Establishment, functional and genetic characterization of three novel patient-derived rectal cancer cell lines

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Abstract

AIM
To establish patient-individual tumor models of rectal cancer for analyses of novel biomarkers, individual response prediction and individual therapy regimens.

METHODS
Establishment of cell lines was conducted by direct in vitro culturing and in vivo xenografting with subsequent in vitro culturing. Cell lines were in-depth characterized concerning morphological features, invasive and migratory behavior, phenotype, molecular profile including mutational analysis, protein expression, and confirmation of origin by DNA fingerprint. Assessment of chemosensitivity towards an extensive range of current chemotherapeutic drugs and of radiosensitivity was performed including analysis of a combined radio- and chemotherapeutic treatment. In addition, glucose metabolism was assessed with \( ^{18} \text{F}-\text{fluorodeoxyglucose} \) (FDG) and proliferation with \( ^{18} \text{F}-\text{fluorothymidine} \).

RESULTS
We describe the establishment of ultra-low passage rectal cancer cell lines of three patients suffering from rectal cancer. Two cell lines (HROC126, HROC284Met) were established directly from tumor specimens while HROC239 T0 M1 was established subsequent to xenografting of the tumor. Molecular analysis classified all three cell lines as CIMP-0/ non-MSI-H (sporadic standard) type. Mutational analysis revealed following mutational profiles: HROC126: \( \text{APC}^{\text{wt}}, \text{TP53}^{\text{wt}}, \text{KRAS}^{\text{wt}}, \text{BRAF}^{\text{wt}}, \text{PTEN}^{\text{mut}} \); HROC239 T0 M1: \( \text{APC}^{\text{mut}}, \text{P53}^{\text{mut}}, \text{KRAS}^{\text{mut}}, \text{BRAF}^{\text{wt}}, \text{PTEN}^{\text{mut}} \) and HROC284Met: \( \text{APC}^{\text{wt}}, \text{P53}^{\text{mut}}, \text{KRAS}^{\text{mut}}, \text{BRAF}^{\text{wt}}, \text{PTEN}^{\text{mut}} \). All cell lines could be characterized as epithelial (EpCAM\(^{+}\)) tumor cells with equivalent morphologic features and comparable growth kinetics. The cell lines displayed a heterogeneous response toward chemotherapy, radiotherapy and their combined application. HROC126 showed a highly radio-resistant phenotype and HROC284Met was more susceptible to a combined radiochemotherapy than HROC126 and HROC239 T0 M1. Analysis of \( ^{18} \text{F}-\text{FDG} \) uptake displayed a markedly reduced FDG uptake of all three cell lines after combined radiochemotherapy.

CONCLUSION
These newly established and in-depth characterized ultra-low passage rectal cancer cell lines provide a useful instrument for analysis of biological characteristics of rectal cancer.

Key words: Patient-derived tumor model; Rectal cancer; \( ^{18} \text{F}-\text{fluorodeoxyglucose} \); \( ^{18} \text{F}-\text{fluorothymidine} \); FOLFOX; FOLFIRI; Personalized medicine

INTRODUCTION
Since two decades our understanding of colorectal carcinoma (CRC) as a heterogeneous disease entity in terms of both molecular carcinogenesis and morphologic multistep pathways has steadily grown\(^{[1]}\). Meanwhile, three major molecular carcinogenesis pathways have been identified: (1) Chromosomal instability (CIN); (2) microsatellite instability (MSI); and (3) CpG island methylator phenotype (CIMP) or epigenetic instability pathways\(^{[2,3]}\). In conclusion, Jass et al\(^{[4]}\) classified CRCs into 5 molecular subtypes primarily by underlying types of genetic instability and presence of DNA methylaton: (1) CIMP-H/MSI-H; (2) CIMP-H/non-MSI-H; (3) CIMP-L/ non-MSI-H; (4) CIMP-0/non-MSI-H (spStd); and (5) CIMP-0/MSI-H. But these molecular subtypes are not distributed equally along the large bowel. Yamauchi et al\(^{[5]}\) could show that clinicopathological and molecular features of CRCs differed depending on the bowel subsite of the tumor.

Recent algorithms for treatment of locally advanced rectal cancer consist of neoadjuvant chemoradiation (nCRT) followed by rectal resection and total mesorectal excision (TME). Currently, beside clinical evaluation, imaging modalities including endorectal ultrasound, computed tomography (CT), magnetic resonance imaging (MRI) as well as \( ^{18} \text{F}-\text{fluorodeoxyglucose} \) (FDG) positron emission tomography-computed tomography (\( ^{18} \text{F}-\text{FDG-PET/CT} \)) are used for staging, assessment of response to nCRT, and restaging after nCRT\(^{[6,7]}\). However,
accuracy of parameters derived from these imaging modalities, especially for predicting a pathological complete response (pCR) after nCRT, is currently limited due to their low sensitivity and specificity.[6,7]

In the last decade, many patient-individual tumor models of CRC could be generated by us and others[8,9]. But no novel rectal cancer models have been published up to now.

