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Brachyury expression predicts poor prognosis at early stages of colorectal cancer

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ABSTRACT

Although survival rates of colon cancer patients diagnosed at an early stage (T1-2N0M0; Dukes A) vary considerably according to the studies cited, several studies indicate development of distant metastases already occurring in a considerable percentage of these patients leading to the death of the patients. This particular high risk group cannot be identified properly as no marker exists to identify these patients. As the Wnt/Win pathway plays a crucial role in metastasis formation in colorectal carcinoma, we analysed whether the transcription factor brachyury critically involved in this pathway may predict metastasis formation in these patients.

The expression of brachyury-homologous (T) was immunohistochemically analysed in 748 patients and the data were correlated with classical and newer prognostic markers in colorectal cancer.

Early stages colorectal cancer patients (T1-2N0M0, Dukes A) showed a significantly decreased survival when brachyury was expressed in the tumour tissue while no correlation was observed in later tumour stages. Hence a subset of colorectal cancers exists in which the ability to metastasise is already present at early stages of tumour growth and this high risk group can now be detected by immunohistochemistry.

1. Introduction

Colorectal cancer is the third most common form of cancer and the second leading cause of cancer-related death in the Western world.[1] As in other cancers, cancer-related deaths in colorectal carcinoma can be attributed to the fact that colorectal cancer metastasises and once generalised metastases have been formed, mostly no cure is possible.

While it was initially proposed that colorectal carcinogenesis is a stepwise process in which metastasis is a relatively

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late mutational event,[2] later data showed that multiple alternative genetic pathways to colorectal cancer exist indicating that the linear model of Fearon and Vogelstein is too simplistic for the molecular events occurring during malignant progression in many colorectal cancer patients.[3] The latter hypothesis implies that cancer patients should exist in which the ability to metastasise is already present at an early stage of tumour development. This point of view is underscored by clinical studies which show that a considerable number of early stage colorectal cancer patients develop metastases later on.[4]

We therefore searched for such a protein that might represent an early genetic marker for metastatic behaviour of colorectal cancer. In our search we concentrated on the Wnt/β-catenin pathway, as proteins of this pathway regulate the epithelial mesenchymal transition (EMT), a process thought to be vital for metastasis formation.[5] EMT is tightly regulated by transcription factors and a particular transcription factor involved in this pathway, namely brachyury, has recently been implicated to play a pivotal role in tumour growth and metastasis.[6] Brachyury, also known as T, is a member of the T-box family, whose expression is regulated by the Wnt signalling pathway which in turn is mediated by the β-catenin/TCF4 complex.[7] This complex has been shown to be expressed in the proliferative compartment of the colonic mucosa, where the intestinal epithelial stem cells reside.

Although initially the expression of the brachyury protein had been identified as a definitive diagnostic marker of chordoma,[8] recent studies showed that brachyury is also expressed in tumours of the small intestine, stomach, kidney, bladder, uterus, ovary and testis, as well as in cell lines derived from lung, colon and prostate carcinomas, however, not in the vast majority of normal tissues tested[9] making it an interesting target as a prognostic marker in cancer.

Until now, the role of brachyury in malignant progression of colorectal cancer has not been investigated. The aim of this study was to evaluate whether brachyury is expressed in colorectal cancer at all, and if, whether it shows a correlation with different stages and grading and finally, whether it may be a suitable prognostic marker for colorectal cancer.

2. Patients and methods

2.1. Patients

Patients with CRC attending surgery between 1975 and 1995 in the Department of Surgery Hannover Medical School were investigated and whose records and tissue sections were available ($n = 748$). Patients age ranged between 33 and 87 with a median age of 63.3. Each case was classified both according to the pTNM system and Dukes classification. Dukes A tumours were confined to the bowel wall (T1N0M0 and T2N0M0), Dukes B tumours extended locally beyond the bowel and Dukes C tumours involved lymph nodes and Dukes D tumours presented with distant metastases. In addition, data for tumour differentiation (grading), tumour size, sex and different known prognostic and predictive markers as well as follow-ups to 300 months (25-years) were also included in the analysis.

