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Histopathological changes induced by selective inactivation of *menin* on the thyroid gland in *RET/PTC3* and *E7* transgenic mice. A study of 77 cases

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Abstract

Multiple Endocrine Neoplasia Type 1 (*MEN1*) does not involve the thyroid gland, but animal studies have shown that mice with inactivation of *menin* could develop thyroid pathologies. The objective was to evaluate if the selective inactivation of *menin* in murine thyroid glands expressing *RET/PTC3* and *E7* oncogenes, might induce an increased index of proliferation and a more rapid development of thyroid hyperplasia and/or tumors. The thyroid glands of 77 mice aged 4–18 months (31 expressing the *E7* oncogene and 25 the *RET/PTC3* oncogene) were analyzed for histological changes and Ki67 proliferation index. Fifty-two mice had selective inactivation of *menin* in the thyroid gland (16 mice with *RET/PTC3* oncogene and 19 mice with *E7* oncogene). As compared to wild type, mice with inactivation of *menin* presented an increased Ki67 proliferation index. Mice presenting the *E7* oncogene showed larger thyroid glands with a pattern of diffuse hyperplasia. Mice expressing the *RET/PTC3* oncogene presented larger thyroid glands compared to the wild type mice but smaller compared to *E7* mice. The lesions in the *RET/PTC3* group were "proliferative papillary cystic changes" (60%), "cribriform" (16%), "solid" (8%) and a combination of these patterns in the rest of the thyroid glands. The inactivation of *menin* in the thyroid gland of young mice does not seem to change the histological pattern, but it influences the proliferation of follicular cells. Further molecular studies especially in aged mice are needed to better understand the correlation between certain oncogenes and the inactive status of *menin*.

Keywords: *menin*, thyroid, RET-PTC3 oncogene, *E7* oncogene, Ki67 antigen.

⊟ Introduction

Multiple Endocrine Neoplasia type 1 (MEN1) is a hereditary syndrome with an autosomal dominant transmission characterized by the development of hyperplasia and tumors in certain endocrine glands (parathyroid glands, pancreas and pituitary gland) due to mutations in the *Men1* gene. Reports found that thyroid pathology could be also present in MEN1 patients in a percentage of 2.6% up to 25%, mostly represented by adenomas, colloid goiters, or more rarely, carcinomas [1]. In contrast, a recent paper showed that *menin* expression is preserved in different types of thyroid cancer and that the incidence of thyroid pathology in MEN1 patients does not differ from that of patients with isolated primary hyperparathyroidism [2]. Both in humans and animals, the functions of *menin* in the thyroid gland are still not completely understood.

Menin, an oncosuppressive nuclear protein, is expressed in all tissues, both endocrine and non-endocrine, but the selective inactivation of *menin* in non-endocrine tissues such as liver, does not induce tumors [3]. Its oncosuppressive properties are explained by its multiple roles among which the most important are the regulation of the cell division cycle, the inhibition of S-phase during

cell division, the regulation of apoptosis and the stability of the genome [4].

In 2003, Bertolino *et al.* obtained knockout mice for *menin* by inactivating the third exon of the *Men1* gene. Homozygous mice were lethal whereas heterozygous *Men1*^{+/-} mice developed MEN1 syndrome similar to what is observed in MEN1 patients, including 6% of mice developing thyroid tumors [5, 6].

Murine models that express *RET/PTC3* (*Re*arranged during *T*ransfection/*p*apillary *t*hyroid *c*arcinoma) and *E7* (human papillomavirus type 16 E7) oncogenes are known to develop thyroid hyperplasia and/or tumors in the thyroid gland [7]. Crossing these mice with those who have a selective inactivation of *menin* in the thyroid gland could offer a further insight of the involvement of *menin* in the thyroid pathology.

Our hypothesis is that the selective inactivation of *menin* in murine thyroid glands that expresses *RET/PTC3* and *E7* oncogenes, respectively, might induce an increased index of proliferation and a more rapid development of uncontrolled thyroid hyperplasia and/or tumors. In the following study, we also analyzed several aspects that can be influenced by the selective inactivation of *menin*

in the thyroid gland: surface of the thyroid gland, Ki67 index of proliferation and histological aspects.

⊟ Materials and Methods

Seventy-seven mice 4 to 18-month-old were included in this study, which was performed in the UMR 1052-INSERM, CRCL "Tumor Escape" Department of the "Claude Bernard" University from Lyon, France. All the animals were raised in a conventional animal facility, with a 12 hours light/12 hours darkness cycle. They were fed with a standard regime with normal iodine content and provided with water *ad libitum*.

On the day of the sacrifice, the animals were anesthetized with 0.1–0.2 mL/100 g body weight of a mixture of anesthetics (0.5 mL 2% Xylazine, 1.25 mL 50 mg/mL Ketamine hydrochloride and 8.8 mL 0.9% sodium chloride).