In this study, we describe the establishment and functional characterization of three patient-derived rectal cancer cell lines along with corresponding patient-derived xenografts (PDX). A broad analysis of tumor biology, genetic alterations, protein expression, and assessment of chemosensitivity towards a range of chemotherapeutic drugs and of radiosensitivity was performed.

In addition, an analysis of the metabolism of the glucose analogue 18F-FDG was carried out. Due to their higher glucose metabolism, 18F-FDG is differentially taken up by malignant cells[10]. This phenomenon can be assessed by molecular imaging with 18F-FDG-PET/CT and may be used to detect tumors' therapy responses, that are either not apparent with other morphological imaging modalities or may precede a significant tumor shrinkage by weeks or months[11]. Furthermore 3-deoxy-3-18F-fluorothymidine (18F-FLT) uptake was analyzed. 18F-FLT is intracellularly trapped when monophosphorylated by thymidine kinase 1 and 18F-FLT-PET uptake reflects activity of thymidine kinase 1 which is elevated during the S phase of the cell cycle. Thus, 18F-FLT-PET uptake mirrors tumor cell proliferation[12].

Considering these aspects, our characterized matched in vitro and in vivo tumor models represent excellent tools for further development of individual response prediction, therapy regimens, and might prove especially valuable to gain additional insights in the tumor biology of rectal cancer.

MATERIALS AND METHODS

Tumor preparation, establishment of PDX and cell line establishment

Primary rectal cancer resection specimens of HROC126, HROC239 and resection specimens of a rectal cancer liver metastasis (HROC284Met) were received fresh from surgery, with informed written patient consent. Tumor samples cut into small pieces (3 mm × 3 mm × 3 mm) were vitally frozen in freezing medium [fetal calf serum (FCS) containing 10% DMSO] at -80 °C for subsequent xenografting. Snap-frozen pieces were stored at -160 °C.

Snap-frozen pieces were stored at -160 °C and frozen down in a master cell bank. The rectal cancer cell lines HROC126, HROC239 T0 and HROC284Met as well as the central CRC cell line HCT116 were cultured in T75 culture flasks using Dulbecco’s modified Eagle medium (DMEM) /Ham’s F12 supplement with 2 mmol/L L-glutamine and 10% FCS (all cell culture reagents were obtained from Pan Biotech, Aidenhagen, Germany).

Histology and immunohistochemistry of original tumors and PDX

Hematoxylin and eosin (HE)-stained primary tumors and corresponding PDX were clinicopathologically staged[13], and additional information was extracted from clinical charts.

Mutational and methylation profile of tumor-associated target genes and determination of CIN levels

Molecular classification was performed as described before[3]. Mutation status of the genes APC, TP53, KRAS, PIK3CA, PTEN and BRAFV600E were analyzed. DNA-methylation was checked using a modified marker panel originally published by Ogino et al[16]. CIN was assessed using the SNP Array 6.0 from Affymetrix (Cleveland, OH, United States).

DNA identity check

Genomic DNA was isolated from cell lines, matched tumor and normal tissue, PDX tissue as well as corresponding B cells using the Wizard® Genomic DNA Purification Kit (Promega Madison, WI, United States). Highly polymorphic short tandem repeat (STR) DNA marker (CSF1PO, TPOX, THO1, vWA, D16S539, D13S317, and D7S820) and the marker amelogenin for gender determination were used by taking advantage of published PCR primers[17].

Generation of peripheral B cell cultures

Peripheral blood mononuclear cells were isolated by density-gradient centrifugation. B-lymphoid cell lines (B-LCLs) were generated via Epstein-Barr virus (EBV)-transformation[18]. Outgrowing B-LCL cultures were harvested, expanded, characterized by flow cytometry, and frozen down in a master cell bank.
**In vitro growth kinetics, ploidy and cell cycle analysis**

Doubling times of HROC126, HROC239 T0 M1 and HROC284Met cells were determined from serial passages. Five times $10^5$ cells were seeded into 25-cm² flasks and viable cells (defined by trypan blue exclusion) were subsequently counted for seven consecutive days. Cultures were fed when needed. Cell cycle and ploidy were determined by flow cytometry (FACSCalibur; BD Biosciences, Heidelberg, Germany) using fixed (70% ethanol) and RNase A digested (100 µg/mL; Sigma Aldrich, Munich, Germany) and propidium iodide (10 µg/mL) stained cells. For each sample, at least 10000 events were measured. Cell cycle analysis was done by taking advantage of the Modfit software (Verity Software House, Topsham, ME, United States) using matched B-LCLs as diploid controls.

