Expansion of Microsatellite in the *Thyroid Hormone Receptor-α1* Gene Linked to Increased Receptor Expression and Less Aggressive Thyroid Cancer[1]

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ABSTRACT

Purpose: **The purpose of this study was to determine whether the length of the THRA1 microsatellite, which resides in a noncoding portion of the *thyroid hormone receptor-α1* gene, affects receptor expression and is linked to clinicopathological parameters in thyroid cancer.**

Experimental Design: **In 30 cases of surgically resected sporadic thyroid cancer, the length of the THRA1 microsatellite was determined by DNA sequence analysis, and expression of thyroid hormone receptor-α1 was assessed immunohistochemically in thin sections cut from tumor blocks. The length of THRA1 and expression of thyroid hormone receptor-α1 were also assessed in seven cancer cell lines. Regression analysis was used to gauge the correlation between the size of THRA1 and receptor expression. Multivariate analysis was used to test for links to the clinical parameters of gender, age, histology, stage, nodal involvement, distant metastasis, extrathyroidal invasion and tumor-node-metastasis (TNM) classification.**

Results: **A statistically significant correlation between the length of THRA1 and thyroid hormone receptor-α1 expression was observed in both cell lines and primary thyroid cancers. Thyroid tumors that displayed higher than average thyroid hormone receptor-α1 expression had expanded THRA1 microsatellites and were less aggressive as judged by TNM ranking. A statistically significant correlation was also found between low thyroid hormone receptor-α1 expression and more aggressive thyroid cancer, as judged by extrathyroidal invasion and nodal involvement.**

Conclusions: **Less aggressive thyroid cancer was found to be linked to increased thyroid hormone receptor-α1 expression and an expanded THRA1 microsatellite.**

INTRODUCTION

Microsatellites comprised of a large number of dinucleotide repeats, although ubiquitous, are not found in the coding portion of genes. Such microsatellites can exhibit very high rates of mutation, the biological consequence of which is not clear. It is known that changes in the size of a $(CA)_n$-repeat microsatellite upstream of the transcription start site of the *aldose reductase* gene are linked to diabetic nephrophy and retinopathy (1–4) and that changes in the size of a $(TA)_n$-repeat microsatellite upstream of the *estrogen receptor* α gene are linked to changes in bone mineral density and osteoporosis (5–7). Changes in the number of dinucleotides in these two microsatellites likely influence promoter function, perhaps by altering the degree of interaction between transcription factors bound on opposite sides of the microsatellite. Our work is novel in that it is the first report where a change in the length of an internal dinucleotide repeat affects gene expression and is linked to a clinical parameter.

The *NR1A1a* gene (8), previously referred to as c-*erbA*α or *TRα1*, encodes the thyroid hormone receptor-α1, which binds the thyroid hormone 3,5,3′-L-triiodothyronine and DNA. In the THRA1 dinucleotide repeat microsatellite, which resides in a noncoding portion of exon 9 of NR1A1a (Refs. 9 and 10; see Fig. 1), we have observed a high frequency (36.5%) of instability in sporadic thyroid cancer (11), and we have postulated that it must have a biological consequence. Given the carcinogenic effect of the NR1A1a antagonist v-*erbA* (12–14), one might expect to find a reduction in NR1A1a expression in cancer with either an increase or decrease in the size of THRA1; surprisingly, this was not the case. In this report, we present data on a significant correlation between thyroid hormone receptor-α1 expression and the size of THRA1, where expansion of THRA1 is associated with increased thyroid hormone receptor-α1 expression. In addition, we report that thyroid tumors displaying lower than average expression of thyroid hormone receptor-α1 with a smaller than average size THRA1 microsatellite were, as a group, more aggressive, as judged by extrathyroidal invasion and TNM[3] ranking.

The *NR1A1a* locus, in addition to producing NR1A1a, can give rise to alternatively spliced variants (*i.e.,* NR1A1b and NR1A1c) and truncated isoforms (*i.e.,* TRΔα1 & TRΔα2) that, although not binding 3,5,3′-L-triiodothyronine or DNA, inhibit ligand-dependent transactivation of NR1A1a (15, 16). Gene

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[3] The abbreviation used is: TNM, tumor-node-metastasis.

