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# Anticancer drug response and expression of molecular markers in early-passage xenotransplanted colon carcinomas

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**Abstract**

Despite some success in the treatment of colorectal carcinomas, novel rational therapies targeting specific cancer-related molecules are under development and urgently needed. These approaches need careful preclinical evaluation in models that closely mirror the clinical situation. Therefore, we established a panel of 15 xenotransplantable tumours directly from fresh surgical material. We showed that both the histology and expression of tumour-associated markers (Epithelial Cell Adhesion molecule (EpCAM), E-cadherin, carcinoembryonic antigen (CEA)) could be maintained during passaging in nude mice. Xenotransplanted tumours were characterised for chemosensitivity and revealed a response rate of 5/15 (33%) for 5-fluorouracil (5-FU), 15/15 (100%) for irinotecan and 8/14 (57%) for oxaliplatin. 5 patients out of 15 were treated with cytostatics because of synchronous metastases. The response to chemotherapy in these patients coincided very closely with the response of the individual xenografts. All of the xenografts expressed the proliferation marker Ki67 and the nuclear enzyme, Topoisomerase IIα (Topo IIα) at the protein level. Most of the xenografts also expressed the tumour suppressor, p53 (9/14) and the nuclear enzyme Topoisomerase Iα (Topo Iα) (13/14) at the protein level. Interestingly, the presence of a K-*ras* mutation in codon 12 (5/15 xenografts) coincided with a low response rate towards oxaliplatin. This observation needs further confirmation using a larger number of tumours. In conclusion, we were able to establish transplantable xenografts suitable to mimic the clinical situation. These well characterised models are useful tools for the preclinical development of novel therapeutic approaches and for investigating translational research aspects.


*Keywords:* Apoptosis; Topoisomerase; Chemosensitivity; *ras* mutations; Cytostatics

## 1. Introduction

Colorectal carcinomas represent the second most frequent cause of cancer deaths in the Western population. Treatment of colorectal cancers generally comprises a combination of the three classical strategies of oncology: Surgery accompanied by radio- and chemotherapy. Advanced colorectal disease is routinely treated with adjuvant therapy such as 5-fluorouracil (5-FU) combined with Leucovorin or Levamisole. Recently, novel, highly efficient compounds like oxaliplatin [1] or irinotecan [2] have been introduced into clinical treatment modalities. Many other molecular therapies are now under investigation including immunotherapeutic agents like Panorex [3] and molecular therapeutic approaches (for a review, see Ref. [4]). At present, the preclinical development of more effective therapeutic strategies to treat colorectal cancer is limited by the availability of well characterised clinically relevant models [5]. Cell lines used for such studies *in vitro* or as xenografts mainly represent poorly differentiated carcinomas which lack similarity to the original tumour and therefore the clinical situation. To better bridge the gap between preclinical and clinical studies, we started a programme to establish colon cancer xenografts directly from surgical material. Serially transplantable tumours

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in nude mice were obtained and used for further studies. The tumours were maintained either *in vivo* by passaging or cryogenically banked in liquid nitrogen. In order that these xenografts reflected the original patient tumour, no more than 10 passages of each tumour in mice were performed. In this report, we have characterised the chemosensitivity of these xenografts, analysed the expression of relevant molecules at both the transcriptional and protein level, and correlated these results with the clinical course of the disease.

## 2. Materials and methods

### 2.1. Tumour samples

Tissues from colorectal carcinomas (35 in total) were obtained from the Surgical Department of the Charité, Robert-Rössle-Clinics Berlin-Buch. All tumours were collected after first obtaining informed patient consent and according to local research ethics and regulations. Pathological characterisation of each sample was performed by experienced pathologists. Table 1 shows the TNM classification of the tumours, and Table 2 the chemotherapy given to 5 of the patients and the course of disease. All samples were obtained from primary tumours, with the exception of tumours 1 and 13 which were secondary metastatic deposits excised from the liver. To ensure sterility, all tumour samples were incubated overnight at 4 °C in a gentamycin solution (100 μg/ml).

### 2.2. Establishment of xenografts

Tumour fragments (5×5 mm) of each sample were surgically implanted into the flanks of anaesthetised nude mice (NMRI: nu/nu, Bomholtgaard, Ry, DK). Once established, tumours were grown to a maximum size of 8×8 $mm^3$ when they were routinely passaged. The *in vivo* growth rate of the xenotransplanted tumours was determined between passages 3 and 5. In order to retain as many features of the original tumour as possible, xenotransplanted tumours were only grown through 10 passages in mice. At several passages, fragments of xenografted tumour were frozen in liquid nitrogen and stored.