**Flow cytometric phenotyping of primary cell line**

Levels of markers expressed on the cells’ surface were determined by flow cytometry with and without interferon (IFN)-γ pre-treatment using a panel of FITC-, PE- or APC-conjugated antibodies: CD26, CD29, CD44, CD49a, CD50, CD56, CD58, CD66acde, CD71, CD73,CD90, CD102, CD166, human leukocyte antigen (HLA)-ABC (Immunotools, Friesoythe, Germany); CD152, CD275, CD278, CD326, HLA-DR, HLA-E, HLA-G (Miltenyi Biotec, Bergisch- Gladbach, Germany), Ki-67 (Biolegend, San Diego, United States) and HLA-A2 (cell culture supernatant clone BB7.2). For HLA-A2, a polyclonal, secondary FITC-conjugated anti-mouse serum was used (Dako, Hamburg, Germany). Sample analysis was done by CellQuest (BD Biosciences).

**Mycoplasma and human viral infection**

Presence or absence of mycoplasma as well as potential polyomavirus infection (JC/BK and SV40) was tested as described before[19].

**Migration and invasion assay**

Tumor cell invasion capacity was tested using the classical Boyden chamber test (8-µm pore size in a 24-well plate format) with Matrigel-coating (both BD Biosciences). Two times $10^5$ cells were seeded in 500 µL serum-free medium per upper Boyden chamber. Medium supplemented with 10% heat-inactivated FCS served as chemo-attractant in the lower Boyden chamber. Three days later, the non-invading cells on the surface of the upper inserts were removed and viability of cells on the lower surface was measured by the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,6-benzene disulfonate (WST-1) test (Roche Diagnostics, Mannheim, Germany). In parallel experiments, for cells’ capacity to migrate were tested using uncoated upper Boyden chambers.

**In vitro chemo- and radiosensitivity analysis**

For chemosensitivity, cells were seeded into 96-well microtiter plates at $5 \times 10^3$ or $1 \times 10^4$ cells/well. When cells reached 30%-40% confluency, cultures were exposed to increasing concentrations of 5-FU, oxaliplatin, irinotecan, combination of 5-FU and oxaliplatin (FOLFOX) and combination of 5-FU and irinotecan (FOLFIRI) (freshly provided by pharmacy of the University Medical Center Rostock). After 72 h, media and therapeutics were refreshed. Following another 72 h, plates were carefully washed and stained with crystal violet (0.2%, 10 min). Finally, drug effects from triplicate wells were determined at the level of 50 % inhibition (IC₅₀) in comparison to control, measured at 570 nm (reference wavelength: 620 nm). For radiosensitivity analysis, cells radiated with different doses up to 60 Gy using a $^{137}$Cs-source were seeded into 96-well microtiter plates in triplicates ($1 \times 10^4$ cells per well and six serial two-fold dilutions). Control cells were not radiated. After 4 and 7 d, triplicate plates were analyzed for total cell growth using crystal violet as described above.

For analysis of combined radio- and chemosensitivity, cells were seeded and exposed to increasing drug concentrations as described above. After three days of exposure, media were replaced and cells were radiated with 50 Gy. Following another three days, triplicate plates were analyzed for total cell growth.

**Western blot**

Western blot was done as previously described[20]. Antibodies specific for the following targets were from Santa Cruz (Heidelberg, Germany): BAX, histone deacetylase 2 (HDAC2), HDAC1, and p53. Anti-survivin was obtained from Novus Biologicals (Cambridge, United Kingdom). HSP90 antibody was provided by Enzo Life Sciences (Lörrach, Germany).

**$^{18}$F-FLT and$^{18}$F-FDG uptake**

For analysis of $^{18}$F-FDG and $^{18}$F-FLT uptake, rectal cancer cells were seeded into 24-well microtiter plates at $1 \times 10^5$ cells/well in complete culture medium. At day 1, cells were either exposed to FOLFOX at an IC₅₀ concentration alone or radiated (50 Gy) alone or exposed to combinations thereof, whereas control cultures were treated with solvent. After three days of exposure, media were removed and substituted by DMEM (Fisher Scientific, Schwerte, Germany) without FCS, glucose, glutamine, and incubation continued for 1 h before $^{18}$F-labeled tracers (0.5 MBq/mL culture medium) were added. Thirty minutes later, incubation was terminated by aspirating the medium and rinsing the cell layer three times with ice-cold PBS. The cells were solubilized with 0.1 mol/L NaOH, and incorporated $^{18}$F activity was determined using a gamma counter (WIZARD2 10-Detector Gamma Counter, Perkinelmer, Waltham, MA, United States). Counts per minute were normalized on total protein level of the cells using a commercial Bradford assay (Bio-Rad Laboratories, Munich, Germany).

$^{18}$F-FDG and $^{18}$F-FLT uptake of treated cells is expressed as percent of controls exposed to the solvent only ($n = 10$ per cell line and experimental condition).
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Table 1  Cell line establishment protocol

| Tumor ID | Direct cell line establishment | Cell line from xenograft | Corresponding xenograft | Paired B-LCL |
|----------|-------------------------------|--------------------------|-------------------------|--------------|
| HROC126  | +                             | -                        | +                       | +            |
| HROC239 T0 | -                            | +                        | +                       | +            |
| M1       |                               |                          |                         |              |
| HROC284Met | +                          | -                        | +                       | +            |

*: Positive; : Negative; B-LCL: B-lymphoid cell line.

