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Authors: J.H. Dolderer, H. Schuldes, H. Bockhorn, M. Altmannsberger, C. Lambers, D. von Zabern, D. Jonas, H. Schwegler, R. Linke, U.H. Schröder

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**Title: HERG1 gene expression as a specific tumor marker in
 colorectal tissues**

Running title: Colorectal tumor biomarkers

J. H. Dolderer^{1+3,*}, H. Schuldes³, H. Bockhorn¹, M. Altmannsberger²,
C. Lambers⁴, D. von Zabern⁵, D. Jonas³, H. Schwegler⁶, R. Linke⁶,
U. H. Schröder⁷

¹ Department of Surgery, Northwest-Hospital, J.-W. Goethe-University,
Frankfurt am Main, Germany,

² Department of Pathology, Northwest-Hospital, J.-W. Goethe-University,
Frankfurt am Main, Germany,

³ Cellular Research Laboratory, Department of Urology, Center of Surgery,
J.-W. Goethe-University Hospital, Frankfurt am Main, Germany,

⁴ Department of Internal Medicine IV, Medical University Vienna, Austria,

⁵ Department of Anaesthesiology, University of Heidelberg, Mannheim, Germany,

⁶ Department of Anatomy O.-von-Guerike-University, Magdeburg, Germany,

⁷ Project Group Neuropharmacology, Leibniz Institute for Neurobiology,
Magdeburg, Germany

*corresponding author

Correspondence to:

Dr. Juergen H. Dolderer, MD

Department of Surgery

Head: Prof. Dr. T. Kraus

Nordwest-Hospital

J.-W. Goethe-University Frankfurt am Main

Steinbacher Hohl 2-26

60488 Frankfurt am Main

Germany

Email: drdolderer@hotmail.com

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Conflict of Interest Statement

The authors have no financial or personal relationships with other people or organisations that could inappropriately influence this work.

ABSTRACT

Introduction: Colorectal carcinomas exhibit a frequent recurrence after curative surgery, which may partially be due to histopathologically inconspicuous minimal residual disease. Reliable markers for tumor cells in colorectal tissue are still missing. Therefore, in this study we compared the predictive value of the putative tumor markers carcinoembryonic antigen (CEA), cytokeratin-19 (CK19) and cytokeratin-20 (CK20) to that of a novel marker, the human ether-a-go-go-related gene (HERG1) K⁺ channel, a suggested regulator of tumor cell proliferation.

Materials and methods: Using RT-PCR we studied HERG, CEA, CK19 and CK20 expression in colorectal carcinomas and non-carcinoma controls. HERG1 immunohistochemistry was performed in a total of 66 specimens, in colorectal carcinoma (n=23), in matched histopathologically negative samples (n=23) taken near the excision site from the same tumor patients and in healthy control biopsies (n=20). In order to verify the relevance of HERG1 for tumor proliferation we studied the effect of HERG1 inhibition in the Colo-205 colon cancer carcinoma cell line using the MTT-assay.

Results: HERG1 was expressed in all tumor samples regardless of their stage and in adenomas larger than 0.4 cm, but absent in small adenomas, sigmoid diverticulitis specimen and healthy histopathologically negative samples, except for one which developed a tumor recurrence. In contrast, CEA, CK19 and CK20 were absent in some tumors. The selective HERG1 inhibitor E-4031 dose-dependently impaired tumor growth in the proliferation assays.

Discussion: Our data indicate that HERG1, but not CEA, CK19 or CK20, is a highly sensitive and reliable tumor biomarker that may constitute a novel molecular target for tumor treatment.

Keywords: Colorectal cancer tumor marker, HERG1, carcinoembryonic antigen, cytokeratin-19, cytokeratin-20, molecular therapeutic agent E-4031

INTRODUCTION

Tumor-associated antigens such as carcinoembryonic antigen, cytokeratin-19 and cytokeratin-20, were proposed to be markers for colorectal carcinomas (CRCs) [1-4], but other studies questioned their reliability [5-8]. Although used as markers to diagnose CRCs, they suffer from low sensitivity as well as low specificity. Furthermore, reliable early indicators for these tumors are lacking and there is no appropriate marker for the detection of early stages or for histopathologically inconspicuous minimal residual disease (MRD) that may be one of the causes for recurrences.

