

Experimental Colorectal Cancer Liver Metastasis

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1. Introduction

With estimated 1 080 000 diagnosed cases each year, which account for 1.1% of all deaths, colorectal carcinoma (CRC) ranks fourth in cancer-related deaths in both sexes worldwide (WHOSIS, 2008). In Europe, CRC is the third most lethal malignancy after lung and stomach cancers in men, and it ranks second after breast cancer in women (WHOSIS, 2008).

CRC progression is characterized by increased growth of the primary carcinoma as well as lymphatic and haematogenic spread. The liver is often the first vascular bed in which disseminating colorectal cancer cells are trapped and therefore is affected in up to 10-20% of CRC patients at the time of presentation (Berney, et al., 1998). Another 40-50% of patients will eventually develop liver metastasis during the course of their illness, which is commonly the cause of death (Bentrem, et al., 2005, Stangl, et al., 1994, Sugarbaker, 1990). At present, liver resection is considered the treatment of choice for suited patients with colorectal liver metastases, offering a five-year survival rate of 25-44% (Choti, et al., 2002, Garden, et al., 2006, Zacharias, et al., 2004) to those 20-25% of patients with isolated liver metastasis (Adson, et al., 1984, Bismuth, et al., 1996, Fong, et al., 1999). Unfortunately, this procedure is feasible only in patients with no signs of irresectable extra-hepatic disease, whereas the median survival is only 9-19 months for patients with unresectable disease who receive systemic chemotherapy (de Gramont, et al., 2000, Giacchetti, et al., 2000, Meyerhardt and Mayer, 2005, Saltz, et al., 2000).

However, the fact that CRC malignancy develops over a long period and can only be efficiently controlled if detected early provokes many efforts to better understand the neoplastic progression of this cancer. It is well known, that there is a continuous shedding of tumor cells from a primary CRC (Chambers, et al., 2002), but not all disseminated CRC cells develop into macrometastases. It was hypothesized that sub-populations of malignant cells evolve a genetic advantage to become "highly metastatic". These clones are skilled to dissociate from the primary cancer, to intravasate into nearby blood and lymphatic vessels, to travel through the lymphatic and hematogenous systems, to survive the immune surveillance, to extravasate into distant tissues forming micrometastases, and to eventually colonize the target organ.

In this cascade, the epithelial-mesenchymal transition (EMT), characterized by the loss of cell-to-cell adhesion and cell polarity (Thiery, 2003), plays a crucial role in different stages;

namely the dissemination of tumor cells as well as their intra- and extra-vasation (Gupta and Massague, 2006). Several known transcription factors, such as Snail, Slug, and Twist, were found to induce EMT on one hand and were implicated with tumor progression and metastasis on the other hand. In line with this, some downstream genes of these regulatory factors are responsible for cell-to-cell adhesion, specially E-cadherin and claudins. For example, up-regulation of Snail induces EMT and down-regulates the transcription of different tight junctions proteins (TJs), such as claudins and occludin (Findley and Koval, 2009). Claudins (CLDNs) form the structural backbone of TJs, and comprise at least 27 members of integral transmembrane proteins (Mineta, et al., 2011) ranging in size between 20-27 kDa (Tsukita, et al., 2001). Recently, the altered expression of various claudins has been implicated in the progression of several human cancers (Cheung, et al., 2005, Hough, et al., 2000, Johnson, et al., 2005, Kominsky, et al., 2003, Long, et al., 2001, Michl, et al., 2001, Morin, 2005, Sanada, et al., 2006, Swisshelm, et al., 2005). In contrast to the published notion that claudin expression would decrease from tumorigenesis as tight junctions are lost during cellular transformation, the claudin status seems to change in a tissue-specific manner. For example, over-expression of *Cldn2* has been correlated to colorectal cancer (Kinugasa, et al., 2007), whereas decreased *Cldn7* expression has been reported in head and neck cancer (Usami, et al., 2006), invasive ductal breast carcinoma (Kominsky, et al., 2003), and metastatic breast cancer (Sauer, et al., 2005). In addition, *Cldn3* and *Cldn4* have been found repeatedly elevated in a variety of cancers including pancreatic ductal adenocarcinoma (Michl, et al., 2003) as well as ovarian, uterine, prostate, and breast cancers (Rangel, et al., 2003). In partial contrast, reduced expression of *Cldn4* and *Cldn5* was detected in hepatocellular and renal carcinomas (Soini, 2005). In CRC, both, up- and down-regulation of claudin4 expression have been described (de Oliveira, et al., 2005, Ueda, et al., 2007), as well as aberrant expression of *Cldn1*.

Another type of cell connection has been named cell-to-extracellular matrix (ECM) contacts. On their disruption, they are presumably also implicated in tumor initiation. It is well known from each stage of malignant progression that tumor cells communicate with their microenvironment and thereby elicit responses from it. This microenvironment is mainly composed of tumor cells, extracellular matrix (ECM), stromal cells, immune cells and microvessels (Farrow, et al., 2008, Jung, et al., 2002). The ECM is a scaffold of extracellular proteins that maintain tissue shape and provide the cellular compartment with structural support (Bosman and Stamenkovic, 2003). However, by influencing cell adhesion, migration, differentiation, proliferation and survival, the ECM is a remodeling network that contributes substantially to tumor progression and metastasis (Engbring and Kleinman, 2003, Ioachim, et al., 2002). Remodeling and deposition of the ECM is mostly regulated by a functional family of extracellular proteins known as matricellular proteins, which contribute to the structural integrity and composition of the ECM (Bornstein and Sage, 2002). One of the most important characteristics of matricellular proteins is their ability to manipulate the integration and turn-over of ECM (Bornstein and Sage, 2002, Kyriakides, et al., 2001). Furthermore, by playing a linker role between the ECM and the cell surface, matricellular proteins can also direct cell fate, survival, adhesion and motility (Bornstein and Sage, 2002, Brekken and Sage, 2000, Kyriakides, et al., 2001).

