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Jón G. Jónasson · Jón Hrafnkelsson

Nuclear DNA analysis and prognosis in carcinoma of the thyroid gland

A nationwide study in Iceland on carcinomas diagnosed 1955–1990

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Abstract The purpose of this study was to ascertain whether DNA ploidy status and S-phase fraction affected the prognosis of patients with carcinoma of the thyroid gland. We reviewed all malignant thyroid tumours diagnosed in Iceland from 1955 to 1990. In all, 494 thyroid carcinomas were diagnosed during that period. By analysing tumour material from paraffin blocks by flow cytometry we were able to evaluate the ploidy status in 424 tumours and the S-phase value in 417 tumours. We detected aneuploid cell populations in 9.7% of papillary carcinomas, 24.3% of follicular carcinomas, 42.9% of medullary carcinomas and 78.6% of anaplastic carcinomas. Some 57% of tumours, mainly papillary carcinomas, had an S-phase value of less then 3%, whereas most of the other histological types of carcinoma, including all the anaplastic tumours, had an S-phase value of ≥3%. Univariate analysis indicated that both ploidy status and S-phase fraction were significant variables. When taking into account known prognostic variables of thyroid carcinoma in a multivariate analysis, however, neither ploidy status nor S-phase value proved significant. We conclude that DNA ploidy status and S-phase values are not independent prognostic factors in thyroid carcinoma.

Key words Thyroid carcinoma · Flow cytometry · DNA ploidy · Prognostic factors

Introduction

Malignant tumours of the thyroid gland are rare, constituting about 1% of malignant tumours diagnosed in the Nordic countries each year [14]. In Iceland, however, the incidence is very high, about 2–3 times greater than in the other Nordic countries and one of the highest in the world [14, 26].

Thyroid carcinomas constitute a heterogeneous group of malignant tumours with regard to biological behaviour and clinical outcome. Histological classification into four main groups of thyroid cancer, identifies the papillary carcinomas at one end of the spectrum, with a generally benign clinical course, whereas at the other end of the spectrum the anaplastic carcinomas are among the most aggressive.

Although patients with the differentiated types of thyroid carcinomas have an overall mortality rate which is low some of these tumours do have a grave prognosis. It is very important to identify these aggressive neoplasms in order to give adequate therapy and to avoid overtreating the more benign lesions.

The prognostic significance of DNA measurements has been demonstrated in many malignant tumours, including breast carcinomas [38], ovarian carcinoma [27] and carcinoma of the endometrium [6, 30]. Investigations of DNA ploidy in thyroid neoplasms have yielded conflicting results. Some studies emphasize the prognostic value of ploidy status [1, 10, 16, 33], whereas other studies conclude that the ploidy analysis does not give significant additional prognostic information [8, 34, 35]. Very little has been published on the significance of the S-phase value in thyroid carcinomas.

The purpose of our study was to analyse the DNA content, including the ploidy status and the S-phase fraction of all carcinomas of the thyroid gland diagnosed in Iceland over a 36-year period and to seek to establish whether these data give prognostic information independent of well-recognized prognostic parameters in thyroid neoplasia.

Material and methods

Information about all patients diagnosed with thyroid carcinoma in Iceland in the period 1955–1990 was obtained from the Icelan-

J. G. Jónasson (✉)
Department of Pathology, University of Iceland,
P.O. Box 1465, 121 Reykjavík,
Iceland

J. Hrafnkelsson
Department of Oncology, University Hospital,
and Icelandic Cancer Registry, Reykjavík,
Iceland

dic Cancer Registry. After histological and clinical revision a total of 494 patients with clinically diagnosed thyroid carcinoma were identified. These patients were treated at different hospitals in Iceland. The follow-up period ended in January 1992, with a medium duration of 10.3 years.

Haematoxylin and eosin-stained sections from all available paraffin blocks of surgical specimens of the tumours were reviewed by one of the authors (JGJ), at the Department of Pathology, University of Iceland.

