Aneuploidy and high S-phase as biomarkers of poor clinical outcome in poorly differentiated and anaplastic thyroid carcinoma

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Abstract. This study aimed to investigate the prognostic influence of DNA flow cytometry and RAS gene mutations in patients with poorly differentiated (PDTC) and anaplastic thyroid carcinoma (ATC). The series consisted of 26 patients with PDTC and ATC, and a median follow-up of 10 months (range 1-138). DNA ploidy and S-phase fraction (SPF) were assessed by flow cytometry on frozen samples. RAS point mutations were detected using PCR techniques. Disease staging and tumour angioinvasion were included as prognostic parameters for survival analysis. Nineteen patients (73.1%) succumbed to the disease (median time 5 months; range 1-45). Eighteen tumours (69.2%) were classified as DNA aneuploid. Median SPF was 5.6% (range 1.9-23.1), which was used as a cut-off value to distinguish between low versus high cell proliferation. Three of 20 (15%) patients presented N-RAS gene mutations in codon 61. DNA aneuploidy was most frequently found in female patients (p=0.034). Kaplan-Meier and Cox regression analyses showed that only DNA aneuploidy (p=0.044 and p=0.055, respectively) and high SPF (p=0.001 and p=0.006, respectively) significantly correlated with worse survival. The results indicate that aneuploidy and high SPF are biomarkers of poor clinical outcome in PDTC and ATC, which may provide useful prognostic information with a potentially therapeutic impact in patient management.

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Introduction

Regarding their histological and biological behaviour, thyroid gland carcinomas constitute a heterogeneous group of malignant tumours (1). The majority present as well-differentiated papillary and follicular thyroid carcinomas, affecting patients who generally have a favourable prognosis and low mortality rate. The highest mortality of the disease is associated with poorly differentiated (PDTC) and anaplastic thyroid carcinoma (ATC) subtypes, which are rare forms of thyroid cancer prevalent mainly in areas of endemic goiter (2,3).

Despite the poor clinical outcome of PDTC and ATC (4,5), it is important to investigate prognostic biomarkers that may aid in the selection of high-risk patients, in whom more aggressive multimodal therapies would be indicated (6,7). However, reports on the identification of prognostic indicators of survival in PDTC and ATC are scarce.

DNA flow cytometry, a commonly used technology in assessing DNA ploidy and the proliferative activity of tumours, has been shown to have prognostic significance in differentiated thyroid carcinomas (8-11). In addition, RAS point mutations, which are known to occur among tumours that originated from the follicular epithelium of the thyroid gland (12), have been associated with aggressive tumour phenotypes and poor prognosis in thyroid cancer (13,14). Nonetheless, the potential clinical usefulness of the two techniques has yet to be clarified in PDTC and ATC.

This study aimed to evaluate the prognostic impact of flow cytometric DNA ploidy and S-phase fraction (SPF), as well as RAS gene mutations on the overall survival of patients with PDTC and ATC.