2.2. Tissue microarray

Multi-tissue blocks of routine formalin-fixed tissues were assembled according to tissue array technology developed in the Department of Pathology, Medical School of Hannover as described previously.[10]

2.3. Immunohistochemistry

For immunohistochemical analysis, 4 μm sections were cut and stained according to standard protocols. In brief, sections were deparaffinised and antigen retrieval was achieved by treating the sections with in a microwave oven in pH 6.0 citrate buffer. Thereafter non-specific binding sites were blocked by 1:10 diluted rabbit serum, followed by incubation with a primary goat anti-brachyury antibody (R&D Systems, #AF2085) which was diluted 1:50 and incubated at 4 °C overnight. After washing in buffer, detection of the binding sites of the primary antibody was carried out using biotinylated rabbit anti-goat antibody followed by incubation with an avidin biotin alkaline phosphatase complex (Zytomed, Berlin, Germany). The enzyme reactivity was visualised using new fuchsin and napthol-AS-biphosphate as a simultaneous coupling reagent. The intensity of the reaction was graded as negative for no staining and positive for immunostaining in tumour cells or staining in intracytoplasmic immunoreactive granules.

2.4. Flow cytometry

To ascertain the specificity of the antibody, human HT29 colon cancer cells were detached with enzyme-free Cell Dissociation Buffer (Invitrogen, Karlsruhe, Germany) at room temperature, fixed by incubation with 2% formaldehyde, 0.5% BSA in PBS and permeabilised with 0.5% saponin, 0.5% BSA in PBS. Cells were washed with PBS between all steps. Polyclonal goat anti-human/mouse Brachyury (R&D, Wiesbaden, Germany) or goat IgG control (R&D) was used at 1 μg/ml for staining of 1×10^6 permeabilised tumour cells in 200 μl PBS, 1% BSA, followed by biotinylated polyclonal rabbit anti-goat (Dako, 1:200) and allophycocyanin-conjugated Streptavidin (BD, Heidelberg, Germany). After washing, cells were subjected to fluorescence assisted flow cytometry on a FACSCalibur (BD). Files were analysed using Win MDI 2.9 software.

In addition to the FACS control, HT29 cells were fixed in formaldehyde, embedded in agar and processed for wax histology in the same procedure as human tissues. Protein extracts were prepared from HT29 cells and subjected to Western blot analysis for brachyury using the same antibody.

2.5. Statistical methods

SPSS 15.0 version (SPSS Inc., IL, USA) was used for data analyses for cross tables, univariate and multivariate analyses as well as Kaplan–Meier 25-year survival outcome for different parameters. Parameters were initially tested separately for brachyury expression in all colorectal adenocarcinoma ($n = 748$) and if appropriate for adenocarcinoma in stage Dukes A ($n = 191$) only.

To ascertain possible correlations between different clinical and pathological parameters and known prognostic and predictive markers to the expression of the brachyury protein (details for these markers see Ref.[11]) data were cross-tabulated and a Chi-square (Likelihood) test was performed. The association of staining for brachyury protein expression with patient survival was evaluated using life tables constructed from survival data with Kaplan–Meier plots. The Log Rank test was applied to test significance of differences between stratified survival functions. The end-point used in the present, retrospective investigation was overall survival, counting death from any cause as the event. Overall survival was measured from date of initial surgery until date of death or, if no event was documented, until date of last information (not counting as the event). A multivariate survival analysis was carried out using the Cox proportional hazard model. All p-values were tested two-sided and $p < 0.05$ was considered as significant.

3. Results

3.1. *Flow cytometry (FACS)*

HT29 cells showed a shift of fluorescence intensity indicating the presence of brachyury expression in these cells (Fig. 1). Formalin fixed wax embedded HT29 cells also clearly showed brachyury expression indicating that this antibody is suitable to detect brachyury expression in paraffin embedded tissue sections. Additionally, brachyury could be detected in Western blots of protein extracts from HT29 cells using the same antibody (data not shown).

3.2. *Selection of CRC cases for brachyury analyses*

TMA sections obtained from 748 CRC cases for which a follow up of up to 300 months (25 years) existed were analysed for brachyury expression. Because of different pathological and clinical behaviour and therapeutic consequences we analysed only adenocarcinomas in different stages and excluded squamous cell carcinoma and colorectal carcinomas of other histological type (adenocarcinoma 86%, mucin-type adenocarcinoma 14%). Nearly 90% ($n = 665$) of the colorectal adenocarcinomas were immunohistochemically positive for brachyury.