Osteopontin (OPN) is an acidic extracellular matrix phosphoprotein of ≈ 298 -amino acids secreted by a wide variety of cancers, which functionally favours tumor progression (Gao, et al., 2003, Weber, 2001). The secreted phosphoprotein binds to the integrins (e.g. ITG- $\alpha\beta 3$ or ITG- $\alpha\beta 5$) and CD44 families of receptors to propagate cellular signals (Agrawal, et al., 2002,

Yeatman and Chambers, 2003). In colorectal cancer, gene profiling studies have identified a positive correlation between advanced or metastatic colon tumors and abundant OPN expression (Wai and Kuo, 2004). Increased OPN expression is associated with tumor invasion or metastasis in cancers of the breast (Tuck, et al., 1999, Tuck, et al., 1998, Tuck, et al., 1997), stomach (Ue, et al., 1998), lung (Chambers, et al., 1996, Shijubo, et al., 1999), prostate (Thalmann, et al., 1999), liver (Gotoh, et al., 2002, Pan, et al., 2003), and colon (Agrawal, et al., 2002, Yeatman and Chambers, 2003). Analysis of the OPN promoter has uncovered multiple consensus binding sites for known transcription factors (Hijiya, et al., 1994).

The main aim of our experimental series was first to generate a model suited to be used for investigating the efficacy of new drugs (Eyol, et al., 2008, Seelig, et al., 2004, Wittmer, et al., 1999). One of the few well-characterized animal models for hepatic colorectal cancer makes use of the rat CC531 cell line. Following topical injection of CC531 cells, liver metastases develop and their growth has been frequently used for studying effects of various anti-cancer treatments (Marinelli, et al., 1991, Oldenburg, et al., 1994, Veenhuizen, et al., 1996).

A second aim was to identify metastasis-related changes in gene expression in tumor cells, which differ from those in the primary tumor and probably play a crucial role in metastasis formation. Therefore, temporal changes in gene expression of CRC cells homing to the liver have been investigated using the above *in vivo* model, which is characterized by a defined onset of metastatic proliferation in rat liver following intraportal inoculation of CC531 tumor cells. This, in turn, permits a close following of the time-dependent modulation of gene expression, as the tumor cells home into the liver and then grow to a lethal size. The technique of re-isolating these tumor cells from rat liver permitted to monitor for the first time the expression of several candidate genes in a time-dependent manner (Georges, et al., 2011).

2. Description of the CC531 rat model

2.1 Generation of the model

Initially, the CC531 cell line was induced by treatment of WAG rats with 1,2 dimethylhydrazine (DMH). Forty weeks after 6 weekly injections of 30 mg/kg DMH, a carcinoma originated in the ascending colon of injected WAG rats. After serial implantation to male rats, the resulting transplantable tumor was described to be moderately differentiated on histological examination (Marquet, et al., 1984).

The majority of animal liver metastasis models available at that time was based on the subcutaneous or intraperitoneal injection of tumor cells (Venditti, et al., 1984). To imitate as closely as possible the physiological metastatic spread of colon cancer, different orthotopic models were developed. These included intraportal (Griffini, et al., 1997, Griffini, et al., 1996, Thomas, et al., 1993) and spleen injections (Fukumura, et al., 1997) resulting in a diffuse outgrowth of tumor cells in the liver, as well as implantation of tumor tissue fragments or cells under the Glisson's liver capsule giving a local, limited, and nodal growth pattern (Aguiar, et al., 1987, Bartkowski, et al., 1986, Kamphorst, et al., 1999). Quantification of tumor growth in the latter models is often done by measuring tumor diameters (Aguiar, et al., 1987, Bartkowski, et al., 1986, Kamphorst, et al., 1999), whereas the diffuse models couldn't be quantified easily. To encounter this obstacle, many efforts have been made, including the 3-D reconstruction of metastases by consecutive serial sections (Griffini, et al., 1997), counting tumor nodules in the liver (Thomas, et al., 1993), or the use of tumor specific

antibodies for immunohistological examination (Thomas, et al., 1993). These assays are protracted and allow only a gross grading of tumor mass or cytostatic-induced loss of tumor load in the liver. The use of reporter genes such as green fluorescent protein (GFP), luciferase, or β -galactosidase (GLB1) for tracing tumor cells has greatly facilitated both, quantification and localization at the single cell level (Chishima, et al., 1997, Dooley, et al., 1993, Zhang, et al., 1994). In view of that, we aimed in our study to develop an orthotopic, diffusely growing liver metastasis model that can be used for diagnostic and therapeutic studies. For this purpose the CC531 rat colorectal cancer cell line with its natural homing into the liver was transfected by the *Glb1* gene.

The stable transfection of these cells with the *Glb1* gene (Fig. 1) allowed quantitation of the tumor cell load at any time after implantation and hence a quick evaluation of the efficacy of therapy that can be used for diagnostic and therapeutic studies (Wittmer, et al., 1999).

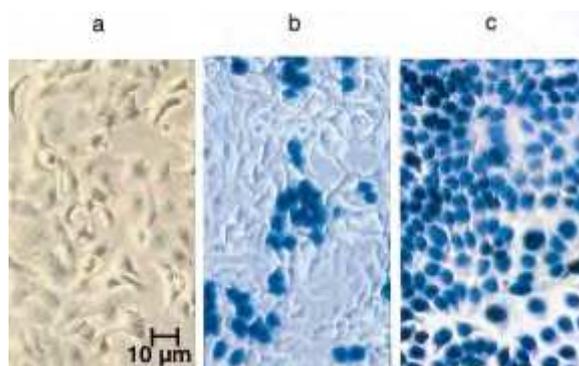


Fig. 1. CC531 cells growing in RPMI-1640 medium were stained by the activity of GLB1 converting X-gal, a chromogenic substrate for GLB1. Parental cells (a) are compared with transfected (b) and subcloned (c) CC531 cells. The magnification is identical for all three photographs (see scale bar)

2.2 Chemosensitivity of the model

2.2.1 Chemoembolization of rat liver metastasis with microspheres and gemcitabine or irinotecan followed by evaluation of tumor cell load by chemiluminescence