Recent studies indicated that members of the ether à-go-go K⁺ channel family [9], such as the human ether à-go-go-related gene (HERG) K⁺ channel, may be regulators of tumor cell apoptosis as well as proliferation [10,11]. In contrast to brain, heart and lactotrophs, where HERG-related K⁺ channels are physiologically expressed [12-15], they have been found to be over-expressed in several tumor cell lines of different histogenesis [16,17]. Furthermore, Cherubini and colleagues found a high frequency of HERG expression in primary human endometrial cancers as compared to normal and hyperplastic endometrium and therefore nominated this protein as a potential indicator of cancerous versus hyperplastic endometrial growth [18]. Moreover, there is evidence that HERG1 gene and HERG1 protein are expressed in CRCs and might regulate tumor cell invasion into other tissues [19].

Because of the inconclusive expression profiles of common tumor markers in CRC we compared HERG1 and CEA; CK19; CK20 expression and

demonstrate that HERG1 is a more sensitive marker for the detection of carcinoma cells in colorectal tissue. As HERG appears to play a crucial role in tumor cell proliferation specific inhibitors might constitute valuable novel therapeutics for the treatment of colorectal carcinomas.

MATERIAL and METHODS

Patients

Tissue specimens from 23 patients with UICC stage [20] I-IV CRC (histologically verified adenocarcinoma) and paired normal colon mucosa (at least 10 cm distant from the primary tumor, orad in case of rectal cancer) were obtained from resections performed at the surgical department of the Nordwest Hospital, Teaching Hospital of the University of Frankfurt am Main, Germany. Histopathological diagnosis, UICC staging and TNM classification were performed using standard procedures. Clinical and histological UICC staging of the tumors is depicted in table 1. All patients had a follow-up period of five years. To avoid heterogeneity of histological carcinoma type, tissue samples were enrolled and analysed only with diagnosed adenocarcinoma. Patients who underwent previous combined neoadjuvant radiochemotherapy were also excluded. Informed written consent was obtained from each patient. Negative control tissues were obtained from another 20 patients, including samples of adenoma, sigmoid diverticulitis and healthy colorectal tissue.

RNA isolation, cDNA synthesis and RT-PCR

RT-PCR was performed on 18 fresh frozen tissue specimen (six male, twelve female; age 51-78, mean 67.7 years) including five tumors from the colon, four from the sigmoid and nine from the rectum using standard RT-PCR techniques [13, 16,21-25]. Histologically negative colon samples from seven patients (three male, 4 female; age 60-68, mean 62.8 years) including 3 tubulovillous colon-adenoma and 4 sigmoid diverticulitis specimen served as controls. To avoid cross contamination, tissue specimen were dissected free from other tissues, and a fresh scalpel was used each time. After excision tissues were immediately frozen in liquid nitrogen and stored at -80°C until extraction of RNA. Total mRNA was extracted from frozen tissue sections with RNeasy system, Qiagen (Hilden, Germany), and RNA-Isolation kit was used according to manufacturer's protocol. The RNA purity was assessed spectrophotometrically at 260 nm and 280 nm wavelengths.

Immunohistochemistry

Immunohistochemistry was performed on tissue specimens from 23 patients (8 male, 15 female; age 51-82, mean 70.3 years) including nine tumors from the colon, four from the sigmoid and ten from the rectum and on 23 matched histopathologically negative samples taken from near the excision site. Histologically negative colon samples from 13 patients (6 male, 7 female; age

34 to 76, mean 58.5 years) including 6 negative control biopsies from healthy persons, 4 tubulovillous colon-adenoma and 3 sigmoid diverticulitis specimen served as controls.

We followed a modified standard protocol using the ABC-technique [26]. Sections were incubated with anti-HERG primary antibody (AB5222, Chemicon, Temecula, CA; 1:500 in PBS) overnight in a moist chamber at 4 °C, in biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA; 1:200 in PBS) for 1 h and in ABC–elite solution (Vector Laboratories, Burlingame, CA; 1:100 in PBS) for 1h at room temperature. The antibody was visualized using diaminobenzidine (0.05 % in PBS and 0.03 % H₂O₂) as chromogen. Some of the sections were counterstained with Mayer's Haemalaun before coverslipping.

Tumor cell culture

The human colon (Colo 205) cell line was cultured at 37°C in a humidified atmosphere in the presence of 5% CO₂ – 95% air. Cells were grown in RPMI 1640 medium with L-glutamine, penicillin (100U/ml), streptomycin (100µg/mL), and 10% fetal bovine serum.