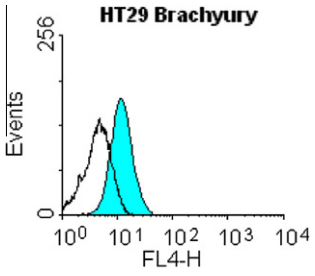

Fig. 1 – Brachyury expression in HT29 cells using FACS-analysis. Brachyury is present in cell line HT29: Intracellular staining with anti-brachyury (filled curve) and isotype control (open curve).

In tissue microarray sections of CRC the brachyury immunostaining was particularly strong in tumour cells (Fig. 2). In some cases a positive brachyury staining could be detected in blood vessels additionally to positively stained tumour cells (Fig. 2).

3.3. *Brachyury expression in correlation with colorectal cancer staging according to Dukes and TNM*

Using cross table analyses most cases negative for brachyury were found in the stage Dukes A while their number continually declined in cases of higher Dukes stages achieving 0% in Dukes D. High brachyury expression correlates significantly with higher Duke stage, AJC stage, tumour grade and lymph node metastasis (Table 1). The correlation between brachyury expression and TNM classification was also analysed. The tumour stage pT1 showed the most negative brachyury cases (16.7%) followed by pT2 (5.1%) and pT3 (8.9%). Accordingly, the percentage of brachyury positive cases rises from 83.3% in pT1 to 84.9% in pT2 and 91.1% in pT3. Due to the few number of cases in pT4 stage ($n = 55$), the Pearson Chi-square test did not show any significance ($p = 0.07$). In contrast, the correlation to N-staging was highly significant with $p = 0.007$. In N0 stage the fraction of cases negative for brachyury was with 13.4% significantly higher in comparison to the stage pN1 with 6.6% and pN2 with 0% negative brachyury cases. The same situation could be found for the TNM classification considering the M-staging. But due to few number of cases for M1 ($n = 79$) the assessment was not significant ($p = 0.063$).

3.4. *Brachyury expression in relation to tumour grading*

Using cross table studies, the correlation between brachyury expression and histopathological grading of the colorectal cancer was analysed. While brachyury negative cases were found to be histologically well differentiated, brachyury positive cases were generally less well differentiated or even undifferentiated ($p < 0.0001$).

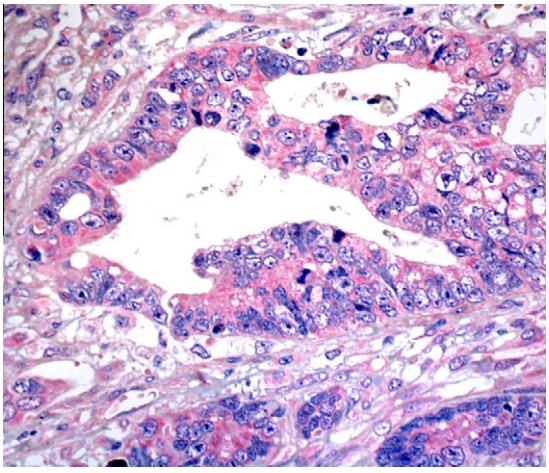

Fig. 2 – Immunohistochemistical detection of brachyury in early colon cancer. Brachyury immunoreactivity is found intracytoplasmatically in tumour cells.

Table 1 – Significancy in cross table for brachyury.

	n	Evaluable	Brachyury (%)	p-Value
Total	890	748	88.9	
Dukes				0.001
A	219	191	82.2	
B	347	314	88.9	
C	245	216	93.5	
D	76	24	100	
T stage				0.07
1	63	54	83.3	
2	221	186	84.9	
3	551	474	91.1	
4	55	34	88.2	
N stage				0.007
0	578	506	86.6	
1	259	213	93.4	
2	43	21	100	
M stage				0.063
0	807	718	88.6	
1	79	27	100	
AJC stage				0.033
T1N0M0	46	43	81.4	
T2N0M0	148	120	80.8	
T3N0M0	274	255	87.8	
T anv. N1-3	202	181	91.2	
M+	26	12	100	
Grade				<0.0001
1	184	152	77.0	
2	596	507	91.9	
3	110	89	92.1	

Significant *p*-values for brachyury in cross tables are given for Dukes, N- and AJC-stages as well as for grading. The T- and M-stages are not significant but show the same tendency as explained in the text.

