ORIGINAL CONTRIBUTION

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Wnt-pathway activation in intestinal-type sinonasal adenocarcinoma*

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SUMMARY

***Background**: Intestinal-type sinonasal adenocarcinoma (ITAC) is an epithelial cancer of the sinonasal sinuses that shows histological similarity to colorectal cancer (CRC) and share chronic inflammation as a possible etiological factor. The Wnt-pathway is one of the most important tumourigenic pathways in CRC. The aim of this study was to investigate if the Wnt-pathway is activated in ITAC.*

***Methodology**: Protein expression profiles of E-cadherin, β-catenin, c-myc and cyclin D1 were analysed by immunohistochemistry in 83 samples of ITAC, organized into tissue microarray blocks.*

***Results**: Nuclear β-catenin expression was observed in 31% of the cases and was twice as frequent in papillary/colonic ITAC compared to solid/mucinous subtypes. Loss of membranous β-catenin staining occurred in 24% and loss of membranous E-cadherin in 6% of the cases and this was more prominent in mucinous types. Strong c-myc and cyclin D1 expression was observed in 30% and 4% of the cases, respectively. Nuclear β-catenin expression was significantly related to poor clinical outcome, independent from established factors as tumour stage and histological type.*

***Conclusion**: The presence of nuclear β-catenin in 31% of patients with ITACs indicated that in a subset of patients, the Wnt-pathway is active and conveys a worse prognosis.*

Key words: ethmoid, sinonasal cavity, adenocarcinoma, Wnt-pathway, tumourigenesis

INTRODUCTION

According to the WHO histological classification [(1)], sinonasal adenocarcinomas are divided in intestinal-type sinonasal adenocarcinomas (ITAC) and non-intestinal-type adenocarcinomas. Five histopathological subtypes of ITAC are recognized: papillary (or PTCC-I), colonic (PTCC-II), solid (PTCC-III), mucinous (alveolar goblet and signet ring), and mixed (transitional) type [(1)]. ITAC represents 8 - 25% of sinonasal tumours and the median age at the time of diagnosis lies between 50 and 60 years [(2)]. Oncological surgical resection combined with radiotherapy is the treatment of choice for most patients with ITAC [(3,4)]. Still, the 5-year survival is low, ranging between 20 - 50% [(5,6)]. The prognosis is better in patients with papillary and colonic type ITAC, in patients without intracranial invasion and in patients with early TNM stage [(7)]. The principal cause of death is local recurrence (40-50%) [(8,9)].

The histological features of ITAC are similar to colorectal cancer (CRC) [(10,11)], hence their denomination 'intestinal-type'. ITAC is strongly related to professional exposure to wood dust [(1,2,4)], however, it is still unknown by what mechanism ITAC develops. It has been suggested that chronic inflammation due to wood dust particles trapped in the middle turbinate may play a role in tumour development [(4)]. Also in a subgroup of CRC, chronic inflammation is known to be involved in tumourigenesis [(12,13)]. The histopathological and etiological similarities could indicate that ITAC and CRC share tumourigenic pathways.

The most important tumourigenic pathway in CRC is Wnt/β-catenin, activated in up to 90% of all tumours [(14)]. The Wnt-pathway functions in two ways. The canonical pathway is important in cell fate determination and has a physiological function in the embryonic development of the neural crest, heart and gastrointestinal tract [(15)]. In most adult tissues this pathway is inactive, except in some stem cells [(16)]. The non-canonical way

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is important in the control of cell movement by the interaction with proteins of the cytoskeleton and tissue polarity regulating the calcium signaling pathway [17]. It promotes the association between the β-catenin and E-cadherin on the cell membrane by the WNT-5a protein increasing the cell adhesión [18].

The central player of this pathway is β-catenin, a protein encoded by the gene CTNNB1, which was originally reported as a protein associated with adhesion molecules like E-cadherin. In normal cells, β-catenin activity is regulated by a large multiprotein 'β-catenin destruction complex' formed by APC, GSK3β, CKIα and Axin [17,19]. In absence of Wnt signals, β-catenin becomes phosphorylated by GSK3β and subsequently ubiquitin-dependent degraded in the proteasome. If β-catenin is released from phosphorylation, it stabilizes and accumulates in the nucleus, where it acts as a transcription factor of target genes like FGF20, DKK1, WISP1, MYC and CCND1 [17,20]. Seventy percent of the sporadic CRC carry APC gene mutations, which results in inefficient β-catenin degradation and up-regulation of β-catenin-mediated transcription of important oncogenes like MYC and CCND1 [21,22].

