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Genetic Alterations in Thyroid Carcinoma Associated with Familial Adenomatous Polyposis: Clinical Implications and Suggestions for Early Detection

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Abstract. Germ-line mutations of the adenomatous polyposis (*APC*) gene, responsible for familial adenomatous polyposis (FAP) were analyzed in 15 patients with FAP-associated papillary thyroid carcinomas: 13 had the mutation between codons 778 and 1309 (exon 15), 1 at codon 593 (exon 14), and 1 at codon 140 (exon 3). Therefore *APC* gene mutations clustered in the genomic area associated with congenital hypertrophy of the retinal pigment epithelium (CHRPE) (codons 463–1387). Ocular patches were documented in 12 patients. In particular, 4 of the 15 patients, all women with a mean age of 23.5 (range 20–32), were found during the study of 15 consecutive kindreds who had undergone systematic screening for extracolonic manifestations. Three of them belonged to the same kindred and were asymptomatic. These four patients were also screened for loss of heterozygosity of *APC* in the thyroid tumoral tissue. No biallelic inactivation of the *APC* gene was found. In contrast, three of these four patients had activation of the *ret-PTC* oncogene. In particular, there was activation of the *ret-PTC1* isoform, a chimeric gene resulting from fusion of a gene named *H4* with the *RET* gene. On histologic examination, three of the four patients showed Hashimoto-like lymphocytic infiltration. Present data suggest that: (1) the incidence of FAP-associated thyroid cancer probably has been underestimated in the past; (2) intensive screening could detect a larger than expected number of thyroid carcinomas; (3) systematic screening is recommended in patients with ocular patches and genetic mutation in exon 15; (4) Hashimoto-like findings do not exclude carcinoma but are a frequent accompanying finding; (5) despite frequent multicentricity and early lymph node involvement, FAP-associated thyroid tumors seem to have an excellent prognosis, in particular those showing *ret-PTC* activation.

An increased predisposition to thyroid carcinoma is well established in certain kindreds with familial adenomatous polyposis (FAP) [1–14]. The *APC* gene, responsible for FAP, maps on chromosome 5q21. FAP-associated thyroid cancers exhibit a marked female preponderance (female/male ratio 20:1) and are more common under the age of 30 [1–14].

Thyroid tumors have also been reported in other familial syndromes [1, 2]. They are the most frequent extracutaneous manifestations of Cowden's disease, being observed in two-thirds of patients. There are case reports describing the association of thyroid carcinoma in patients with Peutz-Jeghers syndrome and ataxia-telangiectasia [1, 2]. In patients with multiple endocrine neoplasia type I (MEN-I), thyroid disease is observed mostly as a benign lesion and far more rarely as a malignancy. Expression of papillary thyroid carcinoma has been observed in two patients from a single kindred with MEN-IIa. In addition to the occurrence of thyroid carcinoma in kindreds with well defined multitumoral genetic disease, the familial occurrence of papillary thyroid carcinoma as a unique pathologic event has also been reported [1, 2, 15–19].

All thyroid carcinomas included in multitumoral genetic syndromes tend to share some common characteristics (e.g., early onset, multicentricity, high frequency of papillary histotype). Commonly an aggressive surgical approach has been invoked for all "familial nonmedullary cancers" [1, 20, 21]. In particular, about 70 patients with FAP-associated thyroid tumors have been reported in detail as case reports [1–14]. Analysis of consecutive series from the largest FAP registers suggested that only 1% of FAP patients had thyroid carcinomas [5, 10]. A papillary histotype was present in more than 90% of cases [4–14]. It has been suggested [14, 18, 19] that FAP-associated thyroid tumors share some unusual and peculiar histologic findings (i.e., increased frequency of the "cribriform pattern"), which could facilitate early detection.

