reported for follicular carcinoma is 2 in 32 analyzable cases.[10-12] Hürthle cell lesions have a median of 7 aberrations each in 3 reported cases of adenomas and 6 cases of carcinomas with CGH detectable aberrations.[13] The collective CGH data from the literature suggests that the genetic complexity of well-differentiated thyroid cancers in general, and PTC in specific, is quite variable and may represent an outcome predictor.

A novel, recurrent, and nonrandom pattern of aberrations was seen in our study population (Figure). Losses were common at 1p, 9q, 17, 19, and 22. Gains were most common at 4, 6q, and 13. Comparison of these findings with those reported for PTC by Chen and Hemmer shows overlap with nonrecurrent copy number increases at 5q.[9,10] A review of published cytogenetic alterations in PTC shows minimal overlap with our findings, but loss of chromosome 22 is identified by both analyses.[2] Comparison of our findings with CGH analysis of follicular adenoma, Hürthle cell adenoma, follicular carcinoma, and Hürthle cell carcinoma shows overlap between the losses at 1p and a gain of chromosome 5, suggesting that these may be early events in the oncogenic pathway taken by the thyroid follicular cell.[9-14] Additionally, overlap with loss of chromosome 22 is seen in all well-differentiated thyroid cancers.[9-14] Although, no overlap in genetic aberrations is identified between the CGH analysis of PTC and anaplastic cancer, significant overlap is observed with an allelotyping analysis of anaplastic carcinomas by Kitamura et al [15] at chromosome 17 and chromosome arms 19p and 22q. Interestingly, loss of genetic information on chromosome 22 also overlaps between well-differentiated cancers and anaplastic carcinomas, suggesting that anaplastic cancers may represent a continuum of disease from well-differentiated cancers.

Analysis of the data showed that the presence of CGH-detectable chromosomal abnormalities was not influenced by age and did not impact on the presence of lymphatic metastasis or recurrence. However, loss of chromosome 22 was unique to younger patients (age < 45 years), occurring in 5 of 13 cases, in contrast to none of the older patients (age > 45 years, P=.04). In addition, a higher rate of lymphatic metastasis was observed in patients with chromosome 22 loss (80%) compared with those without it (16%; P=.02). Studies of follicular cancer have suggested that chromosome 22 loss is a
Screening Papillary Thyroid Cancer

poor prognostic indicator. The study by Hemmer et al identified the loss of chromosome 22 in 35% of cases, identifying its presence in older patients and exclusively in widely invasive tumors.[10,12] They reported that 3 of the 4 patients who died of cancer had chromosome 22 loss. The same group also reported a 46% prevalence of chromosome 22 loss in another study of follicular cancers, confirming its predilection for widely invasive tumors.[10,12] In the current study, loss of chromosome 22 was found to be more common in patients under age 45. These patients had a higher rate of lymphatic metastasis associated with their cancers. Moreover, no increase in the prevalence of follicular variant of papillary cancer was seen in patients with chromosome 22 loss. Although it cannot be confirmed from our data, given the findings in follicular cancers, chromosome 22 loss may represent a poor prognostic indicator in younger patients with PTC.

