Objective: To investigate the expression of connexin 43 (Cx43) in benign vs malignant thyroid tissue for potential use as a diagnostic marker.

Design: Retrospective study.

Subjects: Thyroid specimens were obtained from 50 patients who underwent partial or total thyroidectomy at the New York Eye and Ear Infirmary, New York, New York, between 1999 and 2007. They included goiter (n=5), follicular adenoma (n=15), follicular carcinoma (n=17), papillary thyroid carcinoma (PTC) (n=7) and the follicular variant of PTC (n=6).

Interventions: Tissue sections (5 µm) were immunohistochemically stained for Cx43 with the avidin-biotin-peroxidase method using an automated stainer. The Cx43 membrane staining pattern was evaluated.

Results: Twenty-three of 30 cancer specimens (77%) revealed a loss of Cx43 plaque staining at the cellular membrane compared with only 3 of 20 benign specimens (15%). Among the malignant specimens, loss of Cx43 plaque staining was observed in 11 of 17 follicular carcinomas (65%), 5 of 6 follicular variants of PTC (83%), and 7 of 7 PTCs (100%). In contrast, only 3 of 15 adenomas (20%) and 0 of 5 goiter samples demonstrated loss of Cx43 plaque staining at the cell membrane.

Conclusion: Our data provide evidence that the absence of Cx43 plaque staining is associated with thyroid cancer and thus holds potential clinical utility as a marker for malignant disease.


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Fine-needle aspiration (FNA) is recommended as a diagnostic tool for the initial screening of patients with thyroid nodules. Approximately 10% of thyroid FNAs are considered “suspicious for malignancy,” yet only a small number of these aspirates are confirmed to be malignant on subsequent thyroid excision. A common feature in nondiagnostic aspirates deemed to be suspicious is the presence of a hypercellular, microfollicular architecture with scant colloid in the absence of signature cytologic characteristics of malignancy. Well-differentiated follicular carcinoma cannot be reliably distinguished from a benign adenoma on the basis of an aspirate alone, thus posing a particular challenge.

Studies on thyroid folliculogenesis have shown that the formation of fully functional thyroid follicles is highly dependent on connexin (Cx) gap junction (GJ) proteins.4 Gap junctions are transmembrane channels that are involved in intercellular communication and coordinated secretion between cells. Each GJ is composed of 2 connexons, or hemichannels, which in turn are composed of 6 Cxs. Twenty-one different types of Cxs have been identified to date, and different Cx isotypes may be found within the same GJ.4 Connexins are synthesized in the endoplasmic reticulum, assembled into connexons in the Golgi apparatus, and transported to the cell membrane, where they aggregate into GJs, clump together into plaques, and interact with GJs of neighboring cells. The formation and degradation of GJs are dynamic processes, with a half-life of 1.5 to 5 hours in most tissue types.4 Loss of GJ intercellular communication via Cx downregulation has been implicated in carcinogenesis in a variety of neoplasms; therefore, Cxs have been thought to be a type of tumor suppressor gene.5 Decreased expression of Cx43 in particular has been reported in human prostate, lung, brain, and breast cancers.5 Cx43, Cx32, and Cx26 have been identified in thyrocytes, and animal studies have shown a link between Cx32 expression and thyroid folliculogenesis.5 To our knowledge, no studies to date have examined the expression of Cx43 in thyroid carcinoma.
As Cx family GJ protein expression is associated with thyroid follicular differentiation, its loss may therefore be a biomarker of the malignant phenotype. The objectives of this study were to examine the immunohistochemical expression of Cx43 in normal and neoplastic human thyroid tissue and to determine its potential as a tumor marker for future aspirate studies.

**METHODS**

After institutional review board approval was obtained, patients who had undergone partial or total thyroidectomy at the New York Eye and Ear Infirmary, New York, New York, between 1990 and 2007 for both benign and malignant disease were identified. A total of 50 thyroid patient specimens were collected. Tissues included 5 hyperplastic goiters, 15 follicular adenomas, 17 follicular carcinomas, 7 papillary thyroid carcinomas (PTCs), and 6 follicular variants of PTC (FVPTCs). Clinical staging and prognostic data were obtained from patient medical records. The parameters that were recorded include age at diagnosis, sex, primary tumor size, evidence of lymph node disease or distant metastasis, and evidence of extrathyroidal invasion. Various staging and prognostic indices, including the TNM, AMES (age, distant metastasis, tumor extent, and size), and MACIS (distant metastasis, age, completeness of primary tumor resection, local invasion, and tumor size) systems, were calculated.