The aim of the present study has been to report on 15 patients with FAP-associated thyroid carcinoma whose data were collected during a cooperative study among various European countries (THYROFAP project, within the frameshift of the Biomed II project of the European Community). During the same study, 14 patients with hepatoblastoma, another rare FAP-associated extracolonic manifestation, were also observed, sometimes in the same kindred as those presenting with thyroid carcinoma. In all of these patients—those with thyroid cancer and with hepatoblastoma—

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the specific mutations of the *APC* gene had been detected. In particular, in four patients belonging to the institution of the first author, exaustive genetic analysis of the thyroid tumoral tissue was undertaken, including a search for activation of the *ret-PTC* oncogene [22–26].

Materials and Methods

The 15 patients with FAP-associated thyroid carcinoma were studied during an international cooperative study among some European countries including various FAP registers. Only patients available for genetic analysis were selected.

Four of these patients were observed in a single institution. Only these patients underwent a complete clinical, pathologic, and molecular analysis. Therefore a detailed description is given only for this subgroup of subjects. In particular, three of them, all female, belonged to the same kindred. The extended pedigree of this kindred (23 siblings in four generations) has been reported elsewhere, together with a detailed list of the extracolonic manifestations [27]. The fourth patient was a 20-year-old woman belonging to a small kindred with only two affected siblings (the proband and her mother). Both kindreds belonged to a series of 15 FAP kindreds that were intensively screened for FAP and associated extracolonic manifestations [28–32]. All living patients underwent colonscopy, upper gastrointestinal (GI) endoscopy (supplemented by radiographic examination of the GI tract in selected cases), and multiple biopsies. In addition, patients were screened for osteomas, dental abnormalities, and desmoid tumors. The fundus oculi was examined in all patients. All patients underwent ultrasound examination (US) of the thyroid gland. Fine-needle aspiration (FNA) of nodules larger than 5 mm was performed. Some patients underwent multiple US and FNA procedures. The FNA specimens were examined cytologically according to standard methods. In these four patients, not only was detection of germ-line mutations of the *APC* gene undertaken but also a search for (1) bi-allelic inactivation of the *APC* gene in the thyroid tumoral tissue and (2) activation of the *ret-PTC* oncogene.

Histologic Techniques

All grossly identifiable nodules and the normal thyroid areas were sampled extensively. Sections were routinely stained with hematoxylin and eosin. Immunohistochemistry was carried out using the following monoclonal antibodies: thyroglobulin (Biogenex, San Ramon, CA) diluted 1:500, chromogranin A (Dakopatts, Glostrup, Denmark) diluted 1:200, carcinoembryonic antigen (Immunotech, Marseille, France) diluted 1:10, and cytokeratin AE1/AE3 (Boehringer-Mannheim, Mannheim, Germany) diluted 1:1000. Color was developed using the APAAP method. A polyclonal antibody against calcitonin (Biogenex) diluted 1:200 was also used, and the color was developed with 3,3′-diaminobenzidine tetrahydrochloride.

Search for APC *Mutations in Germ-line Cells*

The DNA was isolated from lymphocytes in 10 ml of EDTA-anticoagulated venous blood by means of proteinase K digestion and phenol-chloroform extraction. The entire coding region (8532 bp) of the *APC* gene was analyzed by the PCR-SSCP method in all patients. The *APC* gene coding region was divided into five overlapping amplification segments, varying in length from 1.8 to 2.5 kb. The primers used for PCR included signals for transcription by T7 polymerase and "in vitro" translation at their 5′-end. Thus PCR products could be transcribed and translated in vitro to research the presence of mutations that resulted in an altered size of the encoded polypeptide. Direct sequencing was performed to identify the nature of the mutations [33, 34].

DNA Extraction and Loss of Heterozygosity (LOH) Analysis for APC *in Thyroid Tumoral Tissue*

Cryosections of 5 to 7 μm were prepared for each tumor tissue, and sections containing predominantly neoplastic cells were used to prepare genomic DNA by the phenol-chloroform method. DNA from the nontumorous part of the same individual was similarly extracted. A PCR was performed. The entire *APC* gene was screened. PCR conditions were 30 seconds at 95°C, 30 seconds at 55°C, and 1 minute at 72°C for 35 cycles. The products were then electrophoresed on 3% agarose gel and stained with ethidium bromide. LOH was defined as the visible change in tumor DNA bands compared with normal tissues with $> 50\%$ reduction of the signal of one allele as measured by densitometry.