These experiments (Seelig, et al., 2004) were performed to determine the potential of hepatic artery chemoembolization (HACE) for reducing the tumor cell load. Seven days after the intraportal injection of CC531-lac-Z cells to male WAG/Rij rats, tumor positive animals were treated by intra-hepatic artery injection with solvent (n=17), degradable starch microspheres (DSM, 30 mg/kg; n=16), DSM plus 5-fluorouracil (5-FU; 90, 60, and 40 mg/kg; n_{total}=43) or DSM plus gemcitabine (Gem; 100, 80, 50, and 10 mg/kg; n_{total}=46). After 3 more weeks the experiment was terminated, the livers were weighed and the number of CC531-lac-Z cells per liver was determined. Injection of DSM reduced the tumor cell load by 21% (T/C%=79), whereas the combination with 5-FU reduced tumor cell number more intensively at 60 mg/kg (T/C%=86), and 90 mg/kg (T/C%=19). None of these effects was significantly different from controls. The combination of DSM plus Gem was well tolerated and significantly (p<0.05) effective at 80, 50 and 10 mg/kg (T/C%= 16, 9 and 26, respectively; Fig. 2).

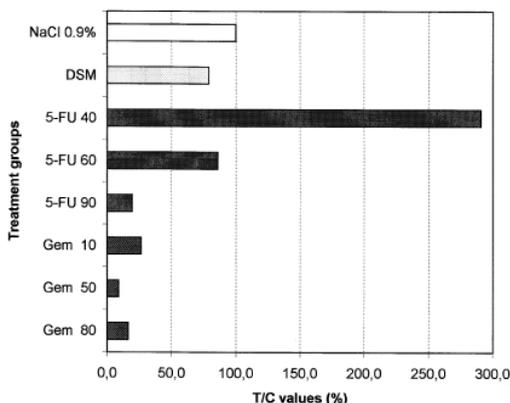


Fig. 2. Comparison of therapeutic efficacy: The mean tumor cell number of treated groups is given in percent of the respective control group (T/C* 100)

Thus, the comparison of HACE with 5-FU or Gem showed that the efficacy of Gem in reducing the hepatic tumor cell load was significantly higher and its therapeutic ratio was greater than that of 5-FU.

In a subsequent experiment, the effect of HACE with irinotecan was compared vs. 5-FU as a standard agent in rat liver metastasis (Saenger, et al., 2004). Briefly, 4×10^6 CC531-lac-Z cells were intraportally injected into male Wag/Rij rats. Irinotecan (10, 30 and 60 mg/kg) and 5-FU (40, 60 and 90 mg/kg) were administered concomitantly with DSM (30 mg/kg) for temporary embolization. The tumor cell load was determined quantitatively using the chemoluminescence assay mentioned above.

HACE with irinotecan induced a complete remission in 44% of the animals and the highest dose reduced the mean tumor cell load by 66% ($P < 0.001$). In contrast, the highest dose of 5-FU caused a reduction of only 18% ($P = 0.026$) and altogether 23% complete remissions were observed in response to 5-FU (Table 1 and Fig. 3). Collectively, HACE with irinotecan had a greater effect than that of HACE with 5-FU, setting the basis for further investigation in clinical trials.

Group no.	Treatment	Mean tumor cell number per liver ^a ± SE ^b	O/E ratio ^c	Mean liver weight (g) ^d ± SE ^b	Increase in tumor cell number ^e	aT _{1/2} ^f	TCD ^g
1A	Untreated	4.1E+09 ± 4.6E+08	—	34.2 ± 2.7	1025	50.4	10.0
1B	Untreated	2.1E+09 ± 3.3E+08	—	28.4 ± 2.7	525	55.8	9.0
2	GEM 50 HACE	5.5E+08 ± 2.3E+08	—	11.1 ± 1.0	136	71.1	7.1
3	GEM 50 IV	1.2E+09 ± 3.3E08	—	15.9 ± 2.0	306	61.0	8.3
4	MTA 30 IV	2.8E+09 ± 7.5E+08	—	22.9 ± 3.6	706	53.3	9.5
5	MTA 60 IV	2.5E+09 ± 4.3E+08	—	17.1 ± 2.8	614	54.4	9.3
6	MTA 90 IV	2.8E+09 ± 3.7E+08	—	21.1 ± 2.1	690	53.4	9.4
7	MTA 30 (IV) + GEM 50 HACE	8.3E+08 ± 1.3E+08	2.19	12.7 ± 0.8	206	65.6	7.7
8	MTA 60 (IV) + GEM 50 (HACE)	3.3E+08 ± 1.6E+08	1.02	8.3 ± 0.6	83	79.0	6.4
9	MTA 90 (IV) + GEM 50 (HACE)	4.2E+08 ± 1.7E+08	1.14	9.6 ± 1.0	105	75.1	6.7
10	MTA 30 (PVCE)	3.3E+09 ± 6.6E+08	—	25.9 ± 3.3	819	52.1	9.7
11	MTA 60 (PVCE)	3.6E+09 ± 6.6E+08	—	29.1 ± 4.0	907	51.3	9.8
12	MTA 90 (PVCE)	3.7E+09 ± 5.7E+08	—	30.8 ± 3.4	918	51.2	9.8
13	MTA 30 (PVCE) + GEM 50 (HACE)	1.4E+09 ± 2.5E+08	2.38	15.7 ± 2.0	260	62.8	8.0
14	MTA 60 (PVCE) + GEM 50 (HACE)	3.1E+08 ± 2.0E+08	0.64	8.4 ± 1.0	77	80.4	6.3
15	MTA 90 (PVCE) + GEM 50 (HACE)	1.2E+09 ± 3.6E+08	2.45	14.6 ± 2.1	300	61.3	8.2

^aDetermined by β-galactosidase assay

^bStandard error of the mean

^cO/E = ratio of observed versus expected treatment effect

^dWet liver weight

^eRatio of final and initial tumor cell numbers

^fApparent tumor cell doubling time (h)

