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ORIGINAL ARTICLE

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Fetal adenomas and minimally invasive follicular carcinomas of the thyroid frequently display a triploid or near triploid DNA pattern

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Abstract The ploidy pattern and the percentage of S-phase cells were investigated by means of flow cytometry using fresh or frozen samples in a series of 143 tumors and tumor-like lesions of the thyroid in an attempt to find whether there is any relationship between the histological characteristics of the lesions and their DNA content. The percentages of aneuploidy cases per category were: nodular goiter, 18.5% (15/81); fetal adenoma (including cases with trabecular/solid growth pattern), 58.3% (14/24); follicular adenoma other than fetal adenoma, 0% (0/18); papillary carcinoma, 11.1% (1/9); and minimally invasive follicular carcinoma, 57.1% (4/7). Regardless of the histological category, aneuploid lesions had a significantly higher ($P<0.001$) percentage of S-phase cells (7.3%) than diploid lesions (4.1%). All the six cases with a DNA content within the triploid range were fetal adenomas, but one was a follicular carcinoma displaying a fetal adenoma-like growth pattern. The other three follicular carcinomas with an aneuploid DNA pattern also displayed foci of fetal adenoma-like growth pattern. Image cytometry of the four aneuploid follicular carcinomas showed similar DNA indexes in the peripheral, invasive foci of the lesions and in the central fetal adenoma-like areas. These results demonstrate that aneuploidy in benign tumors is restricted to adenomas displaying a fetal or fetal/embryonal growth pattern and support the concept that chromosome instability is a major pathway of tumorigenesis in thyroid follicular neoplasms.

Keywords Thyroid · Follicular adenoma · Follicular carcinoma · Flow cytometry · Aneuploidy

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Introduction

Aneuploidy can be defined as an alteration in the chromosome number, which involves gains and losses of whole chromosomes. Tumors with ploidy alterations have been referred to as tumors with "chromosome instability" (CIN). Those showing instability in the repetitive regions of the genome are tumors with so-called microsatellite instability (MIN) [26]. There are a number of gene alterations that can give rise to the CIN phenotype, namely genes that are involved in chromosome condensation, sister-chromatid cohesion, kinetochore structure and function, centrosome/microtubule formation, and dynamics and "checkpoint" genes that monitor the proper progression of the cell cycle [26]. Despite the existence of some interpretative clues, the molecular basis of CIN remains undefined in most human cancers.

The ploidy of thyroid lesions has been extensively studied by several authors [2, 3, 16, 18, 19, 21, 22, 35]. It is generally referred that there is no correlation between aneuploidy and malignancy, because aneuploidy has been observed in benign lesions and diploidy has been observed in malignant tumors [2, 16, 18, 21, 22, 35]. It is also usually accepted that aneuploidy is an adverse prognostic factor in papillary [19, 20, 39], follicular [19, 20], and medullary thyroid carcinomas [19, 20]. It remains, however, controversial whether DNA ploidy is an independent prognostic factor per se [18, 22].

In the benign lesions of the thyroid, the percentage of cases with abnormal DNA content varies from 10% to 22% in goiters [13, 18, 31, 37] and from 18% to 52% in follicular adenomas [8, 18, 22, 34]. The prevalence of abnormal DNA content is particularly high in Hürthle cell adenomas [6, 7, 40]. To the best of our knowledge, no other correlation between the histological characteristics of thyroid tumors and the ploidy pattern has been reported to date.

The cytometric evaluation of DNA content of thyroid tumors has been thoroughly complemented by studies of conventional cytogenetics and fluorescent in situ hybridization (FISH) [2, 4, 9, 16, 32, 37]. Taking the results of

DNA cytometry together with those of conventional and molecular cytogenetics, it has been concluded that follicular adenomas of the thyroid are frequently aneuploid and display often trisomies of chromosomes 5, 7, and 12 [2, 4, 5, 6, 9, 16]. Some of these trisomies can also be detected, less often, in nodular goiters [2, 9, 32] and follicular and papillary carcinomas [2, 17, 32]. We undertook the present flow cytometry study of a series of 143 unselected thyroid tumors and tumor-like lesions in order to find whether there is any relationship between the histological characteristics of the lesions and the ploidy pattern.

