Virchows Arch (2002) 440:498–504
DOI 10.1007/s00428-002-0622-0

ORIGINAL ARTICLE

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Mucoepidermoid carcinoma of the thyroid: a tumour histotype characterised by P-cadherin neoexpression and marked abnormalities of E-cadherin/catenins complex

Received: 21 September 2001 / Accepted: 8 January 2002 / Published online: 21 March 2002

Abstract The cadherin/catenins complex regulates cell–cell adhesion and motility and is believed to have an invasion suppressor role. Primary mucoepidermoid carcinoma of the thyroid (MECT) is a rare tumour characterised by a distinct morphological appearance and a questionable histogenesis. The coexistence of papillary thyroid carcinoma (PTC) foci in many patients with MECT suggests an association between the two tumour histotypes. In an attempt to clarify the putative relationship between MECT and PTC, we analysed tissue from 11 patients with MECT by immunohistochemistry (E-, P- and N-cadherins and α-, β- and γ-catenins). The E-cadherin gene was also analysed by polymerase chain reaction (PCR)/single-strand conformation polymorphism (SSCP). The results were compared with a control group of normal thyroid, classical PTC and the diffuse sclerosing variant of PTC. Compared with normal thyroid and PTC, MECT displays marked abnormalities of the cadherin/catenin complex. Such abnormalities include the consistent neoexpression of P-cadherin and major alterations in the expression of E-cadherin and the three catenins. Our results point to the close relationship between the de novo expression of P-cadherin and the disruption of the cadherin/catenins complex with the squamoid phenotype of MECT.

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Keywords Thyroid · Mucoepidermoid carcinoma of the thyroid · E-Cadherin · P-Cadherin · Catenins

Introduction

Cadherins are single-pass transmembrane proteins that mediate Ca^{2+}-dependent homophilic cell–cell adhesion. In adherens junctions, the cytoplasmic domain of cadherins binds to β- or γ-catenin, which in turn binds to the actin-associated protein α-catenin [24, 39]. Defects in cadherin-mediated adhesion are associated with several characteristics of malignant transformation, such as dedifferentiation, high motility and invasive growth [2, 36].

Loss of or reduction in cell adhesion by abnormalities at the level of expression or function of E-cadherin has often been described in human cancers [6]. In thyroid, a minimal or absent immunoexpression of E-cadherin has been described in poorly differentiated/undifferentiated carcinomas in contrast to a variable degree of immunoreactivity in differentiated carcinomas [5, 33, 37]. In the latter group, a significant association has been described between loss of E-cadherin and unfavourable prognosis [37].

Alterations in β-catenin may also play an important role in the tumourigenesis of several human neoplasias [16, 17, 26]. Cerrato et al. [11] reported the absence of immunoreactivity for β- and γ-catenins in anaplastic carcinomas, whereas papillary thyroid carcinoma (PTC) and follicular carcinoma showed heterogeneously decreased immunoexpression, with a marked down-regulation of γ-catenin. Böhm et al. [4] also reported a decreased expression of α-, β- and γ-catenins in differentiated thyroid carcinomas, with a higher prevalence of γ-catenin down-regulation; moreover, these authors observed that follicular carcinomas showed reduced staining for all the catenins more often than PTC. Mutations in the β-catenin gene, with nuclear localisation of the protein, have been described in poorly differentiated and anaplastic carcinomas by Garcia-Rostan et al. [16, 17].

In recent studies on classical PTC and its diffuse sclerosing variant (DSV), we detected several types of abnormalities in the E-cadherin/catenins complex, such as E-cadherin and β- and γ-catenin down-regulation/aberrant localisation [28]. We have also detected neoexpression of P-cadherin in areas of squamous metaplasia of both PTC and DSV [28].

Despite its rarity, mucoepidermoid carcinoma is one of the most interesting histotypes of thyroid tumours. This peculiar neoplasia was first described by Rathigan et al. [27], who pointed out its (superficial) resemblance to mucoepidermoid carcinoma of the salivary gland because it comprises glandular and solid or squamoid areas, frequently in a cellular, fibrotic stroma. Since then, six similar tumours have consecutively been reported by Fialho et al. [14], Franssila et al. [16], Mizukami et al. [23] and Harach et al. [19] under the name of mucoepidermoid carcinoma of the thyroid (MECT).

