

## Quantitative Analysis of Osteonectin mRNA in Thyroid Carcinomas

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**Abstract.** Our recent study of the gene expression profile in thyroid carcinoma showed an overexpression of osteonectin mRNA, an extracellular matrix protein, in an anaplastic carcinoma. To confirm this, we measured the expression levels of osteonectin mRNA in 84 thyroid normal and tumor tissues, including five anaplastic carcinomas by real-time quantitative reverse-transcription PCR. Increased expression of osteonectin mRNA was observed in anaplastic carcinoma tissue. However, in five anaplastic carcinoma cell lines, no increase was observed in the expression levels of osteonectin mRNA. These findings suggest the possibility that increased expression of osteonectin mRNA in anaplastic carcinoma tissue may be due to its overexpression in stromal cells, but not in anaplastic carcinoma cells.

**Key words:** Thyroid carcinoma, Reverse transcription-PCR, Fine needle aspiration biopsy, Osteonectin  
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GENES that are differentially expressed in benign thyroid tissues and thyroid carcinomas can be directly used as targets for molecular-based diagnosis [1]. It has been shown that aspiration biopsy reverse transcription-polymerase chain reaction (ABRP) can target these genes [2–5]. By relying on 14–15 base cDNA sequences for gene identification, serial analysis of gene expression (SAGE) can easily generate a quantitative transcript profile [6]. We have previously described the use of SAGE in providing gene expression profiles in a normal thyroid tissue and thyroid tumors [7]. By analyzing these profiles, we found the differential expression of osteonectin mRNA. Osteonectin, also called SPRAC (secreted protein acidic and rich in cysteine) or BM-40, is a

glycoprotein involved in many biological processes, such as cell adhesion, differentiation, proliferation, and extracellular-matrix synthesis and breakdown [8].

No study has been done, however, on the expression levels of osteonectin mRNA in each pathological type of thyroid tumor. Thus, in the present study, in order to clarify to what extent osteonectin mRNA is useful for clinical purposes, such as in molecular-based diagnosis, we measured the expression levels of osteonectin mRNA in 84 benign and malignant thyroid tissues. These tissues included five anaplastic carcinomas and 25 RNA samples from fine needle aspiration biopsies (FNABs), and expression levels were obtained by means of real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR).

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## Materials and Methods

### *Extraction of RNA from thyroid tissues*

Tissue samples from thyroid tumors or normal thyroid tissues in the opposite lobe of carcinomas were obtained by surgery. All tissues were frozen in liquid nitrogen immediately after resection. RNAs from 21 normal thyroid tissues, 27 papillary carcinomas, 19 follicular adenomas, 12 follicular carcinomas, and 5 anaplastic carcinomas were extracted according to the method of Chomczynski and Sacchi [9].

### *SAGE protocol*

The SAGE analysis was performed as described previously [7]. Three micrograms of poly A RNA from each tissue was used to synthesize double-stranded cDNA. Sequence files were analyzed by the SAGE software kindly provided by Dr. Kenneth Kinzler of Johns Hopkins University, and the tag sequences were analyzed by the BLAST program of the DNA Data Bank of Japan, Mishima, Shizuoka, Japan.

### *Cell culture*

Five cell lines, ROA, KOA2, MSA, K119 and IAA, derived from anaplastic carcinomas, were cultured in RPMI 1620 medium (Nikken Biochemical Laboratory, Kyoto, Japan) containing 10% fetal bovine serum (Flow Laboratories, Beltsville, MD), 10<sup>5</sup> IU/L penicillin, and 100 mg/L kanamycin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air [10, 11]. Before the cells became confluent, total cellular RNA was extracted as described above.

### *Extraction of RNAs from FNABs*

FNAB samples from 25 thyroid tissues (10 follicular adenoma, 10 papillary carcinoma, 3 follicular carcinoma and 2 anaplastic carcinoma) were obtained preoperatively as previously described [12]. In brief, a syringe with a 22-gauge needle was used to obtain an FNAB from the tissue sample. A sample of the FNAB was prepared on a slide glass for cytological examination, and leftover cells inside the needle were then lysed with a denaturing solution containing 4 M

guanidine thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol into a 1.5-ml tube. The tubes were then stored at 4°C. Total cellular RNA was extracted as previously described.