### 2.3. Chemosensitivity profile

Groups of 6–8 nude mice each (NMRI: nu/nu) maintained under sterile and controlled environmental conditions (22 °C, 50±10% relative humidity, 12-h light–dark cycle, autoclaved food and bedding, acidified drinking water) were randomised either to receive antitumour drug or saline. When tumours were approximately 5×5 mm in size, treatment was initiated with the following drugs: 5-FU (Ribofluor, Ribosepharm, München) 30 mg/kg intraperitoneally (i.p.), every day (qd)) 1–5; 5-FU + leucovorin (Lederle, Wolfrathshausen) 10 mg/kg i.p. 1 h before the 5-FU, qd 1–5; Irinotecan (Campto, Rhone-Poulenc) 15 mg/kg i.p., qd 1–5; Oxaliplatin (Eloxatin, Sanofi-Synthelabo, Berlin) 5 mg/kg i.p., qd 1–5. All drug doses and treatment schedules were optimised in previous studies and represented the maximum tolerated dose (MTD) for the corresponding mouse strain (unpublished data). All drugs were freshly prepared, as prescribed for clinical use, and used at an injection volume of 0.2 ml/20 g body weight. For all chemotherapy studies, therapeutic effects were assessed by twice weekly calliper measurements of the tumour. Tumour volumes (TV) were determined by the formula ($width^2 \times length$) × 0.5 and related to the value of the first treatment day (RTV, relative tumour volume). Treated to control ($T/C$) values of the RTV as a percentage were used for an evaluation of therapeutic efficacy. The following scores were used: negative $= T/C > 50\%$; $+ = T/C$ 36–50%; $++ = T/C$ 21–35%; $+++ = T/C$ 6–20%; $++++ = T/C \leqslant 5\%$. Body weight of the mice was determined twice weekly and the change in body weight (BWC) given as a percentage was used as a measure of toxicity. All animal experiments were performed according to the regulations of the German Animal Protection Law and with the permission of the local responsible authorities.

### 2.4. Immunofluorescence

Expression of the tumour-associated antigens, Epithelial Cell Adhesion molecule (EpCAM) and carcinoembryonic antigen (CEA), in the tumour xenografts were analysed by immunofluorescent analysis of frozen tissue sections. Briefly, 5-μm cryosections of each xenograft were fixed in acetone for 10 min. After washing in phosphate-buffered saline (PBS), non-specific antibody binding was blocked in 5% normal goat serum (Dako A/S, Denmark) for 30 min at room temperature in a humidified atmosphere. EpCAM was detected using a fluorescein isothiocyanate (FITC)-labelled antibody (Ber-EP4; Dako) diluted 1:20 in PBS for 30 min in a humidified chamber; CEA was detected using the CD66e antibody (Dianova, Hamburg, Germany) diluted 1:20 in PBS for 30 mins. followed by a CY3 F(ab')2 fragment labelled antibody diluted 1:400 in PBS for 30 min in a humidified chamber. After washing in PBS, the sections were mounted in fluorescent mounting medium (Dako). Slides were examined using a Zeiss Axioskop fluorescent microscope. Antigen expression was evaluated semiquantitatively as either negative (−), <10% positive cells (±), 10–20% positive cells (+), 20–70% positive cells (++), or >70% positive cells (+++).

Table 1
Characterisation of patients' tumours, response of xenografts to chemotherapy and protein expression

| Sample | Patient pTNM-classification | Tumour doubling time (days ± S.D.) | Response of xenografts ($T/C$) to | | | | Protein expression | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 5-FU | 5-FU + Leucovorin | Irinotecan | Oxaliplatin | Ki67 | | p53 | | Topo I α | | Topo II α | |
| | | | | | | | Staining intensity | % nuclei positivity | Staining intensity | % nuclei positivity | Staining intensity | % nuclei positivity | Staining intensity | % nuclei positivity |
| 1 | yT3N1G3 M1-liver | 6.2 ± 1.6 | – | + | + + + + | + + + | Strong | + + + | Strong | + | Weak | + + + + | Strong | + |
| 2 | T3N1G2 M0 | 10.8 ± 2.4 | + | + | + + + + | – | Strong | + + + + | Strong | + + | Strong | + + + + | Strong | + |
| 3 | T4N2G2 M0 | 17.6 ± 3.5 | + | + | + | – | Strong | + + + | Strong | + + | Weak | + + + | Strong | + + |
| 4 | T4N2G3 M0 | 4.2 ± 0.8 | – | + | + + | + + | Strong | + + | Negative | – | Weak | + + + | Strong | + + |
| 5 | T3N0G2 M0 | 30.9 ± 21.6 | + + | + | + + | – | Strong | + + + | Strong | + + | Weak | + | Strong | + |
| 6 | T3N0G2 M0 | 10.8 ± 2.4 | + | – | + + + | – | Strong | + + | Negative | – | Negative | – | Strong | + + + + |
| 7 | T3N2G3 M1-liver | 10.4 ± 3.6 | – | – | + + + | + + | Strong | + + + | Strong | + + | Strong | + + + + | Strong | + + |
| 8 | T3N2G M1-liver | 11.8 ± 4.5 | (+) | – | + | n.t. | n.t. | | n.t. | | n.t. | | n.t. | |
| 9 | T3N2G3 M1-liver, lung | 8.9 ± 2.7 | – | – | + + | + | Strong | + + + | Negative | – | Strong | + + + + | Strong | + + |
| 10 | T4N1G2 Mo | 32.9 ± 31.0 | – | n.t. | + + + | + + | Strong | + + | Weak | – | Strong | + + + + | Weak | + |
| 11 | T3N2G2 M0 | 12.7 ± 11.8 | + + | n.t. | + + + | + + | Strong | + + | Strong | + | Strong | + + + + | Strong | + + |
| 12 | T4N0G2 M0 | 15.2 ± 8.0 | – | – | + | – | Strong | + + + | Strong | + | Weak | + + + | Strong | + + + + |
| 13 | T4N2G2 M1-liver, lung | 4.3 ± 2.4 | – | n.t. | + + + | + + + | Strong | + + + + | Weak | – | Strong | + + + + | Weak | + |
| 14 | T2N0G2 M0 | 9.7 ± 3.2 | – | n.t. | + | + | Strong | + + + | Weak | + + | Strong | + + + + | Strong | + + |
| 15 | T3aN0G2 M0 | 16.8 ± 7.5 | – | n.t. | + | – | Strong | + + + | Strong | + + | Weak | + + + | Strong | + + |
| Positive response: | | | 5/15 (33%) | 5/10 (50%) | 15/15 (100%) | 8/14 (57%) | | 14/14 | | 9/14 | | 13/14 | | 14/14 |

