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Flow cytometric DNA analysis of 20 bronchopulmonary neuroendocrine tumours

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ABSTRACT: Deoxyribonucleic acid (DNA) content of bronchopulmonary neuroendocrine tumours was measured by flow cytometry in order to investigate correlations between ploidy, S-phase fraction (SPF) and two histological classifications (World Health Organization (WHO), Warren and Gould), and clinical staging.

A paraffin-embedded technique was used on 20 surgical specimens. Cases comprised (according to the classification of Warren and Gould) 7 carcinoids, 3 well-differentiated neuroendocrine carcinomas (WDNC), 6 intermediate neuroendocrine carcinomas (INC), and 4 small cell neuroendocrine carcinomas (SCNC).

DNA aneuploidy was demonstrated in 3 out of 7 of the carcinoids, 3 out of 9 of the WDNCs and INCs, and 2 out of 4 of the SCNCs. A variable SPF was found in each group, except for the SCNCs which showed a constantly high SPF. In our small series, no correlation was noted between high SPF or aneuploidy and metastases.

In conclusion, we observed no diagnostic value of malignancy for DNA aneuploidy. The SCNC group appeared to be an homogeneous group according to the SPF compared to the small cell carcinoma (SCC) group of the WHO classification. This and the prognostic incidence of high SPF need to be further studied.

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Neuroendocrine tumours of the bronchopulmonary tract include very low grade neoplasms, corresponding to carcinoids with rare and late metastases, and very aggressive carcinomas, corresponding to small cell carcinomas. They constitute the extremities of a histological spectrum of neuroendocrine cellular proliferations, which includes two intermediate groups recognized by several authors [1, 2]: GOULD *et al.* [1] defined a group of well-differentiated neuroendocrine carcinomas (WDNC) with frequent metastases, clearly distinct from carcinoids, (previously identified as atypical carcinoids according to the World Health Organization (WHO) classification [3], and a distinct group of aggressive neuroendocrine carcinomas with intermediate cell type (INC). Histological patterns did not allow exact prediction of local nodal or distant metastases and survival [4, 5].

In addition to histological grade and clinical stage, tumour behaviour also depends on proliferative activity and sensitivity to drug therapy. The proliferative activity can be estimated by analysis of H^3-thymidine labelling indices or indirectly by measurement of cellular deoxyribonucleic acid (DNA) content, using flow cytometry or cytophotometry. DNA ploidy and S-phase fraction (SPF) are now demonstrated to be clinically relevant to the prognosis of some malignant diseases [6, 7], such as carcinomas of the prostate, urinary bladder, ovary, melanomas [8], and childhood acute lymphoblastic leukaemia [9]. Flow cytometry using paraffin-embedded tissue [10, 11] allows retrospective study of tumours of rare occurrence, *e.g.* bronchopulmonary carcinoids (1–6% of lung tumours), and tumours rarely resected, *e.g.* small cell neuroendocrine carcinomas (SCNC). Several studies have demonstrated correlations between techniques on fresh and paraffin-embedded tissues [10, 11], meanwhile, good resolution of the histograms had to be obtained.

The purpose of this study was to analyse flow cytometric characteristics, such as ploidy (DNA index) and S-phase fraction in neuroendocrine tumours belonging to the whole spectrum of malignancy. Correlations among flow cytometric characteristics were investigated and an attempt was made to evaluate relationships between these characteristics, histological classifications (Warren & Gould and WHO classifications) and clinical staging.

Materials and methods

Between 1977 and 1988, surgical specimens of 44 bronchopulmonary neuroendocrine tumours were examined at the Department of Pathology of the Hotel-Dieu Hospital. Only 20 cases fixed in 10% formalin were available for flow cytometric study (others were eliminated because of fixation in Bouin's fluid).