Immunohistochemical analysis was performed with the avidin–biotin–peroxidase method. All specimens were fixed in formalin for 24 hours before paraffin embedding and storage. Five-micron sections of each specimen were placed on glass slides, together with a section of normal thyroid tissue from the same patient (internal positive control) and a section of normal thyroid tissue from a different patient (external positive control). One additional section of normal thyroid tissue from the source patient, untreated with primary antibody, was used as a negative control. The specimens were deparaffinized, pretreated with Tris-based buffer (pH, 8) for 30 minutes, and incubated with primary rabbit, polyclonal, anti–Cx43 antibody (Cell Signaling, Danvers, Massachusetts) at a 1:30 dilution for 1 hour at 37°C. An automated stainer (Benchmark XL; Ventana Medical Systems) was used for color development with an indirect biotin-streptavidin system for detection mouse IgG, mouse IgM, and rabbit primary antibodies (iVIEW DAB Detection Kit; Ventana Medical Systems), which includes a biotinylated immunoglobulin secondary antibody composed of goat anti–mouse IgG and IgM, goat anti–rabbit IgG, and a protein block. The secondary antibody incubation was performed for 8 minutes at 37°C. Specimens were then mounted and photographed with a digital microscope. Qualitative changes in staining of Cx43 between benign and malignant thyroid tissue were analyzed by the second author (A.D.P.) and by a senior member of the pathology department (S.M.). Specimens were evaluated based on the intensity of staining of particular cellular structures and compared with staining exhibited on normal tissue within the same slide. The presence or absence of discrete plaques at the cell membrane was recorded.

Functional Cx43 normally demonstrates staining at the cell membrane in close association with tight junctions. Distinct clumping of Cx43 staining at the cell membrane, which was defined as plaque staining, represents the configuration of individual Cx proteins into formed GJs (Figure). Membranous and cytoplasmic Cxs that do not aggregate into plaques are non-functional. Cytoplasmic Cx43 reflects molecules that have not been transported and inserted properly into the cell membrane because of changes in the transformed cell. Because GJ plaques represent the only functional unit, only plaque staining was scored.

For purposes of using Cx43 staining as a possible diagnostic parameter, the loss of Cx43 plaque staining is considered a positive test result and retention of Cx43 staining is considered a negative test result. Specificity was determined by dividing the number of samples that retained Cx43 plaque staining (negative test result) by the number of benign samples. Sensitivity was determined by dividing the number of samples that lost Cx43 plaque staining (positive test result) by the number of malignant samples. Positive predictive value was obtained by dividing the number of malignant samples that lost Cx43 plaque staining by the total number of samples that lost Cx43 plaque staining. Negative predictive value was obtained by dividing the number of benign samples that retained Cx43 plaque staining by the total number of samples that retained Cx43 plaque staining.

The demographic and staging data are shown in the Table. Patient age did not differ significantly between groups ($P = .30$), and, overall, approximately 80% of patients were female, which is consistent with the prevalence of thyroid disease. Approximately 75% of the malignant specimens were T1 or T2 ($\leq 4\ cm$), while 25% were T3 ($>4\ cm$). Only 2 specimens had evidence of nodal metastasis (1 PTC and 1 follicular carcinoma). None of the patients had distant metastasis. Approximately 85% of the benign lesions were 4 cm or less, while 15% were larger than 4 cm.

The immunohistochemical staining pattern for Cx43 at the cell membrane was evaluated qualitatively. Cx43 normally demonstrates staining at the cell membrane in close association with tight junctions. Distinct clumping of Cx43 staining at the cell membrane, which was defined as plaque staining, represents the configuration of individual Cx proteins into formed GJs (Figure). The Cx43 GJ staining pattern of all the specimens is shown below.

### Table: Diagnosis and Loss of Plaques

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Loss of Plaques, No. (%) of Specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goiter</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>Follicular adenoma</td>
<td>3/15 (20)</td>
</tr>
<tr>
<td>Follicular carcinoma</td>
<td>11/17 (65)</td>
</tr>
<tr>
<td>FVPTC</td>
<td>5/6 (83)</td>
</tr>
<tr>
<td>PTC</td>
<td>7/7 (100)</td>
</tr>
</tbody>
</table>

Twenty-three of 30 cancer specimens (77%) revealed a lack of Cx43 plaque staining at the cellular membrane, compared with only 3 of 20 benign specimens (15%).

### Table: Diagnosis and Loss of Plaques

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Loss of Plaques, No. of Specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign</td>
<td>2/20</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>23/30</td>
</tr>
</tbody>
</table>

Among the malignant specimens, loss of Cx43 plaque staining was observed in 11 of 17 follicular carcinomas (65%), 5 of 6 FVPTCs (83%), and 7 of 7 PTCs (100%) (Figure, C and D). In contrast, only 3 of 15 adenoma (20%) and 0 of 5 goiter samples demonstrated an absence of Cx43 plaque staining at the cell membrane. The utility of the loss of GJ plaque staining as a biomarker of malignant disease is summarized in the preceding tabulation.