Cases of MECT merging with classical PTC and DSV of PTC, squamous carcinoma [12] and anaplastic carcinoma of the thyroid [10, 30] have been reported to date. The histogenesis of MECT remains controversial [9, 10, 30], but the coexistence of PTC foci in many patients with MECT and the squamoid differentiation of MECT support the assumption that MECT is related to PTC [9, 10, 30].

Taking this into consideration, we decided to study the immunoexpression of E-, P- and N-cadherins and α-, β- and γ-catenins in 11 patients with MECT and to evaluate the integrity of the entire coding sequence of the E-cadherin gene in this group of tumours. The main objective of this study was to clarify the role of cell–cell adhesion molecules in the acquisition of the MECT phenotype and to make progress in understanding the putative differences and similarities between MECT and PTC.

Material and methods

Tumour specimens

Representative histological slides and paraffin blocks were retrieved from the files of the Departments of Pathology of the Medical Faculty of Porto, Portugal, the University Central Hospital of Helsinki, Finland and the University Health Centre of Rio de Janeiro, Brazil. Some of the patients had been sent for consultation to the Department of Pathology of the Medical Faculty of Porto. No frozen samples were available from any of the patients.

The 11 tumours were independently diagnosed as MECT by two pathologists (KF and MS-S) according to the criteria presented in the WHO booklet [20] and by Rosai et al. [29]. The specimens were also classified according to the co-existence of PTC foci, presence of necrosis, prominence of desmoplasia and/or lymphocytic infiltration, and histological appearance of the adjacent thyroid parenchyma. In two specimens (patients 1 and 6), the immunohistochemical study was performed in metastatic foci because no material was available from the primary tumours.

A series of specimens from 18 patients with PTC and 8 with DSV previously reported [28, 33] was used for comparative purposes.

Immunohistochemistry

Immunohistochemistry was performed using the avidin–biotin–peroxidase (ABC) complex with an additional step for microwave antigen retrieval as previously described [28]. The following antibodies were used: for E-cadherin, HECD-1 (Zymed, San Francisco, CA), diluted 1:200; P-cadherin, clone 56 (Transduction Laboratories, Lexington, UK), diluted 1:75; N-cadherin, clone 3B9 (Zymed, San Francisco, CA), diluted 1:400; anti α-,β-and γ-catenins, clones 5, 14 and 15 (Transduction Laboratories, Lexington, UK), diluted 1:200, 1:1000 and 1:500, respectively.

Normal thyroid was used as a positive control for all the antibodies except for P- and N-cadherins, for which normal skin and normal heart tissue, respectively, were used.

Scoring of immunostaining

Two observers (ASR and MS-S) independently scored the immunostaining using a semi-quantitative approach; the specimens were classified according to the intensity of the immunoreactivity as follows: -, no expression was detectable; ↓↓, the signal was very low; ↓, positive but still weaker than the positive controls; =, the intensity matched the positive control. The immunoreactivity patterns were also classified according to the signal localisation as membranous, diffuse cytoplasmic or granular with perinuclear distribution.

Tumour microdissection and genomic DNA extraction

Genomic DNA was extracted from 10-μm sections of paraffin-embedded material adjacent to those used for histology and immunohistochemistry. In order to reduce contamination from adjacent non-tumoural tissue, including lymphocytes/stromal cells, and to separate different histotypes of tumours in the same section, we selected the areas to microdissect in continuous haematoxylin-eosin-stained sections. After deparaffinisation and proteinase K digestion, the samples were precipitated with 1 volume of isopropanol for 2 h at –20°C, washed in 70% ethanol and eluted in water.

Mutation screening by PCR/SSCP

The analysis was performed for the 16 exons of the E-cadherin coding sequence, including the intron/exon boundaries.