^gNumber of tumor cell doublings

Table 1. Results of hepatic arterial chemoembolization on tumor cell reduction

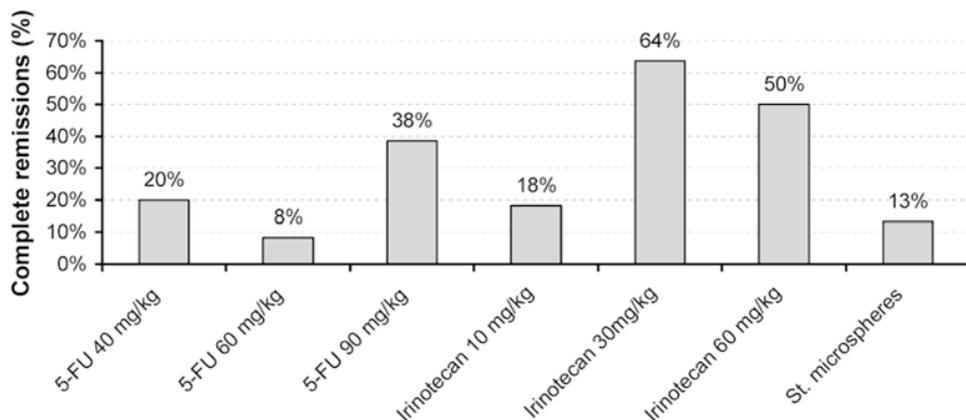


Fig. 3. The columns give the percentage of complete remissions (defined as chemoluminescence signal below that of a healthy control liver) in relation to the respective treatment group

2.2.2 Combination treatment of CC531-lac-Z rat liver metastases by chemoembolization with pemetrexed disodium and gemcitabine

The aim here was to evaluate the combination effect of pemetrexed disodium (MTA; Alimta; LY 231514) and gemcitabine (GEM) administered by hepatic artery and portal vein chemoembolization (HACE and PVCE) in our rat liver metastasis model (Rodenbach, et al., 2005). After implantation of CC531 cells, MTA (30, 60 and 90 mg/kg) was administered locoregionally by PVCE and compared with repeated systemic intravenous injection. GEM (50 mg/kg) was also given locoregionally by HACE as well as systemically. All routes of administration were examined alone as well as in combination. Efficacy of treatment in terms of liver metastases burden was determined with the chemoluminescence assay. Locoregional administration by HACE with GEM was significantly more effective than systemic intravenous bolus treatment ($P=0.03$). Repeated systemic treatment with MTA yielded a slight reduction in tumor cell load that was significant *vs.* control at the medium and high doses (60 mg/kg, $P=0.009$; 90 mg/kg, $P=0.046$) but not *vs.* PVCE. The combination treatment of systemic (60 and 90 mg/kg) or locoregional (60 mg/kg) MTA with HACE using GEM (50 mg/kg) resulted in >80% tumor growth inhibition; this antineoplastic combination effect was maximally additive (Fig. 4). HACE with GEM was superior to systemic intravenous bolus treatment, while PVCE with MTA was ineffective. The optimal *in vivo* regimen of MTA (intravenous or PVCE) preceding GEM (HACE) resulted in a maximally additive tumor growth inhibition indicating that MTA and GEM can successfully be combined and favor further evaluation in patients.

2.2.3 Chemoembolisation of rat colorectal liver metastases with drug eluting beads loaded with irinotecan or doxorubicin

Chemoembolisation with drug eluting beads (DEBs) designed to deliver drug at the target over a prolonged period was tested as a new strategy to reduce the tumor burden of liver metastases (Eyol, et al., 2008). Accordingly, DEBs possessing anionic groups capable of ionically complexing with cationic drugs were synthesized by a suspension polymerization

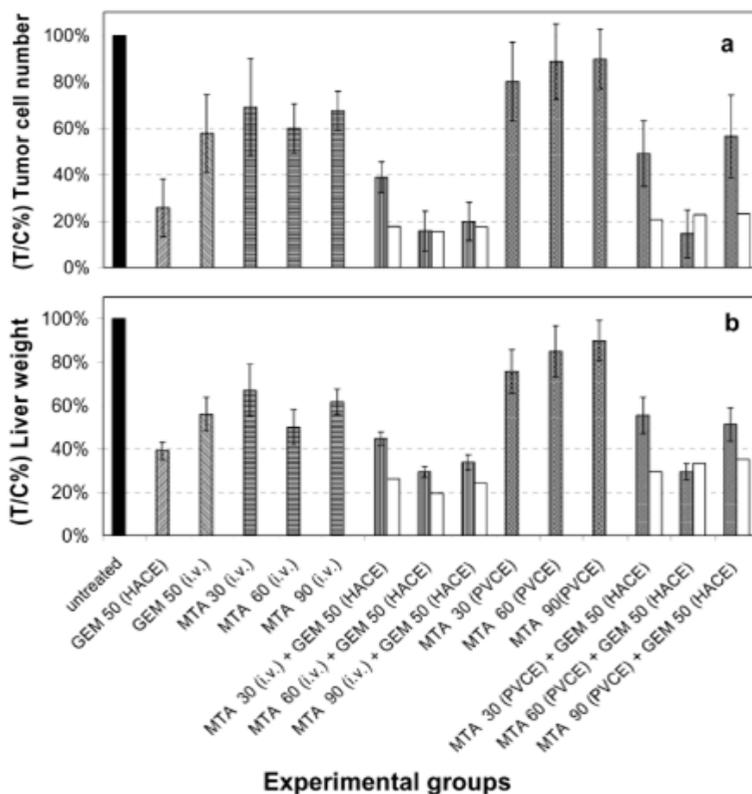


Fig. 4. Summary of treatment effects; columns denote the respective therapeutic efficacy as quotient of treated and control values (T/C%) for (a) tumor cell number determined by β -galactosidase assay and (b) wet liver weight. White columns indicate the expected combination effect. Bars symbolize standard error of the mean

method and were fractionated to produce an average size of 75 μm . The DEBs were loaded with the desired concentration of either doxorubicin hydrochloride or irinotecan hydrochloride prior to administration by immersion in the drug solution, yielding basically 100% loading efficiency (Fig. 5).

