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Regulation of proliferation and apoptosis in sporadic and hereditary medullary thyroid carcinomas and their putative precursor lesions

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Abstract C-cell hyperplasia (CCH) and medullary thyroid carcinoma (MTC) in patients affected by germline mutations of the RET oncogene represent an exceptional opportunity to study the regulation of proliferation and apoptosis during tumour initiation and progression. In 56 specimens [CCH, *n*=1; MTC with CCH, *n*=26; MTC, *n*=20; lymph-node metastasis (LNM), *n*=9] from 46 patients [multiple endocrine neoplasia type 2a (MEN2a), *n*=24; MEN2b, *n*=2; familiar MTC (FMTC), *n*=4; sporadic MTC, *n*=16] and 3 cases of non-neoplastic CCH, proliferation activity (MIB1), the rate of apoptosis [dUTP nick end labelling (TUNEL)] and expression of p53, bcl-2, bcl-x and bax were investigated and compared with clinical data. In MEN-associated CCH and small MTC, bcl-2 was strongly expressed, bcl-x was moderately expressed and bax was only weakly expressed. Advanced tumours and LNM did show a more heterogeneous bcl-2 staining accompanied by an increased bax expression and accelerated proliferation. The rate of apoptosis was extremely low in all investigated tumours. P53 was detectable in three patients with rapidly growing and extensively metastasising MTC. No somatic p53 mutations were found. Hereditary MTC with germline RET mutations at codon 918 (MEN2b) and codon 634 revealed a bias towards a higher proliferation activity at a younger age and are more frequently accompanied by LNM. CCH and MTC are characterised with a preponderance of bcl-2 as a factor blocking the programmed cell death. While MTC, in general, is a slowly growing tumour, a minority of tumours do progress rapidly with high proliferation. The factors leading to an accelerated tumour progression do not seem to take their effect via the regulation of apoptosis. Certain alterations of RET are supposed to have a direct or indirect implication on proliferation and, because of this, an effect on the clinical course.

Keywords Medullary thyroid carcinoma · MEN2 · Proliferation · Apoptosis · bcl · p53

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Introduction

Medullary thyroid carcinomas (MTCs) represent about 5–10% of all thyroid tumours and can occur in an either sporadic or hereditary setting [20, 25]. The so-called "neoplastic C-cell hyperplasia (CCH)" is considered to be a precursor lesion of hereditary MTC and can be evident at a very young age [12, 26].

Sporadic and familiar forms of MTC do show different biological behaviours. Sporadic MTCs usually become clinically evident in the fifth decade of life and show a relatively worse prognosis than C-cell carcinomas associated with MEN (multiple endocrine neoplasia type) 2a and familial (F)MTC. These are mostly tumours discovered during the third and fourth decades of life and have a better prognosis. MTC in a MEN2b setting are tumours found in children and adolescents with the worst clinical outcome [5, 8].

To date, it is not clear whether the different clinical course can be explained exclusively by a close-meshed screening of patients at risk for hereditary MTC followed by an early operation of small, possibly non-metastasising tumours or by different growth kinetics of the tumour itself in different genetic settings.

In general, growth kinetics of tumours are regulated by the balance between proliferation and programmed

cell death (PCD) of tumour cells. In the past, most studies focused on the regulation of proliferation. In recent years, negative growth control has been shown to be essential for the understanding of growth behaviour and the therapeutic effects on human cancers.

Members of the bcl family play an important role in the regulation of PCD. For example, bcl-2 inhibits apoptosis in cancer cells. However, in many tumours, bcl-2 appears to be associated with less aggressive behaviour [24]. In thyroid tumours, the expression of bcl-2 seems to be related to the degree of differentiation with a loss in undifferentiated tumours [19]. It has also been shown that bcl-2 is useful in identifying a subset of MTC which takes on a more aggressive clinical course [21]. Another member of this expanding family is the bax protein, which displays a pro-apoptotic function. Compared with bcl-2, bax is weakly expressed in normal follicular epithelium of the thyroid but predominant in undifferentiated thyroid carcinomas [15]. Bcl-x is an another member of this family and occurs in two isoforms. Similar to bax, bcl-x is weakly expressed in the normal thyroid but upregulated in thyroid tumours. The long form of bcl-x (bcl-x_l) has an anti-apoptotic function, whereas the short isoform (bcl-x_s) promotes PCD [3]. Finally, the tumour suppressor gene p53, which is essential for the regulation of normal cell growth, can arrest the cell cycle in case of DNA damage to allow for DNA repair. If unsuccessful, p53 can eliminate cells via apoptosis. Mutated p53 fails to block cell growth and to induce apoptosis [13].