PCR reactions were based on the conditions reported by Berx et al. [3]. The cycling conditions were 45 cycles of 45 s at 94°C, 45 s at the appropriate annealing temperature and 75 s at 72°C. The sequences of the primers and the annealing temperatures were as previously described [3].

PCR products were diluted 1:1 with a loading buffer (95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol) and denaturated for 10 min at 95°C. Electrophoresis of 10 μl of these samples was carried out in 40% mutation detection enhancement (MDE) gels, run at 180 V for 16 h, either with glycerol (20°C) or without (8°C). The gels were silver stained and transferred to Whatmann paper and dried on a vacuum gel drier.

Sequencing

Both mobility shifts and the corresponding normal bands were excised from the paper and eluted in water. These templates were used for reamplification PCR under the same conditions as previously described. The PCR products were purified and sequenced using the ABI Prism Big Dye Cycle Sequencing kit (Perkin Elmer, Foster City, CA) and an ABI Prism 377 DNA sequencer (Perkin Elmer, Foster City, CA).

Results

Histology

The clinico-pathological characteristics of the specimens from the 11 patients are summarised in Table 1. The his-

Table 1 Clinico-pathological characteristics of 11 patients with MECT

Patient no.	Sex	Age (years)	Tumour material available for immunohistochemistry	Coexistence of PTC foci	Necrotic foci	Desmoplasia	Prominent lymphocytic infiltrate	Adjacent thyroid parenchyma
1	f	20	Three nodal metastatic foci	Yes	Yes	Intense	Yes	Lymphocytic thyroiditis
2	f	25	Primary tumour	No	Yes	Intense	No	Not evaluated
3	f	67	Primary tumour	No	Yes	Intense	No	Not evaluated
4	m	11	Primary tumour and nodal metastasis	Yes	Yes	Intense	No	Not evaluated
5	m	47	Primary tumour	Yes (DSV)	No	Intense	Yes	Not evaluated
6	f	18	Nodal metastasis	Yes	No	Intense	Yes	Lymphocytic thyroiditis
7	f	46	Primary tumour	No	No	Moderate	Yes	Lymphocytic thyroiditis
8	f	34	Primary tumour	Yes	No	Intense	Yes	Lymphocytic thyroiditis
9	f	18	Primary tumour	Yes	No	Intense	Yes	Lymphocytic thyroiditis
10	f	11	Primary tumour	Yes	No	Intense	Yes	Lymphocytic thyroiditis
11	m	47	Primary tumour	Yes	No	Intense	Yes	Lymphocytic thyroiditis

PTC, papillary thyroid carcinoma; DSV, diffuse sclerosing variant of PTC.

Table 2 Immunohistochemical data on E- and P-cadherins and α-, β- and γ-catenins in 11 patients with MECT

Patient no.	E-Cadherin		P-Cadherin		α-Catenin		β-Catenin		γ-Catenin	
	Localisation	Intensity	Localisation	Intensity	Localisation	Intensity	Localisation	Intensity	Localisation	Intensity
1	Cytoplasmic	↓↓	Cytoplasmic	↓↓	–	–	Membranous	↓	Cytoplasmic	↓
	Cytoplasmic	↓↓	Cytoplasmic	↓↓	Membranous	↓↓	Membranous	↓	–	–
	Cytoplasmic	↓↓	Membranous/Cyt	↓	–	–	–	–	Membranous	↓↓
2	Cyt/granular	↓	Cytoplasmic	↓↓	–	–	Membranous	↓	–	–
3	Cytoplasmic	↓↓	–	–	Cytoplasmic	↓	–	–	Cytoplasmic	↓
4	Cytoplasmic	↓	Membranous	=	Membranous	=	Membranous	=	–	–
	Cytoplasmic	↓↓	Membranous	=	Cytoplasmic	↓↓	–	–	–	–
5	Cytoplasmic	↓↓	Membranous	=	Membranous	=	Membranous	=	Membranous	=
6	Cytoplasmic	↓	Membranous	=	Membranous	=	–	–	–	–
7	Granular	NA	Cytoplasmic	↓↓	Cytoplasmic	↓	Membranous	=	Membranous	=
8	Granular	NA	Cytoplasmic	↓↓	Membranous	=	Membranous	=	Membranous	=
9	–	–	Cytoplasmic	↓↓	Membranous	=	Membranous	=	Membranous	=
10	Granular	NA	Cytoplasmic	↓	–	–	Membranous	=	–	–
11	Cytoplasmic	↓↓	Membranous	=	–	–	Membranous	↓	–	–