Search for ret/PTC *Activation*

Search for activation of *ret-PTC* was performed using immunohistochemistry. This method is based on the fact that the *ret* proto-oncogene is not expressed in thyroid follicular cells unless its expression is driven by activating sequences replacing its 5′-portion. Therefore detection of the ret protein, by immunohistochemical analysis, indicates activation of the *ret* gene. The *ret* gene, which is also involved in MEN-IIa and MEN-IIb, encodes a receptor-type tyrosine kinase for neurotropic molecules, belonging to the glial cell line-derived neurotropic factor (GDNF) family. In addition, after RNA extraction from paraffin-embedded samples, RT-PCR according to a previously published procedure [24] was used for subsequent identification of *ret-PTC* expression as *ret-PTC*$_1$, *ret-PTC*$_2$, or *ret-PTC*$_3$. In fact, the *ret-PTC* oncogene derives from fusion of the tyrosine kinase domain of the *ret* proto-oncogene with the 5′-terminal region of other genes. *ret-PTC*$_1$ is fused with another gene, named *H4,* also located on the long arm of chromosome 10, and the 5′-portions of the *ret-PTC*$_2$ and *ret-PTC*$_3$ are represented, respectively, by the regulatory subunit RIα of the cAMP-dependent protein kinase A and the *RFG/ELE*$_1$ gene. Further details of this method have been reported elsewhere [22–26, 31].

Results

Genotype–Phenotype Correlations in the Cumulative Series of 15 Patients

Table 1 shows the genotype–phenotype correlations in the 15 patients with FAP-associated thyroid carcinoma. In particular, in 13 patients with thyroid carcinoma, germ-line *APC* mutations clustered in exon 15. In 14 of the 15 patients mutations were in the genomic area that is usually associated with congenital hypertrophy of the retinal pigment epithelium (CHRPE) (codons 463–

Table 1. Genotype–phenotype correlations in patients with FAP-associated thyroid carcinoma.

Patient no.	Sex	Age (years)	CHRPE	Codon of *APC* gene germ-line mutation	Exon	*ret/PTC* activation	Status/length of survival (years)
1	F	22	+	1061	15	Yes	Alive (2.5)
2	F	20	+	1061	15	Yes	Alive (2.5)
3	F	36	+	1061	15	No	Alive (2.5)
4	F	19	NA	1219	15	NA	Alive (2.0)
5	F	40	NA	1219	15	NA	Alive (13.0)
6	F	20	+	1309	15	Yes	Alive (1.0)
7	F	22	+	1309	15	NP	Dead (5.0)
8	NA	NA	+	593	14	NP	NA
9	NA	NA	+	778	15	NP	NA
10	NA	NA	+	1061	15	NP	NA
11	NA	NA	+	993	15	NP	NA
12	NA	NA	+	976	15	NP	NA
13	NA	NA	−	140	3	NP	NA
14[a]	F	16	+	848	15	NA	Alive (7.0)
15[a]	F	12	+	848	15	NA	Alive (3.0)

All thyroid tumors were of the papillary histologic type.
NA: not available; NP: not performed; CHRPE: congenital hypertrophy of retinal pigment epithelium.
[a]Patient data reported by Kashiwagi et al. [12].

1387) [29]. None was in the desmoid-associated area. CHRPE was documented in 12 patients (Table 1). In contrast, mutations in patients with hepatoblastoma scattered throughout the gene [35, 36]. Interestingly, there were two kindreds with mutations at codon 1061 who had thyroid carcinoma and hepatoblastoma concomitantly. In particular, in one kindred, there were three patients with thyroid carcinoma and one with hepatoblastoma. In addition, thyroid carcinoma and hepatoblastoma were also found in two other patients belonging to different kindreds who had *APC* gene mutation at codon 1061. Therefore this germ-line *APC* mutation, which was the second most frequent *APC* mutation after mutation at codon 1309, resulted in a hot spot for both thyroid carcinoma and hepatoblastoma [35, 36].