S.D., standard deviation; 50-FU, 5-fluorouracil. Evaluation: $T/C$: > 50%–, + 36–50%, + + 21–35%, + + + 6–20%, + + + + 0–5%, n.t. not tested, ( ) borderline. Immunohistology: – negative, + 10–25%, + + 26–50%, + + + 51–75%, + + + + 76–100% positive cells.

### 2.5. Immunohistochemistry

Formalin-fixed paraffin-embedded sections of tumour xenografts (5 μm thick) were dewaxed in xylene and rehydrated to water through graded alcohols. Endogenous peroxidase activity was quenched with freshly prepared 1% hydrogen peroxide for 30 min at room temperature. Non-specific antibody binding was blocked in 5% normal goat serum (Vector Laboratories, CA, USA) for 20 min at room temperature in a humidified atmosphere. Following antigen retrieval, sections were then incubated with primary antibody: anti-human p53 polyclonal antibody (Novocastra, Newcastle, UK) diluted 1:1000 overnight at 4 °C; anti-human Ki67 monoclonal antibody (Pharmingen, Oxford, UK) diluted 1:100 overnight at 4 °C; anti-human Topoisomerase Iα monoclonal antibody (Novocastra, Newcastle, UK) diluted 1:25 for 90 min at room temperature in a humidified atmosphere; anti-human Topoisomerase IIα monoclonal antibody (Dako, Cambridge, UK) diluted 1:100 for 90 min at room temperature in a humidified atmosphere. After washing in PBS, sections were incubated for 30 min at room temperature with either biotinylated, horse anti-rabbit or horse anti-mouse immunoglobulins (0.5% v/v; Vector Laboratories) in PBS, washed in PBS followed by incubation with peroxidase-conjugated streptavidin label for 30 min. The resultant immune-peroxidase activity was developed using diaminobenzidine (DAB) according to the manufacturer's instructions (Vector Laboratories). Sections were counterstained with Harris' haematoxylin and mounted in DPX mountant (Sigma, UK). Appropriate negative controls were performed by omitting the primary antibody and/or substituting it with an irrelevant anti-serum. Slides were examined using a Leica DMRB microscope fitted with a JVC video camera (Leica Microsystems, Milton Keynes, UK) and images collected using AcQuis Bio software (Syncroscopy, Cambridge, UK). Stained sections were independently assessed by three observers and the mean score recorded. Specimens were scored for intensity of staining (negative, weak, strong) and percentage of positively stained cells: (−) < 10%; (+) 10–25%; (++) 26–50%; (+++) 51–75%; (++++) 76–100%.

### 2.6. RT-PCR of E-cadherin

RNA was extracted using the QIAamp RNeasy-Kit (Qiagen, Hilden, Germany) from snap-frozen xenograft tumour samples. The presence of epithelial cells was confirmed by reverse-transcription polymerase chain reaction (RT-PCR) amplifying specifically a 656 bp fragment of human *E-cadherin*. It was performed in a single tube/one-step reaction using the rTth-kit (Applied Biosystems Inc.). Primers corresponded to bases 1984–2640 (5′ primer TCCCAATACATCTCCCTTCACAGC; 3′ primer CCGCTTCCTTCATAGTCAAACACG) of CDH1 (RefSeq NM_004360). Following the initial RT-reaction, cDNA was denatured at 94 °C for 2 min, subjected to 35 amplification cycles (94 °C for 30 s, 62 °C for 30 s, 72 °C for 1 min) followed by a final elongation step of 72 °C for 10 min. DNA products were separated