The relevance of this pathway in ITAC is still largely unknown. The aim of this study is to determinate if the Wnt-pathway is active in ITAC and to what extent. For this, we created a tissue microarray of 83 tumours and evaluated nuclear β-catenin protein expression and its transcriptional targets c-myc and cyclin D1. In addition, we investigated membranous β-catenin and E-cadherin expression. Finally, we correlated all findings to clinico-histopathological and follow up data. We found that the Wnt-pathway is active in one third of all ITAC where it conveys a worse prognosis. Loss of E-cadherin was very infrequent. This expression pattern of β-catenin and E-cadherin better resembles inflammation-related (IBD) than sporadic CRC, which may suggest that chronic inflammation plays a role in ITAC.

MATERIAL AND METHODS

Patients

More than 150 patients with diagnosis of ITAC were treated in our institution since 1980, and 83 of these patients were included in this study; the other 67 cases were excluded because the clinical and follow-up data were not complete or because there was either no material available in the archives of the Pathology Department, or the paraffin block was nearly exhausted. All patients were male and previously untreated at the time of intervention. Informed consent was obtained from all patients, and the study was approved by the ethical committee of our institution. All patients underwent radical surgery and 52 patients (63%) received post surgical radiotherapy. The median age of the patients was 66 year (range 45 - 88 years). Seventy-one patients (86%) have had professional wood dust exposure with a median time of 35 (1 - 60) years. Fifty two percent were smokers. The median follow up was 24 months (range 1-242 months). Six tumours (7%) were papillary, 44 (53%) colonic, 7 solid (8%), 14 (17%) mucinous and 12 (14%) mixed type. The tumour stage according the UICC [23] were 29 (35%) patients stage I, 10 (12%) stage II, 25 (30%) stage III, 11 (13%) stage IVa and 8 (10%) stage IVb. At the moment of the diagnosis 13 patients (16%) had intracranial invasion. A detailed description of all clinical features is given in Table 1.

Tissue microarray

Tissue microarray (TMA) blocks were assembled from formalin-fixed, paraffin-embedded tissues. Areas of interest rich in non-necrotic areas were identified on corresponding hematoxylin and eosin-stained sections and marked with 2 mm circles on the source paraffin block. The source block was cored and a 1 mm core transferred to the recipient master block using the Beecher Tissue Microarrayer (Beecher Instruments, Silver Spring, MD, USA). Three cores from different areas of the same tissue block were arrayed for each case. Four different TMA blocks were constructed with a total of 249 tissue cores with a spacing of 1.5 mm, representing 83 tumours. Three micrometer sections were stained with hematoxylin and eosin and reviewed by one pathologist to determine whether the samples represented the tumour.

Immunohistochemistry

Immunohistochemistry (IHC) was performed using antibodies against β-catenin, E-cadherin, c-myc and cyclin D1. Antibody clone β-catenin-1 (Dako, Glostrup, Denmark) was applied in a dilution of 1/200, with an incubation time of 25 minutes in citrate buffer pH 9.0. Antibody E-cadherin clone NCH-38 (Dako), mouse monoclonal antibody c-myc (Santa Cruz Biotechnology, CA, USA) and mouse monoclonal antibody cyclin D1 (Santa Cruz Biotechnology) were used in a dilution of 1/50, and an incubation time of 20 minutes in citrate buffer pH 9.0. All IHC results were evaluated by two expert pathologists. β-catenin and E-cadherin were considered positive if clear membrane immunoreactivity was present. Special attention was paid to the aberrant nuclear expression of β-catenin. The nuclear expression of c-myc and cyclin D1 was graduated in 4 levels (immunopositivity of: less than 25%, 25-50%, 50-75% and more than 75% of cells).

Statistical analysis

Correlations between the IHC results and clinico-pathological variables were analysed by SPSS 15.0 software for Windows (SPSS Inc., Chicago, IL, USA), using Fischer Exact chi-square test. Multivariate Cox regression analysis was performed for clinical and immunohistochemical factors possibly related to survival, and Kaplan–Meier survival curves were calculated comparing distributions of survival through Mantel-Cox log-rank test. For survival analyses, only died-of-disease was considered as event. Values of $p < 0.05$ were considered significant.

