 ISSN 0172–780X **www.nel.edu**

ORIGINAL ARTICLE

Clinical significance of the insulin-like growth factor I gene promoter (P1) polymorphism in thyroid nodular disease

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Submitted: February 16, 2004 *Accepted: May 5, 2004*

Key words: **thyroid nodule; goitre; thyroid carcinoma; IGF-I gene promoter polymorphism**

Neuroendocrinol Lett 2005; **26**(6):699–703 **PMID:** 16380677 NEL260605A35 © Neuroendocrinology Letters **www.nel.edu**

Abstract

OBJECTIVES: Due to the recent increase of incidence of thyroid nodules and the known risk of malignant transformation, there is an elevated risk of thyroid cancer in Poland. Several approaches, including molecular, have been proposed to support fine needle aspiration biopsy in the early detection of malignant lesions. Although the IGF-I system in thyroid cancer has been studied, little is known about the gene and its promoter structure changes. Our aim was to assess, whether the analysis of the IGF-I gene promoter region and 5'UTR exon 1 structure may be useful in assessing the risk of thyroid carcinoma.
MATERIAL: Our study included 46 patients that underwent strumectomy due to a presence of thyroid nodules.
METHODS: All patients underwent clinical examination and laboratory investigations to assess their thyroid structure and function. Tissues obtained during the surgery were used for DNA extraction, PCR, SSCP and direct sequencing.
RESULTS: Among 46 patients, 14 had a nucleotide difference in one of the examined regions. In our study we revealed no significant difference between carcinomatous and non-carcinomatous groups of patients in terms of presence of nucleotide change, but Fisher's exact test gave a significant result in terms of the efficacy of detecting follicular adenoma. Moreover, the patients with nucleotide change had thyroid glands significantly smaller in volume.
CONCLUSIONS: We conclude that the molecular analysis of the IGF-I gene promoter is thought to be of a functional significance, but probably could not be considered useful in the assessment of risk of thyroid cancer in thyroid nodules.

List of abbreviations and symbols:

FNAB – fine needle aspiration biopsy
MIBI – methoxyisobutylisonitrile
EGF – epidermal growth factor
FGF – fibroblast growth factor
TGF – transforming growth factor
IGF – insulin-like growth factor
5'UTR – untranslated region
bp – base pairs
IGF-I-R – insulin-like growth factor I receptor
FRTL-5 – follicular rat thyroid cell line-5
IR – insulin receptor
TSH – thyroid stimulating hormone
TPO-Ab – anti-thyroperoxidase antibody
T_4 – thyroxine
cc – cubic centimetres
PCR – polymerase chain reaction
SSCP – single strain conformation polymorphism
PPV – positive predictive value
NPV – negative predictive value

Introduction

Thyroid nodules are common in Poland. Nodular goitre can be disclosed in 30–50% of women aged 40 and over, depending on the criteria taken into consideration [1]. Due to the abundance of nodular thyroid disease there is an elevated risk of a neoplastic transformation leading to the development of thyroid carcinomas. The overall risk of carcinogenesis in a nodular goitre is estimated to be as high as 5–12% [2]. The basic procedure in the diagnostics of thyroid carcinoma is still an ultrasound-guided fine needle aspiration biopsy (FNAB). Despite its undisputed potency to discriminate between benign and malignant lesions it has its limitations, such as possible "indefinite" and "indiagnostic" diagnoses. Moreover, it is not possible to distinguish between follicular adenoma and carcinoma by FNAB [2,3].

Therefore several studies have attempted to broaden the view and combine genetic criteria to clinical, ultrasound, and cytological data to increase the possibility for early detection of thyroid malignancies. Proposed techniques include several modifications of thyroid scintigraphy (201 Tl, MIBI), gene expression analysis in cells obtained during FNAB (immunocytochemistry and RT-PCR) (Galectin, CD 26, hTERT) [4,5,6,7]. Among others, different growth factors have been studied intensively (EGF, FGF, TGF, IGFs). Although still controversial, the hypothesis of an increased risk of different types of carcinomas in patients with acromegaly, higher incidence of goitre and thyroid nodules has become a well-established scientific and clinical fact [8,9]. It is known that this phenomenon is caused by increased serum concentration of IGF-I, and gives enough justification for studies concerning the role of IGF-I in thyroid nodular disease and carcinoma. Therefore, the activation of the IGFs system in cancer has emerged as one of the key factors for tumour progression and resistance to apoptosis [10].