Figure 1  Migratory potential and invasiveness of individual rectal cancer cell lines. Invasion and migration of HROC126, HROC239 T0 M1 and HROC284Met were analyzed in comparison to reference cell line HCT116. Cells were subjected to migration assay (migration, grey bars) and matrigel invasion assay (invasion, black bars). Values are mean ± SEM from at least three measurements. After proving the assumption of normality, differences were determined by the unpaired Student’s t-test. Mean group differences were checked by the Kruskal-Wallis test before for multiple comparisons subgroups were tested with post hoc Dunn’s test. All statistics were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, United States).

RESULTS

Clinicopathological characteristics of the primaries and origin of cell lines

We report on three female patients suffering from rectal cancer with an age of 58, 72, and 67 years. The first patient (HROC126) suffered from a carcinoma of the middle rectal third. Preoperative staging revealed an Union for International Cancer Control (UICC) stage II (cT2 cN0 cM0) tumor and a low anterior resection with complete TME was conducted. Postoperative pathological examination showed an UICC stage IIIB (cT3 cN2 cM0) tumor stage. The patient developed after 6 mo a singular liver metastasis that could be resected completely. Subsequently, she developed further irresectable liver metastases and a chemotherapy consisting of FOLFIRI and bevacizumab was administered. Unfortunately, the tumor showed only a partial remission with the additional diagnosis of lung metastases. The patient died 22 mo after the operation due to tumor progression with liver and lung metastases.

Establishment of permanent cell lines and PDX models

In vitro and in vivo approaches were combined as described previously for establishment of matching cell lines and PDX. With this method, direct cell line establishment could be achieved in two out of three cases (HROC126 and HROC284Met) with HROC239 T0 M1 originating from a parallel PDX (Table 1).

Outgrowth of cells in culture occurred immediately. Doubling times of the cell lines were 34.8 h for HROC126, 28.8 h for HROC239 T0 M1 and 38.1 h for HROC284Met. Tumor formation in immunodeficient NMRI nu/nu mice could be observed as fast as 1-3 mo after the tumor engraftment. Histological analysis showed that tumor architecture was preserved in the PDX compared to the original patient tumor architecture (data not shown).

Analysis of invasion and migration revealed a significant reduced infiltrative activity of HROC126 and of HROC239 T0 M1 compared to the reference line HCT116 (t-test, P < 0.05), while infiltrative activity of HROC284Met was comparable to HCT116.

Regarding the migratory activity, all three cell lines (HROC126, HROC239 T0 M1 and HROC284Met) were significantly less migratory active (t-test, P < 0.01) through uncoated Boyden chambers then the HCT116 reference cell line (Figure 1).

Morphology and viral contamination

As determined by phase contrast microscopy, cells of all three HROC lines adhered tightly to the cell culture flasks. The cell lines were growing as monolayers on conventional tissue culture plastic and showed a stable outgrowth as defined by passaging > 40 times. HROC284Met cells proliferated as polygonal cell clusters with more regular dimensions, while HROC126 and HROC239 T0 M1 cells formed tightly packed multicellular islands (Figure 2). Morphology did not change during long term passage (up to 40 passages) (data not shown).
As determined by semi-quantitative PCR, HROC126, HROC239 T0 M1 and HROC284Met cell lines were free of mycoplasma and several other potential contaminants (JC/BK and SV40) which have been described for (colorectal) cell lines (data not shown).

Phenotyping
The epithelial phenotype of all three cell lines was confirmed by high positive immunoreactivity for CD326 (EpCAM) while expression of CD66abcd (CEACAM) differed. HROC126 and HROC239 T0 M1 showed moderate to high expression of CD66abcd, while HROC284Met showed no expression (Figure 2).

A more detailed characterization revealed very heterogeneous expression of adhesion and co-stimulatory factors (moderate to high expression of CD44, CD58 and CD102) (Figure 2). Further analysis showed a high to moderate expression of CD26 and CD29, which have been described as stem cell and metastasis-promoting surface receptors [21,22] (Figure 2).

The proliferation marker CD71 was highly expressed by all three cell lines (Figure 2) reflecting high proliferative activity of the tumor cells.

Regarding HLA molecules that play an important role in specific immune recognition and tumor cell defense, all three cell lines showed a high expression of HLA class I (β2M and pan-HLA-ABC) molecules. HROC126 and HROC284Met showed a moderate expression of HLA class II (HLA-DR), while HROC239 T0 M1 showed no expression. In addition, all three tumor models presented a distinct expression of HLA-E and HLA-G molecules which are involved in immune suppression (Figure 2).