RESULTS

Follow-up

During the time of follow-up, 36 patients (43%) developed local recurrence within a median disease-free time of 12 (1 - 96) months. Thirteen patients (16%) developed distant metastases within a median disease-free time of 10 (1-60) months. Four patients developed both recurrence and metastasis. Thirty-three patients (40%) died of disease, 10 died of other causes (12%) and

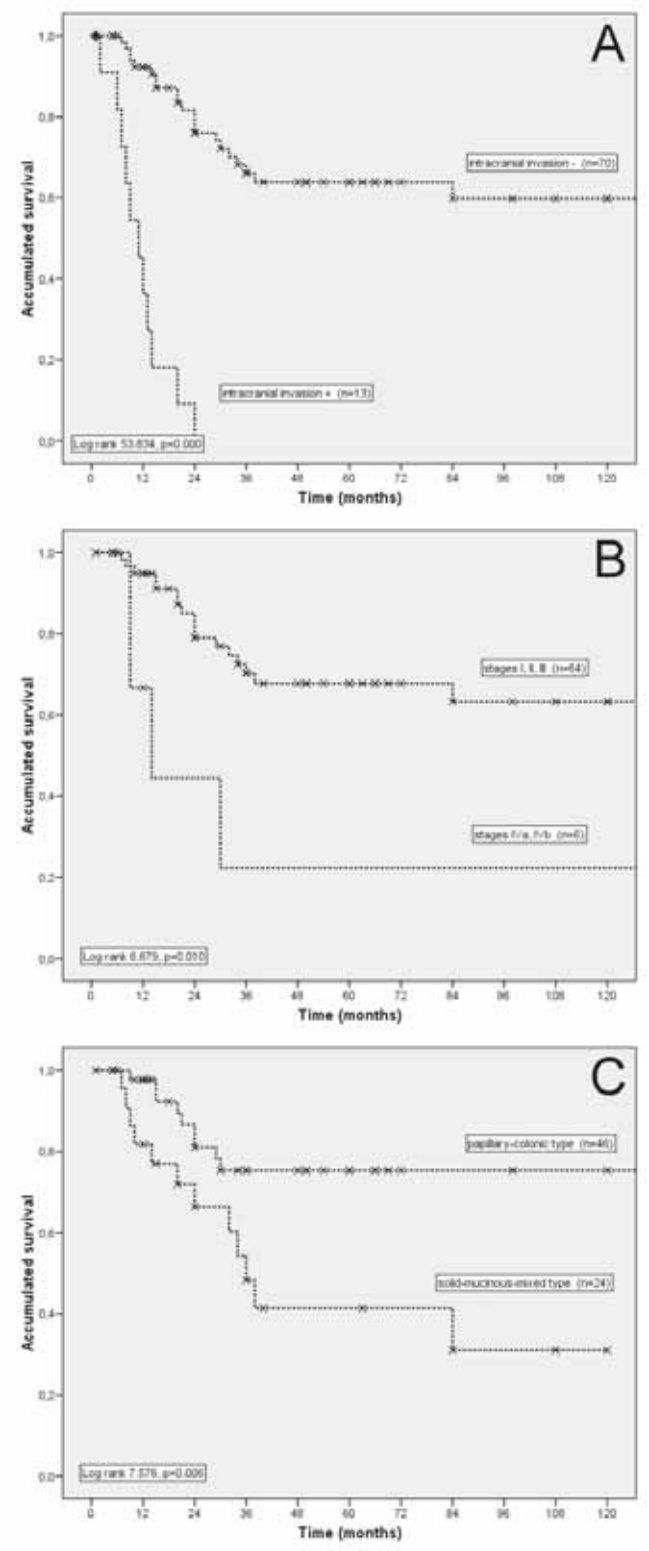

Figure 1. Kaplan Meier survival analysis. (A) Overall survival of 13 patients with intracranial invasion versus 70 patients without intracranial invasion at the time of presentation. (B) Overall survival of the 70 patients without intracranial invasion, according to tumour stage; stages I-III showed significantly better survival than stages IVa and IVb. (C) Overall survival of the 70 patients without intracranial invasion, according to histological type; papillary and colonic type ITAC had a significantly better survival than solid, mucinous and mixed types ITAC.