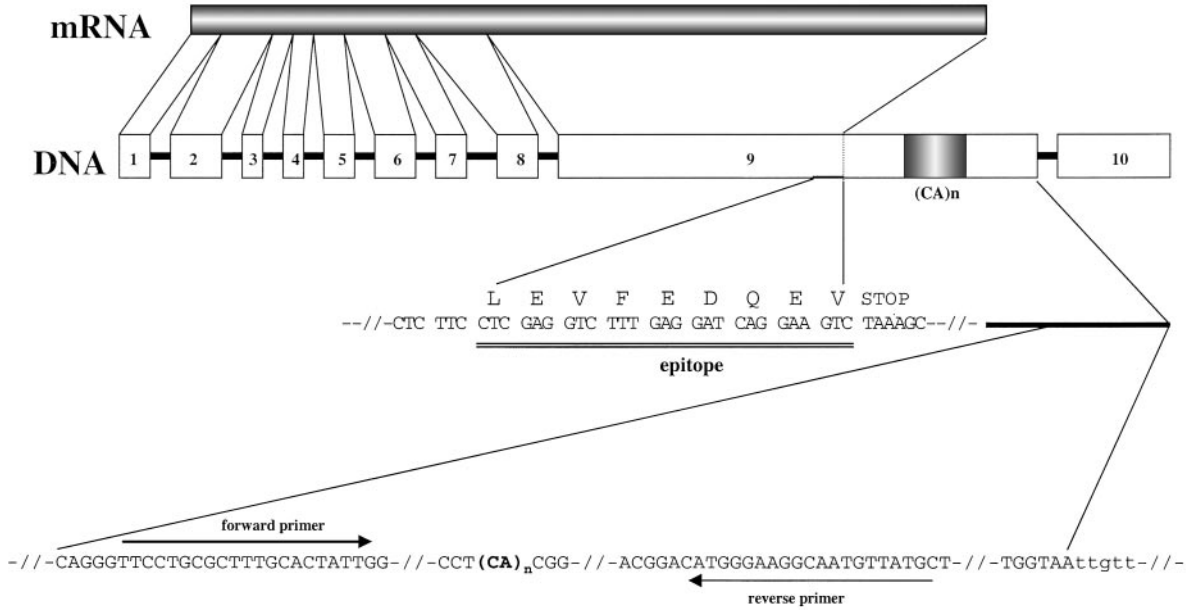

Fig. 1 Schematic representation of the thyroid hormone receptor-α1 (*i.e.,* NR1A1a) locus, showing the PCR primers used to amplify the THRA1 microsatellite and the unique epitope at the extreme COOH-terminal region (amino acids 403–410) of the NR1A1a protein specifically recognized by the NR1A1a antibody used to determine expression. The PCR primers flank the THRA1 microsatellite to generate a 128-bp product.

regulation involving NR1A1a frequently entails formation of a complex with a member of the retinoic acid receptor family or other nuclear proteins to provide cross-talk between different signaling pathways important in cancer (17–21). Given the important role that NR1A1a has played in our understanding of cancer genetics (22, 23), a fuller understanding of factors that modulate NR1A1a expression is of considerable interest.

MATERIAL AND METHODS

Immunochemical Assessment of NR1A1a Protein Expression. The cell lines purchased from American Type Culture Collection were grown to 80% confluence in DMEM, washed, and incubated with radioimmunoprecipitation assay buffer. Cell lysates were sonicated and centrifuged, and the soluble protein concentration was determined by Coomassie Blue staining. The equivalent of 20 μg of protein was denatured in Laemmli buffer, loaded onto a SDS-polyacrylamide gel, and electrophoresed in SDS-Tris-glycine running buffer at 35 mA for 2 h. Molecular weight marker was coelectrophoresed for size determination. Proteins were electrotransferred to polyvinylidene fluoride membrane, which was blocked in 10% powdered milk overnight before immunochemical detection using chemiluminescence (Pierce). The primary antibody anti-thyroid hormone receptor α1 (catalogue number PA1-211; Affinity BioReagents) reacts specifically with thyroid hormone receptor-α1, detecting NR1A1a at the expected M_r 48,000 (24).