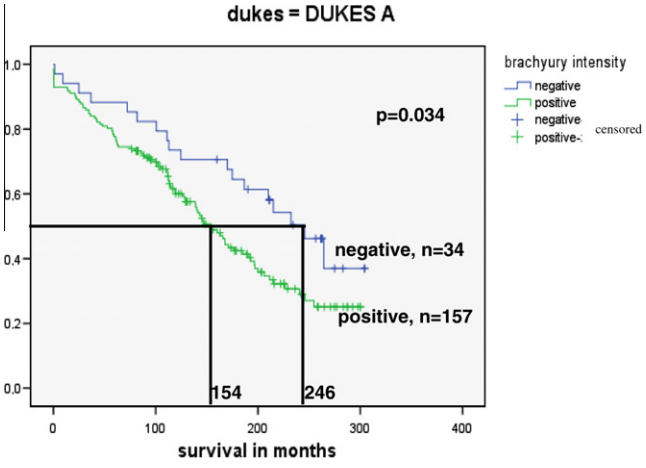

Fig. 3 – Kaplan–Meier survival analyses for brachyury subgroup Dukes A tumours. Patients with negative expression of brachyury (*n* = 34) in colorectal adenocarcinoma have a significant better 25-year survival outcome than patients with positive expression of brachyury (*n* = 157) at the early stage Dukes A (*p* = 0.034).

3.5. *Prognostic relevance of brachyury expression*

While brachyury expression was not significantly associated with prognosis for all stages of CRC (*n* = 748) in the 25-year survival analysis, its expression was found to be significant (*p* = 0.034) in the sub-group Dukes A (*n* = 191) where a positive staining correlated with a poor 25-year survival outcome in Kaplan Meier analysis (see Fig. 3). The significance of this finding was particularly well associated with well differentiated CRC (*p* = 0.048).

3.6. *Brachyury expression in multivariate analyses*

In multivariate analyses (Cox regression) brachyury expression was an independent prognostic marker if brachyury expression was compared with tumour grade in Cox regression analysis in Dukes A (*p* = 0.028) stages only (Table 2).

3.7. *Brachyury expression in relation to tumour location*

Brachyury negative adenocarcinoma had a significantly better 25-year survival outcome when they were located in colon (*p* = 0.029) than in the rectum.

3.8. *Brachyury expression in relation to gender*

Men with colorectal adenocarcinoma stage Dukes A showed a significantly better 25-year survival outcome when brachyury was not expressed (*p* = 0.006). No such correlation could be found for women (*p* = 0.963).

3.9. *Brachyury expression in relation to known prognostic and predictive markers*

Next, we evaluated the brachyury expression in relation to other known prognostic and predictive markers for CRC of Dukes A stage (Table 3). Remarkably, for the most markers there is no correlation with brachyury expression. Even so, low level expression of the cell cycle markers p21 and p27 together with a detection of brachyury in CRC stage Dukes A was significantly correlated with a poorer outcome (*p* = 0.0001 and *p* = 0.027) while p16 expression did not correlate significantly with brachyury expression in CRC (*p* = 0.054) .

3.10. *Brachyury expression in relation to further predictive markers*

Microsatellite instability markers such as MSH2 and MLH1 showed no better 25-year survival for the Dukes A sub-group when brachyury was negative.

In contrast, a positive or strong positive staining for TS but not for TP was found to correlate significantly with a poor outcome in 25-year survival of patients with stage Dukes A when

Table 2 – Cox regression multivariate analysis of brachyury expression in combination with grade and Duke staging.

Factor	p-Value	HR	95% CI
Dukes	<0.0001	1.522	1.352–1.713

In multivariate analyses only Dukes stages are significant for brachyury with a hazard ratio of 1.5.

Table 3 – Significancy of prognostic and predictive markers for brachyury (chi2 likelihood).