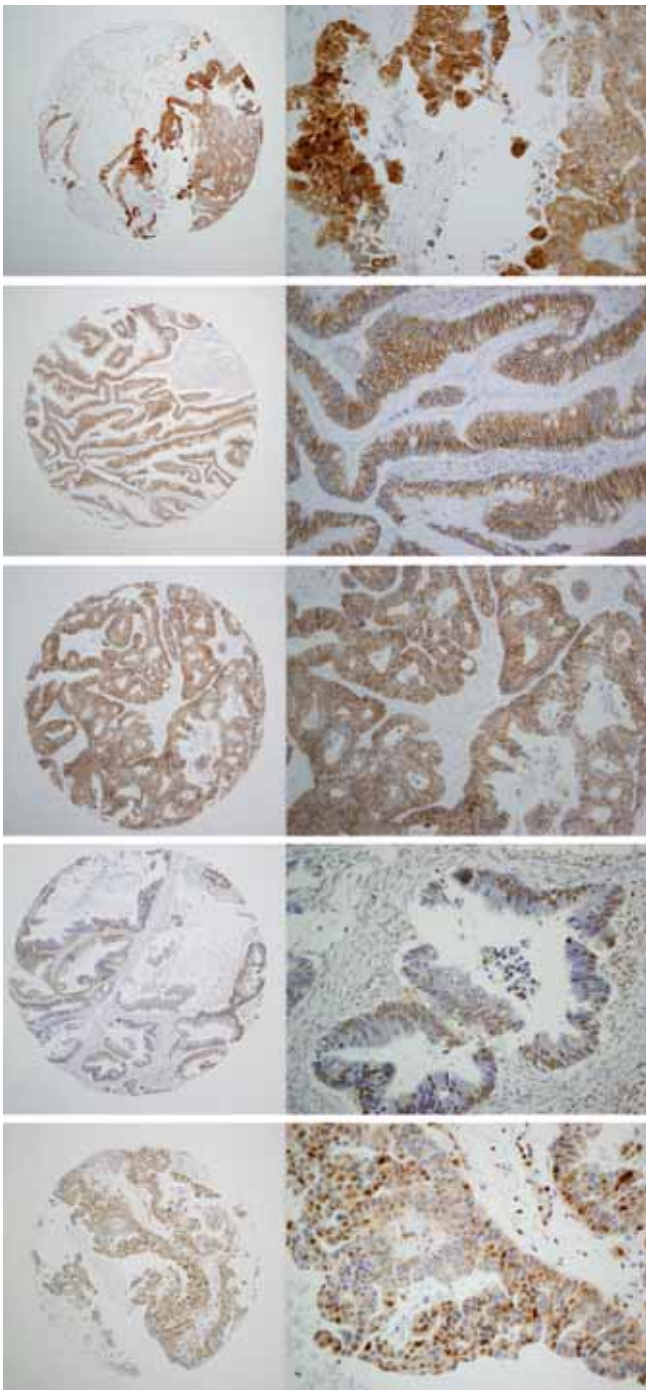

Figure 2. Immunohistochemistry. Photomicrographs of immunohistochemical expression patterns of β-catenin (A, B), E-cadherin (C), c-myc (D) and cyclin D1 (E), each with magnification 100X (left image) and 400X (right image). Sample a) shows a colonic type ITAC with both nuclear and membranous expression of β-catenin, and b) a papillary type ITAC with membranous expression of β-catenin only. Sample c) demonstrates a colonic type ITAC with membranous E-cadherin expression, and samples d) and e) a colonic type ITAC with nuclear expression of c-myc and cyclin D1, respectively.