Materials and methods

Between January 1992 and December 2003, at least one fresh-frozen representative sample was collected for further DNA flow cytometric analysis from surgical resection specimens of patients with thyroid lesions (n=916) who underwent surgical treatment at the Instituto Português Oncologia, Lisbon, Portugal. Of these patients, 26 (2.8%) were diagnosed with
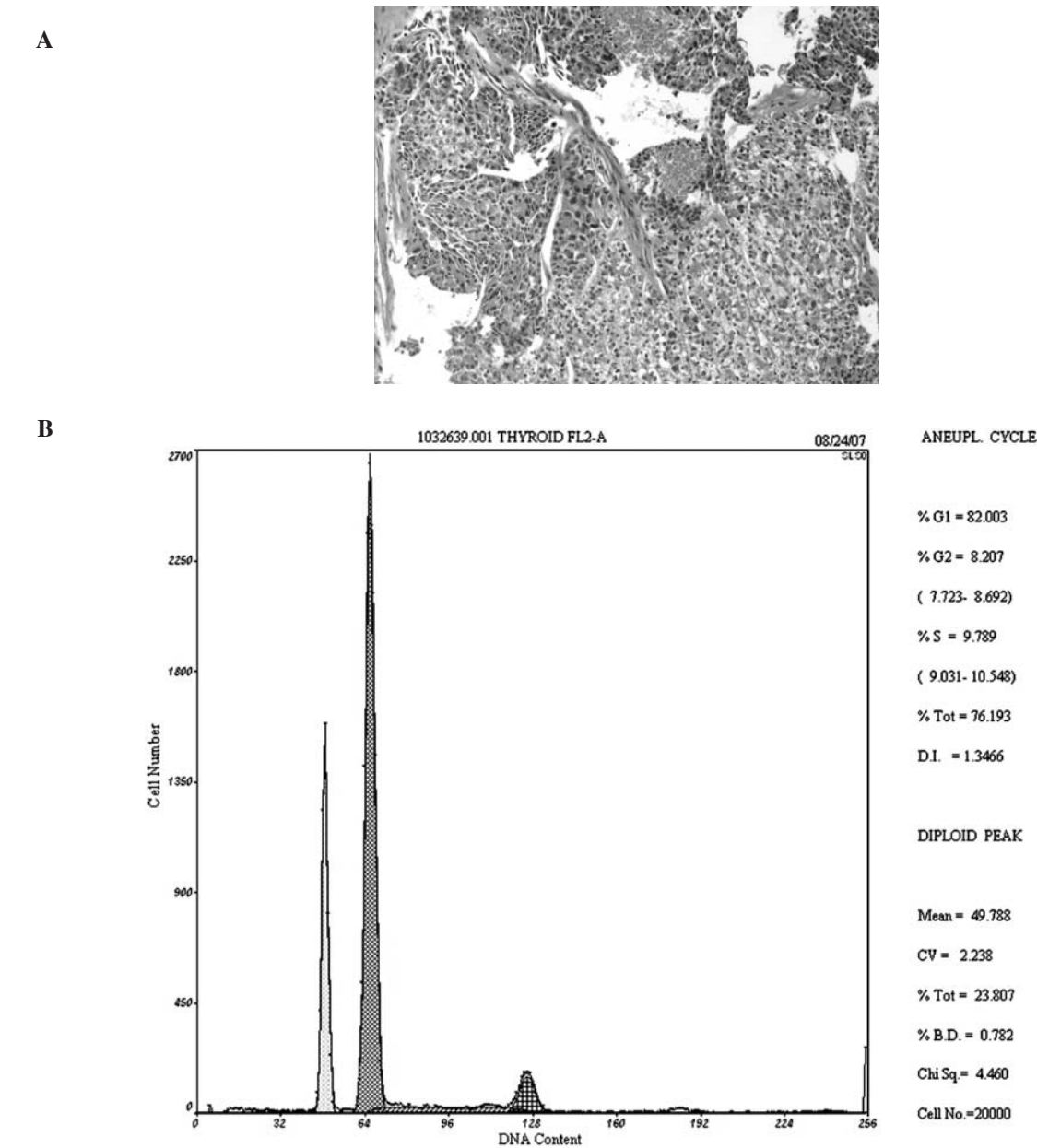
A

B

Figure 1. (A) A haematoxylin and eosin staining sample of a poorly differentiated thyroid carcinoma (original magnification, x100). (B) Its DNA histogram showing DNA aneuploidy (D.I. = 1.35) and high cell proliferation (SPF = 9.8%).

poorly differentiated and anaplastic thyroid carcinoma. Sixteen tumours were poorly differentiated thyroid carcinomas (PDTC) (Fig. 1A), 4 PDTC with minor (<10%) areas of anaplasia (PDTC/ATC), and 6 were exclusively undifferentiated (anaplastic) tumours (ATC). There were 21 women and 5 men, with a female/male ratio of 4.2/1. Median age of the patients was 67 years, ranging from 35 to 80. Table I shows a detailed clinicopathological characterization of the series investigated. Histopathological typing and staging were performed according to the TNM classification system (15). The patients were submitted to total thyroidectomy as primary surgical treatment. Other adjuvant therapies included radioactive [131]iodine (n=15), external radiotherapy (n=5) and chemotherapy (n=1).

The present study was approved by the appropriate institutional review board. Follow-up information was obtained after reviewing clinical records. The end-point of the study was the overall survival, defined as the period from diagnosis to poor survival or the last clinical observation. The median duration of patient follow-up was 10 months (range 1-138). By the end of the study, 19 (73.1%) patients had succumbed to the disease (median time 5 months; range 1-45) and 7 (26.9%) were alive.