The 20 cases comprised 14 men, (mean age 64 yrs, range 41–78 yrs) and 6 women (mean age 40 yrs, range 20–64 yrs). Mean age for all patients was 53 yrs. Surgery consisted of lobectomy (9 cases), bilobectomy (2 cases), pneumonectomy (6 cases) and mediastinal biopsy (3 cases). The slides were reviewed by three researchers (SC, FC and CG). Clinical staging, TNM (T: primary tumour; N: regional lymph node metastasis; M: remote metastasis) was performed according to Union Internationale Contre le Cancer (UICC) 1987 [12]. Information on survival was obtained for 13 patients. The mean follow-up time was 13 months. Five patients were dead at the time of analysis, four because of tumour dissemination and one because of surgical complications.

Flow cytometry was performed on paraffin-embedded tumour tissue of primary site, using the method of HEDLEY *et al.* [10]. Three 30 μ thick sections were cut (more sections for small samples) and a control 5 μ thick section was stained with haematoxylin-eosin. Thick sections were dewaxed in xylene (3×10 min), rehydrated through decreasing concentrations of ethanol (100, 96, 70 and 50%), and washed twice in distilled water. Single nuclei suspensions were obtained by enzymatic digestion with 0.5% pepsin (Sigma Chemical Co., St Louis, MO, USA), in 2 ml NaCl (pH 1.5) at 37°C for 30 min, with intermittent agitation. The samples were centrifuged (×1,000 rpm, 5 min), washed in phosphate-buffered saline (PBS) (pH 7.5), pipetted several times using a fine needle (200 μ diameter) to obtain an homogeneous suspension and filtered through a nylon gauze (pore size, 75 μ). Suspensions were centrifuged once more; the nuclear pellets were resuspended and mixed for 1 h at 37°C with intermittent agitation, in a DNA staining solution of 1 ml of propidium iodide (PI) (Sigma), using 50 $\mu g \cdot ml^{-1}$ Tris/HCl buffer (pH 7.5), and 120 $\mu g \cdot ml^{-1}$ ribonuclease A (RNase A). These suspensions provided 10^5 to 10^6 $nuclei \cdot ml^{-1}$.

The analysis of cellular DNA content was performed on a fluorescence-activated cell sorter (FACS) IV (Becton Dickinson, Sunnyvale, CA, USA). The precision of the instrument was monitored using fluorescent plastic spheres (Odam), with a coefficient of variation of fluorescence less than 3%. The percentage of the S-phase fraction (SPF) was calculated using the "Broadened rectangle" mathematical model (Hewlett-Packard software). The tumour samples contained normal diploid stromal cells, which served as internal control, giving a DNA diploid peak in all histograms. The first peak on the left corresponded in most cases to diploid cells, either from the tumour or from the stroma; the location of the G2M peaks served as control for the position of the diploid and aneuploid peak when a second peak was noted (fig. 1). At least 10,000 to 20,000 nuclei were measured per specimen.

Tumours were categorized as either diploid or aneuploid and divided into two groups according to the SPF, with SPF <38% and SPF >38%; the 38% level corresponded to the mean of overall SPF. The relationship between diploid and aneuploid tumours, SPF, histology and TNM was analysed by Fisher's exact test.