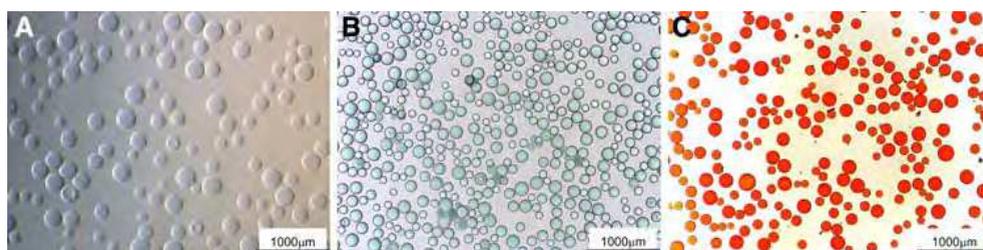


Fig. 5. Aspect of loaded and unloaded beads (a) Unloaded Beads; (b) Irinotecan DEB; (c) Doxorubicin DEB

After injection of CC531 cells as previously mentioned, DEBs loaded with irinotecan or doxorubicin were administered by single injection into the hepatic artery. The resulting reduction in liver tumor burden and the corresponding reduction in liver weight indicated significant anticancer activity (Fig. 6).

Comparing the two agents, irinotecan appeared more advantageous because of its significant activity and excellent tolerability following administration at 2 dosages of either 20 or 30 mg/kg. Doxorubicin showed a narrower activity window, being effective at 4 mg/kg but ineffective at the lower dose of 2 mg/kg. Therefore, HACE with DEBs with either agent may have potential for treating patients with colorectal liver metastasis.

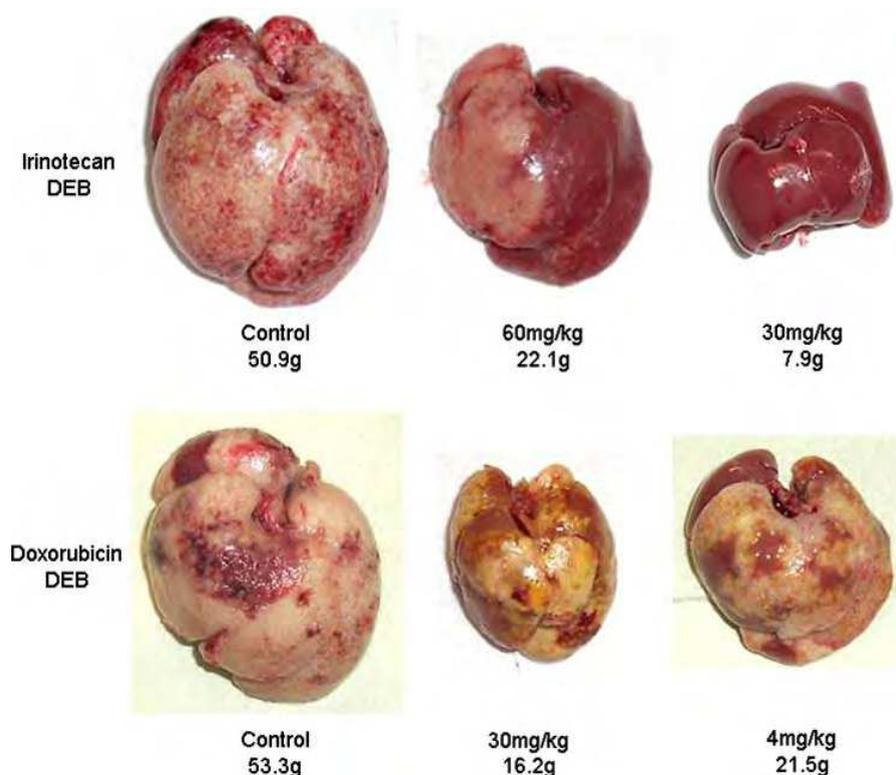


Fig. 6. Gross pathological aspects of implanted control and treated livers using irinotecan DEB (top line) and doxorubicin DEB (bottom line)

3. Search for genes that are involved in colorectal cancer liver metastasis

To identify genes that are involved in the metastatic phenotype of CC531 cells, cDNA microarrays were used to analyze mRNA expression profiles of these cells for changes related to their homing into the liver. Briefly, CC531 cells were intraportally implanted into the liver of Wag-Rij rats and re-isolated after 3, 6, 9, 14 and 21 days (Fig. 7 (A-E)). For the re-isolation purposes, the CC531 cells had been marked with stains for viable cells *i.e.* eGFP and RFP markers.

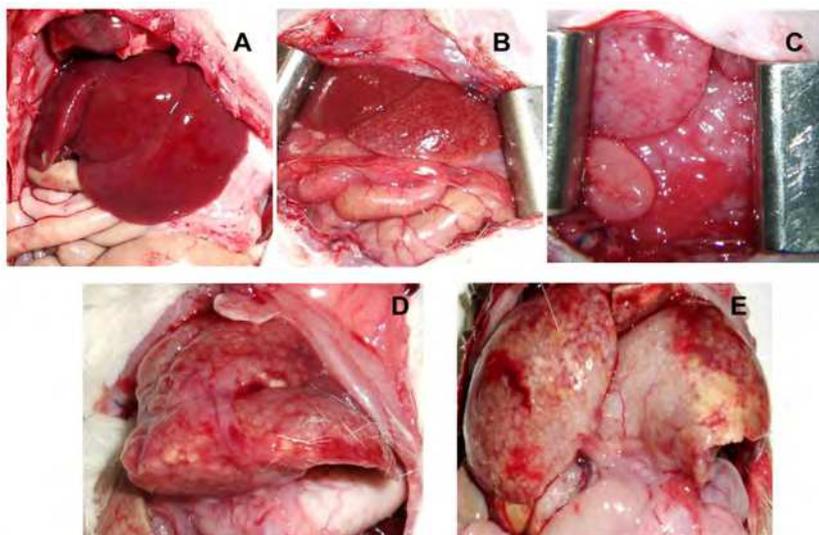


Fig. 7. (A-E). Photographs of rat liver taken at 3, 6, 9, 14 and 21 days after inoculation of CC531 cells, before re-isolation of the metastatic tumor cells

3.1 The re-isolation technique of tumor cells, hepatocytes and Kupffer cells

As mentioned before, the rats were kept for 3, 6, 9, 13 and 21 days after tumor cell implantation (Georges, et al., 2011). Then, the abdominal cavity was opened and a 22 G cannula was inserted into the portal vein, through which the liver was perfused with HBSS medium (20 ml/min, 37° C for 10 min). This medium was replaced with pre-warmed perfusion medium [125 ml HBSS containing CaCl₂ 1M, 0.1% pronase, 100 mg collagenase Type IV (Serva, Heidelberg, Germany), 37 ° C, for the following 10 min] to digest connective tissues. After getting the cells in suspension, they were filtered through a sterile filter (Cell strainer, 70 μm Nylon, BD, Germany) and centrifuged. The resulting cell suspension of liver and tumor cells was transferred into 50ml-tubes and layered carefully onto a Ficoll gradient medium (Amersham pharmacia Biotech AB, Uppsala, Sweden).