Additional immune evasion molecules analyzed included CD73 which was highly expressed by all three cell lines. Lower levels were observed for CD278 [inducible co-stimulator (ICOS)], CD275 (B7-H2) and CD152 [cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)] (Figure 2).

Molecular characterization
To verify the genetic identity of our established tumor models to the parental tumors, a DNA fingerprint was performed. Several highly polymorphic STR DNA markers that covered nine validated STR loci on different chromosomal locations were included into this analysis. We could confirm identity to the parental tumors in all established cell lines and PDX from the three clinical cases included, using this approach. Also, cross contamination of any of the tumor cell lines with other tumor cells could be excluded (data not shown).

Furthermore, the molecular features of the cell lines were determined in direct comparison to the original tumors as well as to PDX tissues. All three cases showed a distinct degree of aneuploidy, absent MSI and no methylation in CIMP-sensitive promoters. Thus, they...
HROC239 T0 M1 is the only cell line that presented a reduced glucose uptake after chemotherapy, whereas HROC126 and HROC284Met were sensitive to single radiation treatment (Figure 5). Only after radiation, a significant reduction of tracer uptake was determined in these two cell lines. In HROC239 T0 M1 cells, the application of FOLFOX at an IC50 dose led to a reduced 18F-FDG uptake. All three tumor models displayed a reduced 18F-FDG uptake after a combined radiochemotherapy.

Combination of drugs, currently administered in intensified therapy regimens, FOLFIRI and FOLFOX showed again a good response of all three cell lines, with HROC284Met being less susceptible to FOLFIRI and FOLFOX than HROC126 and HROC239 T0 M1 (Figure 4).

Next step was evaluating the resistance of the cell lines to γ-radiation. Here, all cell lines showed a remarkable resistance to radiation and even radiation with 50 Gy could not prevent further growth of the cell lines; particularly HROC126 displayed a highly radio resistant phenotype (Figure 4).

At last, as actual neoadjuvant regimens for advanced rectal cancer consist of a combined radiochemotherapy (FOLFOX or FOLFIRI with 50 Gy), this was administered to the cell lines. Furthermore, an individual response of all three cell lines could be observed, with HROC284Met being more susceptible than HROC126 and HROC239 T0 M1. Again, when patient plasma levels are set for reference, measured IC50 concentrations were below those plasma concentrations (Figure 4).

Uptake of 18F-FDG, 18F-FLT and Ki-67 expression
Analysis of 18F-FDG uptake showed an individual response of all three cell lines. HROC239 T0 M1 is the only cell line that presented a reduced glucose uptake after chemotherapy, whereas HROC126 and HROC284Met were sensitive to single radiation treatment (Figure 5). Only after radiation, a significant reduction of tracer uptake was determined in these two cell lines. In HROC239 T0 M1 cells, the application of FOLFOX at an IC50 dose led to a reduced 18F-FDG uptake. All three tumor models displayed a reduced 18F-FDG uptake after a combined radiochemotherapy.

Regarding uptake of 18F-FLT, which might reflect higher tumor cell proliferation activity, we found mixed results. Interestingly, HROC126 and HROC284Met can all be classified as CIMP-0/ non-MSI-H (spStd) type. HROC126 presented wild type APC, TP53, KRAS, and BRAF genes, while HROC239 T0 M1 presented mutations in the APC, KRAS, TP53, and PTEN genes with a BRAF wild type. HROC284Met presented with a mutation in the KRAS and PTEN gene and in addition a BRAF wild type (Table 2).

In vitro drug response and analysis of radiosensitivity
Response testing of individual tumor cell lines to current chemotherapeutic drugs and concomitant radiation therapy has become more and more valuable due to huge heterogeneity of individual tumor responses to established therapy regimens. Thus, sensitivity of the three cell lines to a panel of current chemotherapeutic drugs and their combination was assessed using standard proliferation and cytotoxicity assays.

Analysis of drug sensitivity to single substances of 5-FU, irinotecan and oxaliplatin showed an individual response of all three cell lines, with HROC239 T0 M1 being more resistant to oxaliplatin (IC50 = 2.4 µg/mL) (Table 3). Doses were comparable or lower than achievable plasma concentrations in patients (Table 3).