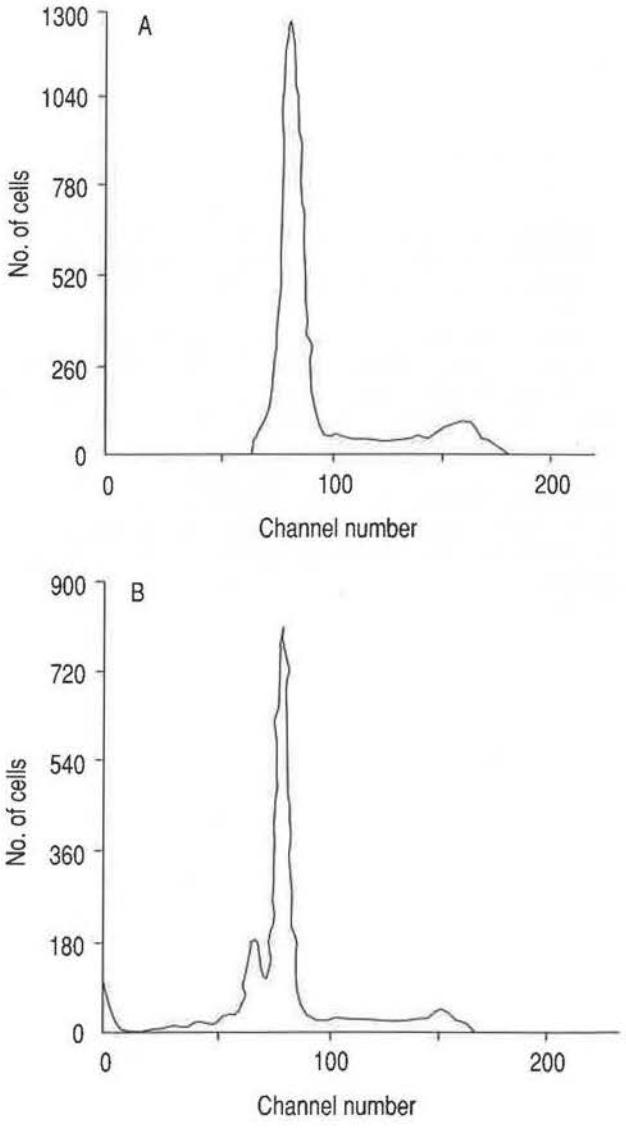

Fig. 1. – A) Deoxyribonucleic acid (DNA) histogram of a diploid neuroendocrine carcinoma of intermediate cell type (INC). The left peak G0 G1 has a coefficient of variation (CV) of 5.4%. The second peak corresponds to the G2+M phase. S-phase fraction of 16±1.5% (SD) is calculated using the broadened rectangle mathematical model. B) DNA histogram of an aneuploid INC. The left peak G0 G1 representing diploid cells has a CV of 5.1%. The second aneuploid peak G0 G1 has a CV of 3.6% and a DNA index of 1.2. The small third peak with a fluorescence intensity twice that of the aneuploid cells G0 G1 peak represents aneuploid cells in the G2+M phase. S-phase fraction is 20±0.5%.

Results

The tumour slides were reviewed according to the classifications of Warren & Gould and WHO: according to the former classification, the 20 cases comprised seven carcinoids (fig. 2), three well-differentiated neuroendocrine carcinomas (WDNC) (fig. 3), six intermediate neuroendocrine carcinomas (INC) (fig. 4), and 4 small cell neuroendocrine carcinomas (SCNC) (fig. 5).

Interpretable DNA histograms were obtained for all tumours. The mean coefficient of variation was 5.3%, with a range of 3.6–7.2%. SPF could be calculated in 18 cases (90%). Because of the small number of patients in each group, flow cytometric data were reported individually, and compared with the histological grade according to the two classifications (figs 6 and 7) and with the clinical data (tables 1 and 2).

Twelve of the 20 cases showed a DNA diploidy, and eight showed DNA aneuploidy. Seven of the DNA aneuploid tumours were hyperdiploid within a range of DNA index from 1.1 to 1.8; one case was hypodiploid with a DNA index of 0.7. DNA aneuploidy was demonstrated in three of the seven carcinoids, in one of the three WDNC, and in two of the six INC. DNA aneuploidy was found in two of the four SCNC. There was no significant difference for ploidy between each group.

Six of the 8 aneuploid cases showed an advanced clinical stage (>T1 N0 M0), but no correlation was found between aneuploidy and advanced clinical stages since 9 of the 12 DNA diploid tumours also had a clinical stage more advanced than T1 N0 M0 (table 2).