Data for the Subgroup of Four Patients with Complete Analysis

Four patients with thyroid carcinoma were observed in the institution of the first author during the study of 15 FAP families who had systematic screening for all extracolonic manifestations. In these cases genetic analysis was more detailed. In particular, *ret-PTC* oncogene, a chimeric oncogene that is restricted to the papillary histotype, was activated in three of the four cases (75%). RT-PCR documented that the isoform was the *ret-PTC$_1$* (i.e., the most frequent of the three isoforms) [31]. In contrast, the wild-type *APC* allele was not lost in the DNA of thyroid tumoral cells. Three of the four patients were completely asymptomatic and were found during the systematic screening. In contrast, the last patient was the index case of a new family. She had a thyroid nodule and underwent FNA and thyroidectomy. FAP was detected in this kindred on the basis of the young age of the patient and the peculiar histologic features of the thyroid specimen, which aroused suspicion of familial polyposis.

All patients were female. Three of them were younger than 22. Three patients had a single nodule, and the other patient had multiple nodules. One of these nodules showed capsular penetration and micrometastasis in a neck lymph node with follicular architecture. An unusual histologic pattern, the so-called cribriform pattern, which has been considered typical for FAP-associated tumors [14, 18, 19], was found in two of the four subjects. One patient had some areas of the "follicular variant" of the papillary histotype [30]. All four patients are alive and disease-free after 36 months (three patients) and 15 months (one patient), respectively. Three of the four patients had a conspicuous lymphocytic infiltration of both tumoral and normal thyroid tissue. Another patient had a germ-line mutation at codon 1061: a 15-year-old girl belonging to a large kindred that contained three members with having thyroid carcinoma and thyroid nodules. In this girl FNA showed groups of lymphocytes with germinative centers, resembling Hashimoto's disease, without evidence of malignancy. This patient has not yet developed overt colonic polyposis and is regularly followed every 6 months for thyroid nodules.

Discussion

Among 15 patients with FAP-associated thyroid carcinoma, 13 had a germ-line *APC* mutation in exon 15 between codons 778 and 1309; 1 patient had a mutation at codon 593 (exon 14); and the last patient had a mutation at codon 140 (exon 3). In particular, *APC* mutation at codon 1061 seems to be a hot spot for both thyroid carcinoma and hepatoblastoma, even if germ-line *APC* mutations in patients with hepatoblastoma were scattered throughout the gene. This finding suggests that in patients with FAP-associated thyroid carcinoma *APC* gene mutations tend to cluster in the genomic area associated with congenital hypertrophy of the retinal pigment epithelium (CHRPE) (codon 463–1387).

The molecular bases for the increased frequency of thyroid carcinoma in FAP patients are still obscure. The occurrence of papillary thyroid carcinoma in three siblings belonging to the same FAP kindred cannot be considered a casual finding [27, 30, 31]. Definitive proof that thyroid tumors are integral to FAP could be obtained following the demonstration of double-hit inactivation of the *APC* gene in FAP-associated thyroid neoplasms (Knudson two-hits hypothesis). In fact, somatic inactivation of the residual allele of the *APC* gene occurs early in colonic polyps and