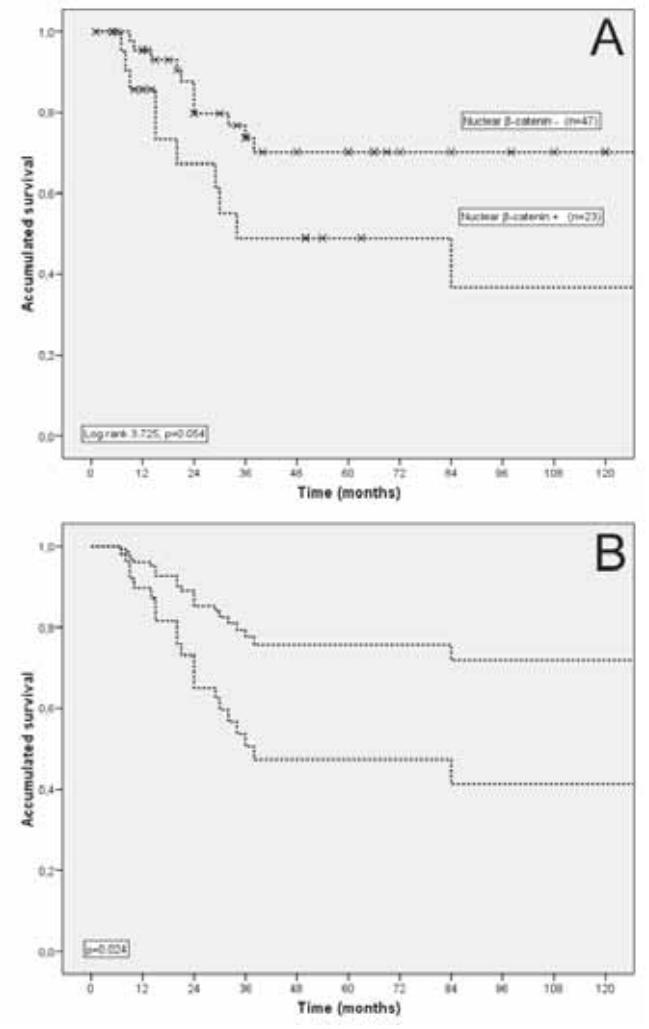

Figure 3. Nuclear β-catenin expression is associated with worse clinical outcome. (A) Kaplan Meier curve of the 70 patients without intracranial invasion showing a worse overall survival of ITAC cases with nuclear expression of β-catenin. (B) Estimated survival curve of the 70 patients without intracranial invasion, adjusted by tumour stage and histological type (based on multivariate Cox regression analysis, Table 4), showing a significantly worse overall survival of ITAC cases with nuclear expression of β-catenin.

38 patients (46%) remained alive without tumour. The five year overall survival was 48% and the 5 year disease-free survival was 53%. Intracranial invasion at the time of presentation was a very strong indicator of poor overall survival (Log rank 53.83, p = 0.000) (Figure 1A). Considering only the intracranial-negative cases (n = 70), tumour stages I - III (Log rank 6.68, p = 0.010) and papillary-colonic type ITAC (Log rank 7.58, p = 0.006) had a more favorable clinical course (Figure 1B, 1C).

Immunohistochemistry

Nuclear expression of β-catenin was present in 26 patients (31%). Sixty-three cases (76%) presented membranous expression of β-catenin and 78 (94%) showed membranous immunoreactivity for E-cadherin. C-myc was expressed in 81 patients (98%), 33 tumours (40%) were scored 1 - 25% positive, 23 tumours (28%) as 25 - 50%, 16 tumours (19%) as 50 - 75% and 9 (11%) as 75 - 100% positive. Cyclin D1 was expressed in 47 patients (57%), 34 tumours (41%) were scored as 1 - 25% positive, 10 tumours (12%) as 25 - 50%, 1 tumour (1%) as 50 - 75% and 2 (3%) as 75 - 100%. Examples of all immunohistochemical stainings are given in Figure 2. The control group, constituted by 7 tissue cores of normal mucosa of paranasal sinuses, showed consistent membranous staining of β-catenin and E-cadherin, and absence of nuclear immunoreactivity of β-catenin, c-myc and cyclin D1.

Immunohistochemical-clinical correlations

Although concerning few cases, high expression (> 50% of nuclei) of cyclin D1 (Fisher Exact test p = 0.028) was significantly related with nuclear expression of β-catenin (Table 3), but c-myc did not (Fisher Exact test p = 0.070). Nuclear expression of β-catenin was in general accompanied by membranous β-catenin expression (Table 3). Loss of membranous β-catenin (10/20 versus 16/63 cases, Fisher Exact test p = 0.039) as well as loss of membranous E-cadherin (4/5 versus 22/78 cases, Fisher Exact test p = 0.053) expression occurred more frequently in mucinous type ITAC.