After complete anesthesia, the animals were positioned on the plastic plate and a bilateral section of the thoracic cavity was practiced in order to expose the heart. Subsequently, the vena cava was cut and the blood was gently collected with a syringe without needle. The blood was gently emptied in a plastic tube and then the tubes were centrifuged at 3500 rpm for 15 minutes. The tubes with the final volume of serum were kept at -80^0C for future analysis of thyroid hormones.

The thyroid gland was removed after incision of the anterior cervical region, exposing the thyroid bed and isolating the thyroid gland from the trachea, adipose tissue, muscles and lymph nodes. We were not able to obtain a precise weight of the thyroids because in some cases we had contamination with lymph nodes, esophagus, trachea or cervical muscles. In contradiction to humans, the parathyroid glands are frequently included in the thyroid gland of mice and have a constant weight. Nevertheless, we estimated the total surface of the thyroid gland. Both lobes of the thyroid gland were weighted, one lobe was fixed in 10% formalin and the other lobe was frozen in liquid nitrogen and conserved at -80^0C.

The surgical and experimental procedures respected the French recommendations for animal experiments.

Murine models and genotyping

As murine models, the *E7* and *RET/PTC3* models with/without inactivation of *menin* were used. As a control group, wild type sv129/C57Bl6 mice (WT) were used.

In the *E7* model (*E7*), the oncogenic properties of human papilloma virus type 16 *E7* are caused by the interaction and direct inhibition of retinoblastoma 1 (RB1) protein, which determines the increased expression of transcription factors that will initiate the cell cycle. Transgenic mice, which express the *E7* oncogene under the control of thyroglobulin, develop since one month old, follicular changes and an uncontrolled growth of the thyroid gland [8], while aged mice develop both papillary and follicular thyroid carcinoma [9].

The *RET/PTC3* transgenic mice express selectively in the thyroid gland the RET (*Re*arranged during *T*ransfection) proto-oncogene under the control of thyroglobulin. These mice were generated by Powell *et al.* (1998) and are known to develop structural changes of the thyroid gland, which vary from hypertrophy, follicular cell hyperplasia, dysplastic follicles, to primary tumors characterized by papillary structures with preserved production of thyroglobulin [7, 8, 10].

We obtained mice with targeted inactivation of *menin* by inactivating the third exon of the *Men1* gene. This was achieved by flanking the exon with two LoxP sequences using a homologous recombination technique [6]. *Menin* was inactivated (floxed) for only one of the alleles (Men$^{+/-}$ *menin* heterozygote) or both (Men$^{-/-}$ *menin* homozygote) in the presence of Cre recombinase selectively expressed also only in the thyroid gland [11].

In our study, the genotype was determined by polymerize chain reaction (PCR) using genomic DNA extracted from the tail tips and/or phalanges. The products of amplification were analyzed by electrophoresis in 2% Agarose gel colored by Ethidium bromide. For the *Men1* gene, we distinguish two alleles, a wild allele observed as a 200 base pair (bp) fragment and a floxed *Men1* gene amplified as a 220 bp fragment. For the *RET/PTC3* and *E7* oncogenes, the fragments of amplification have 600 and 300 bp, respectively.

The mice included in the study had the following genotypes: wild type (WT, $n=4$), Men$^{+/-}$ (*menin* heterozygote, $n=9$), Men$^{-/-}$ (*menin* homozygote, $n=8$), *E7* Men$^{+/+}$ ($n=12$), *E7* Men$^{-/-}$ ($n=11$), *E7* Men$^{+/-}$ ($n=8$), *RET/PTC3* Men$^{+/+}$ ($n=9$), *RET/PTC3* Men$^{+/-}$ ($n=10$) and *RET/PTC3* Men$^{-/-}$ ($n=6$).

For histological examination, the thyroid lobes were fixed in 10% buffered formalin and embedded in paraffin. Four-μm-thick tissue sections of formalin-fixed, paraffin-embedded tissue were prepared according to conventional procedures. Sections were then stained with Hematoxylin–Phloxine–Saffron (HPS) and examined in light microscope by a pathologist not aware of the genotype or the status of *menin* inactivation. The sections were classified in two main categories: normal aspect and hyperplasia/dystrophic features.

The lesions in the hyperplasia/dystrophic group were heterogeneous and consisted of a mixture of cystic changes with proliferative content and a solid pattern, all in an abundant fibrous stroma, creating the pattern described by Jin *et al.* as "Proliferative Papillary Cystic Changes (PPCC) with spindle cells and remodeling" [8]. Other lesions in this group included diffuse hyperplasia, cribriform (Cr) pattern, solid pattern and lesions termed "tumors".

