Abstract. Background: Osteopontin (OPN) and its interacting partner CEA-cell adhesion molecule (CEACAM1) mediate similar biological functions and have been expressed in several types of cancer. Here we investigated the prognostic significance of OPN in thyroid tumours and correlated our findings with the expression of CEACAM1. Materials and methods: 297 human thyroid samples were collected in a tissue microarray and as fresh frozen samples to perform immunohistochemistry and western blotting for OPN and to compare these data with our previously published data on CEACAM1 expression. Results: Nearly all normal samples were negative for OPN. Some thyroid adenomas were weakly OPN positive whereas many carcinomas were strongly positive. In contrast to CEACAM1, which was preferentially expressed in metastatic papillary carcinomas, no associations were found between OPN expression and patient age, gender and tumour size. Conclusion: These results may have future implications for the diagnosis and management of patients with thyroid cancers.

Thyroid tumours are the most common malignancies of the endocrine system. They account for roughly 1% of all new malignant diseases (1) and are the sixth most common malignancy diagnosed between the ages of 20 and 49 years. Tumors derived from thyroid follicular epithelium represent a model of malignant transformation (2) with a spectrum of benign lesions and tumors of varying degrees of malignancy, including papillary (PTC), follicular (FTC) and anaplastic carcinomas (ATC) (3, 4). Tumors derived from C cells are known as medullary carcinomas.

Our understanding of thyroid carcinogenesis has made significant progress. PTCs are characterized by activating mutations of \textit{BRAF} or chromosomal translocations involving the tyrosine kinase domains of the RET receptor or the TRK receptor to heterologous genes (7-9). Various lines of evidence indicate that \textit{BRAF} and \textit{RET/PTC} oncogenes are early events in thyroid carcinogenesis; they are capable of causing thyroid tumors in transgenic mice but are not implicated in metastasis (10).

The majority of differentiated thyroid cancers can be cured by local surgery and those that develop metastases are usually responsive to radioactive iodine therapy. However, there is currently no way to distinguish indolent from aggressive differentiated thyroid cancers.

Based on their identified role as molecules that mediate cell-matrix adhesion and communication, OPN and CEACAM1 have the potential to profoundly influence tumorigenesis and invasion. OPN is a 70-kDa extracellular matrix glycoprotein with an arginine-glycine aspartate-binding motive, which is subject to extensive posttranslational modifications (11). The physiological functions of OPN are best documented in bone where this glycoprotein seems to be involved in osteoblast differentiation and bone formation (11). OPN has been shown to be expressed in a variety of human tissues, including the kidneys, gastrointestinal tract, and breast and has been implicated in mediation of cell-cell and cell-extracellular matrix (ECM) communication through alteration of cell adhesion (12-14). Recently, we showed that OPN regulates invasiveness of the extravillous trophoblast at...
the maternal-fetal interface, and it was suggested that this action is mediated by CEACAM1 (15). The fact that OPN mRNA and/or protein expression levels are increased in many human tumours, including breast (16), lung (17), prostate (18), colon (19), ovary (20) and gastric cancer, has led to the hypothesis that it plays an important role in tumorigenesis, tumour progression, and metastasis formation (21, 22). OPN was first implicated in malignancy by *in vitro* studies detecting increased levels of OPN expression after cell transformation (23) and from the observation that tumour cells with high metastatic potential had increased OPN expression (24-26). In some studies, elevated plasma OPN has been shown to be associated with poor patient survival (27, 28).

In previous work we investigated the expression pattern and functional role of CEACAM1 in thyroid cancer and showed that CEACAM1 promotes invasiveness (29). To study the expression of OPN in thyroid tumours in correlation with clinical data and to find any correlation with the expression of CEACAM1 as diagnostic and prognostic markers, immunohistochemistry of a tissue array and western blotting of fresh frozen tissue were performed.