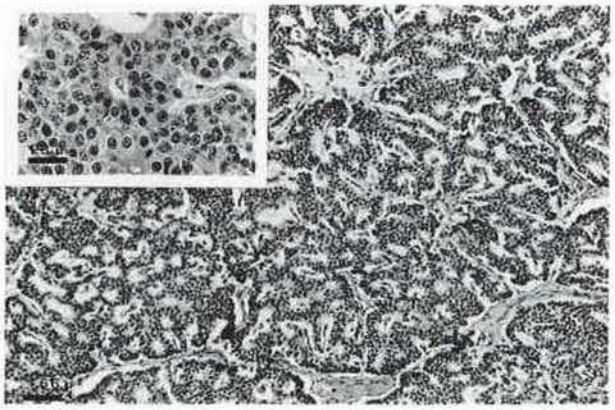

Fig. 2. – Bronchopulmonary carcinoid. Proliferation of trabecular pattern separated by rich vascularized fibroconnective tissue strands (haematoxylin-eosin-safranin (HES), scale bar=66 μ. Inset: cellular monomorphism of medium-sized cells with a rather abundant, finely eosinophilic granular cytoplasm and small round nucleus with clumped chromatin (HES, scale bar=23 μ).

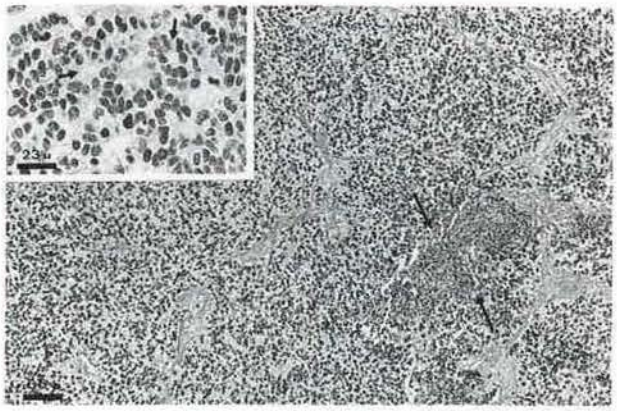

Fig. 4. – Neuroendocrine carcinoma of intermediate cell type (INC). The proliferation shows clusters with an area of necrosis (arrows), (haematoxylin-eosin-safranin (HES), scale bar=66 μ. Inset: rosette-like structures (arrows). Irregular-shaped nuclei with vesicular chromatin. Abundant, finely granular and eosinophilic cytoplasms (HES, scale bar=23 μ).

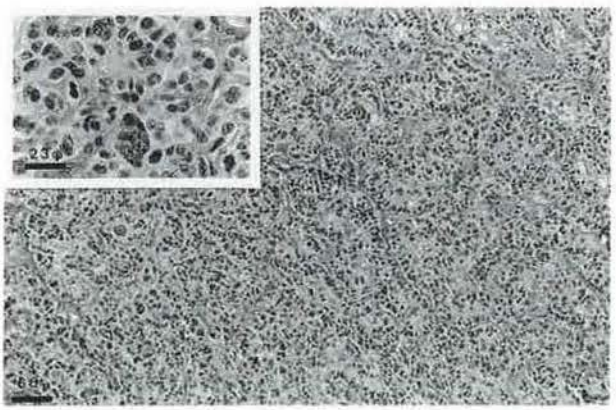

Fig. 3. – Well-differentiated neuroendocrine carcinoma (WDNC). The proliferation shows a trabecular pattern with large ribbons of pleomorphic cells (haematoxylin-eosin-safranin (HES), scale bar=66 μ). Inset: marked cellular and nuclear pleomorphism with abundant pale cytoplasm and irregular clumped chromatin (HES, scale bar=23 μ).