DNA flow cytometry. Flow cytometry was performed on representative fresh-frozen samples of tumour tissue obtained at surgical resection (16). After mechanical disaggregation with scalpels to obtain a monodispersed cell suspension, the samples were treated with 0.05% Nonidet P40 non-ionic detergent (Sigma, USA) for cell lysis, and stained with a propidium iodide solution (Sigma; 50 μg/ml in Tris-MgCl$_2$ buffer), including ribonuclease (Sigma; 1 mg/ml in phosphate-

Table I. Clinicopathological characterization of the whole series.

Variables	No. (%)
Age (years)	
<45	4 (15.4)
≥45	22 (84.6)
Gender	
Female	21 (81.8)
Male	5 (19.2)
Primary tumour	
T4a	8 (30.8)
T4b	18 (69.2)
Nodal status	
N0	17 (65.4)
N1	8 (30.8)
N1b	1 (3.80)
Disease stage	
IVA	6 (23.1)
IVB	16 (61.5)
IVC	4 (15.4)
Tumour angioinvasion	
Negative	3 (15.0)
Positive	17 (85.0)

No., number of patients.

Table II. Characterization of the histopathological subgroups of thyroid carcinomas by DNA ploidy status.

Histopathology	No.	DNA ploidy	
		Diploid (%)	Aneuploid (%)
PDTC	16	7 (43.7)	9 (56.3)
PDTC/ATC	4	0 (0)	4 (100)
ATC	6	1 (16.7)	5 (83.3)

No., number of cases; PDTC, poorly differentiated thyroid carcinoma; PDTC/ATC, poorly differentiated thyroid carcinoma with minor areas of anaplasia and ATC, anaplastic thyroid carcinoma.

buffered saline) for digestion of the double-stranded RNA, for 1 h in the dark at 4˚C. After filtration with a 55-μm nylon mesh, the stained nuclei were analysed on an Epics Profile II flow cytometer (Coulter Electronics, Hialeah, FL, USA). Fluorescent microbeads were used for daily calibration of the instrument (Flow-Check Fluorospheres, Beckman Coulter). DNA histograms were analysed using the Multicycle program (Phoenix Flow Systems, San Diego, CA, USA), which includes a multiple option cell cycle fitting that automatically determines the DNA index. Histograms with a coefficient variation of >6% were not included in the study. The tumours were classified by the dichotomy diploid versus aneuploid according to the DNA ploidy pattern, and whenever possible, the SPF, as a measure of tumour proliferative activity, was also determined. Mixed, non-malignant diploid cells in the same sample analysed were used as internal reference standard.

RAS mutations detection. Fourteen PDTCs, 3 PDTC/ATCs and 3 ATCs were assessed for RAS mutations, while the remaining 6 cases lacked tumour material for reliable analysis.

Total RNA was isolated from frozen samples by TRIzol reagent (Life Technologies Inc., Gaithersburg, MD), according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA, at 37˚C, for 90 min, using oligo (dT) primers (Life Technologies Inc.) and reverse transcriptase (Superscript II, Life Technologies Inc.).

Polymerase chain reactions (PCRs) were performed with 100 ng of cDNA or genomic DNA, 10 pmol of the two primers (forward and reverse), 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 200 μM dNTPs (Amersham Pharmacia Biotech, Uppsala, Sweden), 1.5-2.0 mM MgCl$_2$ and 1 unit of Taq DNA polymerase (Life Technologies Inc.).

Negative controls for cDNA synthesis and PCRs, in which the template was replaced with sterile water, were included in each experiment. The RNA integrity and efficiency of cDNA synthesis were confirmed in each sample by reverse transcription polymerase chain reaction (RT-PCR) for the housekeeping gene phosphoglycerate kinase-1 (PGK) (17).

Primers were designed for cDNA and genomic DNA. PCR products contain the three mutational hot spots codons 12, 13 and 61 from N-, K- and H-RAS genes. The oligonucleotide primer sequences (Invitrogen, Paisley, Scotland) and the corresponding PCR conditions are available upon request.