Materials and methods

Thyroid lesions (143 lesions) were either consecutively collected or retrieved from the files of frozen material of the Department of Pathology of Hospital S. João. The lesions were classified according to Hedinger et al. [15] and Rosai et al. [33] as nodular goiter (*n*=81), fetal adenoma (*n*=24), follicular adenoma other than fetal adenoma (*n*=18), follicular carcinoma (*n*=7), papillary carcinoma (*n*=9), medullary carcinoma (*n*=2), and Graves' disease (*n*=2) in sections stained with hematoxylin and eosin. The following criteria were used in the subclassification of benign lesions:

1. Nodular goiter – the diagnosis of nodular goiter was made whenever there were several nodules or a single nodule without a well-defined capsule. In most cases, the nodules displayed a normofollicular or a macrofollicular pattern of growth with or without papillary hyperplasia [15]. In some cases, the nodules displayed a totally or partially microfollicular (fetal) and/or trabecular/solid (embryonal) pattern of growth [15]. No classification importance was given to the presence of oxyphilic cells and lymphoid infiltration. In cases displaying several nodules, flow cytometry studies were performed in the largest nodule.
2. Fetal adenoma – encapsulated follicular adenomas were classified as fetal adenoma when more than 50% of the lesion was composed of microfollicles, usually dispersed in an abundant, edematous, or hyalinized stroma [15]. In many cases, the microfollicular pattern of growth merged with foci of trabecular/solid adenoma [15]. For the sake of simplicity, all of these cases were classified as fetal adenoma regardless of the size of the trabecular/solid areas.
3. Follicular adenoma (other than fetal adenoma) – all of the remaining encapsulated benign tumors were included in this category. Most of the tumors displayed a predominant or exclusive normofollicular pattern of growth. Both in fetal and non-fetal adenomas, no classification importance was attributed to cell oxyphilia or lymphoid infiltration.

Flow cytometry

Flow cytometry was performed in fresh or frozen samples of the same lesion used for histological diagnosis. The samples were processed according to a previously reported procedure [12], slightly modified by us. Briefly, samples were thawed, and complete cell lysis was assured with NP-40 treatment (final concentration 0.5%), and the isolated nuclei were washed once with ice-cold phosphate-buffered saline. Nuclei of chicken erytrocytes (NCE) were added as an internal standard at a final concentration of 5–10% of total nuclei. Nuclei (1×10^6) were stained with a propidium iodide (PI) solution containing 50 µl/ml of PI, 10 mM Tris, 5 mM $MgCl_2$, and 0.5 mg/ml RNase (DNase free), pH 7.0. Tubes were vortexed, and the samples were incubated, protected from light, for at least 30 min. After incubation, the suspension was syringed through a 27 needle and sieved through a 55 nylon mesh immediately before analysis. Acquisition was performed on a EPICS C flow cytometer (Coulter Electronics, Inc., Hialeah, Fla.). Computer analysis of DNA histograms was done using MPLUS (Phoenix Flow Systems, San Diego, Calif.). This software includes MCYCLE AV, a multiple option cell cycle fitting that automatically determines the DNA index (DI) and cell cycle phase fractions in cell or nuclei populations. The same batch of NCE was used with all of the thyroid samples. The DI of the standard peak (DISP) was calculated using MCYCLE AV, as the ratio of the mean channel number of the standard peak and the mean channel number of a peak labeled as "diploid". The coefficient of variation of DISP measurement intra-sample was less than 1.0% (in most cases, less than 0.5%). The mean coefficient of DISP inter-sample was 2.5%. The median value was 0.341%. For classification purposes, cases were categorized as hypodiploid (DI<0.9), diploid (DI=1.0±0.1), hyperdiploid/hypotriploid (1.1<DI<1.4), triploid (DI=1.5±0.1), hypertriploid/hypotetraploid (1.6<DI<1.9), tetraploid (DI=2.0±0.1), and hypertetraploid (DI>2.1). The S-phase fraction (SPF) value of each cell cycle was considered only if it was within the intra-model and inter-model 95% confidence intervals. Due to these restrictions, the S-phase fraction was not calculated in 5 of the 143 cases.