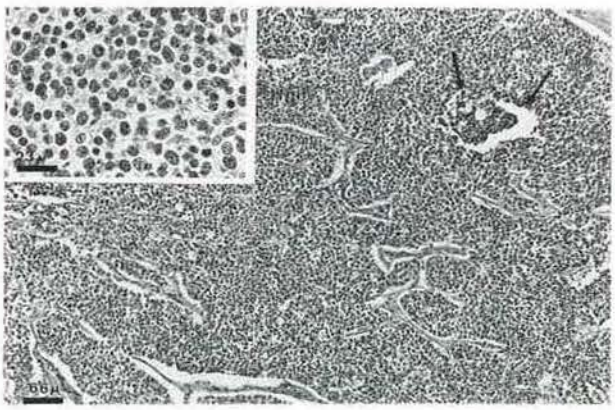

Fig. 5. – Neuroendocrine carcinoma of small cell type (SCNC). Densely packed proliferation of small-sized cells with areas of necrosis (arrows), (haematoxylin-eosin-safranin (HES), scale bar=66 μ. Inset: hyperchromatic small nuclei with dusty chromatin. Scanty and ill-defined cytoplasms. Absence of rosette-like structures (HES, scale bar=23 μ).

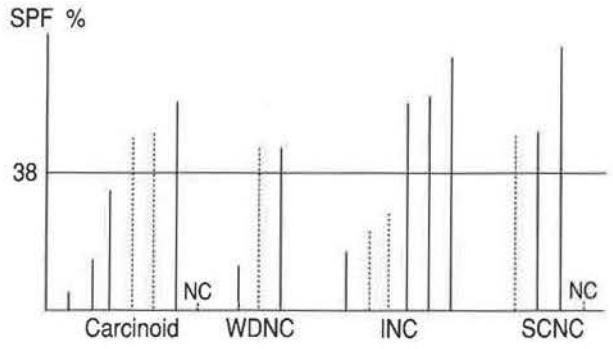

Fig. 6. – Histogram of ploidy and S-phase fraction (SPF) according to the classification of Warren and Gould. WDNC: well-differentiated neuroendocrine carcinomas; INC: intermediate neuroendocrine carcinomas; SCNC: small cell neuroendocrine carcinomas; NC: non-calculated SPF; ——: diploid tumour; · · · ·: aneuploid tumour.

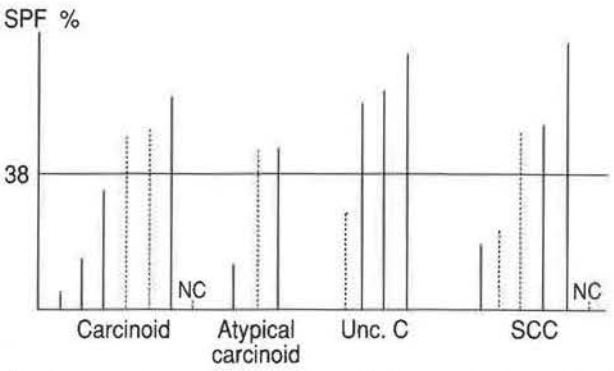

Fig. 7. – Histogram of ploidy and S-phase fraction (SPF) according to the WHO classification. Unc. C: unclassified carcinomas; SCC: small cell carcinomas; NC: non-calculated SPF: —— : diploid tumour; · · · ·: aneuploid tumour.

Table 1. – Clinical and flow cytophotometric data according to the classification of Warren and Gould