PCR products were analysed by electrophoresis in a 2% agarose gel, purified using the GFX PCR DNA and Gel band purification kit (Amersham Pharmacia Biotech), and sequenced automatically (ABI PRISM 310 genetic analyser using the ABI PRISM big dye terminator cycle sequencing ready reaction kit version 2; Applied Biosystems, Foster City, CA).

Statistical analysis. Correlations between variables were evaluated using the Chi-square and Fisher's exact test when appropriate. The cumulative probability of overall survival was estimated according to the Kaplan-Meier method, and the differences between the survival curves were analysed by the log-rank test. A Cox regression model was used to assess the influence of each prognostic parameter on survival. $P \leq 0.05$ was considered as statistically significant.

Results

Overall, 18 tumours (69.2%) were classified as DNA aneuploid (Fig. 1B) and 8 (30.8%) showed a DNA diploid pattern. Table II illustrates the ploidy status of the different histopathological subgroups of thyroid carcinomas. Reliable SPF determination was achieved in 16 of 26 cases (61.5%), mostly in PDTC (13 cases). Median SPF was 5.6% (range 1.9-23.1), which was used as a cut-off value to distinguish between low versus high cell proliferation (Fig. 1B).
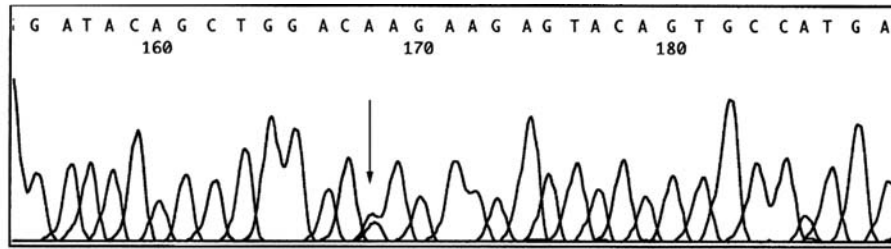
Figure 2. RAS gene mutation in a poorly differentiated thyroid carcinoma (the arrow indicates a mutation in codon 61 of N-RAS, which shows the transition CAA 'CGA, resulting in a *Gln 'Arg* substitution).
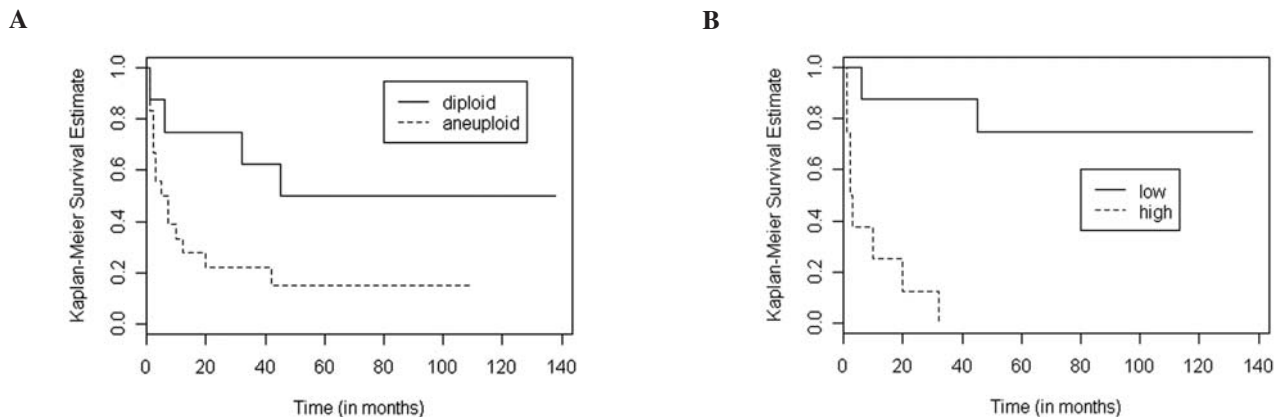

Figure 3. Significant differences between Kaplan-Meier overall survival curves according to (A) DNA ploidy (p=0.044) and (B) SPF (p=0.001) in PDTC and ATC.

Three of 20 (15%) patients, diagnosed with PDTC, presented RAS gene mutations. Two of them were alive at the end of follow-up. The mutations were found in codon 61 of N-RAS (Fig. 2). No mutations were detected in K- and H-RAS nor in the other codons of N-RAS, as well as in anaplastic tumours. Seventeen of 20 (85%) cases showed tumour angio-invasion.