Image cytometry DNA measurements were made in 6-µm sections of the paraffin blocks stained using Feulgen's procedure (acid hydrolysis in HCl 5 N at 26 C for 55 min). Briefly, in each case, 200 nuclei from the lesion and 50 lymphocytes (used as a diploid reference population) were captured using a green filter mounted in a Nikon Optiphot-2, a Nikon XC75 black and white CCD video camera, and a Leica's Q500 Image Workstation (Leica Imaging Solutions Lda, Cambridge, UK). The acquired images were processed using Leica Qwin and analyzed using Leica Qploidy. The integrated optical density (IOD) obtained for the nuclei of the lesion was compared with IOD of the lymphocytes of the same case, leading to a DI (DI=IOD of the sample/IOD of the reference population). For technical reasons, no attempt was made to compare the DIs calculated using flow and image cytometry.

Statistical analysis

The results are expressed as a percentage or as mean±SEM. The statistical analysis was performed using the χ^2 method after the Yates correction and unpaired *t*-test. Two results were considered significantly different if *P* was less than 0.05.

Results

The DNA content of the 143 lesions is summarized in Table 1. Of the benign lesions and the carcinomas, 22% (28/125) and 27% (5/18) had an aneuploid DI, respectively (*P*>0.1; Table 1). Excluding Graves' disease (*n*=2) and medullary carcinoma (*n*=2), the percentage of aneuploid cases per category was: nodular goiter, 18.5% (15/81); fetal adenoma, 58.3% (14/24); follicular adenoma other than fetal adenoma, 0% (0/18); papillary carcinoma, 11.1% (1/9); and minimally invasive follicular carcinoma 57.1% (4/7) (Table 1).

The data on the percentage of S-phase cells are summarized in Table 2. The highest values were observed in a few cases of fetal adenoma and nodular goiter (Fig. 1). Regardless of the histological category, aneuploid lesions had a significantly higher (*P*<0.001) percentage of S-phase cells (7.3%) than diploid lesions (4.1%). The percentage of S-phase cells was not significantly different (*P*>0.1) in benign (4.7%) and malignant lesions (5.7%); the same holds true when fetal adenomas (6.5%)

Table 1 Distribution of the DNA index (DI) in 139 thyroid lesions. The two cases of medullary carcinoma and the two cases of Grave's disease were diploid

	DI<0.9	0.9<DI<1.1	1.1<DI<1.4	1.4<DI<1.6	1.6<DI<1.9	1.9<DI<2.1	DI>2.1
Nodular goiter	0% *n*=0	82.7% *n*=67	9.9% *n*=8	0% *n*=0	5% *n*=4	1.2% *n*=1	1.2% *n*=1
Fetal adenoma	0% *n*=0	41.7% *n*=10	25% *n*=6	20.8% *n*=5	8.3% *n*=2	4.2% *n*=1	0% *n*=0
Follicular adenoma[a]	0% *n*=0	100% *n*=18	0% *n*=0	0% *n*=0	0% *n*=0	0% *n*=0	0% *n*=0
Papillary carcinoma	11.1% *n*=1	88.9% *n*=8	0% *n*=0	0% *n*=0	0% *n*=0	0% *n*=0	0% *n*=0
Follicular carcinoma	0% *n*=0	42.9% *n*=3	42.9% *n*=3	14.2% *n*=1	0% *n*=0	0% *n*=0	0% *n*=0

[a] Follicular adenoma other than fetal adenoma

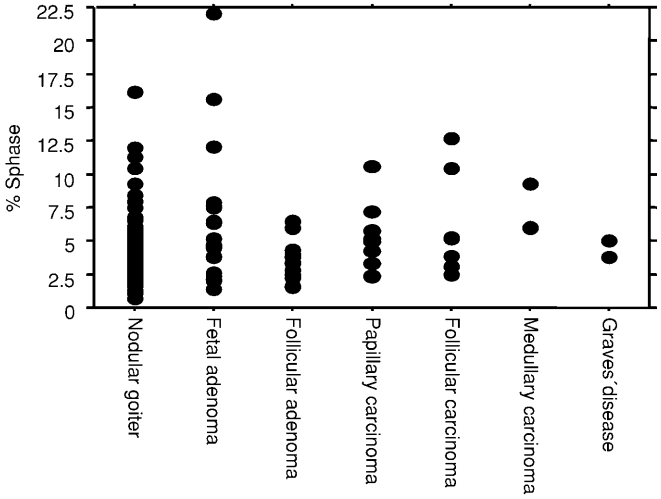


Fig. 1 Scattergram of the percentage of S-phase cells in the different groups of thyroid lesions according to the histological category

were compared with follicular carcinomas (6.1%) (Table 2).