Case no.	Warren & Gould classification (Type of surgery)	Age yrs	Sex	Tumour Size cm	TNM (UICC)	Follow-up outcome	Ploidy/SPF
1	Carcinoid (Lobectomy)	51	M	3	T1N1M0	5 yrs alive	D/31
2	Carcinoid (Lobectomy)	20	F	2	T1N0M0	3 months alive	D/13
3	Carcinoid (Lobectomy)	38	F	3.5	T2N0M0	NF	A/46
4	Carcinoid (Lobectomy)	64	M	1	T1N0M0	8 months alive	D/54
5	Carcinoid (Lobectomy)	57	M	2.5	T1N0M0	NF	A/NC
6	Carcinoid (Lobectomy)	27	F	4	T2N0M0	NF	D/5
7	Carcinoid (Lobectomy)	49	F	5	T2N1M0	NF	A/44
8	WDNC (Lobectomy)	64	F	6.5	T2N0M0	11 months alive	D/41
9	WDNC (Pneumectomy)	70	M	2.5	T1N0M0	7 months alive	A/41
10	WDNC (Lobectomy)	49	M	2	T1N0M0	7 months alive	D/12
11	INC (Pneumectomy)	56	M	5	T2N0MX	NF	D/64
12	INC (Pneumectomy)	64	M	6	T4N1M1	12 months dead	A/25
13	INC (Lung Biopsy)	58	M	-	T4NXMX	NF	D/55
14	INC (Lobectomy)	78	M	1.5	T1N1MX	NF	D/53
15	INC (Lung Biopsy)	49	M	-	TXN3M1	10 months dead	D/16
16	INC (Lobectomy)	56	M	5.5	T2N2MX	1 month dead	A/20
17	SCNC (Pneumectomy)	41	M	3	T1N2M0	33 months dead	D/46
18	SCNC (Lymph node biopsy)	-	F	-	TXN2MX	NF	D/67
19	SCNC (Pneumectomy)	61	M	4.5	T2N1M2	4 months dead	A/44
20	SCNC (Pneumectomy)	59	M	10	T4N2M1	5 months alive	A/NC

WDNC: well-differentiated neuroendocrine carcinomas; INC: intermediate neuroendocrine carcinomas; SCNC: small cell neuroendocrine carcinomas; TNM: primary tumour, nodal metastasis, remote metastasis (clinical staging); UICC: Union Internationale Contre le Cancer; NF: no follow-up; SPF: S-phase fraction; D: diploid; A: aneuploid; NC: non-calculated; X: size of tumour unknown.

Table 2. – Flow cytophotometric data according to the classifications of Warren and Gould, WHO and TNM

Case no.	Classifications		Ploidy (DNA index)	SPF % (SD)	TNM (UICC)
	Warren & Gould	WHO			
1	Carcinoid	Typical C	D	31 (4.4)	T1N1M0
2	Carcinoid	Typical C	D	13 (2.5)	T1N0M0
3	Carcinoid	Typical C	A (1.1)	46 (0.6)	T2N0M0
4	Carcinoid	Typical C	D	54 (0.9)	T1N0M0
5	Carcinoid	Typical C	A (0.7)	NC	T1N0M0
6	Carcinoid	Typical C	D	5 (6.3)	T2N0M0
7	Carcinoid	Typical C	A (1.2)	44 (0.7)	T2N1M0
8	WDNC	Atypical C	D	41 (0.9)	T2N0M0
9	WDNC	Atypical C	A (1.8)	41 (1.5)	T1N0M0
10	WDNC	Atypical C	D	12 (4.4)	T1N0M0
11	INC	Unclassified	D	64 (1.4)	T2N0MX
12	INC	Unclassified	A (1.8)	25 (3.7)	T4N1M1
13	INC	Unclassified	D	55 (0.6)	T4NXMX
14	INC	Unclassified	D	53 (0.3)	T1N1MX
15	INC	SCC	D	16 (1.5)	TXN3M1
16	INC	SCC	A (1.2)	20 (0.8)	T2N2MX
17	SCNC	SCC	D	46 (0.6)	T1N2M0
18	SCNC	SCC	D	67 (0.6)	TXN2MX
19	SCNC	SCC	A (1.2)	44 (0.8)	T2N1M2
20	SCNC	SCC	A (1.3)	NC	T4N2M1

SCC: small cell carcinoma; C: carcinoma; WHO: World Health Organization. For further abbreviations see legend to table 1.