	Cut-off	*n*	*p*-Value
Ki-67	<50%	317	0.162
	>50%	109	
CXCR4	neg	218	0.091
	pos	512	
p16	neg	190	0.054
	pos	549	
p21	neg	84	<0.0001
	pos	142	
p27	<50%	183	0.027
	>50%	561	
MSH2	<5%	25	0.528
	>5%	451	
MLH1	<5%	26	0.276
	>5%	465	
IGF1	neg	507	0.714
	pos	231	
IGF2	neg	594	0.426
	pos	136	
TP	neg	125	0.676
	pos	539	
TS	neg.	66	<0.0001
	pos.	666	
p53	neg	144	0.346
	pos	594	
RB	<50%	228	0.053
	>50%	490	
S100	<50% (neg)	633	0.157
	>50% (pos)	111	
SV40	neg	330	0.299
	pos	393	

To ascertain possible correlations between different clinical and pathological parameters and known prognostic and predictive markers to the expression of the brachyury protein data were cross-tabulated and a Chi-square (Likelihood) test was performed. The cell cycle markers p21 and p27 as well as the predictive marker thymidilate synthase (TS) are significant ($p < 0.05$)
Microsatellite instability genes (MSH2, MLH1), IGF1 and -2 = insulin-like growth factor-1 and -2, TP = thymidine phosphorylase, RB = retino blastoma protein, SV40 = simian virus40.

additionally a strong brachyury expression was present ($p = 0.0001$).

4. Discussion

The present study shows that it is possible to detect metastatic cancers during early stages of colorectal carcinogenesis by using an antibody against the transcription factor brachyury in immunohistochemistry analysis. This finding is of interest for two reasons.

The first reason is molecular as it shows that metastasis initiating cells are present at a very early stage of colorectal cancer carcinogenesis in some cancer patients. This observation from a clinical point of view puts the original hypothesis of stepwise colorectal cancer carcinogenesis by Fearon and Vogelstein[2] into question and corroborates the critique of this hypothesis proposed by Smith et al.[3] Brachyury, the protein indicating metastasis in this report, is a transcription factor involved in the Win/Wnt pathway which is critically involved in the epithelial mesenchymal transition (EMT), which in turn plays a major role in allowing cancer cells to escape from the primary tumour.[5,12,13] However, EMT is a complicated process in which many other transcription factors are involved[6] and as such it is not astonishing that brachyury loses its predictive power during later stages of colorectal cancer in which then these factors take over. That many other factors are involved in the brachyury signalling was already observed in the study by Fernando et al.[6] who showed that the effects of brachyury overexpression on E-cadherin expression were at least partially mediated by slug and not by snail, both factors normally working together in the epithelial mesenchymal transition.

The finding that metastasis initiating cells are present at early stages of development is also of interest for the biology of cancer metastasis. Two different models of metastasis formation have been proposed, namely the linear progression model and the parallel progression model.[14] In the first model metastatic tumour cells develop to full malignant potential within the primary tumour while in the second model tumour cells evade and undergo somatic progression and metastatic growth at a distant site. Both models are under discussion as no direct and incontrovertible evidence exists to support either hypothesis.[14] Our finding that the ability to form metastases is already present at an early stage of colorectal cancer development would argue for a linear progression model and particularly so as the marker chosen is a transcription factor involved in a fundamental molecular step of the metastatic cascade. The observation that a transcription factor is involved during the malignant progression to colorectal cancer metastasis would also fit to the observation that not all disseminated tumour cells progress to full metastases as the local microenvironment at the metastatic site might be involved in the modulation of the effects of the transcription factor.[15] This influence of local factors might explain why some disseminated tumour cells develop into clinically detectable metastases while other disseminated tumour cells remain silent in a single cell status.

In our clinical series the percentage of brachyury expression increased with the clinical stages of the disease, i.e. early stages showed a lower percentage of brachyury positive cases whereas in later stages all cancers were positive for brachyury. This finding is in agreement with Fernando et al.[6] who showed the same tendency of brachyury expression in lung cancer patients where initial stages of lung cancer showed lower expression levels of brachyury than later stages. However, as large numbers of cases were studied in the present study, our findings can be extended to earlier stages as well. Because of the larger number of cases studied we were able to identify brachyury expression as a predictive marker in colon cancer at an early stage. It might therefore be of interest to extend our observation on early stage of lung cancer as well.

The second reason for the high significance of our results is clinical as our results put the concept of early detection of primary cancers into doubt. If the ability to form metastases is already inherent at a very early stage of malignant progression of colorectal cancer, clinical screening methods commonly applied might be less effective than previously thought. In particular, it would be of interest to investigate how many colorectal polyps, which are known to give rise

to colorectal cancer at a high frequency are positive for brachyury expression.

Conflict of interest statement

None declared.

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