The expression pattern of β-catenin did not appear related to age, or to wood dust or tobacco smoking etiology. Papillary and colonic cases showed more frequently nuclear expression of β-catenin (19/50, 38%) than the solid and mucinous tumours (7/33, 21%) (Fisher Exact test p = 0.147) (Table 3).

Patients with nuclear expression of β-catenin showed a tendency towards worse overall survival (Log rank 1.99 p = 0.158), but after taking out the 13 cases with intracranial invasion, the relation became nearly significant (Log rank 3.725 p = 0.054, Figure 3A). Using multivariate Cox regression, we found that nuclear β-catenin was a factor independent from established prognostic indicators as tumour stage and histological type (Table 4). Figure 3B shows the expected survival curve according to the nuclear β-catenin status, estimated from the multivariate Cox regression model of Table 4 (adjusted by tumour stage and histological type.

DISCUSSION

The morphological and etiologic similarities between ITAC and CRC may suggest a common oncological pathway. Working with this hypothesis, several authors compared genetic aberrations of ITAC and CRC [24-27], but their results did not support the hypothesis. Microsatellite instability, observed in approximately 15% of sporadic CRC, is present only in 2% of ITAC and does not appear to be an important pathway of tumourigenesis [28]. Ras mutation that is detected in 50% of CRC, is almost absent (0 - 15% of cases) in ITAC [24-27] and TP53 mutations, present in 75% of CRC, occur in 18-55% ITACs [26,27]. Also β-catenin has been analysed, although in studies with a low number of tumour samples. In this paper, we present data from a relatively large set of tumours, and report the presence of nuclear β-catenin in 26 of 83 tumours (31%). This finding is similar to Frattini et al., [29] who reported membranous, cytoplasmic and nuclear positivity

Table 1. Clinical features according to histological type of ITAC.

		all tumours	papillary	colonic	solid	mucinous	mixed
Total		83	6	44	7	14	12
Age	median	66	66	65	56	68	70
Wood	no	12	0	5	4	0	3
	yes	71	6	39	3	14	9
	median time	35	38	35	10	40	20
Tobacco	no	40	2	20	3	8	7
	yes	43	4	24	4	6	5
Stage	I	29	1	22	1	4	1
	II	10	2	2	0	4	2
	III	25	3	13	2	3	4
	IVa	11	0	5	2	3	1
	IVb	8	0	2	2	0	4
Intracranial	no	70	6	40	5	12	7
	yes	13	0	4	2	2	5
Radiotherapy	no	31	1	17	4	4	5
	yes	52	5	27	3	10	7
Recurrence	no	47	6	26	2	8	5
	yes	36	0	18	5	6	7
	median time to rec	12	na	13	3	14	12
Metastasis	no	70	5	39	6	11	9
	yes	13	1	5	1	3	3
	median time to met	10	15	8	6	8	8
Status patient	alive	40	3	27	1	6	3
	died of disease	33	1	11	6	6	9
	died of other causes	10	2	6	0	2	0

Table 2. Immunohistochemical staining results.

	β-catenin nuclear	β-catenin membranous	E-cadherin	c-myc	cyclin D1
Negative	57 (69%)	20 (24%)	5 (6%)		
Positive	26 (31%)	63 (76%)	78 (94%)		
0% Cells				2 (2%)	36 (43%)
< 25 % Cells				33 (40%)	34 (41%)
25 - 50% Cells				23 (28%)	10 (12%)
50 - 75% Cells				16 (19%)	1 (1%)
> 75% Cells				9 (11%)	2 (3%)

Table 3. Correlations of nuclear β-catenin.

		Nuclear β-catenin		Significance
		negative	positive	
Membranous β-catenin	positive	39	24	p = 0.0253
	negative	18	2	
Membranous E-cadherin	positive	52	26	p = 0.319
	negative	5	0	
c-myc	< 50%	35	22	p = 0.070
	> 50%	21	4	
cyclin D1	< 50%	57	23	p = 0.028
	> 50%	0	3	
papillary/colonic		31	19	p = 0.147
solid/mucinous/mixed		26	7	

Table 4. Multivariate Cox regression survival analysis of nuclear β-catenin, tumour stage and histological type.