Previously, we and others have shown that the development of hereditary MTC is normally supposed to be an age-related long-lasting step-by-step process [12, 14, 26]. Therefore, this model offers the exceptional opportunity to study tumour development and progression from CCH as a precursor lesion to manifest malignant tumours and their metastasis. We have investigated proliferation and apoptosis and its regulating proteins in a large number of MEN2-related thyroid lesions and in sporadic MTC.

Materials and methods

Case selection

Paraffin-embedded material of 59 specimens was available, including 47 total thyroidectomy preparations and nine lymph-node metastases (LNMs) from 49 patients (female *n*=25; male *n*=24). Sixteen MTC patients revealed no hereditary background (primary tumours *n*=15; LNM *n*=5). Three sporadic MTCs harboured a somatic RET mutation at codon 918 [9]. The patients with proven germline mutations of the RET gene can be subdivided into MEN2a (*n*=24), MEN 2b (*n*=2) and FMTC (*n*=4). The mean age for patients with sporadic MTC and MEN was 51 years (range 20–72 years) and 25.6 years (range 3–58 years), respectively.

Three patients with so-called “non-neoplastic CCH”, which was found accidentally or as an accompanying phenomenon not related to sporadic or familiar forms of MTC, served as a control for normal C cells. Clinical data are summarised in Table 1.

Immunohistochemistry

Monoclonal mouse antibodies against Ki76 (clone MIB1, 1:30 dilution; Dianova, Hamburg, Germany), p53 (clone DO-7, 1:200 dilution; Dako, Denmark) and bcl-2 (clone 124, 1: 50 dilution; Dako) and the polyclonal rabbit antibodies anti-bcl-x (directed against bcl-x_s and bcl-x_l; 65186E, 1:1200 dilution) and anti-bax (13666E, 1:1000 dilution, both from Pharmingen, San Diego, Calif.) were used after inhibition of endogenous peroxidase activity. In addition, the antibodies were used for antigen retrieval using microwave heating (3×5 min) and 0.001% trypsin pretreatment (only for bcl-x and bax). The primary antibodies were incubated for 1 h at 37°C except for bcl-2 (1 h at 37°C and 23 h at 4°C). The slides were subsequently incubated with a 1:10 dilution of normal horse serum (Vector, Burlingame, Calif.). The primary antibodies were detected using a biotinylated anti-mouse or anti-rabbit antibody (Dilution 1:100, Vector). Subsequently, an avidin-biotinylated peroxidase complex was applied (Vectastain, Vector) according to the manufacturer’s instructions. Peroxidase activity was visualised using the diaminobenzidine chromogenic substrate. Activated lymph nodes served as an external control, and normal follicular epithelium of the thyroid served as an internal control for MIB1- and bcl antibodies. A glioblastoma was the positive control for p53.

The number of MIB1-positive cells was counted and indicated as a percentage of positive tumour cells. P53 immunoreactivity was regarded as positive if more than 10% of tumour cells revealed a distinct nuclear staining. A weak signal in scattered single cells was not noted. The level of bcl-2, bcl-x and bax expression was subdivided into negative (no staining), weak (less than 10% staining), moderate (less than 50% staining) and strong (more than 50% staining).

In situ apoptotic cell labelling

Terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end labelling (TUNEL) was used for in situ labelling of apoptotic cells. Following deparaffinisation and inactivation of endogenous peroxidase using 3% hydrogen peroxidase, the slides were incubated with 20 µg/ml proteinase K for 20 min at room temperature. After that, the cellular permeability was improved through an incubation with 0.1% triton X100. The following steps were performed according to the manufacturer’s instructions (in situ cell death detection kit, POD; Boehringer, Mannheim, Germany). The slides were incubated with a mixture of fluorescein-labelled nucleotides in the presence of TdT. Incorporated fluorescein was detected using an anti-fluorescein antibody conjugated with horse-radish peroxidase (POD). Finally, the slides were incubated with diaminobenzidine (DAB) and hydrogen peroxidase for 5–10 min at room temperature. “Starry sky” macrophages within the germinal centres of hyperplastic lymph nodes served as an external control.