Table 2
Course of disease and chemotherapeutic response of patients with synchronous metastases

| Sample | Date | Clinical course of disease | Treatment | Response | Coincidence with xenograft results |
|---|---|---|---|---|---|
| 1 | 03/99 | 2 months neoadjuvant CT | 5-FU | Progression of liver metastases | Yes |
| | 05/99 | Rectum and liver resection | | | |
| | 10/99 | Relapse of liver metastases | | | |
| | 10/00 | Relapse of liver metastases | I | Complete remission | Yes |
| | 01/03 | Alive with stable disease | I | Complete remission | Yes |
| 7 | 11/98 | Hemicolectomia | 5 months 5-FU/LV | Progression of liver metastases | Yes |
| | 03/00 | Relapse | 4 months O/I | Partial remission | Yes |
| | 02/01 | Death | 4 months I/5-FU/LV | Partial remission | Yes |
| 8 | 06/99 | Sigma resection | 19 months 5-FU/LV i.a. | Stable disease | (Yes) |
| | 10/01 | Progression of liver metastases | 3 months Capecitabine | Stable disease | |
| | 04/02 | Progression of liver metastases | O/5-FU | Partial remission | (Yes) |
| | 01/03 | Alive with stable disease | | | |
| 9 | 08/09 | Rectum extirpation | 6 months 5-FU/LV i.a. | Partial remission of liver metastases | (No) |
| | 06/00 | Lung metastases | 2 months 5-FU/LV | Progression | Yes |
| | 01/01 | Death | | | |
| 13 | 07/00 | Rectum resection | 4 months O/5-FU/LV | Complete remission of lung, partial remission of liver metastases | Yes |
| | 04/02 | Lung metastases | 7 months 5-FU | Progression | |
| | 10/02 | Alive with stable disease and local relapse | | | Yes |

CT, chemotherapy; O, oxaliplatin; I, irinotecan; 5-FU, 5-fluorouracil; LV, leucovorin; i.a., intra-arterial.

on a 1.75% agarose gel containing ethidium bromide. Amplified DNA fragments were visualised using ultraviolet light. RNA samples from a human breast carcinoma line (MaCa 3366) as a positive control and mouse liver tissue as a negative control were processed in parallel.

### 2.7. *K*-ras *genotyping*

DNA extraction from tissue samples has been performed using the Invisorb Spin Tissue DNA Mini Kit (Invitek). K-*ras* genotyping has been performed using the Invigene K-*ras* genotyping Kit (Invitek). Sample DNA was amplified by mutant-enriched PCR. The principle of this method is to introduce a *Bst*NI restriction site into the amplicons of wild-type (wt)-coding K-*ras* by means of primers containing a mismatch to the wt K-*ras* sequence. An ensuing incubation with *Bst*NI cleaves wt amplicons, but leaves mutant PCR products intact. This way, the ratio of mutant to wt amplicons can be increased by two orders of magnitude. A second amplification step is added to produce the amounts of PCR products required for further analysis. Mutant enrichment was carried out as described except that unlabelled forward primers (5′AAC-TTG-TGG-TAG-TTG-GAG-CT 3′) and 5′ biotin-labelled reverse primers (5′ GTT-GGA-TCA-TAT-TCG-TCC-AC 3′; Biotez) were used in the second amplification step. Amplicons were diluted 1/100 in binding buffer (NaCl, Tris HCl, ethylene diamine tetra acetic acid (EDTA)), and 50 μl was added to each well of a strip of eight streptavidin-coated microwells (Biotez). The samples were immobilised by incubation for 15 min at room temperature, the solution was then aspirated, and 50 μl of NaOH was added for the strand separation. After 15 min at room temperature, the wells were washed three times with PBS containing Tween 20. Single-stranded amplicons were hybridised with 3 pmol of 5′ FITC-labelled probes representing wt-K-*ras* coding for glycine or mutated K-*ras* coding for alanine, arginine, aspartate, cysteine, serine or valine at codons 12 and 13.

After hybridisation, the wells were washed three times with washing buffer (SSC, sodium dodecyl sulphate (SDS)) at 48 °C. Then 50 μl of conjugate (anti-FITC-peroxidase (POD) Biotez) was added and incubated for 30 min at room temperature). After three washing steps, 50 μl of TMB was added to each well and incubated for 10–15 min. The colorimetric results were measured using a microplate reader (TECAN). The results were analysed by DIGEM software (Invitek).

### 2.8. *Statistics*

The non-parametric U-test of Mann–Whitney with a significance level of $P \leqslant 0.05$ was used for the statistical evaluation of tumour growth. The Spearman correlation coefficient was calculated for comparisons of immunohistological and genomic data. All calculations were performed with the Windows program STATISTICA 5.0.

## 3. Results

### 3.1. *Characterisation of carcinomas* in vivo

We implanted a total of 35 surgical samples of colorectal carcinomas subcutaneously (s.c.) into the flanks of nude mice. Of these, 15 (43%) grew progressively over more than three passages and were used as tumour models in the subsequent investigations. The doubling time of these xenografted tumours was determined between passages 3 and 5 and ranged from 4.2 to 32.9 days (Table 1). No correlation was found between the *in vivo* growth rate and clinical tumour stage or presence of metastases (Table 1).