Immunohistochemistry was performed on an automated immunostainer (Ventana Discovery XT, Roche, Meylan, France) using DABmap Kit according to the manufacturer's instructions. Sections were incubated one hour with a rabbit anti-Ki67 antibody (clone SP6, diluted at 1:200, Labvision, Fremont, USA). Staining was visualized with DAB solution, with 3,3'-diaminobenzidine as a chromogen substrate. Finally, the sections were counterstained with Gill's Hematoxylin.

The determination of the proliferation index (Ki67%) consisted in counting Ki67-positive labeled cells out of at least 2000 cells in "hot spot" regions with increased expression of Ki67. The slides were imaged using a computer-assisted system (Histolab 6.2.0$^®$, Microvision Instruments), which recognizes brown marked cells (Ki67-positive) and blue marked cells. Using the same software, we were able to manually determine the surface of the thyroid gland.

Data were analyzed in Microsoft Office Excel 2007$^®$,

using the two-tailed Student's *t*-test. The results are expressed as mean ± standard deviation or median. The statistical significance was considered in case of a $p<0.05$.

⊟ Results

The total thyroid surface for the WT, Men$^{+/-}$, Men$^{-/-}$ mice had a median of 1.260 mm^2, and did not vary between the three groups (1.134 mm^2, 1.393 mm^2 and 1.276 mm^2 respectively). The thyroid lobes had a normal architecture and were formed by small to medium sized follicles lined by cubic cells with a small normal nucleus (Figure 1). The Ki67 proliferation index was increased in the Men$^{+/-}$, Men$^{-/-}$ thyroids *versus* WT ones (1.16% and 1.05% *versus* 0.64%, $p<0.05$).

E7 thyroid glands were larger as compared to the WT ones. The surface had a median of 10.583 mm^2, 8.4 times higher than the WT ones. Within this group, the *E7* Men$^{+/+}$ thyroid glands had a median surface of 12.363 mm^2, while the *E7* Men$^{+/-}$ had a median surface of 11.163 mm^2. The only statistically significant difference was observed in the surface of the *E7* Men$^{-/-}$ thyroids, which were smaller than the *E7* Men$^{+/+}$ ones (median 8.503 mm^2 *versus* 12.363 mm^2, $p=0.037$).

The *E7* thyroids showed a homogenous pattern of diffuse hyperplasia at all ages. The follicles were large, dilated by abundant homogenous colloid. They were lined by flat cells and presented most often at the periphery of the thyroid lobe, papillary projections some of them simple or some containing in their central axis, small follicles. The lining of these papillary structures was formed by tall cells with large, fusiform nuclei, which gave a "palisade like" aspect (Figure 2). The stroma was reduced and there was no fibrosis or inflammation. The thyroid glands of mice older than eight months seemed to present a lesser degree of hyperplasia compared to the 2–4-month-old mice.

The Ki67 proliferation index did not differ between the three groups (13.3%, 12.92% and 12.68%). It was more intense in the proliferative papillae and in the cells separating the smaller follicles within the papillary projections (Figure 2).

The *RET/PTC3* thyroids were 6.5 times larger than the WT ones (8.222 mm^2 *versus* 1.260 mm^2) and the surface of *RET/PTC3* Men$^{-/-}$ thyroids was statistically significant smaller than that of *RET/PTC3* Men$^{+/+}$ thyroids (median 6.235 mm^2 *versus* median 11.163 mm^2, $p<0.05$).
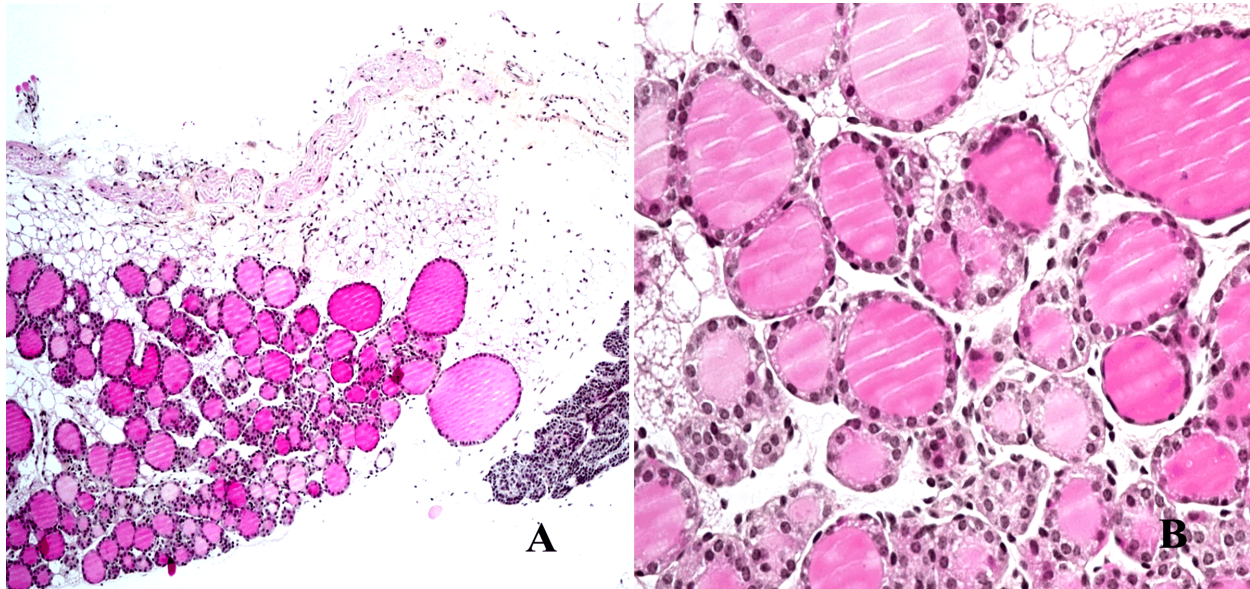