Several important candidate genes can be identified in the regions of gains and losses seen by CGH that merit investigation for their role in PTC pathogenesis. Candidate tumor suppressor genes include p73 at 1p, which has functions similar to p53.[16] Both APC and MCC are candidate genes, located at 5q21q22. Clinical studies have strongly linked the development of thyroid carcinoma with familial adenomatous polyposis coli, which is associated with a germline mutation in APC.[17] Candidates at 9q include TSCI and PTC. TSCI is a gene associated with tuberous sclerosis, mutation which allows unrestricted growth.[18] PTC is a homologue of PTCH or patched in Drosophila. PTC encodes for a transmembrane protein that represses transcription of genes encoding for members of TGF-beta and Wnt family of signaling proteins, both of which have been implicated in cancer pathogenesis, including thyroid carcinoma.[19] The FYN oncogene and MAS1 oncogene are excellent candidates at 6q21 and 6q24-q27, respectively. MAS1 encodes for a membrane receptor that, when activated, modulated a critical component of a growth regulating pathway.[21] On chromosome 17, p53 is an obvious candidate. However, because mutation of p53 is rare in PTC, alternative genes, such as suppressor of TY6 (SUPT6H) on 17q11.2 should be considered.[22] SUPT6H encodes for a variety of proteins having chromosomal activity, including histones and transcription factors. On 13q12, FMS-related tyrosine kinase 1 (FLT1) is an excellent candidate oncogene. Vascular endothelial growth factor, an angiogenesis-promoting protein that has been found to be overexpressed in PTC, has a high affinity binding site for FLT1.[23] Moreover, FLT1 has been found to be essential for blood vessel formation. Suppressor of TY 5 (SUPT5H) is a homologue of SPT5 in yeast.[24] It functions similarly to SUPT6H, making it a strong candidate gene at 19q13.1-q13.2. Candidates at chromosome 22 include neurofibromin 2 (NF2), which when mutated is associated with the development of neural tissue into benign and malignant tumors.[25]

Several aspects of this study merit comment. First, given the potential for selection bias and the small sample size, the generalizability of this study is limited. Next, the retrospective format for the clinical data collection encompasses known limitations based on the availability, content, and accuracy of the medical records. Finally, the resolution
ability of CGH for detecting aberrations is limited to unbalanced alterations, 5 to 10 Mb in size. Accordingly, smaller aberrations, such as point mutations, and balanced aberrations, such as paracentric inversion at 10q that results in RET-PTC mutations, are not detected by CGH. Nonetheless, this study has identified a novel, recurrent pattern of chromosomal aberrations in PTC. Loss of chromosome 22, which has been shown to be a poor prognostic marker in follicular carcinomas and 5q gain, which are pervasive throughout all types of thyroid cancer, merit detailed analysis.

References
Screening Papillary Thyroid Cancer


Acknowledgments
We thank Dr R. S. K. Chaganti for his invaluable guidance.
Discussion

Dr Irving Rosen (Toronto, Ontario, Canada). I really would like to congratulate you on this study. I think it is remarkable evidence of the genetic influence. I speak not as an expert, of course. I am curious to know whether you feel that the changes you have described represent an acquired effect due to some physical injury or whether they represent an inherited tendency. That has an implication, I think, for a general attitude toward thyroid neoplasia.

Dr Shaha. The answer is very simple: I don't know. This has been studied not only in thyroid cancer but renal tumors and lung cancer. We have been looking at it in the head and neck tumors. It is very difficult to say, because in head and neck cancer we can study from dysplasia and carcinoma in situ and invasive carcinoma and we can see the difference. So it is quite likely these may not be inborn genetic alterations and may be due to environmental factors.

Dr Krukowski (Aberdeen, SD). We already know that there is a clear relationship between aneuploidy, papillary cancer, and prognosis. Did you correlate to the changes you observed with ploidy?

Dr Shaha. We did not study the ploidy. But I think it has been studied. The first paper, if I remember right, to this organization was presented by Ken Kohn. He did his work at Karolinska. He showed the difference. We did not look at the ploidy. But I think this essentially says the same thing: it is a DNA expression.

Dr Krukowski. You haven't shown a relationship between the changes you have described and prognosis.

Dr Shaha. That is right. Because the numbers are very small right now. We have already studied 63 cases. Once we have more than 100 cases studied, we may be able to show some difference.

Dr Orlo H. Clark. (San Francisco, Calif). Have you looked at lymph node metastasis and primary tumors? Do they have the same profile?

Dr Shaha. No, we did not look at the lymph node metastasis. These are the specimens from the primary tumors. Now that we know there are some differences, as we have shown in patients below the age of 45, it would be nice in the operating room to take the primary tumor, take the lymph node metastasis, and do the CGH. The technique of CGH does not take much time. We are aware of the technique in our laboratory, so we should be able to look at this more critically. Before I conclude, I would like to thank the first author of this paper, Dr Singh. He is the new faculty on our service, and he has done tremendous work in a very short period of time.