All the carcinomas were classified into main histological groups according to the World Health Organisation classification [18]. Two lymphomas of the thyroid gland were diagnosed in the period in question, but these were excluded from our study. The size of the tumour and the presence or absence of tumour outside the thyroid capsule was recorded, as well as the presence of lymph node or distant metastases.

Clinical variables such as patient's age at diagnosis, gender and time of diagnosis (year and month), were determined. Hospital records were consulted for clinical information on the diagnoses, treatment, clinical outcome and whether patients had died due to their thyroid carcinoma.

All death certificates were checked, and autopsy reports were consulted in selected cases in order to determine whether the thyroid carcinoma was a cause of death. Autopsy was performed in 34% of deaths and in 41% of the patients that died of thyroid carcinoma.

Flow cytometry

An archival representative paraffin block containing an adequate amount of tumour with as little necrosis as possible was selected after examination of all the H & E-stained sections from each tumour. If large areas of normal thyroid tissue were present in the block, these were masked when sections were taken. In sixty-six cases flow cytometric analysis was not performed. In 33 of those cases the tumour was too small (less than 0.5 cm in diameter), in 11 cases only decalcified material was available, in 15 cases only histology blocks from autopsy material was available, in 4 cases the tissue blocks could not be found and in 3 cases the carcinoma diagnosis was based on cytology material alone. Flow cytometry was thus done on 428 thyroid carcinomas.

The DNA content of tumour cells was analysed using a single-cell suspension prepared according to the method described by Hedley et al. [19] and Thornthwaite et al. [44] with some modifications. Sections of 50 μm thickness were cut from paraffin blocks of tumour of DNA analysis and a 3–5 μm section was cut for H & E staining to determine whether there was an adequate amount of tumour material in the block to be evaluated. The 50-μm section was dewaxed in xylene, rehydrated in a sequence of 100%, 95%, 70% and 40% ethanol and washed in distilled water. It was then resuspended in 1.8 ml of trypsin solution (1.25 g trypsin in 500 ml stock solution [trisodium citrate, 3.4 ml Nonidet P40 (0.1% v/v); spermine tetrachloride (1.5 ml) and TRIS (0.5 ml), pH 7.6]. The remaining pieces of tissue were sliced manually with dissecting scissors and placed in a shaking waterbath at 37° C overnight. The supernatant and debris were separated by extraction through a 50-μm nylon mesh and then centrifuged at 2000 rpm for 10 min. Nuclear isolation and DNA fluorochrome stains were combined by adding 1 ml of propidium iodide nuclear isolation medium (PI-NIM).

Nuclear DNA was measured partly using a Leitz MPV flow (Leitz, Wetzlar, Germany) and a Monroe OC8888 microcomputer with S-phase calculations as described by Stål et al. [40]. In a similar number of tumours the DNA content was measured using a FACScan flow cytometer (Becton Dickinson). The S-phase analyses and DNA index assessments were done on a histogram using the RFIT program of the Cell Fit system (Becton Dickinson). In 20 cases both types of flow cytometers were used with comparable results. A total of about 20000 cells were analysed for each tumour.

Inflammatory cells, stromal cells and other normal cells in the tissue served as internal diploid standard [19]. A diploid pattern was defined as a single G_0/G_1 peak on the DNA histogram and an aneuploid pattern as at least one other clearly distinct G_0/G_1 peak with a corresponding G_2M peak [10]. A tumour was considered tetraploid (polyploid) if more than 20% of tumour cells were in the G_2M peak, or if this figure was more than 15% with a clear G_2M peak of its own.

In our analysis we separated ploidy into two groups, diploid and aneuploid, the aneuploid group including tetraploid, polyploid and multiploid histograms. The S-phase values were divided into two groups, <3% and ≥3%.

In four instances we were unable to analyse the histograms for ploidy or for S-phase values, and in an additional seven cases we were unable to analyse the S-phase value.

Cox's regression [11] was applied to assess the predictive power of the various potential risk factors. Hazard ratios were estimated in a simultaneous regression using EGRET statistical software [12]. A *P*-value of less than 5% was regarded as significant. Survival rate corrected for intercurrent death was computed by means of the EGRET software program [12] using the Kaplan-Meier life table technique [28].