### 3.2. *Chemotherapeutic response of xenografts*

The drugs 5-FU (with or without Leucovorin), irinotecan and oxaliplatin, used routinely in the clinic, were used to characterise the chemosensitivity profile of the tumour xenografts. As can be seen from Table 1, only 5/15 (33%) tumours responded significantly to 5-FU. Co-treatment of 5-FU with Leucovorin resulted in increased toxicity (body weight loss and lethality; data not shown) and response in 5/10 tumours (50%). Interestingly, co-treatment with Leucovorin resulted in an improved response compared with 5-FU in only two xenografts, and a decrease in efficacy in one xenograft. Oxaliplatin caused a significant therapeutic response in 8/14 (57%) of the xenografts. In contrast, irinotecan treatment was very effective resulting in a response in 15/15 (100%) of tumour xenografts. Complete tumour remission was observed in 2/14 responsive xenografts and significant retardation of tumour growth in the others. An example of the differential response to the three agents is shown in Fig. 1, using xenograft 14 as the model. No response to 5-FU was observed in this

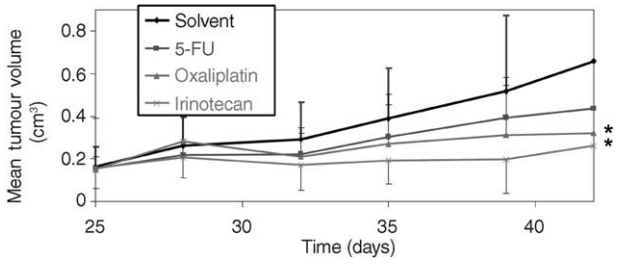


Fig. 1. Growth and chemoresponsiveness of xenograft 14. Tumour fragments were inoculated subcutaneously (s.c.) to six mice per group. Treatment with 5-fluorouracil (5-FU), irinotecan and oxaliplatin started at palpable tumour size (day 25); for doses and schedules, see Material and methods. *Significant to solvent ($P < 0.05$).

model, whereas both irinotecan and oxaliplatin significantly retarded tumour growth (Fig. 1).

### 3.3. Correlation with clinics

Xenograft response to the chosen chemotherapeutic agents was found to be independent of both the growth rate *in vivo* and the clinical stage of the human tumour. In contrast, sensitivity of xenografts to chemotherapy corresponded well with that observed in the clinic (Table 2). Chemotherapy for synchronous metastases was received by 5/15 patients. For instance, patient 1 was following to progression of liver metastases under 5-FU therapy-successfully treated with oxaliplatin and irinotecan and is so far (at 4 years) alive with stable disease. The xenotransplanted tumour derived from this patient also responded very well to treatment with these drugs, but was resistant to 5-FU. Another patient (7) was treated with 5-FU/Leucovorin as first-line therapy. Due to a lack of response, second-line therapy of oxaliplatin and irinotecan was given resulting in a decrease in the levels of the tumour marker CEA and a shrinkage of liver metastases. The corresponding xenotransplanted tumours were found to be 5-FU-resistant, but sensitive to both oxaliplatin and irinotecan. In addition, patients 8 and 9 initially responded to intra-arterial administration of 5-FU/Leucovorin, but became increasingly resistant to systemic 5-FU treatment. The same tumours when grown as a xenotransplant gave only a borderline response to systemic 5-FU treatment. Taken together, these data demonstrate a close correlation between the clinical response and that observed using the xenotransplanted tumours.

### 3.4. Expression of epithelial and tumour-associated markers

Expression of the epithelial marker, EpCAM, and the tumour-related antigen, CEA, were assessed in both the original clinical tumour and samples taken after 2–4 passages as xenografts in the nude mice (Table 3, Fig. 2). All clinical tumours expressed EpCAM and 13/14 were positive for CEA. Passaging of the xenotransplanted tumours resulted in an increase in the number of cells expressing both EpCAM and CEA. In addition, the proportions of areas of histologically malignant tissue were also higher in the passaged xenografts compared with the corresponding original clinical tumour. Overall, the architecture and characteristics of the original tumour were maintained during early passaging in mice (Fig. 2).

All 15 xenotransplanted tumours were tested for *E-cadherin* mRNA, both in the original sample and in several murine passages, by RT-PCR. The presence of human epithelial cells and the stable maintenance during passaging was observed in all samples (data not shown).

### 3.5. Expression of Ki67 and p53 proteins

All xenografted tumour samples demonstrated nuclear positivity for the proliferation marker, Ki67, with expression in >25% of tumour cells (Table 1). These observations support the malignant status and sustained growth of the tumours as xenotransplants. Expression of the tumour suppressor protein p53 was observed in 9/14 (64%) xenografts, with high expression (strong intensity in >25% cells) being observed in 6/9 (67%) tumours (Table 1). The antibody used for p53 can detect both the wild-type and mutated forms. However, it was shown in previous studies that wild-type background levels were not identified with the titres used, whereas high levels of p53 were indicative for the mutated form. Although a positive relationship was observed between the expression of Ki67 and p53 protein ($R=0.69$), no correlation was seen between xenograft doubling time and either Ki67 or p53 protein levels ($R=0.19$ or 0.46), respectively.