Patient Profile and Sample Preparation. Normal and tumor-matched samples were collected at Fukushima Medical University Hospital from 1993 to 1999. After surgical resection, tumor and normal control tissue was classified with regard to histology and stage by a medical pathologist and immediately frozen at −80°C, or 10% formalin-fixed paraffin-embedded tumor blocks were prepared. To obtain purified genomic DNA for PCR amplification reactions, frozen tissue samples were homogenized and treated with proteinase K, and the DNA isolated by phenol-chloroform extraction. For cell lines, cell lysate was treated with proteinase K, and the DNA isolated by phenol-chloroform extraction.

Immunohistochemical Assessment of NR1A1a Protein Expression. From paraffin-embedded tumors, 4-μm sections were cut, mounted on glass slides, and stained by the avidin-biotin complex method using a Vectastain avidin-biotin complex kit (Vector Laboratories) following the manufacturer's protocol. The primary antibody used was the same as that used in our Western blots. Nucleocytoplasmic shuttling of NR1A1a has been reported previously (25), and although a cytoplasmic fraction of NR1A1a is also evident in our tissue preparations, the vast majority of NR1A1a is located in the nucleus. Evaluations of nuclear NR1A1a were carried out on digitized photomicrographs of thin tissue sections using an AlphaImager (Alpha Innotech), which provided a precise assessment of protein expression. The staining intensity of 50 randomly selected nuclei/slide was averaged to provide the reported staining intensity. This process served to remove any bias arising from how cells were randomly cut during the sectioning process and was carried out blind to other results.

THRA1 CA-repeat Assessment. PCR amplification of genomic DNA derived from cell lines or patient matched normal and tumor tissue was performed with the addition of [α-^{32}P]dCTP (EASYTIDES; New England Nuclear Life Science Products), using an initial denaturing step; 30 cycles of 94°C for 40 s, 54°C for 40 s, and 72°C for 40 s; followed by a final extension step of 72°C for 5 min. PCR primers are shown in Fig. 1. PCR products were denatured, loaded on 6% SequaGel

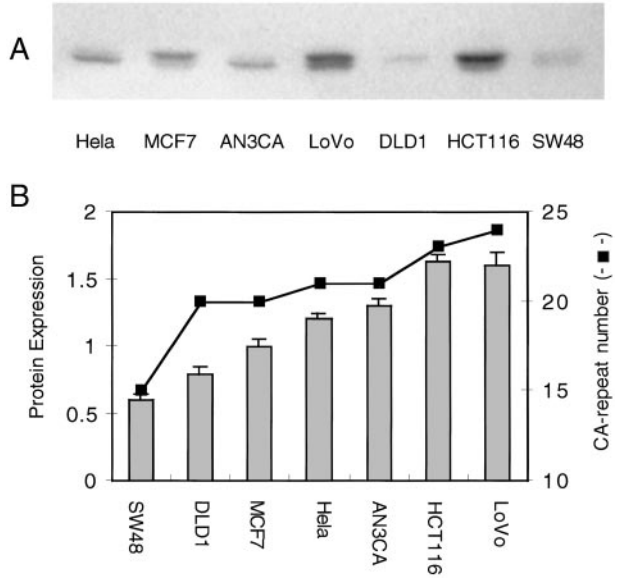

Fig. 2 Immunochemical determination of NR1A1a expression (*A*) with NR1A1a expression and THRA1 CA-repeat number (*B*) measured in cancer cell lines HeLa (cervix), MCF7 (breast), AN3CA (uterus), and LoVo, DLD1, HCT116, and SW48 (colorectal). For quantitation, background subtraction was carried out, and NR1A1a expression was normalized by that in the MCF7 cell line.

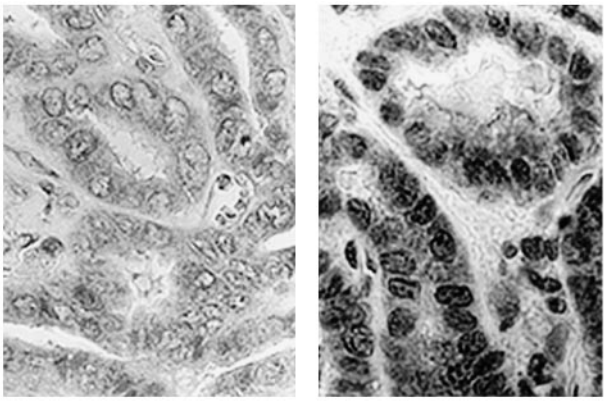

Fig. 3 Digitized photomicrographs of thyroid tumor, thin sections showing low (*left*) and high (*right*) immunohistological staining derived from the NR1A1a antibody.