IGF-I is a 70-amino acid basic polypeptide, encoded by the IGF-I gene (12q22-q24.1) which extends over 90 kilobase pairs and contains 6 exons interrupted by five introns. In mammals, the IGF-I gene transcription is controlled by two promoters, P1 and P2, that are located 5' to the unique leader exon 1 and 2, respectively. The 5' untranslated region (5'UTR) of exon 1 (P1) is the most conserved part of the gene. The 322 nucleotides of human 5'UTR are 95% identical to corresponding portions of rat and chicken genes. It was suggested that this sequence could play an important role in translation control of the IGF-I synthesis (most IGF-I transcripts originate in exon 1, and the IGF-I gene contains a number of binding sites within 300 bp upstream and downstream of the major transcription initiation sites). Many studies have confirmed the extra-hepatic expression of the gene and potential for it to be regulated by numerous agents including hormones (e.g. TSH, FSH, LH, estradiol) [11,12,13].

Recent studies of IGF-system activation in thyroid have revealed local production and secretion of IGF-I and IGF-II in normal thyrocytes (cultured cells) and in the human papillary thyroid carcinoma cell line. Local concentration of both IGFs were higher in carcinomas and nodular goitre than in normal tissue. IGF-I receptor (IGF-I-R) has also been proven to be present in normal and carcinomatous thyroid tissue with elevated binding affinity to ligand in colloid goitre and carcinoma. The IGF-IR were proved to be overexpressed in well-differentiated papillary carcinomas but not in undifferentiated tumours [14,15].

Another study has confirmed exogenous IGF-I to a exert mitogenic effect on FRTL-5 cells in culture. To express the complexity of the IGF-I system one has to give the latest data stating that insulin receptors (IR) and IGF-R/IR hybrids are reported to be overexpressed in all thyroid cancer histotypes [15].

Although the IGF-I system activation in thyroid cancer has been extensively studied, still little is known on the gene and its promoted structure changes with possible influence on regulation of the expression.

Our aim was to assess whether the analysis of the IGF-I gene promoter P1 region and 322 bp 5'UTR exon 1 structure may be useful in diagnosis of thyroid nodular disease and in assessing the risk of thyroid carcinoma.

Material and methods

Our study included 46 patients with different thyroid disorders, aged 18 to 59, mean 34, SD 11.1 ; 6 males (13%) aged 21 to 43, mean 34, SD 13.8 and 18 females (87%) aged 18 to 59, mean 34, SD 10.9. All patients underwent subtotal or total strumectomy, due to the presence of thyroid nodules. In all cases an indication for surgery was suspicion of carcinoma in pre-existing thyroid nodules, regardless of primary thyroid disease.

Tissue fragments were acquired after the final diagnosis had been made by the pathologist.

A post-operative pathological investigation revealed follicular adenoma in 16 cases (34.7%), nodular colloid goitre in 14 cases (30.4%), papillary thyroid cancer in 12 cases (26.1%), Graves' disease in 3 cases (6.5%) and Hashimoto's autoimmune thyroiditis in 1 case (2.2%).

All patients were provided with written comprehensive information describing the study, and a written consent was obtained from each individual.

A clinical examination was performed twice in all patients, before and after strumectomy. A neck ultrasound (Aloka 1100, linear head 7.5 MHz) and laboratory investigations were performed only once, before surgery. An approximate thyroid volume was calculated in cubic centimetres (cc) using Gutekunst's formula.

Laboratory investigations included: serum TSH concentration (ELISA-Brahms), serum free-T4 concentration, and serum anti-thyroperoxidase (TPO-Ab) concentration.

After collection, thyroid tissues were frozen in –70°C with liquid nitrogen and after defrosting were used for further procedures. A genomic DNA was isolated from thyroid tissues by proteinase digestion and phenol extraction, using a DNA isolation kit (Qiagen). Genomic DNAs were used for in vitro amplification by polymerase chain reaction (PCR). The primer sequences for region A were: F – TCATCGCAGGAGAAAAAAGTAT, R –GCTGGGCATGAAGACACAAAC, and for region B were: F – ATGTCTGCGAACCCTGTCACTAA, R – TATTCCATTGCGCAGGCTCTATCT. Regions A and B extend over 299 (–93 to +206) and 329 (–476 to –148) bases, respectively. The PCR reactions were performed in the following conditions: Total volume was 10 µl. The final mixture contained 1 µM of each primer, 200 µM deoxynucleotide triphosphates, 1x PCR buffer [10 mM Tris-Hcl (pH 8.8), 50 mM KCl, 0.1% Triton X-100], 1.5 mM MgCl2 and 1.0 U/25 µl of mixture of Taq polymerase. The samples were amplified for 35 cycles. Each cycle consisted of the following steps: denaturation at 95°C for 60 sec. (first cycle for 90 sec.), annealing at 53.8 and 53°C for 30 sec. (for region A and B, respectively), primer extension at 72°C for 60 sec. an a thermal cycler (Minicycler BIOMETRA). Products of amplifications were analysed in 2% agarose gel stained with ethidium bromide.