Figure 1 – *Normal thyroid gland with small and medium sized follicles, with smooth distribution of the stroma and no fibrosis. HPS staining: (A) ×40; (B) ×400.*
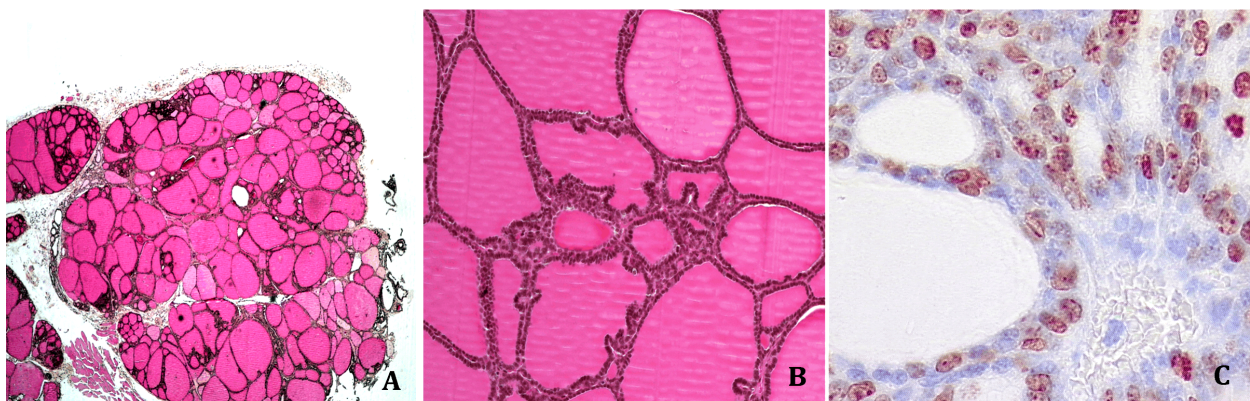

Figure 2 – *(A) Diffuse hyperplasia in all the thyroid lobe of a gland expressing the E7 oncogene (HPS staining, ×40); (B) Small papillary projections on the distended follicular wall lined with cubo-cylindrical epithelium, dense nuclei, "palisade like" pattern (HPS staining, ×100); (C) Brown follicular cells marked for Ki67 proliferation index (Ki67 immunostaining, ×400).*

The lesions in this group were heterogeneous and varied from one mouse to another, and sometimes-different lesions in each lobe of one thyroid gland were noticed. The lesions consisted in "PPCC" pattern (Proliferative Papillary Cystic Changes with spindle cells and remodeling), cribriform (Cr) pattern, solid pattern and lesions termed "tumors".

The PPCC pattern was observed in 60% (15/25) of the *RET/PTC3* mice and was equally distributed in the three groups. This pattern was more evident at the age of 7–9 months (86%), while at the age of 4–5 months was observed only in two cases.

In this type of lesion, the normal architecture was replaced by numerous large cysts lined by flattened follicular cells with exuberant pseudopapillary structures, more or less complex, with or without secondary small follicles. They were lined by tall, columnar cells, "palisade like" with hyperchromatic nuclei, without any morphologic changes specific of papillary carcinoma (ground glass appearance, clearing, grooves) (Figure 3). In rare cases, the follicular cells showed an oncocytic metaplasia made of large eosinophilic cells containing irregular nuclei with rare pseudoinclusion figures (Figure 3, C and D). Another feature seen in some cases consisted of benign dystrophic epidermoid metaplasia. Between the cysts, the spindle cell areas were made of more or less large sheets of fusiform epithelial cells harboring the same nuclear aspect. The cysts and the spindle sheets were situated in a fibrous dense stroma, containing some chronic inflammatory cells, macrophages and cholesterol.