Results

From 1955 to 1990 a total of 494 thyroid carcinomas were diagnosed clinically in Iceland. In 424 cases the ploidy of the tumours was analysed and in 417 cases the S-phase was evaluated.

In Table 1 various known prognostic factors such as sex, age, stage (separated into tumour extent beyond the thyroid capsule, nodal metastases and distant metastases), histological classification, ploidy status and S-phase fraction are analysed within univariate analysis. Overall, 73 (17%) of 424 tumours were aneuploid, and 180 (43%) out of 417 tumours had an S-phase greater than or equal to 3%. In univariate analysis both ploidy and S-phase values proved significant variables for survival.

In Table 2 the results for DNA ploidy are depicted according to the histological classification of thyroid carcinomas. Only 9.7% of papillary carcinomas were aneuploid. The separation of the papillary carcinomas into microcarcinoma (≤1 cm in diameter) intrathyroidal and extrathyroidal carcinoma is shown. A slightly higher proportion (12.5%) of extrathyroidal papillary carcinomas were aneuploid. Overall 24.3% of follicular carcinomas were aneuploid. The separation of those into minimally invasive and widely invasive follicular carcinomas is shown. In Table 3 the S-phase values according to histological classification are depicted. More than two-thirds of the papillary carcinomas had S-phase values below 3%, whereas none of the anaplastic carcinomas had an S-phase value of less than 3%.

Of the 288 patients with diploid papillary carcinomas, 39 (13.5%) died of their disease. Eight (25.8%) of the 31 patients with aneuploid papillary tumours died. For the follicular carcinomas the death rate was very similar for diploid and aneuploid tumors. Six patients (11.3%) with diploid follicular carcinomas two patients (11.8%) with aneuploid follicular carcinomas died of their disease. All the patients with anaplastic thyroid carcinomas died of their disease. We only had nine medullary carcinomas in our series and were able to do ploidy analyses on seven

Table 1 Outcome of patients with thyroid carcinoma according to sex, age at diagnosis, extent of primary tumour, nodal status, presence or absence of metastases, histological classification, ploidy, and S-phase fraction. Results of a univariate analysis are shown

		Total number	Alive	Dead of disease		*P* value
				n	%	
Sex	Male	135	98	37	27.4	
	Female	359	287	72	20.1	0.03
	Total	494	385	109	22.1	
Age	≤45	156	155	1	0.1	
	>45	338	230	108	32.0	<0.001
	Total	494	385	109	22.1	
Extent of primary tumour	TO-3	380	334	46	12.1	
	T4	114	51	63	55.3	<0.001
	Total	494	385	109	22.1	
Nodal status	NO	360	303	57	15.8	
	N1–3	134	82	52	38.8	<0.001
	Total	494	385	109	22.1	
Metastases	MO	461	378	83	18.0	
	M1	33	7	26	28.8	<0.001
	Total	494	385	109	22.1	
Histo-logical classifi-cation	Papillary	374	317	57	15.2	<0.001
	Follicular	74	64	10	13.5	<0.001
	Medullary	9	4	5	55.6	0.07
	Anaplastic	37	0	37	100.0	<0.001
	Total	494	385	109	22.1	
Ploidy	Diploid	354	300	54	15.6	
	Aneuploid	70	36	34	48.6	<0.001
	Total	424	336	88	20.6	
S-phase	<3%	237	207	30	12.7	
	≥3%	180	126	54	30.0	<0.001
	Total	417	333	84	20.1	

Table 2 Results of ploidy analysis according to histological classification

	Total number	Total number measured	Diploid	Aneuploid	
				n	%
Papillary carcinoma	374	319	288	31	9.7
Microcarcinoma	(49)	(23)	(22)	(1)	(4.3)
Intrathyroidal	(174)	(160)	(147)	(13)	(8.1)
Extrathyroidal	(151)	(136)	(119)	(17)	(12.5)
Follicular carcinoma	74	70	53	17	24.3
Minimally invasive	(63)	(60)	(47)	(14)	(23.3)
Widely invasive	(11)	(10)	(7)	(3)	(30.0)
Medullary carcinoma	9	7	4	3	42.9
Anaplastic carcinoma	37	28	6	22	78.6
All carcinomas	494	424	351	73	17.2