Interpretation

The number of apoptotic cells was determined through examination at ×400 magnification under a microscope. Depending on tumour size (minimum for evaluation: 1 mm), 5–20 high power fields (HPF) of non-necrotic areas of the tumour were examined. The number of decorated nuclei and apoptotic bodies was converted into “apoptotic cells per 10 HPF”.

p53 Mutation analysis

Three p53-positive primary tumours were tested for a somatic mutation of the p53 tumour suppressor gene. DNA from paraffin sections was isolated according to a standard method [27]. Through PCR, exons 4–9 of the p53 gene were amplified. We used the 20 oligomer primers described by Mashiyama et al. [17]. Polymerase chain reaction (PCR) conditions were as follows: pre-denaturation

Table 1 Clinical data of all investigated patients and immunohistochemical staining results. *G-mut* germline mutation (codon); *S-mut* somatic mutation (codon); *Dg* diagnosis; *Apop* apoptosis; *LNM* lymph node metastasis; *N.i.* not investigated; – negative; + weak (less than 10% positive); ++ moderate (10–50%); +++ strong (>50%); *FMTC* familiar medullary thyroid carcinoma; *MEN* multiple endocrine neoplasia type; *MTC* medullary thyroid carcinoma; *CCH* C-cell hyperplasia

Patient	Age (years)	Gender	G-mut	S-mut	Dg	pT	pN	pM	Size (mm)	MIB1 (%)	Apop	p53	BCL2	BCL-X	BAX
MEN2a and FMTC															
KA	53	Female	768	N.i.	MTC	1b	0	0	6	2	0	<10	+++	+	+
TI	58	Female	790	N.i.	MTC	1b	1a	0	7	5	1	<10	+++	++	+
KG	60	Male	804	N.i.	LNM	4b	1b	0		5	2	–	+++	++	+
HO	3	Male	634	N.i.	MTC	1a	0	0	1	1	–	<10	+++	+	+
					MTC	1a	0	0	1	5	–	<10	+++	++	+
WJ	5	Male	634	N.i.	MTC	1a	0	0	1	2	–	–	+++	++	++
HN	6	Male	634	N.i.	MTC	1b	0	0	1	2	–	–	+++	++	++
HD	6	Male	620	N.i.	CCH	–	–	–		1	–	–	+++	+	+
RW	7	Male	618	N.i.	MTC	1a	0	0	1	5	–	–	+++	++	+
SM	7	Female	634	N.i.	MTC	1b	x	0	2,5	5	0	<10	+++	++	+
WD	10	Male	634	N.i.	MTC	1a	0	0	1	3	–	–	+++	++	+
SL	11	Male	634	N.i.	MTC	1b	x	0	5	4	0	–	+++	+	+
WS	11	Female	634	N.i.	MTC	1a	0	0	2	2	0	–	+++	++	+
GA	13	Female	634	N.i.	MTC	1b	0	0	5	1	0	<10	+++	+	+
SM	15	Male	618	N.i.	MTC	1a	0	0	3	10	0	<10	++	+	+