Table 2  Molecular characterization and mutational profile of rectal cancer cell lines

| Cell line      | CIMP-number | MSI-status | Ploidy status | TP53 | APC   | K-Ras | PIK3CA | BRAF | PTEN |
|----------------|-------------|------------|---------------|------|-------|-------|--------|------|------|
| HROC126       | 0           | Mss        | Aneuploid     | Wt   | Wt    | Wt    | Wt     | Wt   | Wt   |
| HROC239 T0 M1 | 0           | Mss        | Aneuploid     | Mut  | Mut   | Mut   | Mut    | Wt   | Wt   |
| HROC284Met    | 0           | Mss        | Aneuploid     | Wt   | Mut   | Wt    | Mut    | Wt   | Wt   |

Wt: Wildtype; Mut: Mutated; CIMP: CpG island methylator phenotype; MSI: Microsatellite instability; Mss: Microsatellite stable.
displayed a similar response pattern as shown in the FDG trials. In both cell lines, FLT uptake was elevated after FOLFOX treatment, whereas a single radiation therapy showed a lower thymidine uptake in comparison with single chemotherapy or combination with FOLFOX. In HROC239 T0 M1 cells, a markedly but not significantly higher uptake of FOLFOX treated cultures compared to control was observed. Again, radiation alone displayed a lower number of counts in comparison to FOLFOX treatment or controls.

| Cell line          | 5-FU ($\mu$g/mL) | Irinotecan ($\mu$mol/L) | Oxaliplatin ($\mu$g/mL) |
|--------------------|------------------|-------------------------|-------------------------|
| HROC126            | 0.42             | 0.72                    | 0.3                     |
| HROC239 T0 M1      | 21               | 4.4                     | 2.4                     |
| HROC284Met         | 7                | 4.3                     | 0.68                    |
| Plasma levels (pharmacokinetic) | 50                | 10                      | 2                       |

Values are given as mean, resulting from at least three independent experiments each performed in triplicates. 5-FU: 5-Fluorouracil.

Figure 4 Analysis of chemo- and radiosensitivity. A: Sensitivity of individual rectal cancer cell lines to FOLFOX and FOLFIRI chemotherapy; B: Sensitivity of cell lines to combination of irradiation with 50 Gy and chemotherapy with FOLFIRI or FOLFOX; C: Radiosensitivity of rectal cancer cells. Cells were irradiated to different doses using a $^{137}$Cs-source. Cell viability of all experiments was measured using crystal violet assay. Values represent the mean absorbance at 570 nm ± SEM of $n = 3$ analyses; t-test $^aP < 0.05$ vs control, $^bP < 0.01$ vs control, $^cP < 0.0001$ vs control.
To gain additional information on the proliferation status of the cell lines under treatment, a flow cytometric analysis of Ki-67 expression was performed. In all treatment groups (FOLFOX, radiation, combined FOLFOX and radiation), HROC126 and HROC239 T0 M1 showed a significantly reduced expression of Ki-67 compared to untreated controls. HROC284Met revealed a significantly reduced expression of Ki-67 in the radiation and combination group (FOLFOX and radiation) and a markedly reduced activity in the FOLFOX group (Supplementary Figure 1).

**DISCUSSION**

In the last decade, many patient-individual tumor models of colonic cancer were established by our lab and others[8,9]. These models have shown to be extremely helpful in decoding colorectal cancers’ molecular heterogeneity[9,24] and identification of novel biomarkers[25], druggable targets and novel treatment strategies[26]. But no novel rectal cancer models have been published up to now. Here, we describe the establishment and functional characterization of three patient-derived rectal cancer cell lines that could be established from fresh patients’ tumor samples for the first time.

These established cell lines reproduce the original molecular signature of the tumor in a perfect way. In addition, PDX models of the original tumors could be established. PDX models show high morphologic and molecular correspondence with the analogous patient tumor, facilitating pharmacologic studies to predict clinical response[27,28].

While HROC126 and HROC239 T0 M1 were obtained from locally advanced rectal cancer primaries, HROC284Met was acquired from a liver metastasis. Thus, the established rectal cancer cell lines may represent valuable models for advanced rectal cancer that requires multimodal therapy according to international guidelines[29].

Regarding growth kinetics and to some aspect morph-
ology, the three cell lines displayed some variations. While migratory activity was comparable between all three cell lines, HROC284Met showed a significantly higher migratory activity compared to HROC126 and HROC239 T0 M1. This fact might reflect the worse clinical outcome of HROC284Met in terms of a metastatic and rapid progressive disease.

The epithelial phenotype of all three lines was confirmed by high positive immunoreactivity for CD326 (EpCAM); while expression of CD66abcde (CEACAM) was heterogeneous with HROC284Met showing even no expression of this tumor antigen. Further characterization revealed very heterogeneous expression of adhesion and co-stimulatory factors (moderate to high expression of CD44, CD58 and CD166 but low to absent expression of CD49a, CD50, and CD102). Interestingly, a high to moderate expression of CD26 and CD29, which have been described as stem cell and metastasis-promoting surface receptors\(^{[21,22]}\) could be shown. Reflecting the high proliferative activity of the tumor cells, the proliferation marker CD71 was highly expressed in all three cell lines.

As tumor immune escape mechanisms play a more and more important role in clinical oncology, we analyzed several key molecules of immune escape. All three cell lines express high levels of CD73, which has been described as immune-evasion molecule and overexpression was observed in various tumor tissues\(^{[30]}\). Interestingly, a moderate expression of CD152 (CTLA-4) could be demonstrated, especially in HROC126 and HROC284Met. CTLA-4 is an immunoglobulin superfamily receptor and was the first immune-checkpoint receptor that was clinically targeted using blocking antibodies\(^{[31]}\). This finding suggests that the rectal cancer tumor models could also help to develop immune checkpoint inhibiting strategies and to optimize immune-therapeutic applications.