All samples were analysed by single strain conformation polymorphism (SSCP). Products from each PCR reaction (10 µl reaction mixture) were denaturated chemically (mixed with formamide and loading buffer), and thermally: denaturated at 95°C for 5 minutes, and placed on ice. Then the samples were run in 10% polyacrylamide gel with 0.5x TBE buffer in 200 V for 12 hours, silver stained and dried.

The fragments of DNA with altered gel migration pattern were cloned into pGEM T-Easy Vector System (Promega, Mannheim, Germany), according to the

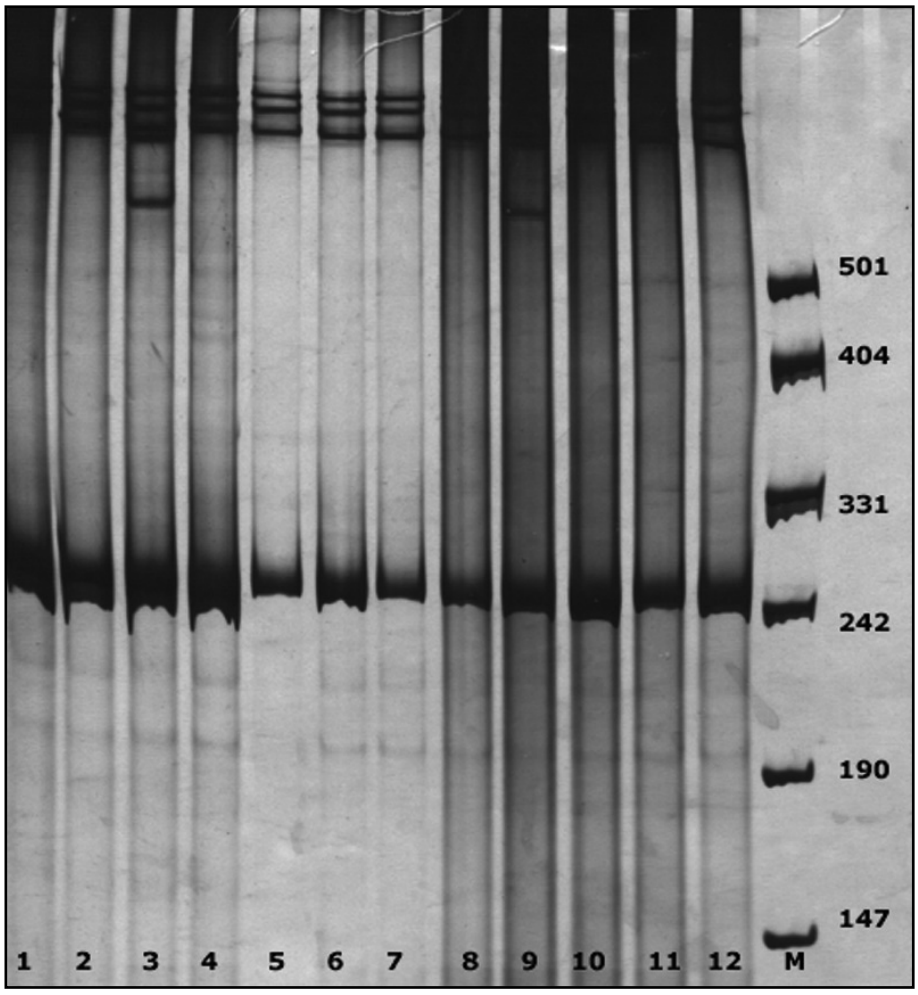

Figure 1. The result of SSCP (fragment A) of the selected group of patients.
Patients number 3 and 9 with an altered gel migration pattern.

Table 1. The results of the molecular analysis of patients with altered the IGF-I gene promoter structure.

Patient's No.	Age	Sex	Diagnosis	SSCP Fragment A Migration	SSCP Fragment B Migration	Sequencing
3	23	M	GD	+	+	na
9	21	F	PTC	+	–	na
13	40	F	NCG	+	–	na
14	37	F	FA	+	–	na
15	35	F	PTC	+	–	na
16	40	F	NCG	+	–	na
17	20	F	NCG	+	+	–363 (A–G) –383 (C–T)
22	29	F	NCG	+	+	
32	56	F	NCG	+	–	–281 (T–A)
34	21	F	PTC	+	–	na
38	22	F	PTC	+	–	na
40	19	F	NCG	+	+	–383 (C–T)
41	22	F	HAT	+	+	–373 (C–T) –383 (C–T)
46	42	F	NCG	–	+	na

FA Follicular Adenoma;
PTC Papillary Thyroid Cancer;
HAT Hashimoto's Autoimmune Thyroiditis
– unchanged gel migration pattern in SSCP
NCG Nodular Colloid Goitre;
GD Graves' Disease;
+ altered gel migration pattern in SSCP

instructions of the manufacturer, and then automatically sequenced.