The results are expressed in comparison with those obtained in normal follicular cells, and in the case of P-cadherin with normal skin. =, the intensity of the staining matched the positive control; ↓, reduced expression; ↓↓, very reduced expression; –, no expression at all; NA, not applicable.

tological appearance of a typical case of MECT (patient no. 8) is shown in Fig. 1a.

Immunohistochemistry

The results obtained in the 11 MECT patients regarding P-cadherin and membrane immunoreactivity for E-cadherin and the three catenins are summarised in Table 2.

E-Cadherin. Tissue sections from the 11 MECT patients were examined with HECD-1 antibody that recognises the extracellular domain of E-cadherin. The results are summarised in Table 2.

In the "normal" adjacent thyroid parenchyma, strong homogenous staining along the intercellular borders of the epithelial cells was observed; a similar pattern was seen, focally, in the co-existent foci of PTC. In contrast, all the MECT specimens displayed a very abnormal pattern of expression. Six displayed reduced expression restricted to the cytoplasm, and four showed a very strong signal with a granular perinuclear localisation (Fig. 1b–d). The remaining specimen (patient 9) had no E-cadherin expression (Table 2). The granular pattern of immunoreactivity in four patients and the absence of expression in one patient cannot be attributed to technical problems, e.g. poor preservation, because the pattern of immunoexpression was membranous in the co-existent foci of PTC and in the adjacent thyroid parenchyma (Fig. 1e).

P-Cadherin. In contrast to the absence of P-cadherin expression observed in normal thyroid and in the co-existent foci of PTC, positive immunoexpression was detected in ten of the 11 MECT patients (Table 2). A clear cell–cell border pattern was observed in four patients (Fig. 1f), whereas there was weak to moderate staining in the cytoplasm in six patients (Fig. 1f). Patient 3 was negative for P-cadherin expression (Table 2).