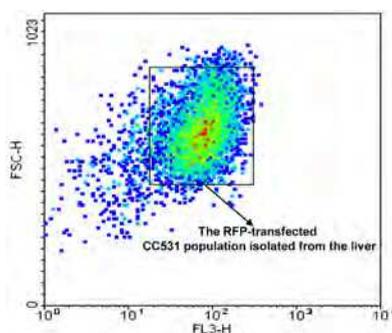


Fig. 8. The density plot illustrates the CC531 population which was obtained by FACS sorting. The marker protein RFP was used for isolating CC531 cells without contaminating liver cells

After centrifugation, the tumor cells were obtained from the top of the interface and resuspended in RPMI medium. To obtain a high purity of isolated tumor cells, CC531 cells were subsequently isolated by fluorescence-activated cell sorting technology using red fluorescent protein (RFP) as marker (Fig. 8).

Afterwards, the pure cells were pelleted and snap frozen at -80°C . An aliquot of the cells, which were isolated on day 21, was used for re-culturing CC531 cells *in vitro*. These cells were propagated every 3 days, but two time points (14 and 22 days after tumor cell explantation) were chosen for subsequent microarray analysis, PCR, and Western blot.

For the isolation of rat hepatocytes (HCs) and Kupffer cells (KCs), the same perfusion method was performed as described above. However, to separate parenchymal (PCs) from non-parenchymal cells (NPCs), cell suspensions were gently pelleted and the resulting pellet, containing mainly hepatocytes, was taken up in Maintenance-Medium without FCS. Trypan blue exclusion (1 part trypan blue: 2 parts cell suspension) was used for cell counting and assessing their viability. 4×10^7 hepatocytes with 95% viability were usually obtained from one rat liver. Afterwards, the 25%/50% two-step Percoll gradient was used to isolate Kupffer cells as pure as possible.

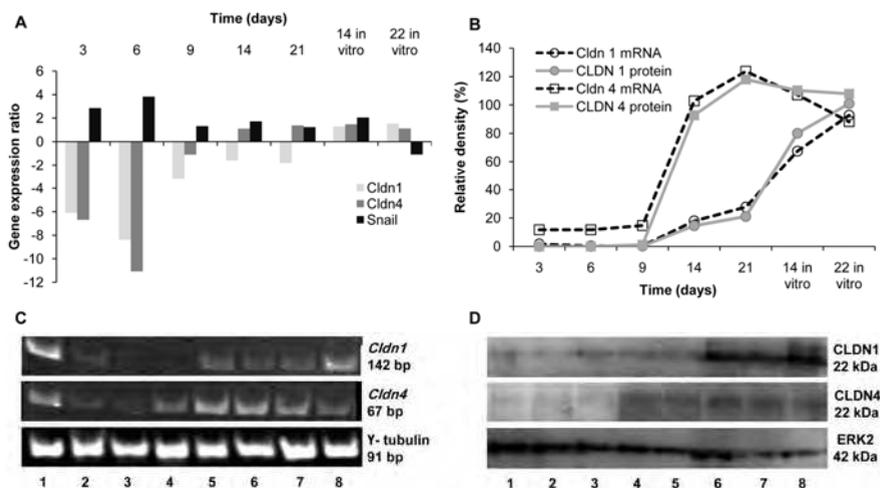


Fig. 9. Down-regulation of *Cldn1* and *Cldn4* in CC531 cells homing into the liver.

(A) Expression profile of claudins (1, 4) and Snail in CC531 cells as shown by microarray analysis. The values represent the gene expression in isolated metastasizing cells in comparison to the expression in cells growing *in vitro*. (B) The diagram represents the mRNA or protein expression levels in re-isolated CC531 cells in % of the expression detected in control cells (100%). Values were calculated using the pixel density of each PCR/or Western blot band normalized to the corresponding value of γ -tubulin (*Tubg1*) or ERK2, respectively. (C) Expression of claudins (1, 4) in CC531 cells as shown by RT-PCR. Lane 1: control CC531 cells, lanes 2-6: CC531 cells isolated from the liver after 3, 6, 9, 14 and 21 days, respectively, lanes 7, 8: CC531 cells re-isolated after 21 days and cultured *in vitro* for 14 and 22 days, respectively. (D) Expression of CLDNs (1, 4) in CC531 cells as shown by Western blot. Lanes 1-5: CC531 cells isolated from the liver after 3, 6, 9, 14 and 21 days, respectively, lanes 6, 7: CC531 cells isolated after 21 days and cultured *in vitro* for 14 and 22 days, respectively, lane 8: control CC531 cells

The cDNA microarray results showed that compared to control CC531 cells, claudin1 and claudin4 were among the ≥ 8 -fold initially down-regulated genes (Fig. 9A).

Interestingly, both genes were at first down-regulated with a nadir (8 or 11 fold down-regulation) on day 6, followed by gradual up-regulation within the observation period. These results were confirmed with RT-PCR (maximum down-regulation of 80% on day 6 for both genes; Fig. 9B) and Western blot (specific bands below detection limit, >90% inhibition on day 6; Fig. 9C). It is noteworthy that the transcription repressor gene *Snail* showed an inverse modulation: an increased expression during the first week (up to 3.8 fold) with the peak of its expression corresponding to the nadir of *Cldn1* and *Cldn4* down-regulation (Fig. 9A).