After the results of a molecular analysis were obtained, patients were divided into two groups: with or without nucleotide change, respectively. The statistical evaluations were carried out with Statistica for Windows 5.1G software (StatSoft Inc. USA). The significance was considered as $p<0.05$

Results

Among 46 patients 13 (28.3%) had a nucleotide change in region A whilst only 6 (13.0%) in region B. Nucleotide changes were the most frequent in patients with immune-related thyroid diseases (treated as a whole), 2 (50%) in both, A and B regions. In patients with colloid nodular goitre, 6 (42.8%) and 4 (28.6%) had nucleotide change in the region A and B, respectively. In the largest group of patients, those with follicular adenoma nucleotide changes were found very rarely: only 1 patient (6.25%) in region A and none in region B. Regarding the patients with papillary carcinoma, 4 (30%) had a nucleotide change in the region A, and none had in the region B.

The presence of some of the nucleotide changes was confirmed by direct sequencing. In three samples (colloid nodular goitre) there was a C – T transition in the –383 upstream transcription starting site, moreover one had additional transition (A – G) in –363. A –281 transversion T – A was found in the patient with colloid nodular goitre, and one patient with Hashimoto's thyroiditis had –373 transition (C – T). The results of molecular analysis are shown in table I.

The two-sided Fisher's exact test revealed no significant difference between the carcinomatous and non-carcinomatous group of patients in terms of presence of nucleotide change ($p=1.0$). The test sensitivity was 0.28, specificity 0.75, positive predictive value (PPV) 0.33, negative predictive value (NPV) 0.7. The two-sided Fisher's exact test was also used to assess the efficacy of detecting follicular adenoma, and revealed a positive result ($p=0.016$). In this case the test sensitivity was 0.07 , specificity 0.53, PPV was 0.06, and NVP 0.57.

Clinical and biochemical assessment revealed that 36 patients were euthyroid (78.3%), 6 (13%) were hyperthyroid and 4 (8.7%) were hypothyroid. Among the group with an altered gene structure only one patient was hypothyroid (7.1%), whilst the remaining 13 (92.9%) were euthyroid. The patients without nucleotide change were also mainly euthyroid (23–71.9%), 6 (18.7%) were hyperthyroid and 3 (9.4%) were hypothyroid.

As for thyroid structure, the patients with nucleotide change had thyroid glands smaller in volume. The mean thyroid volume in the aforementioned group was 17.8 cc (SD 7.08), compared to 26.4 cc (SD 11.92) of those without alternation in the gene structure. The mean difference of 8.64 cc was considered very significant ($p = 0.004$) in unpaired T-test with Welch correction.

Discussion and Conclusions

Nucleotide differences in the promoter region P1 of the IGF-I gene are very common in patients with thyroid diseases. However, its clinical relevance still remains unclear.

Analysis of the clinical data combined with the molecular analysis of the IGF-I gene promoter P1, allows nucleotide differences to be seen as functional. They could result in abolishing the binding capacity of specific transcription factor or changing its affinity to the binding element that could play an important role in a translation control of the IGF-I synthesis. The eventual effect of the influence on a local IGF-I gene expression could be clinically manifested as a decrease in a thyroid volume. However, these nucleotide differences probably cannot be considered useful in the assessment of risk of thyroid cancer in patients with thyroid nodules.

In our study we confirmed the frequent presence of IGF-I polymorphism in thyroid which is consistent with other authors [16]. There was no difference in the frequency of nucleotide differences between patients with malignant and benign thyroid disease. Together with the fact that the patients with nucleotide differences had significantly smaller thyroids, this would support the view that the changes had an inactivating influence on the gene expression. This hypothesis can be indirectly confirmed by the study of Obrepalska-Steplowska et al. The authors found similar gene structure changes in a group of growth-deficit children with diminished serum IGF-I concentration [17].

The results of our study are not eligible to be compared to those concerning IGF-I gene promoter polymorphism in breast cancer because of the different gene fragment being examined. However, we have to state that the results of the studies mentioned above are not clear, but mainly tend to confirm the link between CA repeat IGF-I gene promoter polymorphism and cancer susceptibility [18,19,20].

More problematical is the interpretation of the results regarding follicular adenoma. It is known that follicular lesions utilise different pathways in their pathogenesis, which may explain the contrast between papillary lesions [21]. Although in our study Fisher's exact test on the efficacy of detecting follicular adenoma was significant, its very low sensitivity denies the usefulness of the technique. We conclude that further studies should be carried out on this topic, including a larger group of follicular adenomas as well as carcinomas.

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