Table 3
Expression of epithelial (EpCAM) and tumour-specific (CEA) marker in original patient tumour and xenograft passages

| Sample | Original/murine passage | EpCAM | CEA |
|---|---|---|---|
| 1 | 0[a] | + | + |
| | 1 | +++ | + |
| | 2 | +++ | ++ |
| 2 | 0 | ++ | ++ |
| | 2 | +++ | ++ |
| 3 | 0 | + | (+) |
| | 2 | ++ | (+) |
| 4 | 0 | + | + |
| | 2 | +++ | +++ |
| | 4 | +++ | ++ |
| 5 | 0 | +++ | ++ |
| | 2 | +++ | ++ |
| 6 | 0 | + | – |
| | 2 | ++ | (+) |
| 7 | 0 | (+) | + |
| | 2 | ++ | + |
| 8 | n.t. | n.t. | n.t. |
| 9 | 0 | + | + |
| | 2 | +++ | ++ |
| | 3 | +++ | +++ |
| 10 | 0 | (+) | + |
| | 2 | ++ | ++ |
| 11 | 0 | + | ++ |
| | 2 | +++ | ++ |
| 12 | 0 | (+) | + |
| | 2 | ++ | ++ |
| 13 | 0 | (+) | + |
| | 3 | + | ++ |
| 14 | 0 | ++ | ++ |
| | 2 | ++ | ++ |
| 15 | 0 | ++ | + |
| | 4 | +++ | ++ |

[a] 0, original patient sample; – to +++ see Materials and methods; n.t., not tested; EpCAM, Epithelial Cell Adhesion molecule; CEA, carcinoembryonic antigen.

### 3.6. Expression of Topoisomerase Iα and IIα

The expression of the Topoisomerase Iα and IIα (Topo Iα and Topo IIα) in the xenografted tumours is of interest since irinotecan acts by inhibiting Topo I activity. As shown in Table 1, 13/14 xenotransplants expressed Topo Iα protein. This profile correlates well with the high response rates of the xenografts to irinotecan ($R = 0.79$). Surprisingly, one tumour (6) did not demonstrate such a relationship. Tumour 6 did not appear to express Topo Iα, but responded very well to treatment. Therefore, other mechanisms of irinotecan action may have to be taken into consideration when studying the sample. All samples were positive for Topo IIα protein (Table 1). As none of the chemotherapeutic agents included in the present study were typical Topo IIα inhibitors, no correlation to response could be drawn.

### 3.7. *K*-ras *genotyping*

We were interested in potential K-*ras* mutations detectable in the transplantable colorectal carcinomas and analysed it using a specific genotyping test kit. The data are summarised in Table 4. Point mutations in codon 12 (concerning amino acids Ser, Asp, or Val) and codon 13 (Asp) were observed in 5/15 (33%) and 2/15 (13%) of the xenotransplants, respectively. Although no