**Materials and Methods**

**Thyroid tissue specimens and tissue microarray.** For the tissue array construction, formalin-fixed, paraffin-embedded tissues from 230 patients who underwent thyroid surgery at the University Health Network (UHN) Hospitals between 2001 and 2004 were used as described previously (29). From the surgical specimens of these 230 patients, a total of 297 distinct samples were obtained, including 94 benign samples and 203 malignant samples (Table I). Data on tumour characteristics and prognostic factors were obtained from surgical pathology reports. All patient samples and related data analyses were performed in accordance with research ethics approval at the University Health Network in Toronto.

**Immunohistochemistry.** Immunohistochemistry was performed on 4 μm sections cut from the paraffin blocks of the tissue array blocks and mounted on 3-aminopropyl-triethoxysilane-coated slides, deparaffinized in xylene, and rehydrated in graded alcohol to Tris-buffered saline [50 mmol/l Tris, 150 mmol/l NaCl (pH 7.4)]. Sections were then microwave-heated in 10 mM citrate buffer at pH 6.0. Endogenous peroxidase and biotin activities were blocked, respectively, using 3% hydrogen peroxide and Vector’s avidin/biotin blocking kit (Vector Laboratories Inc., Burlingame, CA, USA). Sections were treated for 10 min with protein blocker (ID Labs Inc., London, ON, Canada) and then incubated overnight with anti-OPN mouse monoclonal antibody (Akm2A1) (Neomarkers) at a dilution of 1:200. Slides were then reacted with biotinylated secondary antibody (ID Labs Inc.) for 30 min and HRP-conjugated Ultra Streptavidin Labeling Reagent (ID Labs Inc.). Color development was performed with DAB (3,3’-diaminobenzidine tetrahydrochloride containing 0.03% H₂O₂) (Sigma, Chemical Co.) solution, counterstained with Mayer’s hematoxylin, dehydrated and mounted in Permount (Fisher, Ontario, Canada).

**Table I. Statistical analysis of OPN expression in thyroid tissue.**

<table>
<thead>
<tr>
<th>Thyroid tissue</th>
<th>N</th>
<th>OPN N (%)</th>
<th>OPN intensity Med (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal thyroid</td>
<td>23</td>
<td>+</td>
<td>5 (22)</td>
</tr>
<tr>
<td>Chronic thyroiditis</td>
<td>18</td>
<td>+</td>
<td>18 (78)</td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>4</td>
<td>+</td>
<td>9 (50)</td>
</tr>
<tr>
<td>Follicular adenoma</td>
<td>35</td>
<td>+</td>
<td>1 (25)</td>
</tr>
<tr>
<td>Hurtle cell adenoma</td>
<td>7</td>
<td>+</td>
<td>3 (75)</td>
</tr>
<tr>
<td>Papillary carcinoma</td>
<td>141</td>
<td>+</td>
<td>114 (81)</td>
</tr>
<tr>
<td>Medullary carcinoma</td>
<td>4</td>
<td>+</td>
<td>3 (75)</td>
</tr>
<tr>
<td>Hurtle cell carcinoma</td>
<td>4</td>
<td>+</td>
<td>1 (25)</td>
</tr>
<tr>
<td>Insular/Anaplastic Ca</td>
<td>4</td>
<td>+</td>
<td>4 (100)</td>
</tr>
<tr>
<td>Papillary Ca LN Met</td>
<td>35</td>
<td>+</td>
<td>25 (72)</td>
</tr>
<tr>
<td>Medullary Ca LN Met</td>
<td>3</td>
<td>+</td>
<td>3 (100)</td>
</tr>
<tr>
<td>Anaplastic Ca LN Met</td>
<td>4</td>
<td>+</td>
<td>4 (100)</td>
</tr>
</tbody>
</table>

N, Number; Med, median; IQR, interquartile range.

**Microscopic evaluation.** Histological and immunohistochemical evaluation was performed independently by two pathologists. Positive staining was based on the distribution and intensity of distinct membrane or cytoplasmic signals in each core using the following criteria. The percentage of cells staining was scored in each core as 0 (0%), 1 (1-50%), and 2 (51-100%) and then averaged for each sample to form an distribution score. The intensity of signal was scored in each core as 0 (no signal), 1 (weak), 2 (moderate), and 3 (marked) and then averaged for each sample to form an intensity score. The total of the distribution score and intensity score was summed for each sample into a total score (TS) indicated as negative (TS≤2), weak positive (2<TS≤3), or positive (TS>3). In addition, the immunohistochemical localization pattern was noted.