Table 3 S-phase values according to histological classification

	Total number	Total number measured	S-phase <3%	S-phase ≥3%	% ≥3%
Papillary carcinoma	374	317	218	99	31.2
Follicular carcinoma	74	68	17	51	75
Medullary carcinoma	9	7	2	5	71.4
Anaplastic carcinoma	37	25	0	25	100
All carcinomas	494	417	237	180	43.2

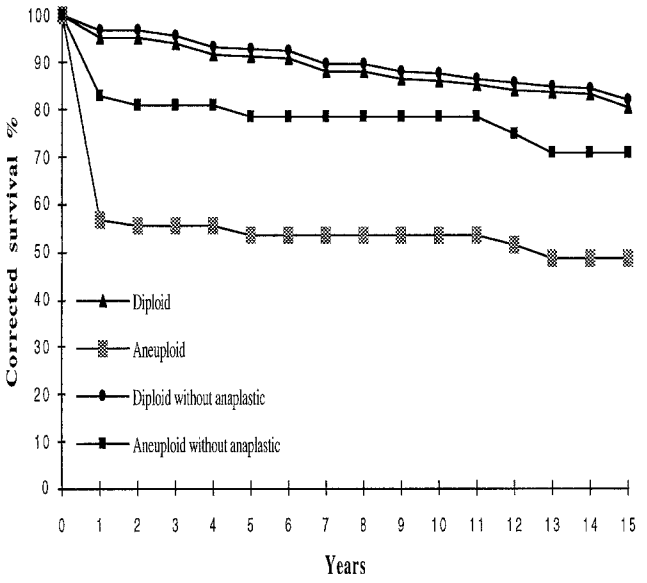

Fig. 1 Survival corrected for cause of death other than thyroid neoplasia in 424 patients with diploid or aneuploid carcinomas, with and without the inclusion of the anaplastic carcinomas

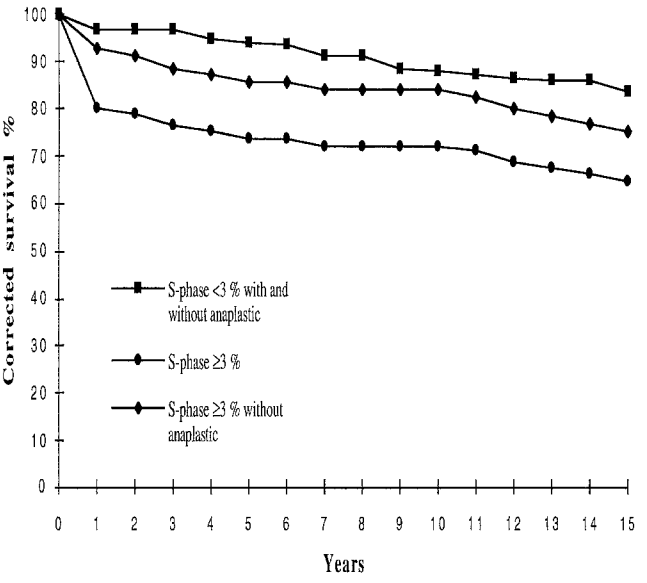

Fig. 2 Survival corrected for causes of death other than thyroid neoplasia in 417 patients with an S-phase value of <3% or ≥3% in their thyroid carcinomas, with and without the anaplastic carcinomas

Table 4 Factors associated with survival in patients with thyroid carcinoma (*ca.*) (Cox's proportional hazard model, simultaneous regression; DNA ploidy and S-phase fraction included)