Next, two experiments were performed to explain the initial down-regulation of *Cldn1* and *Cldn4*; these included co-culture of CC531 cells with isolated rat hepatocytes or Kupffer cells and the physical forces' effect experiment:

3.2 Co-culture/two compartment model

Briefly, this model is based on a two-compartment system in which hepatocytes or Kupffer cells, plated in the lower compartment, are co-cultured with CC531 tumor cells growing in the upper compartment, with the two cell types being separated by a porous membrane (0.4 μm pore size). This system, preventing a direct contact between the two compartments, allows the cells to be only indirectly influenced by molecules secreted from the cells in the other layer, respectively.

No down-regulation effect on claudin expression was noticed, whereas both genes were up-regulated after co-culture with hepatocytes (Fig. 10).

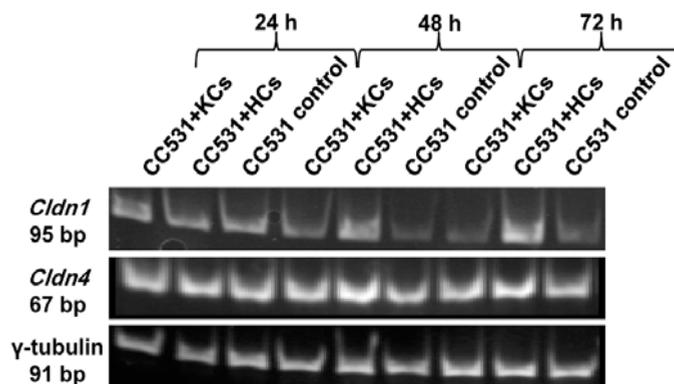


Fig. 10. Expression of claudins (1, 4) in CC531 cells co-cultured for 24 to 72 h with Kupffer cells (KCs) and hepatocytes (HCs) in comparison to the housekeeping gene γ -tubulin as shown by RT-PCR

3.3 Physical forces' effect experiment

2×10^6 CC531 cells were seeded in 25 cm^2 flat-bottom flasks or into round 50 ml glass bottles (Steiner GmbH, Siegen Eiserfeld, Germany), which were rotated on a roller (Stovall Life Science Incorporated, Greensboro, NC USA) at a speed of 1rpm, preventing the cells from adhesion to each other and onto the flask bottom. After 24 h, the cells in flat flasks and half

of the cells in round bottles were harvested for PCR analysis; the other half of cells was seeded in flat flasks till the next day to investigate the influence of adhesion status on claudin expression and then harvested after determining their viability under the microscope. This procedure was done daily for 3 days after seeding the cells (Fig. 11).

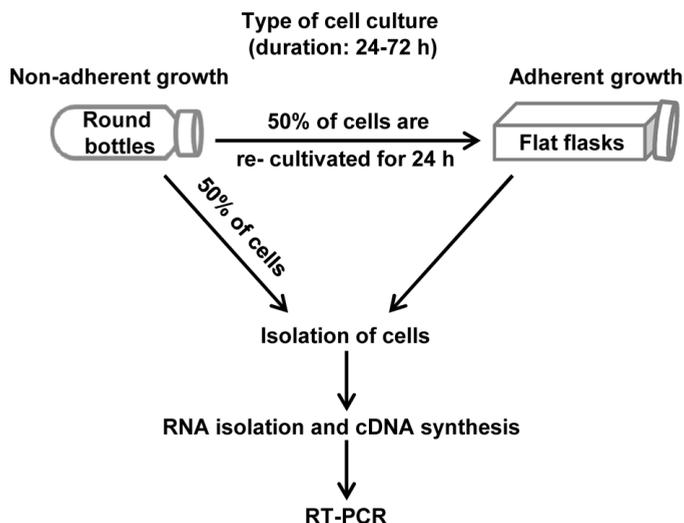


Fig. 11. Scheme indicating the experimental procedure for assessing the physical force's effects

As shown in Fig. 12, no change in *Cldn1* expression was noticed either in CC531 cells growing continuously in flat flasks or in round bottles. On the contrary, *Cldn4* mRNA expression was 2- and 1.8- fold higher in CC531 cells growing in flat flasks than their counterparts growing in round bottles at 48 and 72 h after seeding the cells, respectively.

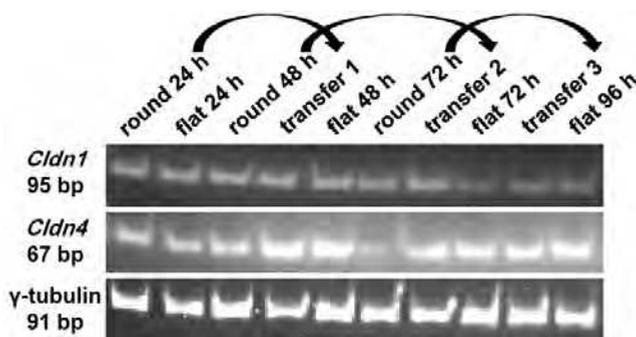


Fig. 12. Expression of claudins (1, 4) in CC531 cells harvested from round and flat flasks as shown by RT-PCR. Lanes 1, 3, 6: CC531 cells harvested from round bottles after 24, 48 and 72 h, respectively. Lanes 2, 5, 8, 10: CC531 cells harvested from flat flasks after 24, 48, 72 and 96 h, respectively. Lanes 4, 7, 9: CC531 cells harvested from flat flasks after being transferred from round bottles after 24, 48 and 72 h, respectively

Furthermore, transferring tumor cells from a non-adhesive state in round bottles to growing in flat bottom flasks for 24 h caused >2.5-fold increased expression of *Cldn4*, whereas no effect on *Cldn1* expression was noticed. Accordingly, the physical conditions and the adhesion status of the cells affected differently the expression of *Cldn1* and *Cldn4*, suggesting a direct relationship with the latter, but not with the former gene.

3.4 Small interfering RNA (siRNA) knockdown experiments

CC531 cells cultured in 6-well-plates were transfected with 100 nM siRNA or negative control using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. The cells were harvested at 24, 48 and 72 h after treatment. As shown by RT-PCR in Fig. 13A, exposure to siRNA species directed against *Cldn1* and *Cldn4* caused reduced expression of mRNA to 24% and 15%, respectively.