cancers, hepatoblastomas, and desmoid tumors of FAP patients. In the present series, at least in the four patients who underwent this analysis, there was no loss of heterozygosity for the *APC* gene in the thyroid tumoral tissue. On the contrary, in these particular thyroid tumors there was a high rate (75%) of activation of the *ret-PTC* gene, a chimeric oncogene that is restricted to the papillary histotype and is currently considered a marker of indolent behavior [35]. If the observation that the wild-type *APC* allele was not lost and there was a high rate of *ret-PTC* activation in FAP-associated thyroid tumors is confirmed in larger series, it suggests that whereas for colonic polyps and liver tumors the impact of the inherited genetic alteration plays a basic role, during the development of thyroid carcinoma the germ-line mutation of the *APC* gene confers only a generic susceptibility to thyroid cancer; hence perhaps other factors, namely modifier genes, sex-related factors (female/male ratio 20:1), or environmental factors such as radiation are also required for the phenotypic expression [36, 37]. In fact, a high rate of *ret-PTC* activation (66.6%) was observed in thyroid tumors that occurred after radiation exposure of children in Belarus as a consequence of the Chernobyl nuclear disaster [38, 39].

In three of the four patients with extensive genetic analysis it was found that loss of function of the *APC* gene coexisted with activation of the *ret* oncogene. Obviously, coexistence does not mean cooperation. However, cooperation between these two genes could be hypothesized and deserves further evaluation. Two additional findings observed in these four patients were (1) the absence of germ-line mutations of the *ret* oncogene (data not shown); and (2) the absence of microsatellite instability in the thyroid tumoral tissue (data not shown).

The clinical relevance of the present data concerns: (1) early diagnosis of FAP-associated thyroid tumors; (2) the natural history of these tumors; and (3) treatment. Cumulative data suggest that: (1) the incidence of FAP-associated thyroid cancer probably has been underestimated in the past; (2) intensive screening of all affected siblings by ultrasonography could detect a larger than expected number of thyroid carcinomas; (3) the occurrence of papillary thyroid cancer with a cribriform pattern in a young girl should arouse the suspicion that we are in the presence of a FAP syndrome; (4) in members of a known FAP family, systematic screening is recommended in patients with ocular patches (CHRPE) and genetic mutation in exon 15. In fact, in the present series germ-line mutations of the *APC* gene in patients with thyroid cancer clustered in this exon. If this finding is confirmed, it could restrict the range of patients at risk and be of major importance in terms of cost-effective screening. In addition, the occurrence of Hashimoto-like findings or lymphocytic infiltration cannot be considered a negative finding, excluding the presence of cancer. On the contrary, it must be recognized as a frequent concomitant finding, being present in three of our four cases with papillary thyroid carcinoma.

The last comment concerns the natural history of the entire group of nonmedullary familial thyroid carcinomas. Obviously, all of them tend to be multifocal and produce early lymph node involvement. However, they can no longer be considered a unique entity. On the contrary, they must be regarded as a heterogeneous syndrome, including different diseases with different pathologic findings, a different natural history, and maybe requiring a different treatment [32]. FAP-associated thyroid tumors, in particular those with *ret-PTC* activation, despite early lymph node infiltration, seem to have a good prognosis. Obviously, this conclusion cannot be drawn on the basis of only four patients with a disease-free interval of less than 3 years. However, this view is supported by our accumulated findings and by cumulative data from the literature. In one hand, all thyroid tumors not associated with FAP but showing *ret-PTC* activation had a relatively indolent behavior (our group has documented a good prognosis in a series of more than 200 cases). On the other hand, most of the 70 FAP-associated tumors reported in the literature [1–17], regardless of *ret-PTC* activation, did not produce distant metastases, and the patients had a mean survival of more than 10 to 15 years without recurrence [32].

Genetic studies of inherited multitumoral syndromes play a key role in gaining better insight into mechanisms of cancer development and thyroid cancerogenesis. International cooperation is mandatory for an exhaustive analysis of the few available cases of rare manifestation, such as FAP-associated thyroid tumors.