**Protein isolation and Western blot analysis of human thyroid tissue.** Western blotting analysis using anti-rabbit specific OPN monoclonal antibody (Neomarkers) was performed on normal and tumour samples for which fresh-frozen material could be obtained. Tissues were lysed in lysis buffer (0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 1% Nonidet P-40 and 1 x PBS) containing proteinase inhibitors (100μg/ml phenyl-methylsulfonyl fluoride (PMSF), 69 μg/ml aprotinin (Sigma, St. Louis, MO, USA) and 1 mM sodium orthovanadate. Total tissue and cell lysates were incubated on ice for 30 min, followed by micro-centrifugation at 10000 xg for 10 min at 4°C. Protein concentrations of the supernatants were determined by
the Bio-Rad protein assay. Fifty μg of protein were mixed with 2 × SDS buffer, boiled for 4 min and separated by 10% SDS-polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membranes (Bio-Rad Laboratories). Five percent non-fat milk in 1 × TBST (tris-buffered saline with 0.1 Tween-20) was used to block non-specific binding. Primary antibodies and antisera were directed against OPN (rabbit monoclonal anti-OPN antibody; Neomarkers), which was used at a concentration of 1:200, and against actin (mouse monoclonal anti-actin antibody; Sigma), which was used at a concentration of 1:500. After washing three times in 1 × TBST, blots were exposed to the secondary anti-rabbit antibody (IgG-HRP; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:2000. The ECL chemiluminescence detection system (Amersham, Buckinghamshire, UK) was used for visualisation and densitometry for quantification of band intensities.

Statistical analysis. The statistical analysis was performed using SPSS 13.0. To compare proportions, appropriate variants of Chi-square tests were used and the comparison of means of the quantitative variables (OPN protein expression, CEACAM1 staining) was analysed using non-parametric statistics (Kruskal-Wallis and Mann-Whitney U). P-values lower than 0.05 were considered statistically significant.

Results

Immunohistochemistry. Immunohistochemistry was performed on paraffin-embedded human thyroid tissue arrays with an anti-OPN-specific monoclonal antibody OPN (AkM2A1). There were 297 thyroid samples, of which 23 were samples of normal thyroid tissue and 71 benign lesions: 18 chronic lymphatic thyroiditis (CLT), 10 hyperplastic nodules (Hyper), and 43 follicular thyroid adenomas (FTA) including 7 Hurthle cell adenomas (HurA). There were 203 malignant lesions including 141 papillary thyroid carcinoma (PTC), 7 conventional follicular thyroid carcinomas (FTC) and 4 Hurthle cell follicular carcinomas (HurC), 4 medullary thyroid carcinomas (MTC), and 4 poorly differentiated or anaplastic thyroid carcinomas (ATC). In addition lymph node metastases of 35 papillary thyroid carcinomas (PTCmet), 3 medullary (MTCmet) and 4 anaplastic thyroid carcinomas (ATCmet) were investigated.

Figure 1A provides examples of OPN positivity for the different thyroid tissues and figure 1B and figure 1C show the levels of intensity (boxplots represent median,
Figure 2. Immunohistochemical localisation of OPN in thyroid tissue. A: Normal thyroid gland (N) showing no OPN staining. B: Moderate OPN expression of epithelial cells in a case of chronic lymphocytic thyroiditis (CLT); notice the staining of some lymphocytes. C: Follicular (FTA) and D: Hurthle cell adenoma (HurA). E and F: Strong OPN expression in membranes and cytoplasm of papillary thyroid carcinomas (PTC) (E, low magnification; F, high magnification).