Proliferation assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was performed for the analysis of the tumor cell proliferation as described by Mosmann [27]. Briefly, the Colo 205 colorectal cancer cells were harvested with 0.05% trypsin/EDTA and counted. The cells were seeded into 96-well

plates at 10^4 viable cells per well and left to attach for 24 h. After 24 h the medium was replaced by a medium containing methanesulphoanilide (E-4031; Alomone Labs, Israel) at the concentrations indicated in Results. After 0-3 days 20 μ l MTT (5mg/ml in PBS) was added and the cells were incubated for 2 h at 37°C. The medium was then removed and 100 μ l dimethyl sulphoxide (Sigma) was added to each well for 10 minutes. Formazan formation was analyzed in five wells per experiment at a wavelength of 570 nm in an ELISA 96-well microtitre plate reader (Comtek, Germany). The absorbance of the untreated control was taken as 100 % and the IC₅₀ value was determined.

RESULTS

Sensitivity Testing

We used the human colorectal cancer cell line (Colo 205) as positive control to determine the sensitivity of the RT-PCR. Dilution series ranging from 10^6 to 10^0 Colo 205 cells were added to 10^7 lymphocytes isolated from healthy volunteers. After the extraction of total RNA, RT-PCR was performed. The experiments were performed in triplicate and showed that the primers were able to detect an amount of mRNA equivalent to one tumor cell in 10^6 lymphocytes.

Identification of CEA-, CK19- and CK20-mRNA

RT-PCR analysis of CEA, CK19 and CK20 mRNA in 18 fresh-frozen colorectal carcinoma samples (for details see Table 1) and in 7 normal tissue samples revealed that these markers were only expressed in part of the specimen with no significant dependence on the degree of the tumor stage (Table 1). Evaluating 18 patients with CRC we detected CEA-mRNA in 17 patients (94.4% sensitivity), CK19- in 14 patients (77.8%) and CK20- in 17 patients (94.4%). Correlations between the CEA, CK19 and CK20 expression in the colorectal carcinomas and various clinical and pathological characteristics are shown in Table 1.

Adenoma and sigmoid diverticulitis samples were used as histologically negative controls. In the adenomas a 100% expression of CEA, CK19 and CK20 was observed, whereas in the sigmoid diverticulitis samples there was a 50% expression of the three markers. These results demonstrate a specificity of 28.6% of all three markers.

HERG expression analyzed by RT-PCR

HERG1 gene expression was tested in 18 primary human colorectal carcinomas and 7 in histologically negative controls by reverse transcription-PCR. As shown in Table 1, the HERG1 gene was expressed, in contrast to the other markers, in all of the colorectal carcinoma examined (100% sensitivity) independent from anatomical location and tumor grade. Three patients with tubulovillous adenoma and four patients with sigmoid diverticulitis served as

negative controls. There was no HERG1 expression observed in the negative control samples (100% specificity).

The PCR band related to the HERG1 transcript was easily detectable in representative samples from colorectal adenocarcinoma, whereas a corresponding band was missing in adenomas and histopathologically negative control samples. The amplified DNA fragment was 386 bp for the HERG1 primer pairs.

HERG protein detection by immunohistochemistry

Tumor diagnosis, PCR studies and immunohistochemistry were conducted in different laboratories. Immunohistochemistry with an anti-HERG1 antibody was performed blinded and confirmed the presence of HERG1 protein in all carcinoma samples (Table 1 & Fig. 1). We observed two distinct staining patterns: normally the antibody was concentrated at the cytoplasm-vacuole border of the goblet cells but in some instances exhibited a scattered distribution in the cytoplasmic compartment of the epithelial cells (Fig. 1a- 1c). In contrast we did not detect antibody staining in the 13 control biopsies, except for adenomas larger than 0.4 cm (Pat No. 6 & 7). 22 of 23 histopathologically negative samples taken from near the excision site were also negative for HERG1 (Fig. 1d). One histopathological inconspicuous sample (patient No. 19) exhibited HERG1-positive staining at the very bottom of some crypts (Fig. 1e & 1f) and this patient experienced a locoregional recurrence two years later. HERG1 positive staining of single cells was observed in adenomas larger than

0.4 cm. Therefore it can be speculated that HERG1 might be suited for detection of precancerous phenotype.