The cribriform pattern, 16% (4/25) of the cases, was made of microfollicles joined together, sharing a part of their wall, forming large areas without stroma. The cells were round or polygonal, with mild anisokaryosis but without real atypia (Figure 4).
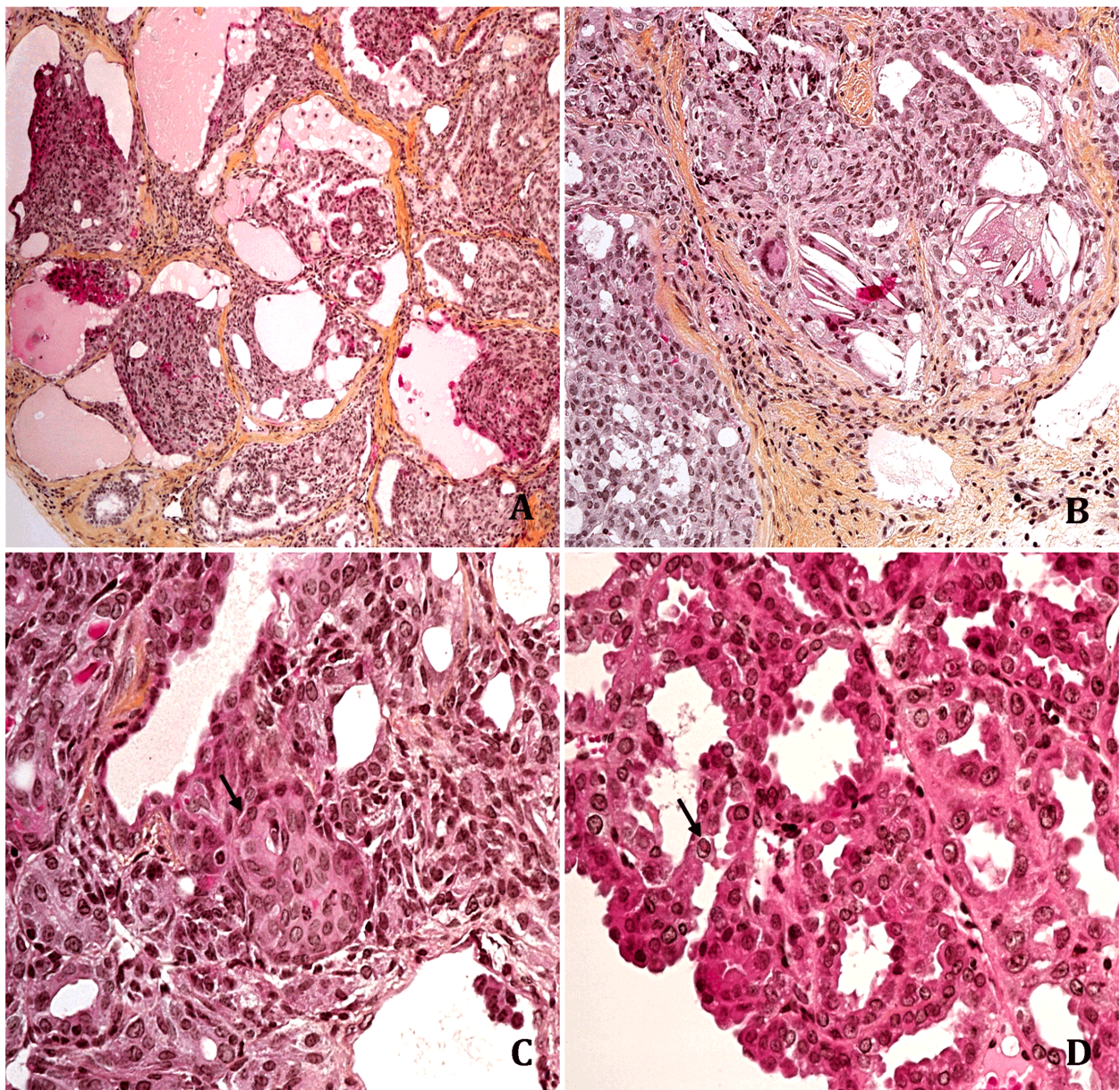