Figure 3. Immunohistochemical localisation of OPN in thyroid tumours. Immunohistochemical detection of OPN in tissue samples of A: papillary thyroid carcinoma (PTC) with cytoplasmic epithelial staining of the tumour cells; B: Hurthle cell carcinoma (HurC) with strictly membranous staining; C: medullary thyroid carcinoma (MTC) with tumour cells invading the surrounding tissue; D: metastases of a papillary thyroid carcinoma (PTCmet) infiltrating a lymph node.
interquartile range and outliers for the different thyroid lesions). Eighteen of 23 (78%) normal samples were negative (Figure 2A). This contrasts with our recent report that CEACAM1 was undetectable in all normal samples (29), and statistical analysis confirmed this is a significant difference ($p<0.001$). At one of 50% (9/18) of the CLT samples showed weak OPN positivity. Staining was identified in epithelial cells in a membrane and cytoplasmic distribution and there was also positivity in leucocytes (Figure 2B). Similarly, 51% (18/35) of the FTA (Figure 2C) and 57% (4/7) of the HurA (Figure 2D) were slightly OPN positive but negative for CEACAM1 ($p<0.001$).

In the group of malignant thyroid tumours, PTC showed strong expression of OPN in 81% (114/141) of the samples and positivity was confined to tumour cells (Figure 2, Panel E and F), as opposed to CEACAM1 that exhibited positivity in only 14% (20/141) of cases ($p<0.001$). In addition PTC had a significantly higher intensity for OPN (Med: 6.0 [IQR: 3.9]) than for CEACAM1 (Med: 1.3 [IQR: 2.3]) ($p<0.001$) (Table I; Figure 1B; Figure 1C). Forty-three percent (3/7) of FTC (Figure 3A) and 100% (4/4) of HurC (Figure 3B) were OPN positive, whereas all of the FTC were negative for CEACAM1 (Table I, Figure 1A). In the group of MTC (Figure 3C) immunostaining with OPN resulted in 75% (3/4) positivity. Only 25% (1/4) of the samples in both groups were positive for CEACAM1 (Table I, Figure 1A). There was no significant difference with reference to the intensity of OPN staining in PTC lymph node metastases (Figure 3D), which showed the same OPN expression pattern as the primary lesions (Table I; Figure 1A). As previously described, CEACAM1 was preferentially expressed in metastatic PTC compared to primary tumours (29). However, there was a significant difference ($p=0.0001$) between the intensity of OPN (Med: 6 [IQR: 7]) and CEACAM1 expression (Med: 2.3 [IQR: 2.3]) in lymph node metastases (Table I; Figure 1B). Interestingly, there was an increase of the OPN intensity for MTC (Med: 9 [IQR: 1.5]) and ATC lymph node metastases (Med: 12 [IQR: 0.1]) in comparison

**Figure 4. Western blot analysis of OPN expression in thyroid tissue.** A: OPN up-regulation in thyroid carcinomas; 7 papillary (PTC) out of 30 thyroid carcinomas and 7 normal (N) thyroid samples. Equal amounts of proteins (50 μg) were immunoblotted with anti-OPN monoclonal antibody. Anti-actin monoclonal antibody was used as a control for equal loading. Migration of size markers is indicated in kDa. B: Representation of densitometric analysis of OPN expression relative to actin levels derived from seven separate experiments.
to their primary lesions (Med: 6.75 [IQR: 7.1] and Med: 10.5 [IQR: 5.3]) respectively (p=0.15) (Figure 1B; Table I).

No associations were found between OPN expression and patient age or gender.

**Immunoblot analysis of OPN on human primary thyroid tissue.** Protein lysates were harvested from 30 normal human thyroid glands and from 30 thyroid carcinomas including papillary and follicular subtypes. Densitometric analysis of the blots was performed, and OPN levels were normalized to actin expression. Western blot analysis resulted in a strong OPN expression in the group of carcinomas whereas the expression was decreased in normal samples (p<0.001). No difference could be seen between the two types of carcinomas.

As shown in Figure 4A, the OPN protein (molecular mass, ~65 kDa) was strongly expressed in all carcinomas, however the expression level in normal tissue was considerably lower. Figure 4B represents the intensity of the OPN expression relative to actin levels and a significant higher expression of OPN (Med: 142 [IQR: 92]) in thyroid carcinomas compared to normal thyroid tissue (Med: 102 [IQR: 15]) (p<0.0001). The results are the average of seven independent experiments.