Proliferation assay

The effect of the HERG inhibitor E-4031 was tested on Colo 205 carcinoma cell proliferation. All cultures presented normal logarithmic growth rates during the first 2 days of cell proliferation, whereas cultures treated with E-4031 (1, 5 or 10 μ M) exhibited a significant reduction in cell numbers compared to the control group ($p < 0.05$; IC_{50} 1 μ M) on the third day (Fig. 2).

DISCUSSION

Reliability of CEA, CK19 and CK20 as colorectal carcinoma markers

CEA, CK19 and CK20 have been considered to be markers for colorectal carcinomas [3,4,21]. In line with other studies questioning their usefulness [5-8] we show that neither CEA, nor CK19 or CK20 is a reliable tumor marker in colorectal tissue. CEA was not detected in a stage I carcinoma but was expressed in all tubulovillous adenomas and two sigmoid diverticulitis specimen. In line with our findings Bhatnagar and co-workers [29] showed that CEA may be absent in moderately or poorly differentiated tumors using quantitative enzyme immunoassay and immunocytochemistry and the group of Boucher [5] demonstrated that CEA is not only expressed in colonic adenocarcinomas but also in normal adjacent colonic mucosal tissues. CK19 and CK20 were absent

in stage III tumor samples but present in some control tissues, confirming previous findings that CK19 and CK20 can also be found in normal mucosa but may be missing in some tumors [6,7,8,28]. This limits their use as tumor markers by means of sensitivity and specificity. A combination of these markers would not greatly improve the discrimination between cancerous and non-cancerous colorectal tissue as in some tumors (e.g. pat. No 2) only one marker is expressed and combinations of two markers can be found in non-cancerous tissue. Cytokeratins, such as CK19 and CK20 are used as surrogate markers indicating histogenetic origin of a tumor. For example strong CK20 and lack of CK7 staining indicates intestinal differentiation and thus can be used in lung or liver metastasis to distinguish metastasized CRCs from other adenocarcinomas. As we confirmed that CRCs do not necessarily express these markers their presence or absence even in combinations is of limited predictive value for metastases of CRC origin.

Relevance of HERG as tumor marker and therapeutical target

In contrast to these established markers HERG1 expression is a tumor marker of clinical significance in CRC because of its high sensitivity and specificity. HERG1 gene expression as evaluated by reverse transcriptase PCR and the intratumoral HERG1 protein expression as assessed by immunohistochemistry was found in all carcinomas regardless of their stage. In contrast, the HERG1 protein was absent in all control biopsies and in all of the matched histologically negative tissue samples from tumor patients, except for the one who developed a locoregional recurrence. This suggests that HERG1 expression has no central

role in function or maintenance of normal healthy colorectal epithelium and thus apparently is a reliable tumor marker in CRC. Our data support the view suggested by recent *in vitro* studies that ether à go-go K⁺ channels, including the HERG1 K⁺ channels, are involved in carcinogenesis. These K⁺ channels were found to be highly conserved in various tumor cell lines, to confer a transformed phenotype in transfected mammalian cells and to favour tumor progression in immune-depressed mice [16-18].

In accordance with the findings of Lastraioli and coworkers we detected HERG in all metastatic carcinomas, but contrary to their observations not only 75% but all nonmetastatic carcinomas were positive for HERG1 on the mRNA and the protein level [19]. The discrepancy between the two studies may be due to methodological differences such as different primers and antibodies. In addition, carcinomas sometimes did not exhibit a focal but rather a diffuse pattern of HERG1 expression (Fig. 1b).

HERG1 expression apparently precedes the histopathological signs of a colorectal tumor as very few HERG1 positive cells could already be detected in otherwise unsuspecting colorectal tissue of a patient with a later diagnosed locoregional recurrence (Fig. 1e) and in large adenomas, which are known to hold the danger of tumor development. This indicates that HERG could not only be used as a marker to reliably distinguish between cancerous and non-cancerous tissue but also to exclude inconspicuous minimal residual disease at the borders of a carcinoma and to detect mutated cells that do not yet exhibit a morphologically distinguishable tumorous or precancerous phenotype at the very onset of a tumor.