All cases with a DNA content within the triploid range (*n*=6) were fetal adenomas, but one was a follicular carcinoma displaying a fetal adenoma-like growth pattern (Table 2; Fig. 2). The other three follicular carcinomas with an aneuploid DNA content also displayed foci of fetal adenoma-like growth pattern (Fig. 3, Fig. 4, and Fig. 5). Two of these four follicular carcinomas were composed of oxyphilic cells (Fig. 2 and Fig. 3). The study using image cytometry of the four aneuploid follicular carcinomas showed similar (triploid or near triploid) DIs in the peripheral, invasive foci of the lesions and in the central fetal adenoma-like foci (Table 3).

Table 2 Percentage of S-phase cells in 134 thyroid lesions. For technical reasons, the S-phase fraction was not calculated in four nodular goiters and one follicular adenoma

	% of S-phase cells (mean±SEM)
Nodular goiter (*n*=27)	4.5±0.3
Fetal adenoma (*n*=24)	6.5±1.0
Follicular adenoma (*n*=17)	3.5±0.3
Papillary carcinoma (*n*=9)	5.6±0.8
Follicular carcinoma (*n*=7)	6.1±1.5

Table 3 DNA indexes (DIs) of the four aneuploid follicular carcinomas with areas of fetal adenoma-like growth pattern calculated using image cytometry. No attempt was made to compare the DIs calculated using image cytometry and flow cytometry (see Materials and methods)

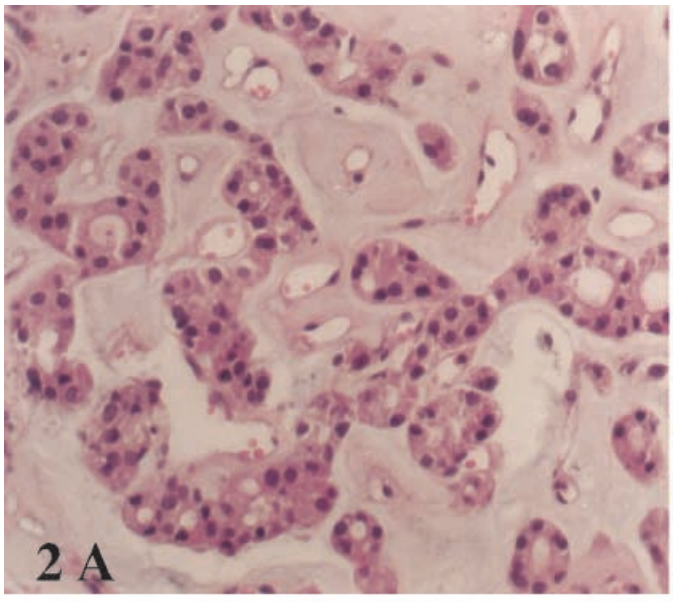
	Peripheral, invasive foci of the tumors	Fetal adenoma-like areas
Case 1 (Fig. 2A, B)	DI=1.6	DI=1.5
Case 2 (Fig. 3)	DI=1.8	DI=2.1
Case 3 (Fig. 4)	DI=1.7	DI=1.5
Case 4 (Fig. 5A, B)	DI=1.4	DI=1.6

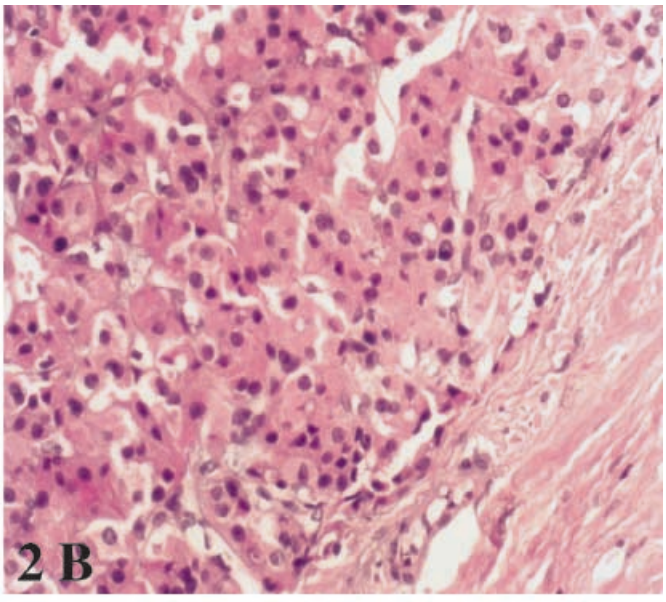
Fig. 2 Minimally invasive follicular carcinoma (capsular and vascular invasion not shown) composed of Hürthle cells and displaying fetal adenoma foci (**A**), together with solid foci (**B**). Both components had a DNA content within the triploid range. Hematoxylin and eosin (**A** and **B** ×660) ▶