DN	17	Female	634	N.i.	MTC	2b	1	0	40	30	2	>10	+++	+	++
					MTC	2b	1	0	40	20	2	>10	+++	++	+
					LNM	2b	1	0		15	1	>10	+++	+	+
DT	20	Female	634	N.i.	MTC	1b	1a	0	8	15	0	–	++	++	++
SY	23	Female	634	N.i.	MTC	1b	0	0	7	3	0	–	+++	++	+
HD	24	Female	634	N.i.	MTC	1b	0	0	6	5	1	–	+++	++	+
LY	27	Male	634	N.i.	MTC	1b	1b	0	9	10	0	<10	+++	++	++
HS	27	Male	634	N.i.	MTC	1b	1a	0	7	3	1	–	+++	++	+
					MTC	1b	1a	0	7	2	1	<10	+++	++	+
HM	31	Female	620	N.i.	MTC	1b	0	0	5	2	0	–	+++	+	+
HU	33	Female	790	N.i.	MTC	1b	0	0	9	1	0	–	+++	+	+
DE	34	Male	634	N.i.	MTC	1b	1	0	5	10	5	–	++	++	++
RS	34	Female	634	N.i.	MTC	1b	0	0	7	1	0	–	+++	++	+
DM	41	Male	634	N.i.	MTC	2b	1b	0	8	2	1	–	+++	++	+
PG	47	Female	634	N.i.	MTC	2b	0	0	22	2	4	–	++	++	+
WK	51	Male	634	N.i.	MTC	1b	1a	0	7	1	11	–	+++	++	+
WM	54	Male	634	N.i.	MTC	4b	1	1	17	3	0	<10	+++	+	+
					LNM	4b	1	1		15	6	–	+++	++	++
MEN2b															
GC	10	Female	918	N.i.	MTC	4b	1	1	22	20	0	<10	+++	+	+
					LNM	4b	1	1		15	1	<10	+++	++	++
LC	29	Female	918	N.i.	MTC	3b	1	0	20	5	2	<10	+++	+	++
Sporadic MTC															
BM	20	Male	No	No	MTC	4a	1b	1	54	50	5	>10	+++	+	++
					LNM	4a	1b	1		70	15	>10	+++	+	+
SB	33	Male	No	No	MTC	3a	0	0	50	5	2	–	+++	++	+
WR	34	Female	No	N.i.	LNM	3	1	x		1	0	–	+++	++	++
MR	35	Male	No	N.i.	MTC	2a	0	0	35	10	10	–	+++	+	+
JI	41	Female	No	918	MTC	3	0	0	50	5	0	<10	+++	++	++
OW	42	Male	No	N.i.	MTC	4	1b	1	60	20	0	<10	++	+	+
					LNM	4	1b	1		20	2	<10	+++	++	+
FJ	49	Female	No	N.i.	MTC	2a	0	0	17	2	1	<10	+++	+	++
HG	53	Female	No	N.i.	MTC	2a	0	0	13	3	2	<10	+++	++	+
GH	56	Female	No	No	MTC	1b	0	0	11	1	1	<10	+++	++	+
ZH	58	Female	No	N.i.	MTC	1a	0	0	8	2	0	<10	+++	+	+
KE	59	Female	No	N.i.	MTC	2a	0	0	16	5	2	<10	+++	+	++
DG	64	Male	No	918	MTC	4a	1	1	60	15	3	<10	+++	+	+
AH	66	Male	No	N.i.	MTC	4a	1	1	30	40	1	>10	++	++	++
					LNM	4a	1	1		40	15	>10	++	++	+
LE	66	Female	No	No	MTC	1	0	0	8	1	0	–	+++	+	++
MG	69	Female	No	918	MTC	4a	1b	0	50	5	10	–	++	+	++
					LNM	4a	1b	0		20	15	–	++	+	++
SA	72	Female	No	N.i.	MTC	2a	x	x	14	1	10	–	+++	++	+
Non-neoplastic CCH															
BE	58	Male	No	N.i.	CCH					–	–	–	+	–	+
BG	72	Male	No	N.i.	CCH					–	–	–	++	+	–
NS	68	Male	No	N.i.	CCH					–	–	–	+	–	+