Regarding HLA molecules that play an important role in specific immune recognition and tumor cell defense, expression of HLA class I molecules was preserved in all three cell lines. HROC126 and HROC284Met cells were additionally found to be positive for HLA class II molecules. This provides a basis for further immunological analysis aiming at identification of immunogenic epitopes from shared or individual neo-antigens.

Lately, tumor sidedness has emerged as an important prognostic and predictive element in the treatment of CRC\(^{[32]}\). Clinico-pathological and molecular features of CRCs differ depending on the bowel subsite of the tumor\(^{[33]}\). The molecular profiles of all three rectal cancer cell lines were associated with the CIMP-0/ non-MSI-H (spStd) type\(^{[33]}\). This finding is not surprising as approximately 70% of all sigmoid and rectal cancers show the CIMP-0/ non-MSI-H (spStd) subtype\(^{[34]}\). This fact may account for the differences in the clinico-pathological features of right sided and left sided or rectal cancers\(^{[35]}\).

Regarding the tumor specific mutational analysis, a BRAF wild type could be detected in all three cell lines. This is not unexpected as Salem et al\(^{[37]}\). analyzed 1445 rectal tumors and found a BRAF mutation in only 3.2% of all examined tumor samples. Likewise, the observed mutations of Kras, TP53 and APC in HROC239 T0 M1 and HROC284Met correlate with the frequent mutation of these genes in rectal tumors ranging from 50%- 66%. Of interest, a PTEN mutation was found in HROC239 T0 M1 and in HROC284Met, while several studies could show that PTEN mutations are rare in rectal tumors\(^{[38]}\).

Current guidelines recommend nCRT in locally advanced rectal cancer\(^{[29]}\). Hence, cell lines were tested against a panel of current chemotherapeutical regimens. In addition, analysis of radiosensitivity and sensitivity of combined radio- and chemotherapy was evaluated, particularly with regard to the known differing clinical response rates to nCRT. In principle, all cell lines were susceptible towards these regimens as well as to the single substances (5-FU, irinotecan, oxaliplatin) or combinations of drugs (FOLFOX, FOLFIRI) which are currently administered in intensified therapy regimens. Of note, doses were comparable or lower than plasma concentrations measurable in patients.

Analysis of sensitivity to sole radiation showed a remarkable resistance of HROC126 even at high radiation doses, while HROC239 T0 M1 and HROC284Met displayed a moderate sensitivity. As shown above, HROC239 T0 M1 and HROC284Met exhibited a PTEN mutation. It is known that the PI3K/PTEN/AKT/mTOR signaling pathway participates in drug resistance, tumorigenesis and progression of cancer\(^{[36]}\). These alterations have been implicated as modulators of clinical outcomes in patients with esophageal cancer who underwent CRT\(^{[37]}\). In particular, a loss of PTEN decreases CHK1 and TP53 activity by regulating their protein levels and promoting genomic instability in tumor cells\(^{[38]}\). In rectal cancer, a recent study by Peng et al. could show that patients with a PTEN mutation displayed a better response to CRT than those with a wild-type genotype\(^{[39]}\). This might contribute to a more distinct sensitivity to radiation of HROC239 T0 M1 and HROC284Met in contrast to the PTEN wild type HROC126.

Concerning p53 and its target gene induction, we see no clear correlation between p53 status and the sensitivity of our cell models to drugs and γ-irradiation. This is not due to a lack of correlation between p53 expression and activity. The low to undetectable levels of p53 in HROC126 and HROC239 T0 M1 cells seems to be a marker for active wild type TP53 or a loss of TP53, respectively\(^{[22]}\). While HROC126 cells express various p53 target genes, HROC239 T0 M1 carry very low levels thereof (Figure 3). The mutation of TP53 in HROC284Met agrees with TP53 mutations in advanced cancers and metastases\(^{[40]}\). The high levels of survivin, which is a target of mutant p53 in combination with nuclear factor kappa B (NF-kB) p65, and a suppressor of chemosensitivity\(^{[41]}\) is not reflected by our assays assessing cellular sensitivity to drugs and γ-irradiation. Thus, additional factors need to be considered when molecular markers are used to predict the responsiveness
of cancer cells to drugs and γ-irradiation. Although the class I HDAC HDAC2 is frequently dysregulated in MSI colorectal cells[23], we see no loss of HDAC2 in our systems. This might be due to the fact that a loss of HDAC2 only affects a portion of HDAC2 positive cells in culture (Krämer et al, unpublished observation).