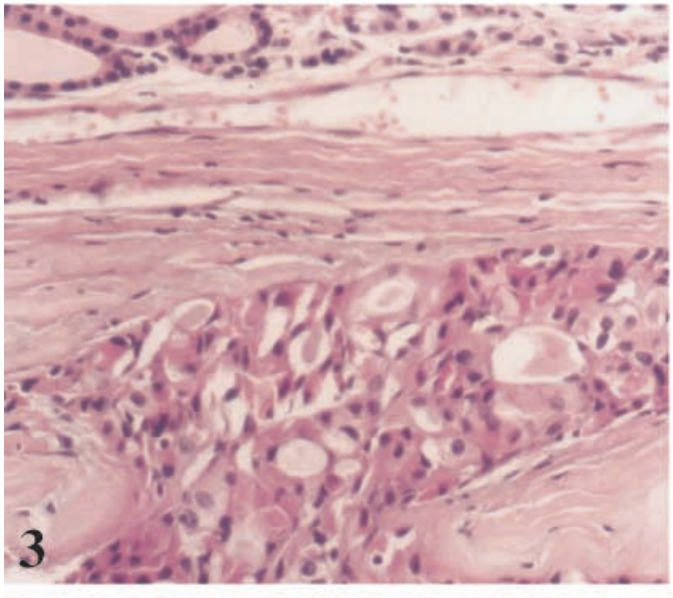
Fig. 3 Invasive periphery of a minimally invasive follicular carcinoma composed of Hürthle cells and displaying a hypotriploid DNA content. Hematoxylin and eosin (×660)

Fig. 4 Focus of fetal adenoma growth pattern in a minimally invasive follicular carcinoma with a hypotriploid DNA content. Hematoxylin and eosin (×330)

Fig. 5 Minimally invasive follicular carcinoma (capsular and vascular invasion not shown) with a predominant trabecular growth pattern with fetal adenoma foci (**A**) and displaying a hypotriploid DNA content (**B**). Hematoxylin and eosin (**A** ×330; **B** ×660)