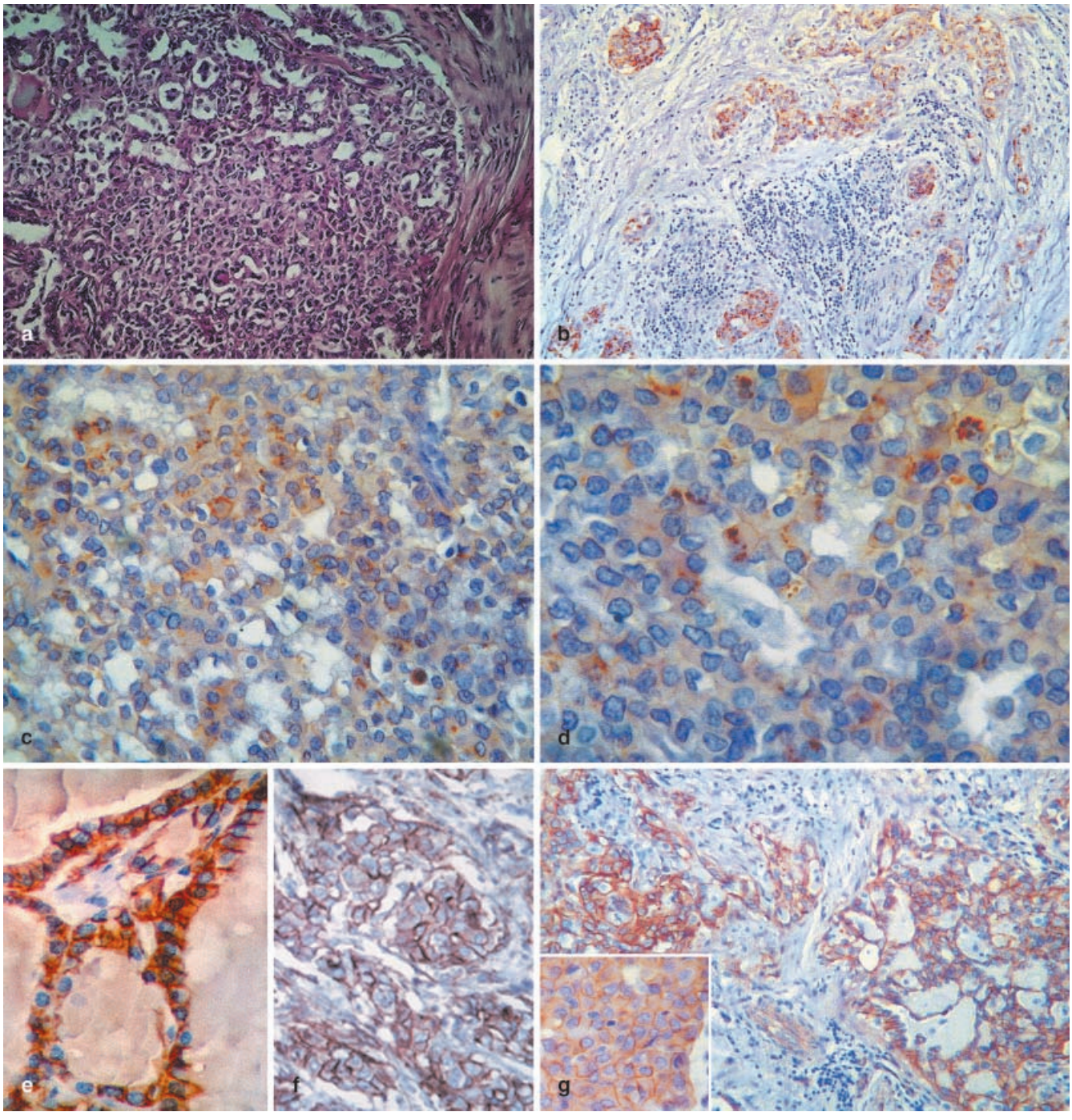

Fig. 1 **a** The histological appearance of tissue from patient no. 8 is characterised by nests of neoplastic cells forming glandular spaces and solid, squamoid areas involved by a fibrotic stroma. H&E, ×100. **b** Low-power magnification of tissue from patient no. 8 showing intense desmoplasia, prominent lymphocytic infiltration and E-cadherin granular perinuclear staining. ×100. **c,d** E-Cadherin granular perinuclear staining in tissue from patient no. 7. ×200 (**c**), ×400 (**d**). **e** The adjacent thyroid parenchyma in patient no. 7 displays a normal membranous E-cadherin staining pattern. ×400. **f** Strong P-cadherin membranous staining in tissue from patient no. 4. ×400. **g** The membranous positivity for β-catenin can be seen in this low-power magnification of a representative sample from patient no. 5. ×150. *Inset*, membranous and cytoplasmic staining for β-catenin in this area of tissue from patient no. 2. ×400

N-Cadherin. The 11 MECT patients displayed no immunoreactivity for N-cadherin, which was not present in the adjacent thyroid parenchyma or in the co-existent foci of PTC.

α-Catenin. MECT displayed a similar pattern of expression regarding both intensity and localisation to that of normal thyroid in four of the 11 patients (Table 2). In two patients (nos. 3 and 7), immunoreactivity was reduced and diffuse in the cytoplasm. The remaining four

specimens were completely negative for α-catenin, except for one of the nodal metastases in patient 1, which displayed weak membrane positivity (Table 2); in these four patients, most of the co-existent foci of PTC exhibited normal α-catenin expression.

The specimens from patient 4 presented different α-catenin expression profiles in the primary tumour and in the metastasis; the former retained normal protein expression, while the latter displayed marked down-regulation accompanied by translocation of the signal to the cytoplasm (Table 2).

β-Catenin. Nine of the 11 MECT patients displayed β-catenin at sites of cell–cell contact (Table 2); the intensity of the signal usually matched that of the positive control or was slightly reduced (Fig. 1g). Staining was usually homogeneously spread throughout the tumour section. Patients 3 and 6 and the metastasis in patients 1 and 4 displayed no immunoreactivity (Table 2), despite the normal pattern of β-catenin expression in the co-existent focus of PTC in patient 6.