Analyzing the combination of chemo- and radiotherapy, once more an individual response of all three cell lines became evident. Identification of novel mechanisms leading to radiation-resistance might help to overcome this and might thus improve therapy efficacy[41] particularly as different responses to nCRT are associated with differences in long-term outcomes including disease-free survival[45]. Therefore, this novel set of rectal cancer models represents an ideal tool for further research also in this area.

At last, we analyzed uptake of 18F-FDG and 18F-FLT in the cell lines. Rosenberg et al[43] reported that the metabolic response to nCRT in rectal cancer can be correlated with histopathological response using 18F-FDG PET. Analysis of glucose uptake revealed a good response of all three cell lines. While HROC126 and HROC284Met showed a significantly reduced 18F-FDG uptake in radiated cultures, HROC239 T0 M1 showed a significantly reduced uptake after FOLFOX treatment and in the chemoradiation group. In addition, the combination of FOLFOX and radiation resulted in all three cell lines in a significantly reduced 18F-FDG uptake compared to solitary FOLFOX treatment. This finding emphasizes the possible use of 18F-FDG PET for detection of early tumor responses and restaging after nCRT.

Many anti-cancer drugs target cell proliferation as primary or secondary target. These changes can be investigated using 18F-FLT PET after chemotherapeutic treatment. However, 18F-FLT changes following chemotherapy are inconstant and depend on the tumors and specific treatments[44]. In two out of the three novel rectal cancer cell lines, FLT could be predictor for response to a chemotherapy regimen, where an increased FLT uptake was determined.

After radiation, all three cell lines showed a significantly reduced uptake of 18F-FLT in comparison to FOLFOX, reflecting an expected decline of cell proliferation after radiation. The combined RCT treatment displayed again a significantly higher uptake of 18F-FLT for two of the three cell lines. This marked retention of 18F-FLT might be triggered by 5-FU that interferes with endogenous thymidine synthesis and can cause rapid accumulation of thymidine kinase 1 levels[45]. Of note, Hong et al[46] reported that the 18F-FLT flare observed during 5-FU infusion was associated with poor treatment response in patients with mCRC. Hence, more clinical studies are needed to define the role of 18F-FLT PET after FOLFOX treatment.

In summary, the enormous strength of the present study lies in the establishment and characterization of the first pairs of matching in vitro and in vivo patient-individual rectal cancer models (cell lines + PDX). They represent ideal tools for further development of personalized medicine concepts of rectal cancer. However, given the small sampling size of n = 3, the number of rectal cancer cases successfully transferred into models must be considered as a limitation. Similarly, the molecular and functional data provided could be expanded.

ARTICLE HIGHLIGHTS

Research background
It is well known that ultra-low passage and in-depth characterized patient-derived tumor models are highly desirable for basic research and for predicting individual responses to current or novel therapy regimens.

Research motivation
To establish individual tumor models of rectal cancer from patient-derived tumor samples to gain further insights into the biological behavior of rectal cancer.

Research objectives
Main objective of the study was the establishing and profound characterization of new patient-derived rectal cancer cell lines with corresponding patient-derived xenograft models that allow testing of drug response, translational and basic research.

Research methods
Establishment of cell lines could be achieved by direct in vitro culturing and in vivo xenografting with following in vitro culturing. Profound analysis of morphological features, invasive and migratory behavior, phenotype, molecular profile including mutational analysis, and protein expression was done. Responsiveness to current chemotherapeutic drugs was evaluated and sensitivity to radiation and combined radio-chemotherapy was examined. At last the positron emission tomography (PET) tracers 18F-fluorodeoxyglucose (FDG) and 18F-fluorothymidine were used to assess glucose metabolism and proliferation activity respectively.

Research results
Three individual ultra-low passage rectal cancer cell lines could be established. In vitro and in vivo experiments demonstrated that all cell lines retained their malignant properties. Molecular analysis classified all three cell lines as sporadic type (CIMP-0/non-MSI-H). Mutational analysis revealed an individual mutational profile of each cell line (HROC126: APCwt, TP53mut, KRASmut, BRAFmut, PTENeo, HROC239 T0 M1: APCwt, P53mut, KRASwt,BRAFmut, PTENeo and HROC284Met: APCmut, P53wt, KRASmut, BRAFwt, PTENwt). The cell lines demonstrated a heterogeneous response to chemotherapy, radiation and combined radio-chemotherapy. Interestingly, analysis of glucose metabolism showed a markedly reduced uptake of the PET tracer 18F-FDG after combined radio-chemotherapy of all three cell lines.

Research conclusions
Taken together, this study describes the development and in-depth characterization of three patient-derived rectal cancer cell lines that could be established from fresh patients’ tumor samples for the first time. These powerful matched in vitro and in vivo models provide useful tools not only to perform basic research to better understand the biology of rectal cancer, but also to test and establish novel therapy regimens.

Research perspectives
This descriptive study exemplifies the methodology and characterization of rectal cancer cell lines obtained directly from patients’ tumor material. This is an important step to extend the abilities of personalized tumor therapy in the near future.

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