In conclusion, the present study demonstrated that HERG1 expression was notably associated with CRC and was absent in healthy control samples whereas the expression of CEA, CK19 and CK20 varied significantly in tumors and control tissues. Our data suggest that the high sensitivity and specificity renders HERG1 expression a reliable tumor marker in colorectal tissue while CEA, CK19 and CK20 are of limited value. In addition, anti-HERG1 antibodies could be a useful tool to verify the absence even of very early stages of residual tumor and to identify patients at high risk for tumor recurrence. Furthermore, the HERG1 ion channels could not only be used as novel prognostic factors but may also constitute novel molecular targets for future therapies of colorectal carcinomas. This notion is supported by our findings that HERG K⁺ channels are important for tumor growth and/or survival as they are expressed in colorectal carcinomas but not in non-cancerous tissue and because Colo 205 cell proliferation ceased in the presence of the channel inhibitor E-4031.

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TABLE**Table 1:**

Pat. No.	Staging - TNM	Localisation	PCR	PCR	PCR	PCR	Immuno	Immuno
			CEA	CK19	CK20	HERG1	HERG1 Tumor	HERG1 paired Colon tiss
1	III -T4,N1,M0	Sigmoid	pos	pos	pos	pos	pos	neg
2	III -T3,N1,M0	Colon	pos	neg	neg	pos	pos	neg
3	II -T3,N0,M0	Colon	pos	neg	pos	pos	pos	neg
4	IV -T3,N2,M1	Rectum	pos	pos	pos	pos	pos	neg
5	I -T2,N0,M0	Sigmoid	neg	pos	pos	pos	pos	neg
6	IV -T3,N2,M1	Rectum	pos	pos	pos	pos	pos	neg
7	III -T2,N1, M0	Rectum	pos	neg	pos	pos	pos	neg
8	III -T3,N1,M0	Colon	pos	pos	pos	pos	pos	neg
9	III -T3,N2,M0	Rectum	pos	pos	pos	pos	pos	neg
10	I -T2, N0,M0	Rectum	pos	pos	pos	pos	pos	neg
11	II -T3,N0,M0	Colon	pos	pos	pos	pos	pos	neg
12	IV -T3,N2,M1	Sigmoid	pos	pos	pos	pos	pos	neg
13	II -T3,N0,M0	Rectum	pos	pos	pos	pos	pos	neg
14	II -T3,N0,M0	Colon	pos	pos	pos	pos	pos	neg
15	III -T3,N2,M0	Rectum	pos	pos	pos	pos	pos	neg
16	I -T1,N0,M0	Sigmoid	pos	pos	pos	pos	pos	neg
17	III -T3,N2,M0	Rectum	pos	pos	pos	pos	pos	neg
18	II -T3,N0,M0	Rectum	pos	neg	pos	pos	pos	neg
19	IV -T3,N2,M1	Colon					pos	pos
20	III -T3,N1,M0	Colon					pos	neg
21	II -T3,N0,M0	Rectum					pos	neg
22	II -T3,N0,M0	Colon					pos	neg
23	III -T3,N2,M0	Colon					pos	neg

LEGENDS

Table 1: Detection of mRNA of the tumor markers carcinoembryonic antigen, cytokeratin-19 and cytokeratin-20 and HERG by RT-PCR as well the HERG antigen expression in colorectal carcinomas and matched histopathologically negative samples taken from near the excision site. The gradings of the colorectal cancers were G2 in all samples except for the samples from patients No. 1 (G2-3), No. 4 (G3) and No. 9 (G2-3).

Fig. 1 A-F: Immunohistochemical detection of HERG1 protein in colorectal epithelium carcinoma and normal colon tissue.

Neoplastic colorectal carcinoma cells showing an intense and focal HERG1 positive staining (with counterstaining (A) and without counterstaining (B)). The HERG1 antibody is concentrated at the cytoplasm-vacuole border of the goblet cells (arrow). Normal colorectal mucosa resulted in a negative HERG1-specific staining (with counterstaining (C) and without counterstaining (D)). Positive HERG1-specific staining at the bottom of a crypt in a probe diagnosed as carcinoma-negative probe (arrow). Evidence of possible minimal residual diseases of the colorectal carcinoma (E). Higher resolution of Fig. 1E with the positive HERG1 staining at the bottom of the crypts (arrows) (F).

Fig. 2: The effect of the selective HERG1 inhibitor E-4031 on Colo 205 carcinoma cell proliferation. E-4031 was applied in 3 different concentrations (1,

5 and 10 μM). After 3 days E-4031-treated cultures exhibited a significant reduction in cell numbers compared to the control group ($p < 0.05$). (OD x 100: Optical Density x Factor 100 used for more accurate calculation).