**Discussion**

In this study, we investigated the expression pattern of the glycoprotein OPN in thyroid tumours and its correlation with the expression of adhesion molecule CEACAM1 (as previously described (29)). Several studies have defined OPN and CEACAM1 as two important glycoproteins, playing a role in basic cellular processes, such as neovascularisation and tissue remodeling (31). Recently we showed that OPN and CEACAM1 may act as a functional complex and cooperate in regulating the invasive process of benign and neoplastic cells of the endometrium as well as of the placenta (32, 33). OPN is overexpressed in many tumour types (14) by various signaling proteins, such as growth factors (34) and oncogenes (35, 36).

The distinction between benign and well-differentiated malignant thyroid neoplasms poses numerous problems. In addition, only a small group of patients with well-differentiated thyroid carcinomas will develop metastasis and require more aggressive management. These controversies create a clinical dilemma for the rational therapy of low-risk thyroid malignancies. Giuarino et al. (37) suggested that OPN might be used as a diagnostic and prognostic marker for PTC and additionally investigated the OPN-CD44v6 axis as a possible molecular target for aggressive PTC. Our data report the expression of OPN and compare the results with the profile of CEACAM1. OPN was weakly expressed in a small number of normal thyroid glands and OPN expression was increased in thyroid adenomas. A significant up-regulation of OPN was seen in papillary, follicular, insular/anaplastic and medullary carcinomas. In comparison, CEACAM1, exhibited a lower incidence of expression in all types of thyroid carcinomas, however, it was preferentially expressed in metastatic papillary carcinomas with a clear dissociation between CEACAM1-mediated metastatic potential and growth that could clarify the mechanism of spread in small tumours (29). Although 25/35 of papillary, all medullary and all anaplastic carcinoma metastases were positive for OPN, no significant associations were found between OPN expression and prognostic markers such as tumour size, multifocal growth, extrathyroidal extension, vascular invasion, lymph node metastasis or distant metastasis as well as gender or age.

Several studies have defined an important role for OPN in carcinogenesis and metastasis. OPN binds to cells through integrins and the hyaluronic acid receptor CD44 (38). Singhal et al. (27) and Fedarko et al. (28) reported that OPN is up-regulated in several pathologic contexts, including vascular remodeling, and cancer. CEACAM1 is an adhesion molecule belonging to the immunoglobulin gene family that is specifically expressed in epithelial tissues such as colonic mucosa (39). There are several groups who have implicated a possible role for OPN and CEACAM1 in regulating the normal processes taking place at the maternal–fetal interface during implantation and placentation (15, 40). Coppola et al. (31) showed a correlation of OPN protein expression and pathological stage across a wide variety of tumour histologies, such as colon carcinoma. They also found that only 26% of 154 lymph node-negative breast tumours had OPN staining in the tumour cells themselves. CEACAM1 generally acts as a growth suppressor, because the expression of CEACAM1 was shown to be lost or significantly down- or dysregulated in carcinomas (41). Tumour growth was strongly reduced when CEACAM1 was transfected into a nonexpressing colorectal carcinoma cell line (42). We have recently shown an increased invasiveness of CEACAM1-transfected melanoma cells (43). As we showed as well, CEACAM1 is strongly expressed by the extravillous trophoblast and might be involved in the molecular mechanisms controlling this process and for differentiating them from those implicated in tumour progression (40). Our study of thyroid cancer cells clarified this apparent contradiction by identifying a clear dissociation between CEACAM1 inhibitory effects on cell proliferation and its role in promoting cell invasion and migration.

Overall, in this study we show that OPN is expressed in benign and malignant thyroid tumours with increasing expression correlating with advancing malignancy. Despite this pattern of expression, we could not show relevance for OPN as a prognostic marker for patients with malignant
thyroid tumours, whereas CEACAM1 reactivity was associated with metastatic potential. Therefore CEACAM1 remains a better prognostic marker for metastatic disease in papillary thyroid carcinoma.

The relationship between these two proteins is important for understanding the process of carcinogenesis, and these results may have future implications for the diagnosis and management of patients with thyroid cancer.

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References


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