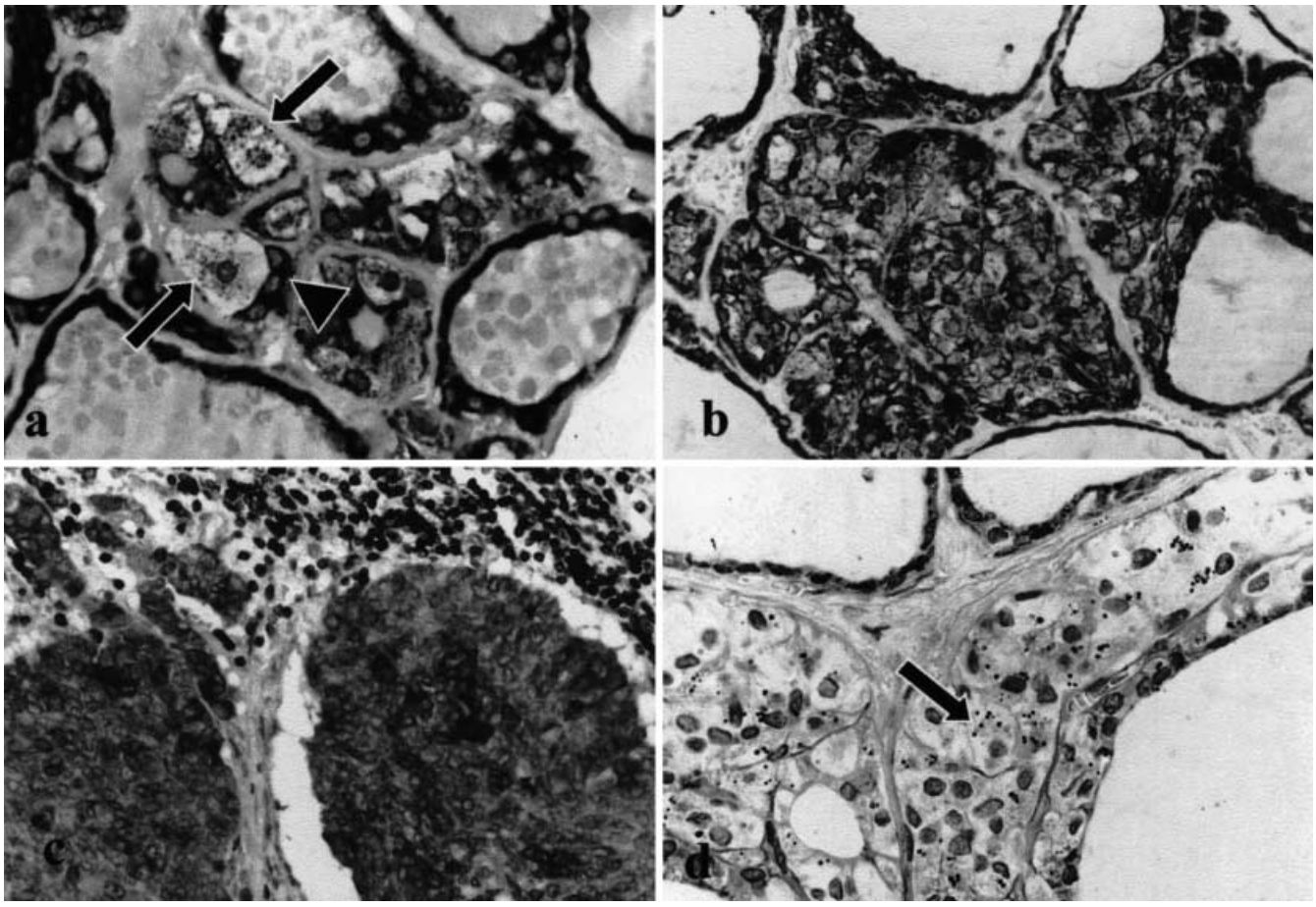

Fig. 1 **a** Very weak bcl-2 immunostaining of non-neoplastic C-cells (*arrow*) compared with strongly decorated adjacent follicular epithelium (*arrowhead*), ×400. **b** Neoplastic nodular C-cell hyperplasia (CCH) associated with multiple endocrine neoplasia type (MEN) 2a with clear cytoplasmatic expression of bcl-2 in hyperplastic C cells and adjacent follicular epithelium, ×200. **c** Moderate to strong bcl-2 expression in a manifest medullary thyroid carcinoma (MTC). Lymphocytes (*above*) are strongly positive, ×200. **d** Weak and heterogeneous expression of bcl-x in neoplastic CCH (*arrow*), ×400

at 92°C for 5 min and 35 cycles of denaturation at 92°C for 1 min, annealing for 30 s at 56°C (exons 8–9), 58°C (exon 5), 62°C (exons 4, 6, 7), DNA synthesis for 1 min at 72°C, post-synthesis for 5 min at 72°C and storage of the PCR products at 4°C.

After purification of the PCR products (Quiagen, Hilden, Germany), they were amplified using a cyclic PCR [2 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 56°C (exons 8–9), 58°C (exon 5) or 62°C (exons 4, 6, 7), 1 min at 72°C; and 5 min at 72°C] with the sense and antisense PCR primers (Dye Terminator Kit; Perkin Elmer, Foster City, Calif.). Sequencing products were run on a 373 ABI sequencer.

Statistical analysis

Differences between groups of patients were analysed using the χ^2 or the Fisher's exact test for categoric variables and the unpaired Student's *t*-test for continuous variables (SPSS software, Chicago, Ill.). The level of significance was set at a *P* value of 0.05.

Results

BCL family

Bcl-2 was strongly expressed in normal follicular epithelium and in CCH related with MEN (Fig. 1b, Table 1). By contrast, non-neoplastic C cells showed clearly a less intensive staining than the follicular epithelium (Fig. 1a, Table 2). A moderate and focal reduction of staining intensity was detected in nine tumours. These tumours were larger (mean 25.7 mm vs 14.8 mm) and revealed increased proliferation (MIB1 mean 18.0% vs 8.3%; Fig. 2) and a slightly increased but nevertheless very low rate of apoptosis (two and one-half cells vs five and one-half cells per 10 HPFs). Bcl-x was found to be weakly expressed in hyperplastic C cells (Fig. 1d). MTC revealed a heterogeneous but altogether weak staining pattern also (Fig. 2d).

Bax showed a weak expression in CCH and the majority of MTC. Tumours (21%) showed an increased staining for bax (Fig. 2c), obviously coincidental with a significantly higher proliferation (mean 17.3% vs 5.6%; $P<0.001$), tumour size (mean 14.1 mm vs 22.0 mm; not significant) and apoptosis (2.5 vs 3.1). A tendency to an inverse correlation between bcl-2 and bax expression is perceptible ($P=0.027$). Hyperplastic C-cells revealed no or very weak bax expression.