	Hazard ratio	95% confidence limits	*P*-value
Year of diagnosis[a]	0.95	0.93–0.97	<0.001
Age[a]	1.06	1.04–1.08	<0.001
T4 vs others	3.7	2.1–6.5	<0.001
Nodal status (N0 vs N1–3)	2.1	1.3–3.3	0.002
Distant metastasis (M0 vs M1)	2.4	1.2–4.9	0.02
DNA ploidy (diploid vs aneuploid)	1.4	0.8–2.5	0.24
S-phase fraction (<3% vs ≥3%)	1.1	0.6–2.0	0.75
Anaplastic ca. (vs papillary ca.)	15.2	5.2–44.6	<0.001
Medullary ca. (vs papillary ca.)	4.0	1.0–15.8	0.05

[a] Continuous variables

of those tumours. None of the four patients with diploid tumours but all three patients with aneuploid medullary carcinomas died of their disease.

In Fig. 1 the survival curves, for all diploid and aneuploid tumours, corrected for intercurrent death, are shown. The curves for diploid and aneuploid tumours when the anaplastic carcinomas are excluded are also shown. DNA aneuploidy is seen to be associated with shorter survival. The separation between the ploidy curves is less distinct with anaplastic carcinomas excluded.

In Fig. 2 the survival curves for the S-phase values of thyroid carcinomas are shown in the same manner as for ploidy in Fig. 1, with and without the anaplastic carcinomas, and using the S-phase value of 3% as a cut-off point. The curves for S-phase <3% with and without the anaplastic carcinomas coincide, as no anaplastic tumours had such a low S-phase value. As in Fig. 1, the outcome of patients with S-phase values ≥3% is less poor when the anaplastic carcinomas were excluded.

In both Fig. 1 and Fig. 2, the separation of the curves occurs mostly in the first 2–3 years after the diagnosis; thereafter the curves are more or less parallel.

In a multivariate analysis using Cox's regression methods [11], histological type (anaplastic and medullary), increasing patient's age, tumour extension outside the thyroid capsule, positive lymph nodes and distant metastases were significant negative prognostic factors. This is shown in Table 4. We also found a similar significant negative correlation in our analysis between survival and the time during the study period when the tumour was diagnosed. Included in the table are results for both ploidy status and S-phase values, although neither proved to be an independent prognostic factor. The strongest negative prognostic parameter in this analysis was anaplastic histological type, bearing a risk of death 15.2 times greater than papillary types of carcinoma, and tumour extent beyond the thyroid capsule, with a 3.7 times greater risk of death, from the disease than intrathyroid tumours. Patient's age at diagnosis and the year of diagnosis were analysed as continuous variables and were found to be highly significant factors on multivariate analyses. Patient's sex was a borderline significant factor in univariate analysis, but did not prove significant in multivariate analysis.

Multivariate analysis was also done on all tumours with the exclusion of anaplastic carcinomas, and on papillary carcinomas alone. In neither of these analyses was DNA ploidy status or S-phase value an independent prognostic factor.

Discussion

In this study we evaluated all thyroid carcinomas clinically diagnosed in Iceland over a 36-year period. The very high incidence of thyroid carcinoma in Iceland is mainly accounted for by papillary carcinomas [26]. In our material, which excludes carcinomas diagnosed incidentally at autopsy, papillary type of carcinoma comprised 76% of thyroid malignancies. The sex ratio and age distribution of various histological types of thyroid tumours are comparable to those in other countries.

Long-term patient follow-up in tumour studies is usually difficult to obtain, sometimes making statistical analyses impossible. This is, however, not a problem in Iceland, where the population is well defined and patients are followed-up by a limited number of clinicians at very few institutions. The mean duration of follow-up in our series was 10.9 years.

We were able to analyse the DNA content of thyroid carcinomas for ploidy status in 86% and for S-phase fraction in 84% of tumours. In the past 10–15 years large number of papers have been published on nuclear DNA content of tumours of various organs of the body. For some tumours DNA ploidy status and/or S-phase fraction are now accepted as independent prognostic variables [6, 27, 30, 38]. Although a number of studies have been performed on DNA analyses in thyroid tumours (both benign and malignant), as well as non-neoplastic conditions, these are derived from a limited number of institutions and researchers, and only a few investigators have published series exceeding 150 patients. The overall results have been very variable, regarding both percentage of aneuploidy in various thyroid lesions and ploidy status of malignant tumours. In the early days of thyroid ploidy research, investigators were hopeful that ploidy status would help distinguish follicular carcinomas from follicular thyroid adenomas or adenomatoid nodules [3]. More recently a number of researchers have published evidence to the contrary [15, 16, 32].