Figure 3 – *(A) Proliferative papillary cystic changes (PPCC) in a thyroid gland expressing the RET/PTC3 oncogene, with cysts lined by flattened follicular cells and fibrous tissue; (B) PPCC pattern with dense fibrous tissue and cholesterol crystals; (C) Malpighian metaplasia in a cribriform region with spindle cells (black arrow); (D) Oncocytic metaplasia with mild anisokaryosis and a pseudoinclusion (black arrow), but no definite characteristics of papillary thyroid carcinoma. HPS staining: (A) ×100; (B) ×200; (C and D) ×400.*
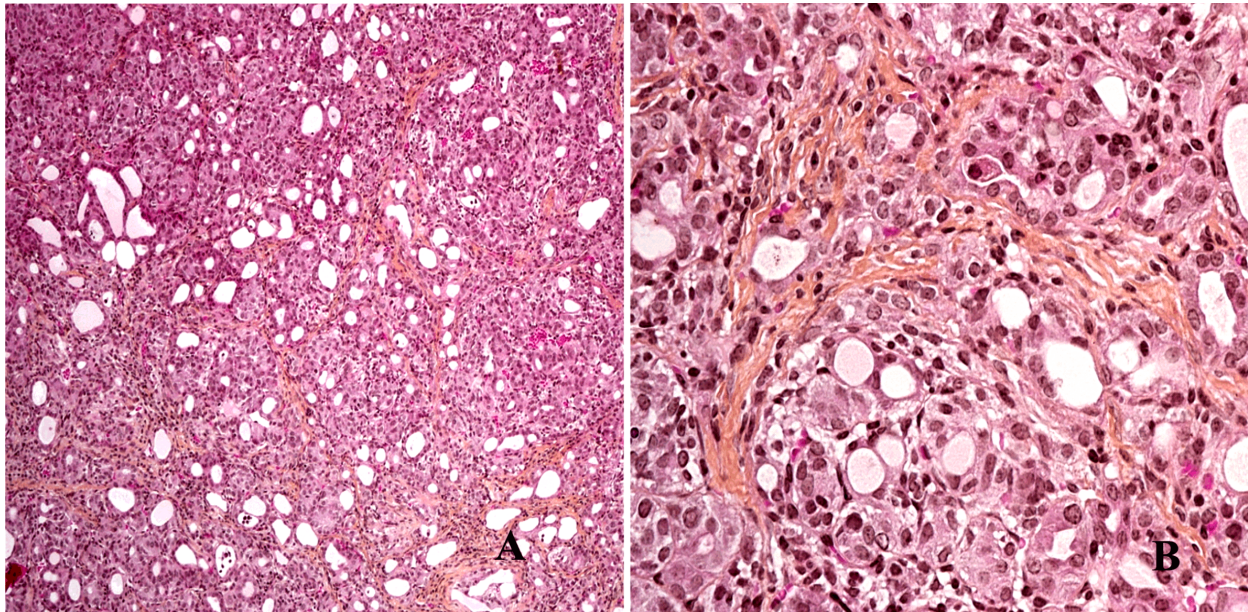
Figure 4 – *(A) Cribriform pattern, areas of dense follicular cells with microfollicles sharing a part of their wall, forming areas without stroma; (B) Round or polygonal cells with mild anisokaryosis without real atypia. HPS staining: (A) ×100; (B) ×400.*

The solid pattern, 8% (2/25) of the cases, was made of dense areas without any follicular or papillary formations. Like the cribriform pattern, it could be seen in loosely distributed areas, often associated with the PPCC growth (Figure 5). In the "tumor" lesions, we found the same pattern. This pattern was present in 10–12-month-old mice, while the "cribriform" pattern was found in 4–5-month-old mice.

The lesions termed "tumors", 8% (2/25) of the cases, consisted of macroscopically visible nodules, 1–5 mm in diameter, not or partially circumscribed. Microscopically, the architectural pattern of growth was trabecular or solid. The cells were fusiform, round or polygonal, with abundant basophilic cytoplasm and showed obvious atypical nuclear features with mitoses. Necrosis foci were seen in the center of the lesions (Figure 5). No characteristic features of any type of thyroid carcinoma were found.

A combination of cribriform/solid and PPCC/solid was found in 4/25 (16%) of the thyroids of 8–12-month-old mice.

We observed that independent from the *menin* status, the changes in the morphology of the thyroids expressing the *RET/PTC3* oncogene, progressed with ageing from cribriform to PPCC, PPCC/cribriform, PPCC/solid and in older mice to solid type. The degree of fibrosis did not correlate with the age of the mice.