2 A


2 B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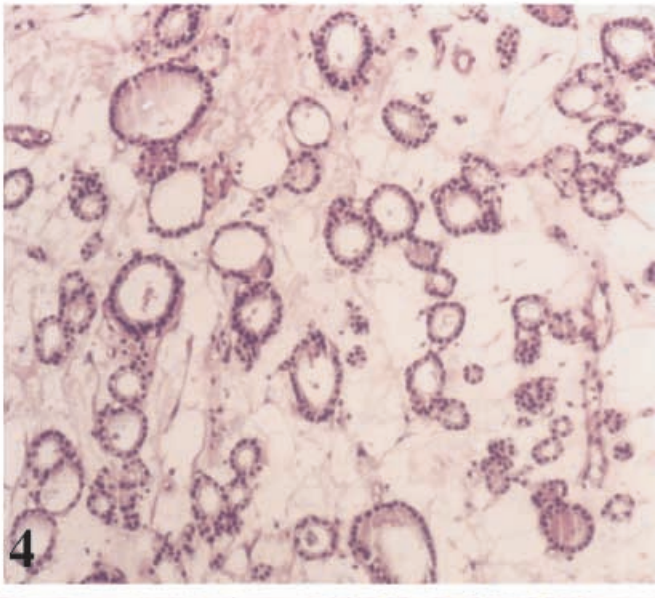
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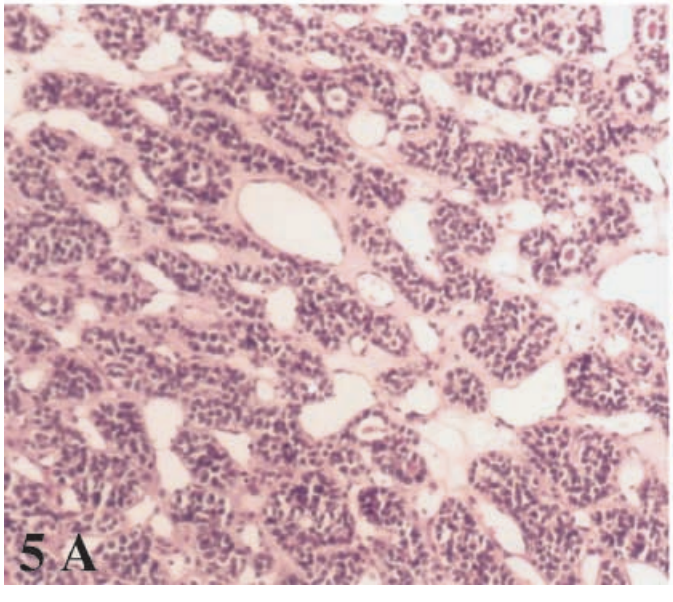
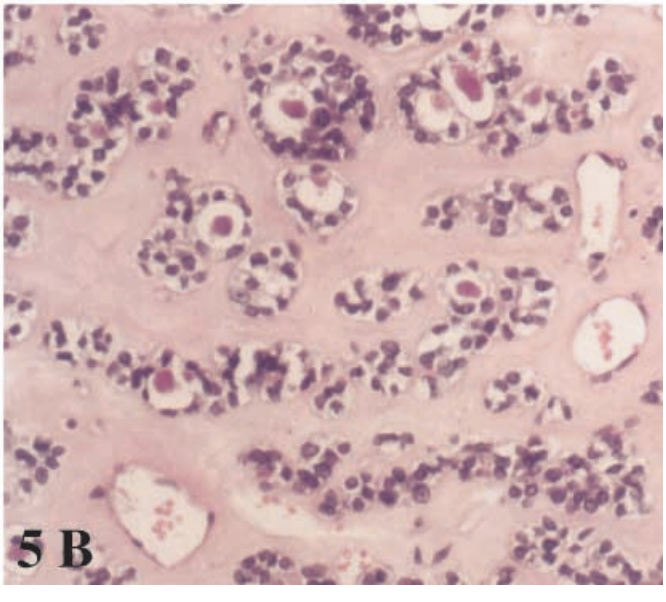

5 A


5 B

Discussion

In order to reduce technical artifacts as much as possible, namely those caused by the utilization of paraffin-embedded fragments, the flow cytometry study was restricted to cases from which fresh and/or frozen representative samples of the lesions had been collected and appropriately handled. Our results on the ploidy of thyroid lesions fit with most of the data on record. Aneuploidy can be detected both in benign and malignant lesions [2, 16, 22]. Within the group of benign lesions, aneuploidy is more frequent in adenomas than in nodular goiters [2, 11, 18, 22, 30, 33]. In the group of malignant tumors, aneuploidy is more frequent in follicular carcinomas than in papillary carcinomas [17, 21, 22, 31].

The results on the percentage of S-phase cells concur also with most of the previously reported data. Aneuploid lesions have a significantly higher percentage of S-phase cells than diploid lesions [11]. Benign lesions do not differ significantly from malignant tumors with regard to the percentage of S-phase cells [23]. There are a few cases of nodular goiter and fetal adenoma that display a higher percentage of S-phase cells than well-differentiated carcinomas [36]. The most interesting finding of the present study is the high frequency of aneuploidy in a subset of follicular adenomas. We have shown that aneuploidy in benign thyroid tumors is restricted to fetal and fetal/embryonal adenomas.

In the group of fetal adenomas, which includes many cases displaying a mixed fetal–embryonal pattern of growth [15], there were 58.3% aneuploid tumors in contrast to 0% in the group of normo or normo/macrofollicular adenomas. We have also observed aneuploidy in 18.5% of cases of nodular goiter, a percentage which is in accordance with the previously reported range of 10–22% [13, 18, 30, 37]. Most of the aneuploid nodules in the present series displayed either papillary hyperplasia or foci of fetal–embryonal growth pattern (data not shown) and may represent, according to some authors, true neoplasms rather than hyperplastic lesions [1].

The percentage of aneuploidy observed in our series of fetal adenomas (58.3%) is higher than those reported to date on follicular adenomas in general (18–52%) [11, 18, 22, 34]. This result reflects the individualization of fetal adenomas. In fact, if we put together all of the adenomas, the percentage of aneuploidy (33.3%) falls within the range on record. Follicular carcinomas constitute the other group of lesions displaying a high percentage of aneuploidy (57.1%). This finding fits with most of the results of other series that show a range from 15% to 65% of aneuploidy in follicular carcinomas [8, 18, 22, 35].