The results of the ploidy analysis in our study show an overall low proportion of aneuploidy in thyroid malignancies (Table 2). We detected aneuploid cell populations in 9.7% of papillary carcinomas, 24.3% of follicular carcinomas, 42.9% of medullary carcinomas and in 78.6% of anaplastic carcinomas. The reported percentage of aneuploidy of papillary carcinomas varies considerably but most studies report under 30% [21, 23, 24, 36, 41, 45, 47]. Some studies, however, report over 60% aneuploidy in papillary carcinomas [8, 17, 31]. In some publications the rate of aneuploidy was similar for follicular adenomas and papillary carcinomas [8, 21, 31, 36], and in two of these reports the rate of aneuploidy in nodular goiter was around 10% [21, 31].

The rate of aneuploidy in follicular carcinomas also varies, with some reports of around 30% aneuploidy [22, 46], but most around 60–65% [9, 15, 16, 21, 23, 31, 36]. The range of reported rate of aneuploidy for medullary carcinoma is from 17% [39] to 57% [23]. Much less variation is seen in reported rates of aneuploidy for anaplastic carcinomas, ranging from 68% to 100% [29, 36, 42].

The obvious discrepancy among studies on the rate of aneuploidy in tumours and non-tumour conditions of the thyroid gland is difficult to explain but could be due in part to differences in interpretation of histograms and/or the cytometry technique used. In this study we used the S-phase value of 3% as a cut-off point. This gave a fairly even separation into two groups, with 57% of tumours having and S-phase value of less than 3% and 43% of tumours having an S-phase value of ≥3%. Most of the papillary carcinomas had a low S-phase value, but most of the other histological types of tumours, including all the anaplastic carcinomas, were in the high S-phase group. In Table 1 it can be seen that 30.0% of patients in the high S-phase group died of disease, compared to 12.7% in the low S-phase group. The results for ploidy were 48.6% death of disease for aneuploid tumours against 15.6% for diploid tumours. In a univariate analysis using Cox's regression, both ploidy status and S-phase values proved significant ($P<0.001$).

When known prognostic factors of thyroid carcinoma, namely age, tumour stage and histological type, as well as the year of diagnosis, were taken into account multivariate analysis was performed (Table 4), DNA ploidy status and S-phase values did not prove to be independent prognostic variables. We also analysed our results with cut-off points other than 3% for the S-phase values, with similar results. It is worth emphasizing that of the seven patients with medullary carcinomas analyzed, all three with aneuploid tumours died of their disease but the four with diploid tumours did not succumb to their carcinoma. These findings are of interest, although the numbers are too small to support statistical analysis.

In the literature on the prognostic value of ploidy analysis in thyroid neoplasia there are a number of positive reports [1, 2, 10, 13, 16, 17, 33, 35, 43, 45]. Others are not supportive [5, 7, 8, 22, 34, 36, 37, 39, 41, 42]. When analysing the reports in favour and against ploidy as a prognostic factor, we could not detect any difference in results between the papers reporting a high rate of aneuploidy and those with a lower rate of aneuploidy. Although the number of reports on the ploidy of thyroid tumours is considerable, relatively few studies with multivariate analyses have been published and only very few have had more than 100 patients [4, 8, 17, 23, 43].

Other studies have, like ours, been unable to detect prognostic significance of the S-phase value in thyroid neoplasia [24, 45].

There is clearly a need for more and larger studies on the DNA content and prognosis in thyroid carcinomas.

The main advantages of our study are the relatively high number of patients and the fact that it is a nationwide study in a well-defined population. We conclude that, although clearly significant in a univariate analysis, DNA ploidy status and S-phase values of thyroid carcinomas were not significant in a multivariate analysis and are therefore not regarded as independent prognostic variables.

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