The Ki67 proliferation index did not vary between the three groups (12%, 14.51% and 16.7% respectively) and also it did not statistically vary between the different histological patterns despite the fact that in the "cribriform" pattern it seemed more increased in comparison to the PPCC and "solid" one (45.57% *versus* 15.24% and 16.01%).
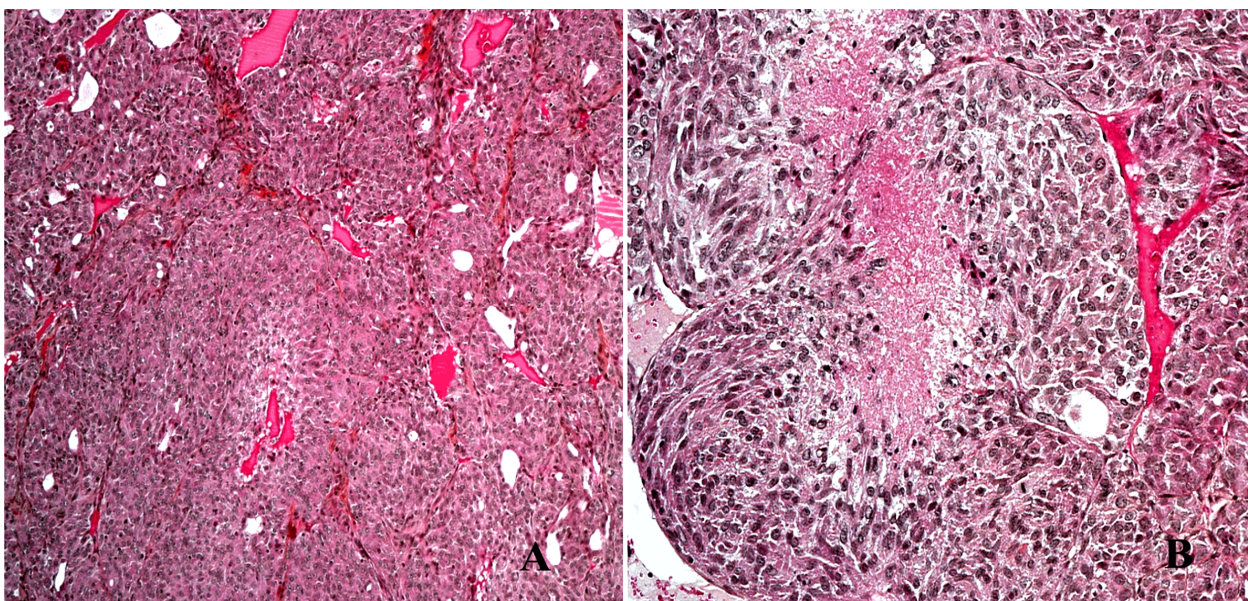

Figure 5 – *(A) "Tumor" lesion in a thyroid gland expressing the RET/PTC3 oncogene, solid architecture, monomorphic basophilic cells with necrosis around the distended vessels; (B) Necrosis foci in the center of the lesion. HPS staining: (A) ×100; (B) ×400.*

Discussion

Our hypothesis was that the selective inactivation of *menin* in the thyroid gland of mice expressing the *RET/PTC3* and *E7* oncogenes could induce a more rapid tumor formation. The results of the present study show that the rate of thyroid "tumor" formation is lower than that described in the literature.

Follicular thyroid adenomas have been reported in mice with loss of *menin* expression. However, in the WT mice models with *menin* inactivation, we did not notice any tumor formation despite the increased Ki67 proliferation index. An explanation could be the fact that the oldest mice in this group were 18 months, while in the study of Bertolino *et al.* lesions such as hyper/dysplasia (6.5%) and tumors, "solid follicular cell carcinomas" (6.5%) were observed in 24-month-old mice with selective heterozygous inactivation of the *Men1* allele [6]. Harding *et al.* also reported that mice older than 12 months, with loss of *menin* expression, developed thyroid follicular adenomas in more than 10% of cases [12]. Another possible explanation for this different result could be the method we used, the targeted selective inactivation of *menin* in the thyroid gland as compared to the knockout of *Men1* gene used in the other studies which leads to complete inactivation of *menin* in all the organs.

The transgenic *E7* mice models are prone to develop tumors in endocrine tissues, including the thyroid gland [13]. Mice develop thyroid goiters due to thyroid cell proliferation and accumulation of the colloid secondary to the inactivation of RB1 protein, while in older mice, the presence of tumor nodules resembles the follicular and papillary carcinomas [14]. In our study, we did not notice any tumor development in the *E7* mice, the only statistically significant difference was observed in the surface of the *E7* Men$^{-/-}$ thyroids, which were smaller than the *E7* Men$^{+/+}$ ones.

Powell *et al.*, first reported in 1998 the presence of papillary carcinoma and metastasis in 55% of the *RET/PTC3* thyroids of mice older than three months and in 31% of mice younger than three months. The definition for the papillary thyroid carcinoma was "disorganized cellular nodules (>50–100 cells) bordering or extending into the thyroid follicle with some or no papillary structures and/or large regions of tumor cells (5–10% or more of thyroid size) devoid of follicular or papillary structures", a definition no longer accepted by the *World Health Organization* (WHO) thyroid cancer classification [7, 15].