### *Real-time quantitative RT-PCR*

Reverse transcription was performed using either 1 µg of total RNA from the tissues or whole RNAs from FNABs in an RT mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, 3 mM MgCl<sub>2</sub>, 0.5 mM dNTPs, 200 U M-MLV reverse transcriptase (Gibco, Gaithersburg, MD), 2 U/µl RNase inhibitor (Takara, Shiga, Japan), and 2.5 µM oligo dT (Gibco) in a total volume of 20 µl at 37°C for 60 min. Real-time quantitative PCR (TaqMan PCR) using an ABI PRISM 7700 Sequence Detection System and a TaqMan PCR Core Reagent Kit (PE Biosystems, Foster City, CA) was performed according to the manufacturer's protocol. One microliter of the first strand cDNA was used in the following assay. The two primers and one TaqMan probe used for the quantification of osteonectin, beta-actin and thyroglobulin mRNAs were [13–15] [ONF (0.5 µM): 5'-TTGCCTGAGGCTGTAAGTGA-3' (base 1511–1530)], [ONR (0.5 µM): 5'-TCCCTAGAGCCCCTGAGAAG-3' (base 1741–1760)], and [ON-TM (10 pmol): 5'-FAM-AGGATGCGCTGACCACTTCCCAGAGAACTG-TAMRA-3' (base 1671–1700)]; [ACF (0.5 µM): 5'-TGGACATCCGCAAAGACCTG-3' (base 901–920)], [ACR (0.5 µM): 5'-CCGATCCACACGGAGTACTT-3' (base 1047–1066)], and [AC-TM (10 pmol): 5'-FAM-CACCACCATGTACCCTGGCATTGCC-TAMRA-3' (base 947–971)]; and [TGF (0.5 µM): 5'-GAGAAGAGCCTGTCGCTGAA-3' (base 7980–7999)], [TGR (0.5 µM): 5'-CAGCTCACTGAACTCCTTGT-3' (base 8128–8147)], and [TG-TM (10 pmol): 5'-FAM-TGAGTTCTCACGGAAGTACCCA-TAMRA-3' (base 8054–8076)], respectively. The conditions for the TaqMan PCR were as follows: 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 1 min. A recombinant pGEM T-vector (Promega, Tokyo, Japan) containing either osteonectin, beta-actin or thyroglobulin cDNA were constructed by PCR-cloning with the

same set of primers used in TaqMan PCR and were used as standard samples. The amplification plots of the PCR reaction were used to determine the threshold cycle ( $C_T$ ). The  $C_T$  value represented the PCR cycle at which an increase in reporter fluorescence ( $\Delta Rn$ ) above the line of the optimal value (optimal  $\Delta Rn$ ) was first detected. The initial copy number of the target mRNA was calculated by a plot of the  $C_T$  against the input target quantity.

#### Statistical analysis

Statistical analysis of differences between the groups was carried out using the Mann-Whitney  $U$  test.  $P$  values of  $<0.05$  were considered significant.

### Results

According to SAGE analysis, the tag count of osteonectin mRNA was greatly increased in anaplastic carcinoma than in normal thyroid tissue and differentiated tumors (Table 1).

The expression levels of osteonectin mRNAs in thyroid tissues were measured by real-time quantitative RT-PCR. The relative expression levels of osteonectin to beta-actin were significantly increased in anaplastic carcinoma compared to differentiated tumors (Fig. 1). Further, the expression levels of osteonectin mRNA relative to thyroglobulin mRNA were measured. These levels were greatly increased in five anaplastic carcinoma, and increased values were also observed in papillary carcinoma (Fig. 2).

The relative expression levels of osteonectin mRNAs in relation to thyroglobulin mRNA in FNABs were also measured by real-time quantitative RT-PCR. Greatly increased values were observed in aspirates from two anaplastic carcinoma (Fig. 3).

Further, the expression levels of osteonectin mRNA were measured by real-time quantitative RT-PCR using RNAs from cell lines derived from anaplastic carcinoma. No increase in osteonectin mRNA levels