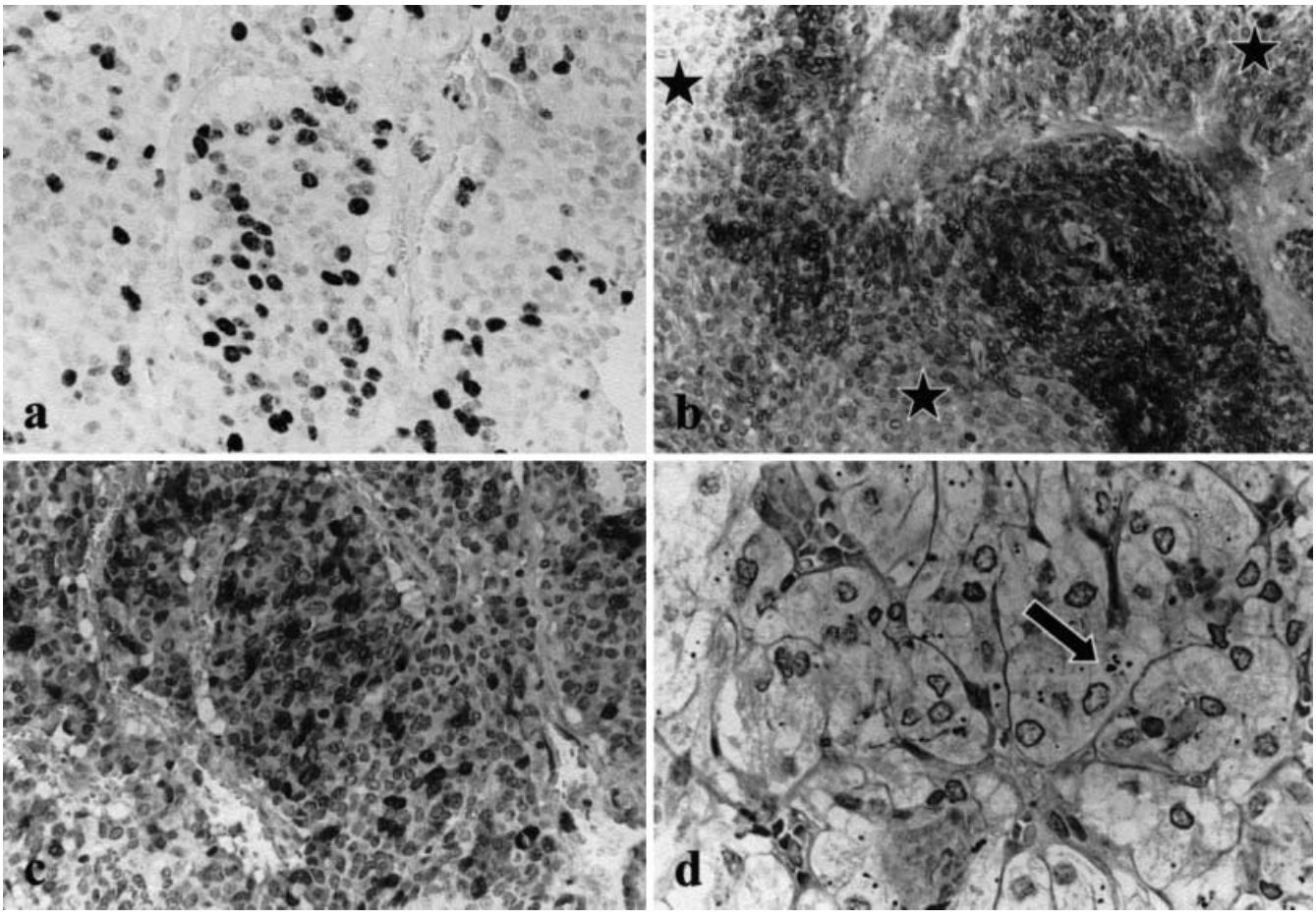

Fig. 2 Corresponding areas of one sporadic medullary thyroid carcinoma (MTC; pT4). **a** High proliferation indicated through MIB1 decoration, ×200. **b** Heterogeneous bcl-2 expression in this manifest MTC with clear focal weakening of bcl-2 immunoreactivity (★), ×200. **c** Increased number of scattered bax-positive cells, ×200. **d** Predominant weak and heterogeneous expression of bcl-x with scattered dark intracytoplasmatic granules (*arrows*), ×400