γ-Catenin. Four of the 11 MECT patients had a normal pattern of expression in terms of both intensity and localisation (Table 2). Two specimens (patients 1 and 3) displayed strong to moderate cytoplasmic immunoexpression, together, in patient 1, with very weak membranous staining (Table 2). Patients 2, 4, 6, 10 and 11 showed no immunoreactivity at all (Table 2), despite the normal pattern of γ-catenin expression in most of the co-existent foci of PTC.

Molecular analysis of the E-cadherin gene by PCR/SSCP

All the 16 exons, including exon/intron boundaries, of the E-cadherin gene were analysed in the 11 specimens from MECT patients.

Normal SSCP patterns were obtained in MECT for all the amplicons studied.

With the exception of a single case of DSV (which had the missense mutation A592T), we did not detect any E-cadherin mutations in the DSV and PTC patients previously studied [28, 33].

Discussion

It has been suggested that disturbances in cell–cell adhesion, namely in the expression of E-cadherin [5, 28, 33, 37] and α-,β- and γ-catenins [4, 11, 16, 17, 28], may play a pathogenic role in thyroid carcinogenesis.

Primary MECT is a rare thyroid tumour characterised by a distinct morphological appearance and a questionable histogenesis [9, 10, 15, 23, 27, 29, 30].

Despite the ongoing controversy on the putative cell of origin of MECT [9, 10, 30], it is widely accepted that MECT share most of the epidemiological and clinicopathological features of PTC (predominance of young females, nodal metastisation, indolent biological behaviour) and often co-exist with foci of classical PTC [29, 30]. Some of the histological features of MECT (squamoid differentiation, lymphoid infiltration, desmoplastic response, decreased or absent thyroglobulin immunoreactivity) are commonly present in DSV of PTC [29, 30], thus reinforcing the evidence for a relationship between MECT and PTC.

In the present study, we defined the immunoexpression pattern of cadherins/catenins in MECT and evaluated the integrity of the E-cadherin gene. We also compared the results obtained in the study of MECT with those previously obtained in the study of classical PTC and DSV [28, 33].

The first interesting result regarding MECT concerns the neoexpression of P-cadherin, a protein not present in the normal thyroid gland [32]. In the present study, we observed positivity for P-cadherin in ten of the 11 MECT patients. Moreover, this expression was present throughout the tumoural tissues regardless of the prominence of squamoid differentiation in contrast to its absence in the co-existent PTC foci. This observation contrasts with our previous results in PTC and DSV, in which we observed P-cadherin expression but solely in foci of squamous metaplasia [28]. Our results suggest that P-cadherin expression is associated with squamous differentiation of the thyroid gland and that such differentiation is a hallmark of MECT.

Recent publications on the expression of P-cadherin and breast cancer [25, 34] showed an important role for this molecule in breast carcinogenesis. In this model, expression of P-cadherin is associated with poor survival and constitutes an independent prognostic indicator [34]; it is also associated with an infiltrative growth pattern [34]. This may argue in favour of a role for P-cadherin in the acquisition of more aggressive behaviour in breast tumours. Since most MECT follow an indolent clinical course [1, 30], our finding of an almost universal positivity for P-cadherin suggests that in this thyroid tumour histotype, as in PTC and DSV, P-cadherin is a marker of epidermoid differentiation rather than a marker of clinical aggressiveness.

The mechanisms that regulate P-cadherin expression are mostly unknown. The sequencing of the 5'-flanking region of P-cadherin gene has shown an Sp-1 binding site and a CpG island [22]; cytosine methylation of this region has been described in P-cadherin-negative prostate cancer cell lines, thus leading to the conclusion that methylation might play a role in this setting. However, the in vitro results have not been confirmed by in vivo findings, in which a lack of expression in cancer specimens was not associated with methylation of the promoter [22].