(National Diagnostics), and electrophoresed on a standard vertical DNA sequencing gel apparatus at 150 W for 2 h. Gels were dried and exposed to Fuji HR-H film. DNA was recovered from single bands in the gel, and a second PCR amplification was performed using the same primers and PCR conditions. The second PCR products were run out on 2% Nusieve-agarose gel (FMC) and stained, and the PCR products were purified with a QIAquick gel extraction kit (Qiagen) following the manufacturer's instructions. DNA sequence was determined using a PRISM BigDye Primer Cycle Sequencing kit (ABI) and our forward PCR primer as the sequencing primer on an ABI 377 automated sequencer.

Statistical Analysis. To assess the significance of relationships between THRA1, the expression of NR1A1a, and various clinical parameters, multivariate analysis involving Student's *t* test, χ^2 test, and McNemar's test was used. $P < 0.05$ was considered statistically significant. All tests were performed with Statview Version 5.0 software (SAS Institute).

RESULTS

To determine how NR1A1a expression is associated with the size of THRA1, immunochemical blot analysis was first carried out on the soluble protein fraction present in whole cell lysates prepared from different cancer cell lines. A representative blot on the panel of cell lines is shown in Fig. 2*A*, with a digital determination of the amount of NR1A1a protein in each cell type, normalized by the expression measured in MCF7, plotted from lowest to highest shown in Fig. 2*B*. SEs were in the range of 5% for the normalized amounts of NR1A1a, based on three independent blots. The number of CA-repeats in THRA1, determined by DNA sequence analysis (26), ranged from 15–24 in the panel of cell lines (Fig. 2*B*). THRA1 normally is comprised of 18 CA-repeats (9), which matches what we measured in the microsatellite instability-negative cell line MCF7. To test for a correlation, linear regression analysis was carried out, which yielded a correlation coefficient *R* of 0.93, with a *P* of 0.0028, indicating that in this panel of randomly selected cell lines, NR1A1a expression is proportional to THRA1 CA-repeat number.

To test for a relationship between THRA1 and NR1A1a in thyroid cancer, we looked at a panel of 30 thyroid tumors for which we had both paraffin-embedded tumor blocks and purified DNA prepared from fresh tissue. These 30 samples are a subset of 76 cases we previously analyzed for microsatellite instability (11) and were chosen blind from the original 76 cases. Patients were 28 females and 2 males, and samples were 25 papillary and 5 follicular cases, all of which were sporadic primary thyroid carcinomas. As in the case of the cell lines, the number of THRA1 CA-repeats was determined by DNA sequence analysis. NR1A1a expression was assessed immunohistochemically in thin sections cut from the tumor blocks (27). Representative examples of low and high NR1A1a expression are shown in digitized photomicrographs generated for computer densitometric determination of staining intensity (Fig. 3). The levels of nuclear NR1A1a expression plotted against the number of THRA1 CA-repeats are shown in Fig. 4. To test the statistical significance of the correlation, linear regression analysis was carried out, which yielded a correlation coefficient *R* of 0.91 with a *P* of <0.0001, indicating that in sporadic thyroid cancer, expansion of THRA1 is strongly linked to increased nuclear expression of the thyroid hormone receptor-α1.