Table 2 Summary of the results. *PT* primary tumour; *LNM* lymph-node metastasis; *N.i.* not investigated; *weak* less than 10% positive; *moderate* 10–50% positive; *strong* >50% positive; *HPF* high power field; *FMTC* familiar medullary thyroid carcinoma; *MEN* multiple endocrine neoplasia type; *MTC* medullary thyroid carcinoma; *CCH* C-cell hyperplasia

		MEN2a and FMTC CCH *n*=1 PT *n*=29 LNM *n*=3	MEN2b PT *n*=2 LNM *n*=1	Sporadic MTC PT *n*=15 LNM *n*=5	Nonneoplastic CCH *n*=3
BCL2	Strong	29 (88%)	3 (100%)	15 (75%)	0
	Moderate	4 (12%)	0	5 (5%)	1 (33%)
	Weak	0	0	0	2 (67%)
BCLX	Moderate	22 (67%)	1 (33%)	9 (45%)	0
	Weak	11 (33%)	2 (67%)	11 (55%)	1 (33%)
	Negative	0	0	0	2 (67%)
BAX	Moderate	7 (21%)	2 (67%)	9 (45%)	0
	Weak	26 (79%)	1 (33%)	11 (55%)	2 (67%)
	Negative	0	0	0	1 (33%)
MIB	% Positive cells	5.8	13.3	11.6	N.i.
P53	>10% Positive cells	3 (9%)	0	4 (20%)	0
Apoptosis	Per 10 HPF	1.5	1.0	4.7	N.i.

Table 3 Comparison of age, tumour size, proliferation, lymph-node metastasis (LNM)- and M status in relation to sporadic medullary thyroid carcinoma (MTC) and different RET mutations in hereditary MTC (exclusively primary tumours). *M* distant metastasis; *mut* mutation

	Age (average in years)	Size (average in mm)	Proliferation (average/median in %)	LNM	M
Sporadic MTC					
n=15	49.6	31.1	11.6 (5)	5/15	4/15
Hereditary MTC					
634 mut (*n*=20)	22.3	9.1	5.7 (3)	11/20	1/20
918 mut (*n*=2)	19.5	21.0	12.5	2/2	2/2
other mut (*n*=7)	29.0	5.2	3.7 (2)	2/7	0/7

Proliferation

Sporadic (9 of 20) and hereditary (10 of 36) MTC and LNM revealed more than 5% Ki-67 positive cells. These tumours were clearly larger (mean 30.5 mm vs 11.5 mm, P=0.013) and showed a higher frequency of LNM (83% vs 26%, P<0.001) and distant metastases (41% vs 3%, P<0.001).

P53

Only seven samples of three patients showed a strong nuclear expression of p53 in more than 10% of the cells in primary tumours and metastases (Table 2). These tumours revealed a clear tendency to grow more rapidly (37.9% vs 5.8% MIB1 positive cells on average), to be larger (41.0 mm vs 14.1 mm on average) and to be more aggressive with LNM in all and distant metastases in two out of the three patients. No somatic p53 mutation could be confirmed for these three patients.

Apoptosis

The number of apoptotic cells proved using the TUNEL assay was extremely low with zero to five positive cells per 10 HPFs in most tumours. A marginally higher frequency was found in the close vicinity of amyloid deposits. There is a weak association between a decreased bcl-2 expression and marginally increased rate of apoptosis and no relation to bax expression (statistically not significant).

RET mutation

Considering the spectrum of germline and somatic RET mutations, the two MEN2b-associated tumours behaved the worst with fast-growing and early metastasising tumours in young adults. Interestingly, MTCs with germline mutations at codon 634 also revealed at a young age relatively large and fast-growing carcinomas with LNM in more than 50% compared with the other RET mutations associated with MEN2a and FMTC (difference statistically not significant; Table 3).

Discussion

In general, MTCs are considered to be slow-growing tumours. The different settings of MTC are assumed to lead to a varying time of onset and progression of tumour development. Recently, we and others have been able to show that CCH, which is regarded as a precursor lesion of familiar MTC, occurs at a very young age [5, 12]. However, manifest tumours usually become clinically evident rarely before the third and fourth decades of life. Few patients show a very rapid progressing course with large metastasising tumours as early as the second decade of life or even earlier [7]. To date, it is not clear whether a germline mutation of the RET gene itself is sufficient for the development of CCH and the progression to MTC.