Another consistent finding in MECT was the abnormal E-cadherin expression, with the protein being diffusely distributed in the cytoplasm or accumulated in granules with a perinuclear localisation. We also observed absence of E-cadherin in one patient (no. 9). The cytoplasmic localisation of E-cadherin has been associated with alterations of the phosphorylation status of

members of adherens junctions with concomitant loss of connection of E-cadherin with the cytoskeleton [18]. The granular pattern suggests an accumulation of E-cadherin in Golgi vesicles, which might indicate a deficiency in protein transport to the cellular membrane (such a deficiency may be due to a defect in the actin polymerisation process, which is known to be important for the correct localisation of the E-cadherin/catenin complex [35]).

We observed a dissociation between the expression of E-cadherin and that of α-, β- and γ-catenin, with a loss of membrane-associated E-cadherin and occasional retention of the normal localisation of the catenins (e.g. patients 5, 8 and 9). A similar observation was reported by Cerrato et al. [11] and Rocha et al. [28] in some patients with follicular and papillary carcinoma of the thyroid. These findings indicate that β- and γ-catenins can establish connections with membrane-associated molecules other than cadherins; this possibility has already been demonstrated with regard to the association between β-catenin and c-erbB2 [31], MUC-1 [38] and EGF-R [21].

α-Catenin was down-regulated or even absent in seven MECT patients; three of these specimens were totally negative for α-catenin; absence of α-catenin immunoexpression has not been observed in PTC or DSV [28]. In the three specimens negative for α-catenin, there was also absence of expression for γ-catenin (patients 2, 10 and 11). Three of the four specimens with normal immunoreactivity for α-catenin also showed normal expression of both β- and γ-catenin (patients 5, 8 and 9); these three specimens apparently normal for the three catenins had major abnormalities of E-cadherin expression (apart from expressing P-cadherin; Table 2).

β-Catenin expression was absent in two patients and partially absent in two other patients. The remaining seven specimens displayed normal immunoexpression. This finding is similar to those reported in DSV [19, 28]. The normal membranous expression of β-catenin in patients with abnormal E-cadherin might be due to the binding of β-catenin to P-cadherin, although De Boer et al. [13] described a higher affinity of P-cadherin to γ-catenin than to β-catenin. In contrast to Garcia-Rostan et al. [16, 17], we did not find nuclear immunoreactivity for β-catenin in any MECT patients (also absent in PTC and DSV).

γ-Catenin expression was lost in five MECT patients and reduced and abnormally localised in two additional ones. These alterations, which outnumber those detected in PTC and DSV [28], may reflect methylation of the gene promoter resulting in silencing of the gene or a structural alteration like the one detected in a gastric carcinoma cell line [8].

We did not observe major differences in the immunostaining pattern between the primary tumour and the nodal metastasis in the single patient (no. 4) in which both tissues were available. This finding contrasts with the results reported by Bukholm et al. [7] in breast carcinoma, but its validity is obviously limited by the size of the sample.

In summary, like DSV on a smaller scale, MECT displays marked abnormalities of the cadherin/catenins complex compared with normal thyroid and PTC. Such abnormalities may be divided in two groups: (1) consistent neoexpression of P-cadherin and (2) marked abnormality of E-cadherin expression and/or of several of the three catenins. Such alterations apparently do not depend upon mutations, since there are usually a heterogeneous pattern of expression and slight differences when primary tumours are compared with metastases or metastases are compared with one another ([28], present study).

On the basis of the data of the present study together with previously obtained data in classical PTC and DSV [28], it is tempting to suggest that, like MECT, the DSV variant of PTC differs from classical PTC due to major and variable alterations of the cadherins/catenins complex. It remains to be found whether such phenotypic alterations reflect structural genetic hits or, as appears more likely from the available data, whether they merely reflect epigenetic changes. The aetiopathogenic mechanism or mechanisms involved in the acquisition of the phenotypic features of MECT also remain to be clarified.

Acknowledgements We would like to thank Dr. Geert Berx for his excellent advice and for useful discussions. This study was supported by PRAXIS XXI grants (ASR, PS, JCM and VM) and by the PRAXIS project P/SAL/13167/1.

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