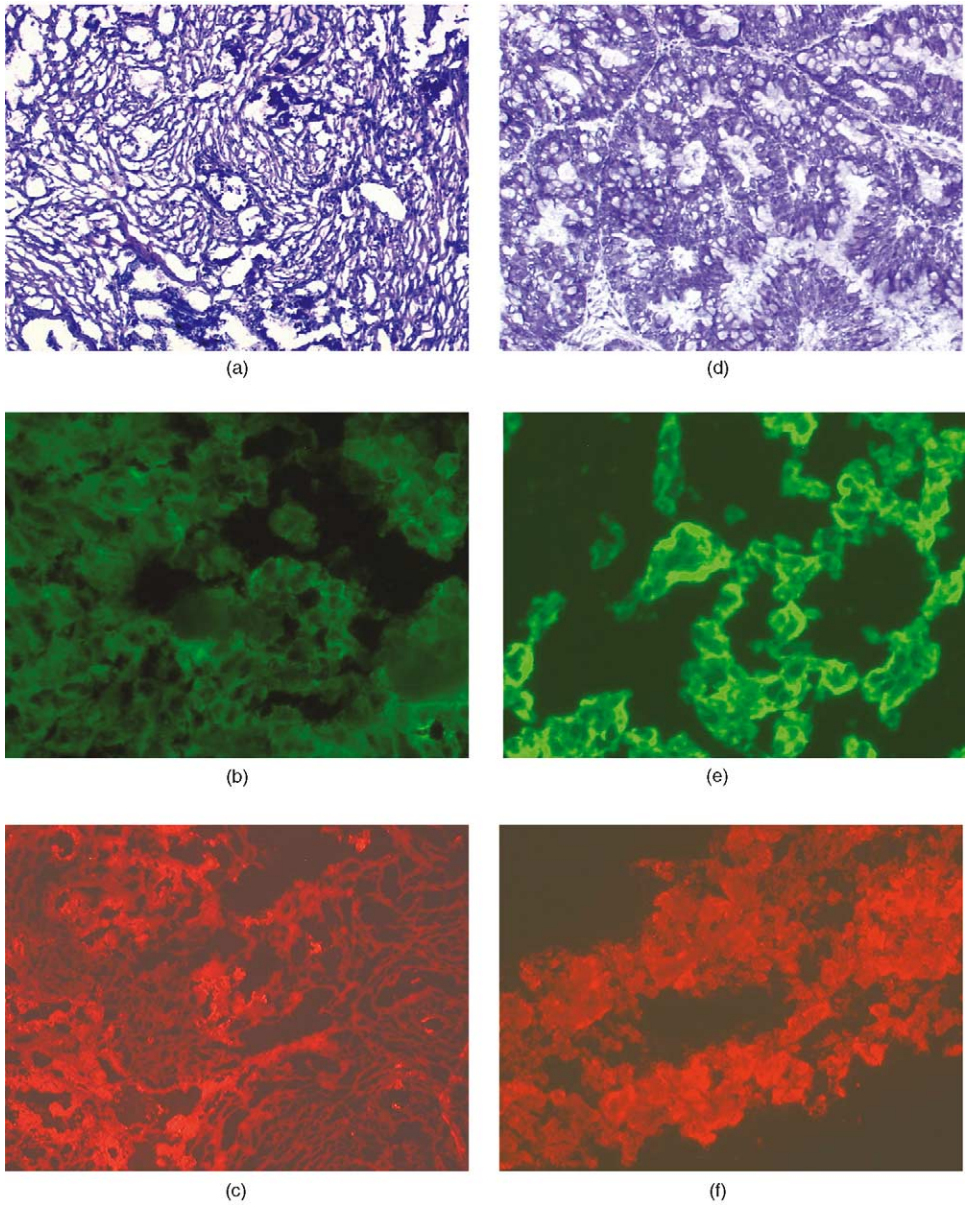


Fig. 2. Representative examples of histological and immunohistological views of tumours: (a–c) original tumour from patient; (d–f) second passage in nude mice; (a + d) haematoxylin/eosin staining in patient 4; (a) 100-fold, (d) 200-fold magnification; (b + e) immunofluorescence of EpCAM, patient 2, 100-fold magnification; (c + f) immunofluorescence of CEA, patient 4, 100-fold magnification.

Table 4
Mutations in K-*ras* gene

| Sample | Mutation | Amino acid |
|---|---|---|
| 1 | n.m. | |
| 2 | n.m. | |
| 3 | Codon 12 | ser |
| 4 | n.m. | |
| 5 | n.m. | |
| 6 | Codon 12 | asp |
| 7 | Codon 13 | asp |
| 8 | n.m. | |
| 9 | Codon 12 | asp |
| 10 | Codon 13 | asp |
| 11 | n.m. | |
| 12 | Codon 12 | val |
| 13 | n.m. | |
| 14 | Codon 12 | ser |
| 15 | n.m. | |

n.m., no mutations; ser, serine; val, valine; asp, aspartate.

relationship between codon 13 mutations and chemotherapeutic response could be drawn, xenografts containing mutations in codon 12 had a poor response to oxaliplatin treatment.

## 4. Discussion

The aim of our study was to establish preclinical models of colorectal carcinomas that maintained a high correlation with the characteristics of the clinical disease by transfer of fresh surgical material directly to nude mice, thereby avoiding any intermediate cell culturing. Using this strategy, we intended that the accompanying tumour micro-environment would be maintained and support the individuality of the original sample. We were successful in establishing 15 xenotransplantable tumour lines out of the 35 patient samples tested. This take rate of 43% in successive passages correlates with similar reports in the literature [6]. Neither the take rate nor the growth rate (tumour doubling time) in the nude mice was dependent upon the pathological tumour stage at the time of diagnosis. The lack of take in 57% of tumour samples is probably due to only a minimum of the transplanted malignant cells surviving immunological attacks (mainly by NK cells) in the nude mice.