In the present study, we showed, in accordance with Matias-Guiu et al. [18] and Basolo et al. [2], a very slow proliferation activity in CCH and small MTCs. Larger C-cell carcinomas revealed either a slightly or, in a minority of cases, a clearly increased proliferation. By contrast, the rate of apoptotic cell death detected using the TUNEL assay was extremely low at any stage of the disease. The most apoptotic cells were found in central stroma-rich areas with tumour cells walled in amyloid and have to be regarded as a phenomenon of tumour regression. While Wang et al. [22] found apoptosis in more than 5% of tumour cells in 6 of 21 MTCs, the very low rate of POD was recently confirmed by Basolo et al. [2].

Bcl-2 can prolong cell life by suppressing PCD in various tissues [24]. The high level of bcl-2 expression in neoplastic CCH and MTCs could explain the low rate of apoptosis in these kinds of tumours. In contrast to the CCH associated with MTC, the three cases with so-called “non-neoplastic” CCH revealed a clearly diminished bcl-2 level when compared with MEN-associated CCH and normal follicular epithelium. This condition refers to a bcl-2 alteration as a very early event in tumourigenesis of MTC since extended multifocal CCH is regarded as a precursor lesion for familiar forms of MTC [26].

Previously, a high frequency of bcl-2 expression was described in different neuroendocrine tumours that do not only share their derivation from the neural crest but also grow slowly in a less aggressive manner like MTC [23]. Other than the indolent tumour growth possibly influenced by bcl-2 in those tumours, sole alterations of bcl-2 were shown to be sufficient for the development of

low malignant follicular lymphomas. Furthermore, the predominance of bcl-2 could explain the poor response of MTC to chemotherapy and irradiation, which do act via an induction of apoptosis in the tumour cells [24].

The ratio of bcl-2 and bax ultimately decides whether or not a cell will undergo apoptosis. The loss of bcl-2 and an increased expression of bax leads to a higher rate of apoptosis as shown, for example, for undifferentiated thyroid carcinomas [15]. In our study, we found a slightly decreased bcl-2 and moderately increased bax expression in some more aggressive MTCs. Beyond that, we saw at least a marginal increase of apoptosis in those tumours with diminished bcl-2 expression, while bax expression was without any convincing effect on the rate of PCD. Viale et al. [21] described a complete loss of bcl-2 in MTC as a strong independent prognostic indicator. In concordance with Pollina et al. [19] and Wang et al. [24], we found bcl-2 to be detectable in all investigated tumours. Therefore, the feasibility of bcl-2 as a prognostic marker remains doubtful.

Mutations of the tumour suppressor gene p53 are extremely rare in MTC. According to our knowledge, a polymorphism in intron 2 and one mutation in exon 4 (codon 49) are the sole alterations of the p53 gene in MTC [10, 11, 28]. In contrast to Wang et al. [22], we found three patients with a strong nuclear reaction of p53 in 10–70% of the tumours cells. Through direct sequencing, we could rule out a somatic mutation in exons 4–9 of the p53 gene in these MTCs. Since all of p53-positive MTCs were large metastasising tumours with a high proliferation activity, a direct or indirect effect of a functional alteration of p53 protein cannot be ruled out [1].

The early onset and rapid progress of MTC in an MEN2b setting and the special indolent course of FMTC when compared with sporadic MTC is well known. MEN2a patients showed a better prognosis despite the fact that tumours developed mostly two decades or more earlier than their sporadic counterparts. Taking this into consideration, we have to assume, at least partially, that the RET mutation itself determines the further course of the disease. This assumption is supported by our observation that, in a MEN2a setting, germline mutations at codon 634 lead to a more aggressive course when compared with the other FMTC- and MEN2a-specific RET mutations. Recently, it has been shown that mutations at codon 630 and codon 634 activate RET more strongly than mutations at codons 609, 618 or 620 [4]. A decision whether mutations at codon 634 are linked with a worse outcome would require a long-term follow-up.

Somatic RET mutations have been described in sporadic and familiar forms of MTC also [6, 16]. In our study, three sporadic tumours that harbour a somatic RET mutation at codon 918 [9] were included. The role of somatic RET mutations for the progression of either sporadic or hereditary MTC is still obscure.

The presented results mark a high bcl-2 expression as an early and consistent event for the tumourigenesis of sporadic and hereditary MTC, which leads to a neglectable low rate of apoptosis and prolonged cell life. Alterations of bax as an opponent for the regulation of PCD is supposed to play a role in a limited number of advanced tumours. The spectrum of the hereditary RET mutation by itself is assumed to have a lasting effect on proliferation and the biological course of the disease, independent of the regulation of apoptosis. High bcl-2 expression is the likely reason for the very limited response to adjuvant chemotherapy or irradiation of MTC. Therefore, an alternative approach using gene therapy to influence the regulation of bcl-2 could be promising.

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