The absence of staining as an indicator of malignant disease has a sensitivity of 0.76, a specificity of 0.85, a
positive predictive value of 0.88, and a negative predictive value of 0.71. These data are highly significant (Fisher exact test, \( P < .001 \)). Of particular interest is the loss of GJ plaques on follicular thyroid cancer cells compared with follicular adenomas.

The loss of staining as an indicator of follicular carcinoma vs follicular adenoma has a sensitivity of 0.65, a specificity of 0.80, a positive predictive value of 0.79, and a negative predictive value of 0.67 (Fisher exact test, \( P = .02 \)).

### COMMENT

A significant number of thyroid operations are performed for benign disease as a result of limitations in our current diagnostic techniques. While FNA is a highly useful tool in the analysis of thyroid nodules, its utility is restricted by the finding of suspicious, nondiagnostic pa-
thology. Improved diagnostic markers are needed to help distinguish benign from malignant lesions before definitive surgical treatment.

Differentiating follicular carcinoma from benign follicular lesions presents a particular challenge that typically precludes FNA as a stand-alone diagnostic tool and requires macroscopic tissue architecture assessment to demonstrate invasive features. Attempts to predict the likelihood of malignancy on a nondiagnostic aspirate are based on degrees of cellularity, follicle formation, and colloid production. Benign-appearing lesions generally exhibit a macrol follicular architecture, whereas malignancy is associated with a microfollicular, trabecular, or solid appearance. Key findings suggestive of follicular carcinoma include a significant proportion of microfollicles (small groups of 6 to 10 follicular cells in a ring with or without a small amount of central colloid), trabecular cells, cellular crowding, and overlapping clusters of follicular cells.

Many studies have supported the idea that decreased expression or dysfunction of GJs may play an important role in carcinogenesis. Intercellular communication of GJs is usually decreased in cancer cells, and restoration of this communication in neoplastic cells by means of Cx transfection has led to normalization of growth and differentiation patterns both in vivo and in vitro. Mice knockout studies have also shown that Cx-depleted cells are more susceptible to tumor induction by chemicals and radiation. Connexin 43, which is the most widely expressed Cx in humans, is present in at least 46 cell and tissue-specific gene expression.

Connexin 32 and Cx43 are known to be present in thyrocytes, where they comprise homotypic GJs (consisting of only 1 Cx type) that reside in different regions of the cell membrane. Proper thyroid folliculogenesis appears to have an association with GJ intercellular communication and Cx expression. Studies have demonstrated thyroid-stimulating hormone–induced folliculogenesis in monolayered cells in association with an increase in Cx expression. Porcine thyrocyte studies have focused on Cx32 as a key player in follicle formation, as it is present in follicular structures but is lost in a cellular monolayer. The role of Cx43 in human thyroid folliculogenesis and thyroid function is unknown.

In this study, the formation of Cx43 plaques at the cell membrane indicates the gathering of individual Cx proteins into formed GJs. Also, 76% of the malignant specimens, compared with only 15% of the benign specimens, demonstrated a lack of plaque staining at the cell membrane, indicating a failure to form Cx43 GJs. In particular, plaque staining was absent in 83% of follicular carcinoma specimens, compared with only 20% of follicular adenoma specimens. This is of significant interest as there are currently no biomarkers to differentiate follicular carcinoma from adenoma on FNA biopsy. As a result, all thyroids that contain neoplasms with follicular architecture (carcinoma or adenoma) are surgically excised. Therefore, there is less concern regarding false-positives (all of which are currently removed) compared with false-negatives, which might argue against surgery, despite the true presence of cancer. This pilot study used a small number of samples, and we expect that sensitivity, specificity, and positive and negative predictive values will change as the study is expanded.

Loss of Cx43 plaque expression appears to be associated with thyroid cancer and loss of normal follicular architecture. Additional studies are required to investigate the qualitative and quantitative aspects of Cx43 staining of FNAs and to correlate the aspirates with pathology specimens to determine the clinical utility of Cx43 staining as a marker of malignant disease. Staining of serial FNA biopsy sections for other Cx molecules, such as Cx32 and Cx26, may further contribute to the diagnostic specificity and sensitivity of altered Cx expression in follicular carcinoma.

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Author Contributions: All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Darr, Patel, Tiwari, and Geliebter. Acquisition of data: Patel, Komorowski, McCormick, Schantz, and Geliebter. Analysis and interpretation of data: Patel, Yu, Schantz, and Geliebter. Drafting of the manuscript: Darr, Patel, Yu, Schantz, and Geliebter. Critical revision of the manuscript for important intellectual content: Patel, Komorowski, McCormick, Tiwari, and Geliebter. Statistical analysis: Yu, Schantz, and Geliebter. Obtained funding: Tiwari. Administrative, technical, and material support: Darr, Patel, Komorowski, McCormick, and Geliebter. Study supervision: McCormick, Schantz, and Geliebter.

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Additional Contributions: Fred Moy, PhD, New York Medical College, provided guidance in statistical analysis.

REFERENCES