The successfully established xenografts within a limited number of murine passages (we performed not more than 10) maintained their histological features compared with the original sample. All tumours expressed epithelial markers (EpCAM, E-cadherin) and were positive for CEA, a molecule used as clinical tumour marker [7,8] and as a target for therapies [9]. During passaging of transplants in nude mice, we observed a well-known increase in malignancy [6], detectable by an elevation of EpCAM and CEA protein expression and a microscopically identifiable number of tumour cells, without any change in the original histological picture. These quantitative changes stress the necessity of keeping the passaging of tumours to a minimum in order to help maintain the heterogeneity of the clinical samples. We registered a high coincidence between the outcome of clinical treatment and response of xenografts to three cytotoxic drugs used for the treatment of advanced stages of colorectal carcinomas. 5 out of 15 patients were treated with at least one of these drugs because of the presence of synchronous metastases. The outcome of the xenograft study correlated in each case with the clinical course of disease and showed coincident sensitivity or resistance. Despite the relatively low number of cases, we propose that a xenograft assay for the individual estimation of drug responsiveness of colorectal carcinomas should be performed. Although such a test of primary high-risk tumours would take 3–4 months, the results would be provided in sufficient time before a decision for clinical treatment of metachronous metastases or recurrences in an individual patient had to be made. A prediction assay would optimise the therapy in a proportion of the patients and could avoid unnecessary therapies with associated high toxicity. A similar correlation between the chemoresponsiveness of xenografted and clinical malignancies has also been reported by Winograd and colleagues and Hoffmann and colleagues [6,10]. A limited relationship between xenograft response levels and phase II response rates was shown by Johnson and colleagues [11]. They compared 39 compounds which were tested within the National Cancer Institute (NCI) drug development programme and found that only non-small cell lung xenografts were predictive of clinical efficacy. However, it should be noted that the NCI study used xenografts that were derived from cell lines that had been cultured for a long period of time, confirming our opinion that a direct *in vivo* establishment and use of early passages is preferable.

The high response rate of xenografts to irinotecan corresponded well with the expression of the Topo I protein, the main target for this drug. It should be noted that there are interspecies differences in the metabolism and pharmacokinetics of irinotecan. For instance, the level and activity of carboxylesterases is higher in mice possibly leading to a higher amount of the active metabolite, SN38 [12]. Presently, a vast number of clinical studies are ongoing comparing camptothecin-containing regimens with the standard therapy of 5-FU. Initial results [2] show increased response rates, improved time to progression and survival of an irinotecan-containing combination compared with 5-FU alone. This correlates with our data; in our approach, irinotecan was distinctly superior to 5-FU in a large number of the xenotransplants.

In a few studies, a correlation between Topo I expression and response to camptothecin therapy has been investigated. No correlation was found by Ohashi

and colleagues [13], a weak correlation by Jansen and colleagues [14] and a good correlation by Kigawa and colleagues [15], when studying mRNA levels in ovarian and sarcoma xenograft lines. In all studies, the effect of the rate of drug metabolism and elimination on the activity of Topo I inhibitors in malignancies was discussed. These rates may partly explain why one of our xenograft lacked coincident Topo I protein expression and response to irinotecan and, as such, warrant further investigation.

Oxaliplatin, a third-generation DNA-damaging agent [16], has been introduced into the treatment modalities for colorectal cancer as a single medication [17] or in combination with 5-FU [18]. So far, it has not yet been decided which strategy (single versus combination) would be the most promising clinical use of oxaliplatin [19]. With the help of a xenograft panel with different sensitivities, such as in this study, a variety of combination studies could be performed to try to answer this question.

The p53 suppressor gene has been investigated as a tumour marker, as a regulator of apoptosis, as a predictor of prognosis or chemoresponsiveness [20,21]. It is known that approximately 50% of tumours carry mutations in the gene leading to the expression of a protein without anti-oncogenic potential. As wt-p53 is detectable in cells in only minute amounts (due to a high rate of elimination), a high level of p53 protein is consistent with malignancy. p53 protein was detectable in 9/14 xenografts tested in our study. The expression level (% of positively stained nuclei) showed only a borderline correlation with the tumour doubling time of the xenografts ($R = 0.46$). There was also no correlation with the response to any of the cytotoxics tested. This confirms data from the literature showing a lack of coincidence between p53 expression and chemotherapeutic responsiveness in clinical ovarian [22] and breast cancers [23].

Mutations in the K-*ras* gene were analysed to see if they could be useful as potential predictive markers for a chemotherapeutic response. K-*ras* is an oncogene which is mutated in 30–50% of colorectal carcinomas and as such is used as a marker for early diagnosis. Mutations appear mainly in codons 12 and 13 and are detectable with specific oligonucleotide probes. The detection of mutations in 7/15 transplantable colorectal carcinomas in our study with the prevailing mutation being in codon 12 coincides with the frequencies. reported in literature [24–26]. We observed at least a tendency of correlation between the appearance of point mutations in codon 12 (in 5/15 samples) and a low or absent response of the xenografts to oxaliplatin. This observation has not been described in the literature, but will have to be confirmed using an extended number of clinical samples. It has been reported that patients with K-*ras* mutations have lower survival following marimastat treatment [27], while others [28] showed no influence of K-*ras* mutations on the response of patients to 5-FU therapy.

We are aware that our data need to be investigated further on a molecule-by-molecule basis. On the other hand, we have shown that the relatively complex xenograft system is suitable for an analysis of mutual interrelationships between marker expression and tumour characteristics.

To summarise, our relatively comprehensive study on the molecular characterisation of colon cancer xenografts in early passages revealed a high coincidence in histology and chemotherapeutic response with the original tumours from which they were derived. The newly established models could therefore be valuable tools for the rational preclinical development of target-oriented therapeutic approaches.

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