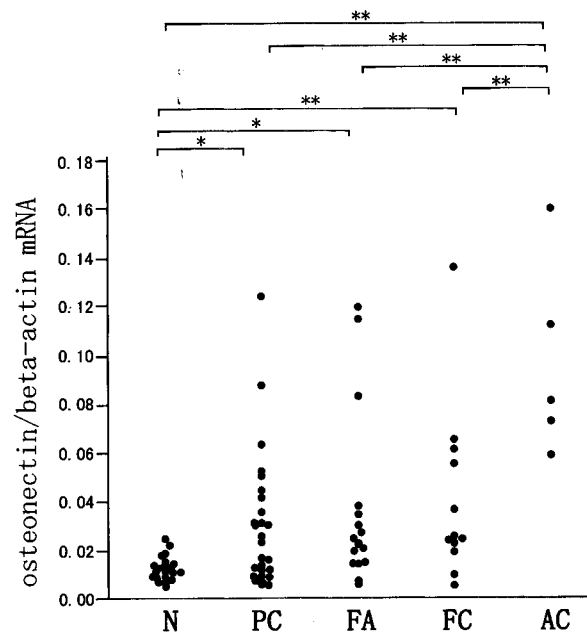


Fig. 1. Expression levels of osteonectin mRNA relative to beta-actin mRNA.

N, PC, FA, FC, and AC represent normal thyroid, papillary carcinoma, follicular adenoma, follicular carcinoma, and anaplastic carcinoma, respectively. The results are shown with mean for duplicate determinations.

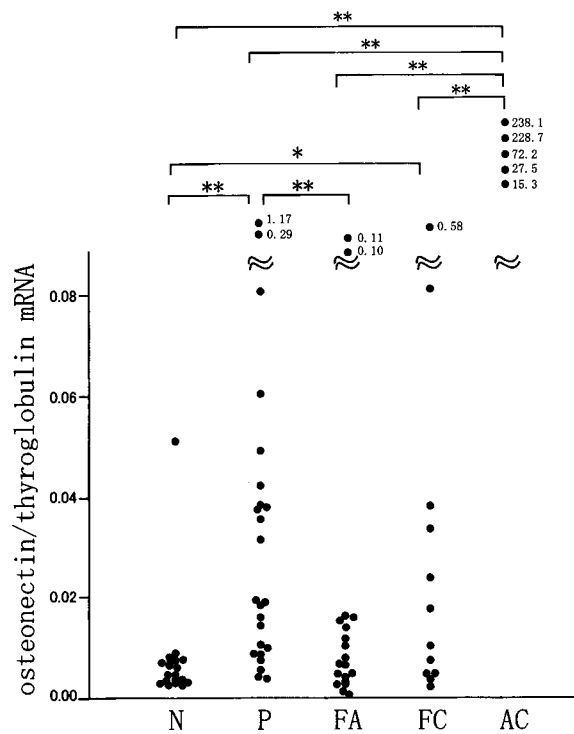
compared to those in differentiated tumors was observed in the cell lines, suggesting that the increased expression of osteonectin mRNA in anaplastic carcinoma tissues may not be due to its overexpression in anaplastic carcinoma cells (Table 2).

### Discussion

Osteonectin is a secreted glycoprotein that modifies cell-extracellular matrix interactions. Osteonectin is expressed in normal tissues showing high rates of cellular proliferation and matrix remodeling such as that occurring in morphogenesis, wound healing, embryogenesis, and angiogenesis [16, 17]. Osteonectin

Table 1. Tag counts of osteonectin in thyroid tumors.

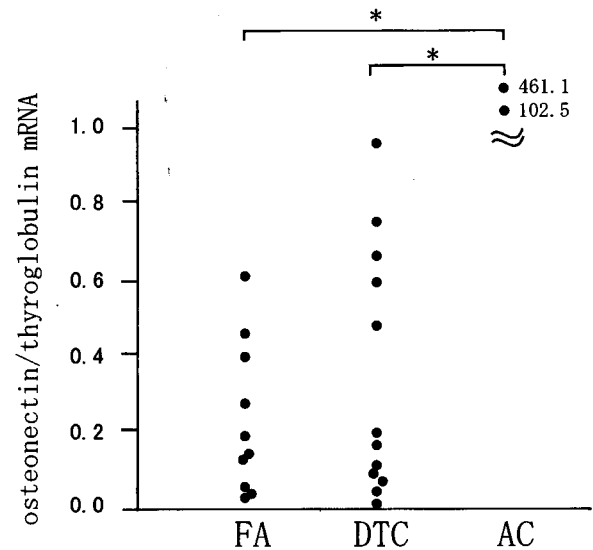
Tissue	normal thyroid	follicular adenoma	papillary carcinoma	follicular carcinoma	anaplastic carcinoma
Tag count (osteonectin/total tag)	0/5411 (0.00%)	2/5030 (0.040%)	4/6435 (0.062%)	3/5275 (0.057%)	60/7124 (0.84%)