Résumé

Les mutations «germ-line» du gène de la polypose adénomateuse du côlon, responsable de la polypose colique familiale (FAP) ont été analysés chez 15 patients ayant une FAP associée à un cancer papillaire de la thyroïde: 13 avaient une mutation entre les codons 778 et 1309 (exon 15), 1 au codon 593 (exon 14) et 1 au codon 140 (exon 3). Les mutations du gène APC se trouvaient regroupées dans la région génomique en rapport avec l'hypertrophie congénitale de l'épithélium pigmentaire de la rétine (CHRPE) (codons 463–1387). Des taches oculaires ont été identifiées chez 12 patients. Parmi 15 membres d'une même famille, quatre, toutes les femmes, d'un âge moyen de 23.5 ans (extrêmes: 20–32), ont eu des tests de dépistage systématique pour des manifestations extracoliques. Trois étaient asymptomatiques. Ces quatre patients ont également été testés pour la perte d'hétérozygosité du gène APC dans leur tissu thyroïdien tumoral. Aucune inactivation biallèlique n'a été retrouvée. Au contraire, 3 des 4 patients avaient une activation de leur oncogène ret-PTC, un gène chimérique résultant de la fusion d'un gène appelé H4 et le gène *ret*, en occurrence le gène ret-PTC1 isoforme. A l'examen histologique, 3 des 4 patients avaient une infiltration lymphocytaire comme on en voit dans la maladie de Hashimoto. Nos données suggèrent que: (1) l'incidence de la FAP associée au cancer de la thyroïde est probablement souś-estimée; (2) une recherche plus poussée pourrait détecter plus de cancers de la thyroïde; (3) un dépistage systématique est recommandée chez les patients ayant des taches oculaires et une mutation génétique en l'exon 15; (4) la découverte d'une infiltration lymphocytaire (de type Hashimoto), retrouvée fréquemment, n'exclut pas un cancer, (5) malgré la multiplicité fréquente et un envahissement ganglionnaire précoce, les tumeurs de la thyroïde associées à la FAP semblent avoir un excellent pronostic, en particulier, celles qui ont une activation ret-PTC.

Resumen

Las mutaciones de la línea genética del gen de la APC (poliposis adenomatosa del colon) fueron analizadas en 15 pacientes con carcinoma papilar de tiroides asociado con APC. Trece presentaban la mutación entre los codones 778 y 1309 (exon 15), 1 en el codon 593 (exon 14) y 1 en el codon 140 (exon 3). Por consigui-

ente, las mutaciones del gen de la APC se concentran en el área genómica asociada con la hipertrofia congénita del epitelio pigmentado de la retina (HCEPR) (codones 463–1387). Se documentó la presencia de manchas oculares en 12 pacientes. En particular, se encontraron 4 de 15 pacientes, todos femeninos, con edad promedio de 23.5 años, rango (20–32), en el curso del estudio de 15 familiares consecutivos sometidos a tamizaje para la detección de manifestaciones extracolónicas. Tres de ellos pertenecían a la misma familia y eran asintomáticos. Estos cuatro pacientes también fueron tamizados para detectar pérdida de heterocigocidad del gen de la APC en el tejido tumoral tiroideo. No se encontró inactivación bialélica. Por el contrario, 3 de 4 pacientes presentaron activación del oncogen PTC-*ret*, un gen quimérico que resulta de la fusión de un gen denominado H4 con el gen RET. En el examen histológico, se encontró que 3 de 4 pacientes exhibían infiltración linfocítica similar a la de la tiroiditis de Hashimoto. Estos datos sugieren que: 1) la incidencia del cáncer tiroideo asociado con APC posiblemente ha sido subestimada en el pasado; 2) el tamizaje intensivo posiblemente podría detectar un número mayor de carcinomas tiroideos de lo que se pueda esperar; 3) se recomienda el tamizaje sistemático en pacientes con manchas oculares y mutación genética en el exon 15; 4) la presencia de cambios histológicos similares a los de la tiroiditis de Hashimoto no excluyen la existencia de carcinoma, sino que representan hallazgos concomitantes frecuentes; 5) a pesar de frecuente multicentricidad y de invasión ganglionar linfática precoz, los tumores tiroideos asociados a APC parecen tener un pronóstico excelente, en particular aquellos con activación RET-PTC.

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