DNA aneuploidy was most frequently found in female patients (17 of 21 cases), while 4 of 5 cases in men were DNA diploid (p=0.034). No other statistically significant correlations were found between variables.

Kaplan-Meier survival estimates revealed significant differences in relation with DNA ploidy (p=0.044) (Fig. 3A) and SPF (p=0.001) (Fig. 3B). The Cox regression analysis indicated that DNA aneuploidy showed a trend (p=0.055) and high SPF (p=0.006) which were significantly correlated with worse survival (Table III). Patients with anaplastic tumours (ATC; n=6) or minor areas of anaplasia (PDTC/ATC; n=4) succumbed to the disease, and showed an aneuploid pattern in 9 of 10 cases. In the subset of poorly differentiated thyroid cancer (PDTC; n=16), 2 of 7 patients (28.6%) with diploid tumours and 6 of 9 (66.7%) with aneuploidy also died of the disease.

Table IV illustrates the clinicopathological, molecular and therapeutic characteristics of a group of 7 patients with a favourable clinical course, who were alive at the end of follow-up. They are, in general, younger patients (median age 50 years; range 35-73) with histopathological diagnosis of poorly differentiated thyroid carcinoma (PDTC) and low proliferative (SPF<5.6%) diploid tumours.

Discussion

In thyroid gland carcinoma, as in every malignant tumour type, morbidity and mortality are higher in advanced disease stages. In our study, patients with PDTC and ATC were included in Stage IV of the disease. Therefore, their markedly poor prognosis is not surprising. Although there are some clinical and histological differences between them, we combined the two entities for prognostic and statistical purposes due to their rarity and similar disease stages. Despite the adverse clinical context, promising research on newer potentially therapeutic targets (18), anti-neoplastic drugs (19,20) and multimodal treatment approaches (6,7), led us to investigate putative prognostic biomarkers to better plan patient management in PDTC and ATC. However, due to few existing reports (21), we have had difficulty in the inter-laboratory comparison of the data.

It has been reported that within each histopathological subtype of thyroid carcinoma, the presence of DNA aneuploidy and a high DNA index are associated with worse prognosis (8,22). Some authors (9) have even added the nuclear DNA content parameter to the AMES risk group classification, to improve the prognostication of papillary thyroid cancer. In

Table III. Cox regression model for overall survival in PDTC and ATC.

Variable	Hazard risk	95% Confidence interval	P-value
Disease stage			
IVA[a]	1.00	-	-
IVB	2.74	0.76-9.84	0.12
IVC	3.51	0.78-15.9	0.10
Tumour angioinvasion			
Negative[a]	1.00	-	-
Positive	1.17	0.26-5.23	0.84
DNA ploidy			
Diploid[a]	1.00	-	-
Aneuploid	3.04	0.98-9.43	0.055
S-phase fraction			
Low[a]	1.00	-	-
High	19.1	2.28-159	0.006
RAS mutations			
Negative[a]	1.00	-	-
Positive	0.27	0.03-2.11	0.21

[a]Reference category; P-value, Wald test.

Table IV. Clinicopathological, molecular and therapeutic characterization of the group of patients alive at the end of follow-up.

Case no.	Age	Gender	Diagnosis	Ploidy	SPF	RAS	Angioinvasion	Stage	Therapy
5	56	Female	PDTC	D	3.7%	Neg	Pos	IVB	TT+[131]I
9	35	Female	PDTC	D	5.4%	Neg	Pos	IVB	TT+[131]I
11	45	Female	PDTC	D	2.6%	Neg	Nd	IVA	TT+[131]I
12	73	Male	PDTC	D	1.9%	Neg	Pos	IVB	TT+[131]I
18	68	Male	PDTC	A	5.4%	Pos	Pos	IVB	TT+[131]I+Ra
21	50	Male	PDTC	D	3.6%	Pos	Neg	IVA	TT+[131]I
26	50	Female	PDTC	A	Nd	Neg	Pos	IVA	TT+[131]I