**Fig. 2.** Expression levels of osteonectin mRNA relative to thyroglobulin mRNA.

N, PC, FA, FC, and AC represent normal thyroid, papillary carcinoma, follicular adenoma, follicular carcinoma, and anaplastic carcinoma, respectively. The results are shown with mean for duplicate determinations.

induces expression of type 1 plasminogen activator inhibitor and matrix metalloproteinases (MMPs), which facilitate degradation of the basement membrane and extracellular matrix [18, 19]. This degradation is a process also essential for invasion and metastasis in human malignancies. Downregulation of osteonectin in human melanoma cells is accompanied by a significant decrease in MMP-2, resulting in a



**Fig. 3.** Expression levels of osteonectin mRNA relative to thyroglobulin mRNA in FNABs. FA, DTC, and AC represent follicular adenoma, differentiated carcinoma, and anaplastic carcinoma, respectively. The results are shown with mean for duplicate determinations.

reduction in cellular adhesive and invasive capacities *in vitro* and a loss of tumorigenicity *in vivo* in nude mice [20].

Many studies have identified an overexpression of osteonectin in a wide variety of human malignancies, including melanoma, glioma, meningioma, and esophageal, colorectal, breast, renal, and hepatocellular carcinomas [21–28]. As such, researchers have reported that overexpression of osteonectin correlates with tumor progression and that its overexpression correlates with prognosis. Therefore, inappropriate expression of osteonectin in tumor tissues seems to contribute to tumor progression, invasion, and metastatic capacities.

**Table 2.** Expression of osteonectin mRNA in thyroid tumors and cell lines.

Tissue	Number	Osteonectin/beta-actin mRNA ( $\times 10^{-2}$ ) (mean $\pm$ SD)
Normal thyroid	21	1.12 $\pm$ 0.49
Papillary carcinoma	27	3.05 $\pm$ 2.75
Follicular adenoma	19	3.37 $\pm$ 3.31
Follicular carcinoma	12	3.93 $\pm$ 3.55
Anaplastic carcinoma	5	9.60 $\pm$ 4.02
Cell line	5	3.93 $\pm$ 2.96

The cellular origin of osteonectin in malignant tumors is different in each malignancy. In some tissues, it is expressed predominantly by tumor cells, whereas in others it is the product of surrounding stromal cells. For example, osteonectin is expressed primarily by tumor cells in melanoma, breast carcinoma, and malignant cells of mesenchymal cell origin, such as those in osteosarcoma and malignant fibrous histiocytoma [29]. In colon and hepatocellular carcinomas, it is distributed exclusively in tumor-associated stromal cells [25, 28].

In the thyroid, increased expression of osteonectin mRNA has been observed in all histological types of thyroid tumors, especially in anaplastic carcinomas. However, the expression levels of osteonectin mRNA in cell lines derived from anaplastic carcinomas are almost the same as those in differentiated tumors. Further, in our preliminary study using in situ hybridization with a specific probe for osteonectin mRNA, no intense staining of osteonectin mRNA has been observed in tumor cells in anaplastic carcinoma (data not shown). Based on the above, increased expression of osteonectin mRNA in anaplastic carcinoma may not be due to its overexpression in tumor cells. Rather, as in colon or hepatocellular carcinoma, the mRNA may be overexpressed by stromal fibroblasts or vascular endothelial cells. The greatly increased relative expression levels of osteonectin mRNA in relation to thyroglobulin mRNA in anaplastic carcinoma may be caused primarily by the

great decrease in thyroglobulin mRNA levels, not by an increase in osteonectin mRNA levels.

One of the purposes of the present study was to determine if measurement of osteonectin mRNA in FNABs is useful for preoperative molecular-based diagnosis of thyroid carcinoma. As shown in Fig. 3, the relative expression level of osteonectin mRNA in relation to thyroglobulin mRNA can be used in distinguishing anaplastic from differentiated carcinomas. False positive results may be obtained, however, when the connective tissues together with tumor cells are aspirated by FNAB. This aspiration is not likely to occur frequently, however, as our previous report showed that contamination by stromal cells is a rather rare event in thyroid FNAB [30]. Nonetheless, osteonectin mRNA is not superior target of ABRP than the previously used oncofetal fibronectin (onfFN), as it can not be used in the differential diagnosis of follicular tumors and papillary carcinomas, which can be easily diagnosed by ABRP detection of onfFN [2].

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