The available data on the ploidy of follicular adenomas and follicular carcinomas, together with the lack of MIN in most of these lesions [25, 38], point to CIN as a major pathway of tumorigenesis in this setting. This assumption is supported by our observation of four aneuploid minimally invasive follicular carcinomas displaying similar DIs in both peripheral and central fetal adenoma-like areas.

Apart from demonstrating that malignant transformation does occur in lesions looking like common fetal adenomas, our results raise a number of interesting issues from a cytogenetic standpoint. First, all of the lesions with a DNA content within the triploid range were fetal adenomas, but one was a follicular carcinoma with a fetal adenoma growth pattern. These findings fit with the well-known high prevalence of trisomies of chromosomes 5, 7, and 12 in follicular adenomas [2, 5, 9, 16, 30, 37], although one cannot identify DNA triploidy with (true) chromosome trisomy.

Second, the observation of aneuploidy in four minimally invasive follicular carcinomas with foci of fetal adenoma growth pattern and with a DNA content similar to that observed in many fetal adenomas (one triploid and three hypotriploid lesions) suggests the existence of a continuum of DNA content in these two groups of lesions. In contrast with other models of human carcinogenesis in which numerical chromosome abnormalities appear to parallel histological progression [10], our data support the assumption that the main differences between follicular carcinomas and follicular adenomas reside in subtle cytogenetic alterations (translocations or loss of heterozygozity) [24, 27, 29, 41] rather than gross numerical changes, as it was recently demonstrated by Kroll et al. [24]. These authors showed that the translocation t(2;3) (q13;p25), leading to formation of *PAX8_PPARγ*, was associated with the malignant transformation of follicular tumors. Finally, it is tempting to use the frequency and type of the DNA content abnormalities observed in the present series of follicular tumors to address the issue of the mechanisms leading to polyploidization in general and triploidization in particular.

According to Giaretti [14], the triploidization that can be observed in colo-rectal carcinoma would derive, in the first place, from endoreduplication of a hypodiploid cell, leading from a DI=0.8 to a DI=0.5 to a DI=3.2 by abnormal mitoses that would then divide “asymmetrically” into two daughter cells with DI values between 1.5–1.8 [14]. Another model of triploidization was advanced for testicular germ cell tumors by Oosterhuis et al. [28]. Germ cell tumors consistently show higher chromosome numbers than normal cells and usually stay in the triploid range. Polyploidization might be an early event, resulting in a tumor with a (near) tetraploid content, followed by chromosome loss [28].

Our data are obviously insufficient to clarify the mechanism underlying the aneuploidization of thyroid follicular tumors. Nevertheless, putting the aforementioned hypotheses [14, 28] together with the spectrum of the distribution of the DNA content in our series of fetal adenomas (Fig. 6), we would also consider, as a possible explanation for the aneuploidization in these tumors, the existence of an initial and highly unstable step of tetraploidization, followed by a gradual loss of chromosomes. However, if this was the case, we would expect a higher number of cases with a DI ranging between tetraploidy and triploidy (Fig. 6). Detailed FISH and comparative genomic hybridization (CGH) studies of a large series of fe-

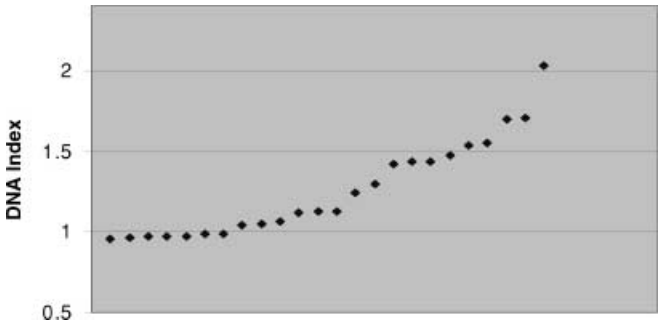


Fig. 6 Distribution of the DNA index in 24 fetal adenomas

tal adenomas and minimally invasive follicular carcinomas are necessary to progress the understanding of the aneuploidization pattern observed in these lesions and to detect any consistent cytogenetic and/or molecular genetic difference(s) between benign and malignant tumors.

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