Jin *et al.* reported tumor formation in 28% of the *RET/PTC3* mice 6 and 10-month-old [8]. In our study, we report in 8-month-old mice, an incidence of 8% "solid" pattern and 16% "cribriform/solid" and "PPCC/solid" patterns neither of them with extra-thyroid extension. We did not notice any types of thyroid carcinoma. The "solid" lesions described in our study could resemble to the human type of poorly differentiated thyroid carcinoma because of the presence of necrosis and mitoses but the degree of malignity is uncertain, as also mentioned by Jin *et al.* [8]. In the study of Jin *et al.*, they reported that the pattern of "PPCC" occurred at the age of two months and completely disappeared by the age of 10 months, while the "cribriform" pattern was more evident in the 6–10-month-old mice [8]. In our study, on the contrary, we observed the appearance of the PPCC pattern at the age of 7–9 months, while the "cribriform" pattern was observed at younger ages. We could explain this difference by the wild type strain used for crossing the transgenic mice (sv129/C57Bl6 in our study and C57Bl6 in Jin *et al.* study) as further detailed and by the fact that the age of the mice was not so advanced to allow proper "tumor" development.

The architectural disorganization of the thyroid gland to this pattern appeared in older mice and it seemed to be caused in our study by the presence of the *RET/PTC3* oncogene and not by the selective inactivation of *menin*.

The lower incidence of thyroid tumors in our study, might also be explained by the fact that as opposed to Jin *et al.* study, our wild strain sv129/C57Bl6 increased, but a lower level, the risk of tumor development [8, 16]. In addition, judging by the observation that co-expression of *RET/PTC3* and human papilloma virus type 16 *E7* oncogenes does not enhance the neoplastic transformation or the malignant phenotype [17], we can suppose that the inactivation of *menin* in the presence of *RET/PTC3* oncogene might not induce a more aggressive histological pattern. An argument to support this theory is the presence of an increased Ki67 proliferation index only in the Men$^{+/-}$ and Men$^{-/-}$ thyroids *versus* the WT ones. The inactivation of *menin* in the presence of the *RET/PTC3* and *E7* oncogenes did not determine a difference in the Ki67 proliferation index in these groups of transgenic mice.

The increase in the Ki67 proliferation index in the Men$^{+/-}$ and Men$^{-/-}$ thyroids is explained by the onco-suppressive properties of *menin* and its role in the cell cycle division and apoptosis and also by the up-regulation of cyclin D and cyclin-dependent kinases involved in proliferation [10]. *Menin* is capable to directly interact with the DNA by its C-terminal region [4]. Loss of this sequence of *menin* results in a failure to suppress the cell proliferation and no longer blocks the G2-M and G1-S phases during the cell division cycle. Another protein that interacts with *menin* is the activator of S-phase kinase (ASK), a component of the cell division cycle, which seems to induce cell proliferation in the absence of *menin* expression [18]. Another property of *menin* is its interaction with JunD, an activator protein 1 (AP-1) transcription factor, considered to be antimitogenic [4]. In the presence of *menin*, JunD acts as a growth suppressor while in the absence of *menin* acts as a growth promoter protein [19]. Nevertheless, these properties of *menin* do not explain in our study, the reduced surface of the thyroids expressing the *RET/PTC3* and *E7* oncogene. We suppose that the result might be explained by a different mechanism of action, presumably the expression of the oncogenes or the combination of their expression with the *Men1* gene inactivation. In the study of Bertolino *et al.*, they observed that in knockout cell cultures for *Men1* gene, there was a more accelerated senescence in the liver cells with absence of differentiation, but when restoring the expression of

menin, they observed a recovery in the normal growth and differentiation of cells [5]. We could also suppose that a similar mechanism of *menin* inactivation could lead to a decrease in the surface of the thyroid gland.

One of the limitations of our study is the lack of hormonal status evaluation, since thyroid-stimulating hormone (TSH) stimulation is known to induce a "solid" pattern in the *RET/PTC1* severely hypothyroid mice from Powell *et al.* study [7, 20], while in euthyroid mice the same oncogene can induce cystic thyroid tumors [21]. We can assume that the mice in our study were euthyroid or had a moderately degree of hypothyroidism because the hybrid background we used (sv129/C57Bl6) is not prone to the development of congenital hypothyroidism or morphological alterations of the thyroid gland in the early life [16]. In addition, severely hypothyroid mice do not survive more than one month without proper thyroid hormone replacement. Another limitation of our study is the reduced number of mice, especially the aged ones, which are prone to develop more aggressive features of thyroid histology according to additional observations from our laboratory (results not shown).

⊟ Conclusions

Our preliminary results show that the selective *menin* inactivation in the thyroid gland of transgenic mice does not induce thyroid tumors as described by other groups, a result which could be due to the different approach used knowing that in knockout *Men1* mice, the expression of *menin* is also lost in other endocrine organs besides the thyroid. Because of the reduced number of mice, we cannot completely exclude the possibility of later tumor development in aged mice with selective inactivation of *menin*. Further molecular studies of genes involved in proliferation are needed, especially in a larger number of transgenic old mice in order to better understand the correlation between the expression of *RET/PTC3* and *E7* oncogenes with the inactive status of *menin*.

Conflict of interests

The authors declare that they have no conflict of interests.

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