Multivariate analysis was performed to test for links between expression of NR1A1a and the clinical parameters of gender, age, histology, stage, nodal involvement, distant metastasis, extrathyroidal invasion, and TNM classification. A statistically significant correlation was found with extrathyroidal invasion when the 30 thyroid cancer cases were divided into two groups based on NR1A1a staining (namely, 14 high staining cases and 14 low staining cases, with 2 cases scored to have identical staining defining the midpoint). The high staining

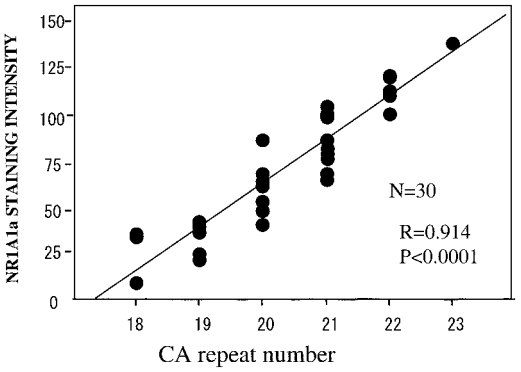

Fig. 4 Linear regression analysis on THRA1 CA-repeat number *versus* nuclear NR1A1a immunohistochemical staining intensity assessed in 30 thyroid tumor samples. Staining intensity is given in pixel grayscale units of 0–255, from photomicrographs digitized at 8-bit resolution.

group consisted of 10 cases of papillary carcinoma and 4 cases of follicular carcinoma. In the low staining group, there were 13 papillary carcinoma cases and 1 follicular carcinoma case. In the high staining group, there were 11 cases with no extrathyroidal invasion, whereas in the low staining group, there were 10 cases showing extrathyroidal invasion. A χ^2 test yielded a *P* of 0.02, indicating that the correlation is statistically significant with greater than 95% confidence. Given the requisite extrathyroidal invasion inherent in a TNM classification of T_4 (28), a significant correlation between low expression of NR1A1a and T_4 TNM tumors was also found, with a *P* of 0.02. A significant correlation was also found between low expression of NR1A1a and nodal involvement, with a *P* of 0.05. Interestingly, in the subset of tumors with above normal NR1A1a expression, there where six T_1 TNM tumors, all of which had larger than normal size THRA1 microsatellites. T_1 TNM tumors are nonaggressive, being small and slow-growing, with a 10-year survival rate of 100%. From our data, elevated NR1A1a expression and a larger than normal size THRA1 microsatellite emerge as biomarkers of less aggressive thyroid cancer.

DISCUSSION

The mechanism providing for a contraction of THRA1 observed in aggressive thyroid tumors is of particular interest because mutation of dinucleotide microsatellites is normally biased toward expansion (29). It is well established that within tumors there can be considerable genetic heterogeneity and that particular mutations persist because they impart a cancerous advantage, whereas other mutations are not observed because they severely limit growth or bring about cell death. It has been observed that thyroid hormone receptor β is expressed in relation to the degree of cellular differentiation in thyroid cancers (30). In anaplastic thyroid cancer, there is a loss of NR1A1a expression, indicating that loss of differentiation is correlated with loss of NR1A1a expression. We postulate that reduction in the size of THRA1, with a concomitant reduction in NR1A1a expression, fosters the development of cells able to survive outside the hormone milieu found in the thyroid and therein advance extrathyroidal invasion involving a loss of dependence on NR1A1a. Our data suggest that such an association is also true for thyroid hormone receptor-α1. No correlation between NR1A1a expression and the size of the microsatellites TSHR, D2S123, D11S912, D2S115, D2S339, P53, RET, or BAT-26 on chromosomes 14, 2, 11, 2, 2, 17, 10, and 2, respectively, that we previously tested (10) was observed, indicating that the size of THRA1 is of special significance to the level of NR1A1a expression.

With regard to how the size of THRA1 affects NR1A1a expression, the simplest way would be through an isolated effect on the generation, processing, or stability of RNA for NR1A1a. However, given the position of THRA1 near a splice junction and the number of spliced variants and truncated isoforms originating from the NR1A1a locus that interact to govern transcription in the NR1A1a locus, much more complicated scenarios are certainly possible. Given that regulation of mRNA is highly idiosyncratic and often abnormal in cancer (31, 32) and that our best algorithms are now only 50% accurate in predicting splice sites (33), the determination of precisely how the size of THRA1 modulates NR1A1a expression is an important, although likely complicated, subject for future study. From our work, the THRA1 microsatellite in the *thyroid hormone receptor-α1* gene distinguishes itself as an exemplary locus in which to better understand the biochemical and clinicopathological correlates that arise in cancers exhibiting instability of dinucleotide repeat microsatellites.

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