	Hazard ratio (95% C.I.)	Significance
Tumour stage (stages I-III versus IVa+b)	5.16 (1.61-16.53)	p = 0.006
Histological type (pappillary-colonic versus solid-mucinous-mixed)	3.95 (1.60-9.76)	p = 0.003
Nuclear β-catenin	2.69 (1.14-6.37)	p = 0.024

C.I. : confidence interval

in 8 of 20 ITACs (40%). Conversely, Yom et al., [30] did not find any mutation in APC or CTNNB1 in 8 ITACs, while Pérez-Ordoñez et al., [31] found only membranous staining for β-catenin and E-cadherin in 10 ITACs studied.

The proportion of cases with nuclear β-catenin is lower in ITACs (31%) than in sporadic CRC (up to 90%). However, Aust et al., [32] reported a lower nuclear β-catenin expression in tumours arising from inflammatory bowel disease (IBD) compared to sporadic tumours, 48% versus 81%, respectively. This may indicate that the Wnt-pathway is less important in tumours that have an etiology in chronic inflammation, which could concur with our finding of a relatively low proportion of ITACs with nuclear β-catenin. Interestingly, nuclear β-catenin expression occurred almost twice as frequent in the more 'intestinal-like' types of ITAC, i.e. the papillary and colonic subtypes, as opposed to the solid and mucinous types (38% versus 21%). Moreover, we found nuclear β-catenin to be related to worse prognosis, independently from established factors as tumour stage and histological type. This may concur with IBD-related CRC, in which, as opposed to sporadic CRC, nuclear β-catenin is considered a late event and therefore progression-related [33]. This finding may be clinically important, especially for identifying patients with tumours of stage I-III or of papillary and colonic subtypes having an adverse prognosis.

As reported in CRC [21,22], we found that the expression pattern of c-myc and cyclin D1 was related to that of nuclear β-catenin. However, also many tumours with no nuclear β-catenin showed overexpression of c-myc and cyclin D1. This may be explained by gene copy number gain or amplification of c-myc (at chromosome band 8q24), which is indeed frequently found in ITAC [34,35]. On the other hand, cyclin D1 can be regulated through the EGFR pathway, which has also been reported to be frequently activated in ITAC [36].

The majority of cases in our study demonstrated membranous β-catenin staining together with E-cadherin, and also the loss of membranous β-catenin (20/83 cases, 24%) was related with loss of the membranous expression of E-cadherin (Fisher Exact Chi^2: p = 0.011), although the latter was an infrequent event (5/83 cases, 6%). Both loss of membranous β-catenin and E-cadherin occurred mostly in mucinous type ITAC, which is in agreement with the fact that this type of ITAC generally shows little cellular adhesion. In sporadic CRC, loss of the membranous expression of β-catenin and of E-cadherin are much more frequent events (around 80% and 65%, respectively) and occur simultaneous with nuclear expression of β-catenin, while in IBD-related CRC, this relation is less obvious [32]. In our series of ITAC, we did not find this relation: only 2/26 cases with nuclear β-catenin also showed loss of membranous β-catenin and 0/26 showed loss of E-cadherin. Again this may indicate that ITAC resembles more inflammation-related CRC than sporadic CRC. Loss of membranous β-catenin and E-cadherin have been related to the development of distant metastases [37], however, we could not confirm this in our series of ITAC.

In conclusion, nuclear β-catenin was detected in 31% of ITAC tumours indicating that in this subset the Wnt-pathway is active. Importantly, nuclear β-catenin was found to be a prognostic factor independent from the tumour stage or the histopathological type. When comparing to CRC, it would appear that the expression pattern of β-catenin and E-cadherin better resemble inflammation-related (IBD) than sporadic CRC, which may suggest that chronic inflammation plays a role in ITAC.

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AUTHORSHIP CONTRIBUTION

Study design: MAH and JLL. Patient collection: JLL and CAM. Clinical data collection: JLL, CAM and JPDM. Sample processing for IHC: JPE and JPDM. Immunostaining and histopathological evaluation: BV, MFF and JPDM. Overall data interpretation: JPDM, PMC and MAH. Writing manuscript: JPDM and MAH. Critical lecture and correction manuscript: JPE, JLL and CAM.

CONFLICT OF INTEREST

The authors declare no conflicts of interest concerning this manuscript.

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