Analysis of the S-phase or of the S+G2M phase showed similar histograms. Eleven of the 18 cases with calculated SPF, had a high SPF (>38%); similarly distributed in carcinoids, WDNC and INC groups. All of the SCNC showed a high proliferative activity (fig. 6). When comparison was made with the SCC group of the WHO classification, only 3 out of 5 of SCCs had a high SPF (fig. 7).

High SPF was observed in 2 of the 4 tumours with a low clinical stage (T1 N0 M0) and in 9 of the 14 more advanced stages: no correlation was found between high SPF and advanced clinical stage. No correlation was noted between high SPF and flow aneuploidy.

Discussion

In our study, DNA aneuploidy was not related to high grade of malignancy, since three of the seven carcinoids were DNA aneuploid. There was no significant difference in the frequency of flow aneuploidy between the groups of neuroendocrine tumours whatever the classification used. A variable proliferative activity was demonstrated in each group, whatever the classification used, except in the SCNC group (according to the classification of Warren & Gould) which had a constantly high SPF. DNA ploidy and SPF showed no correlation with one another and with clinical stage.

Numerous authors have studied the relationship between DNA ploidy or SPF and survival or occurrence of metastases in non-small cell lung carcinomas (NSCLC), with various results: several [13–16] reported a longer survival for patients with a diploid DNA content than with DNA aneuploidy, but others [17–19] did not find prognostic value for DNA ploidy; a significant relationship between SPF and survival was observed in two studies [16, 17], but absent in two others [18, 19]. TIRINDELLI *et al.* [20], found a significantly different prognosis for survival according to the level of the DNA index.

Eleven cytometric studies [2, 21–30] have reported data on small cell carcinomas (SCC), five others [25, 29, 31–33] have analysed typical and atypical carcinoid tumours, but only three reports [2, 29, 30] analysed cases belonging to the whole spectrum of neuroendocrine carcinomas.

The frequency of DNA aneuploidy in our cases of SCC or SCNC (50%) was in the low range of previously reported data, from 59–83% [21, 23–25, 30] and 96% [20]. One group [28] reported a very low frequency (14%), which may be related to a restrictive selection of the tumorous tissue, eliminating diploid control cells. Studies of typical carcinoids demonstrated, as we did, DNA aneuploidy in 15% [23], 18% [32] and 32% [31]. The absence of diagnostic value for malignancy of DNA aneuploidy was also observed for endocrine tumours of other sites, *e.g.* parathyroids [34], or thyroid [35, 36]. Meanwhile, some studies noted an increase in the frequency of DNA aneuploidy with the potential of malignancy between typical and atypical carcinoids [29, 31], or between typical carcinoids and SCC [23, 29]. JACKSON-YORK *et al.* [30], also demonstrated a significant increase between WDNC and INC groups.

We could not conclude on the prognostic value of DNA ploidy and proliferative activity because of our small number of cases as for other studies [2, 29, 33] and insufficient information on survival for tumours such as carcinoids. Among the five deceased patients, two belonged to the SCNC group and three to the INC group with similar survival, and three of them showed DNA aneuploidy. WDNC and INC seemed to be the most interesting groups for analysis of flow cytometric data as seen in the study of JACKSON-YORK *et al.* [30], who found a prognostic value of the DNA ploidy, however, this was less apparent in another study [31]. The prognostic significance of the DNA ploidy was inconsistantly noted in SCC, being present in some works [25–27], and absent in others [2, 23, 24, 30], this inconsistency is probably explained by the very poor prognosis and histological classification used (WHO classification [23–27]). SPF was analysed in only two studies [24, 25] and was found to be of prognostic interest by JOHNSON *et al.* [25]. This variable needs to be studied in more cases to evaluate its prognostic value and to test whether SCNC is effectively a more homogeneous group of tumours according to the classification of Warren & Gould than the SCC group of the WHO classification.

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