PDTC, poorly differentiated thyroid carcinoma; SPF, S-phase fraction; D, diploid; A, aneuploid; Neg, negative; Pos, positive; Nd, not determined; TT, total thyroidectomy; ^{131}I, [131]iodine and Ra, radiotherapy.

our study on PDTC and ATC, as in others (22-24), DNA aneuploidy was found in a high proportion of tumours (69.2%), occurring nearly always in anaplastic carcinomas, and most frequently in female patients (only 1 of 5 men presented aneuploid tumours). Jónasson et al (25), in their large flow cytometric study on paraffin material of 494 thyroid carcinomas, showed that aneuploidy was present in 22 of 37 (78.6%) anaplastic tumours. In agreement with this series, in our study, where 9 of 10 cases showed aneuploidy, patients with anaplastic thyroid carcinomas (or minor areas of anaplasia) succumbed to the disease. The same adverse clinical outcome occurred in 6 of 9 patients (66.7%) with aneuploid poorly differentiated thyroid cancer. Therefore, our results indicate that DNA aneuploidy is associated (or shows a strong trend to be associated) with worse overall survival (Fig. 3A; Table III), adding meaningful prognostic information in PDTC and ATC.

Although the assessment of proliferative activity has been used as an important approach in determining the biological aggressiveness and metastatic potential of the neoplasms (26), little is known about the prognostic significance of SPF in thyroid carcinomas. Studies showed that in papillary (10) and medullary (11) thyroid carcinomas a high SPF correlates with poor outcome. In contrast with well-differentiated thyroid carcinomas, where the growth fraction is usually low (in our lab the median SPF for papillary, follicular and medullary thyroid carcinomas gradually increased from 2.4 to 3.6 and to 4.4%, respectively; unpublished data), the SPF in PDTC and ATC was consistently higher, with a median value of 5.6%. Similarly, by using other methods to determine cell

proliferation, Erickson *et al* (27) and Soares *et al* (28) showed that the percentage of Ki-67 and PCNA expression was higher in anaplastic and poorly differentiated/undifferentiated thyroid carcinomas, respectively. In our study, a high SPF showed a significant correlation with worse prognosis and a higher proliferative activity paralleling a higher aggressive clinical course (Fig. 3B; Table III).

Despite the apparent methodological advantage of using fresh-frozen tumour samples, SPF determination was not reliably assessed in 10 cases, and thus, the results should be viewed with caution. This drawback is due to technical reasons related with background debris, DNA multiploid clones and overlapping populations in DNA histogram analysis. Based on our previous DNA flow cytometry experience, this appears as a recurrent limitation of SPF analysis not only in the thyroid gland but also in other types of human cancer. The small number of SPF cases evaluated may explain the wide 95% confidence interval for the hazard risk of SPF in the Cox regression model (Table III).

In our study, RAS gene mutations were detected in a few cases (15%), and always in codon 61 of N-RAS, which is in keeping with previous studies reporting that mutations in K-RAS or in other codons of N- and H-RAS are rare in thyroid gland tumours (29). In contrast to others (14), who showed the association between RAS mutations and tumour aggressive behaviour, we failed to demonstrate the predictive value of RAS mutations in our series. It should be noted, however, that 2 of the 3 patients with the mutation were alive at the end of the follow-up (Table IV). This apparently favourable characteristic appears to be corroborated by the fact that no RAS mutations have been detected in the most aggressive anaplastic carcinomas.

Despite the gradually higher hazard risk observed in our study from Stage IVA to IVC of the disease (Table III), we did not find statistically significant differences in pathological staging, which may be related with the small sampling size.

A detailed characterization of the group of patients alive at the end of follow-up revealed that they are typically younger (median age 50 years), with poorly differentiated thyroid carcinoma and low proliferative diploid tumours (Table IV). The finding reinforces the view that older age, extent of the anaplastic component and high cell proliferation are adverse features associated with poor overall survival.

In conclusion, our data suggest that, even in these histopathological subtypes of thyroid cancer which usually display a poor clinical outcome, treatment may be individualised according to prognostic biomarkers. Thus, the complementary usefulness of DNA flow cytometry as a reliable prognostic tool in the clinical setting may pinpoint high-risk patients, who are potential candidates of newer and more aggressive multimodal therapies. Future clinical studies should therefore focus on identifying effective adjuvant therapies for this patient group.

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