To further investigate a possible interdependence of these two genes, the expression of *Cldn4* and *Cldn1* was investigated in CC531^{si.Cldn1} cells and CC531^{si.Cldn4} cells, respectively. Expression of *Cldn4* was down-regulated by 50% in tumor cells transfected with siRNA against *Cldn1* (Fig. 13B), whereas inhibition of *Cldn4* did not exert the same effect on *Cldn1* expression (data not shown).

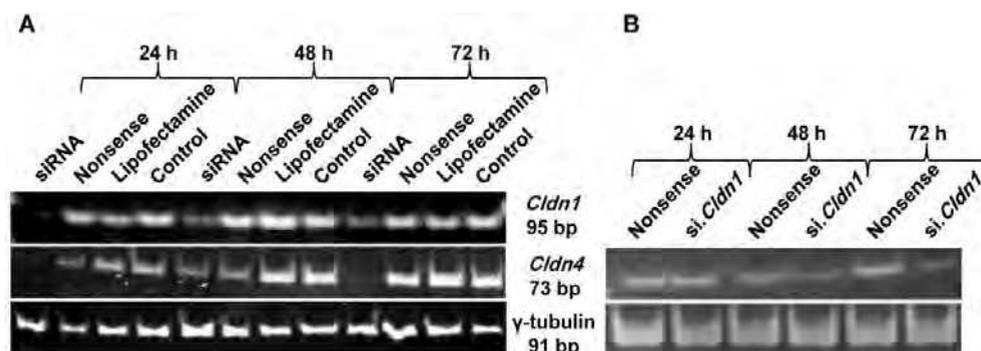


Fig. 13. (A) Down-regulation of claudins (1, 4) in CC531 cells after siRNA transfection as shown by RT-PCR. (B) Down-regulation of *Cldn4* after 24-72 h in CC531^{si.Cldn1} cells (compared to CC531^{nonsense} cells) as shown by RT-PCR

The effect of *Cldn1* and *Cldn4* knockdown on cell growth (MTT assay), cell migration and colony formation (Adwan, et al., 2004, Georges, et al., 2011) was investigated as well. These *in vitro* experiments showed significantly increased migration and decreased clonogenic growth of tumor cells ($p < 0.05$), but no effect on cell proliferation was noticed (Fig. 14).

3.5 Expression of CLDN1 and CLDN4 in neoplastic human CRC tissues

For the immunohistochemical (IHC) analyses of CLDN1 and CLDN4, 32 primary CRC tissue specimens with adjacent non-neoplastic tissue and 8 liver metastases were obtained from the Institute of Pathology, University of Heidelberg.

The patients had a median age of 65 years and were classified into UICC stages II (n=24) and IV (n=8) and graded as G2 (n=25) and G3 (n=7). The histopathological analysis revealed that the expression of CLDN1 was high in 91% (n=30) and that of CLDN4 in 85% (n=28) of all tumor specimens. Comparing the CLDN expression related to UICC stages, CLDN1 and

CLDN4 had significantly lower expression in stage IV than in stage II ($p=0.01$ and $p=0.05$, respectively). In line with this, liver metastases showed lower expression of CLDN1 and CLDN4 than in the corresponding primary carcinomas (Fig. 15). This difference was significant for CLDN1 ($p<0.05$) but not for CLDN4.

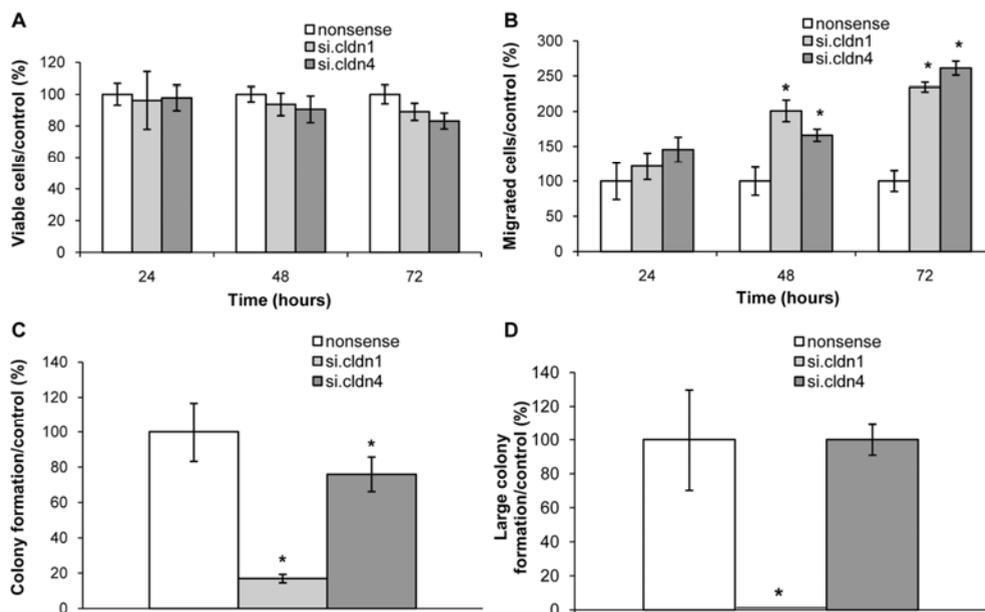


Fig. 14. Knockdown effects of *Cldn1* and *Cldn4* on cellular functions of CC531 cells .
(A) Proliferation of CC531 cells in response to si.*Cldn1* or si.*Cldn4*. **(B)** Increased migration of CC531 cells in response to si.*Cldn1* or si.*Cldn4*. **(C)** Inhibition of colony formation of CC531 cells in response to siRNA down-regulation of claudins (1, 4). **(D)** Inhibition of large colony formation of CC531 cells in response to siRNA down-regulation of *Cldn1* or *Cldn4*. Data ($n=3$) are shown as means \pm S.D. in % of nonsense-transfected cells, an asterisk denotes a significant difference to control cells ($p<0.05$)

3.6 Correlation of CLDN4 or CLDN1 expression with prognosis in CRC patients

For real-time PCR analysis, 67 sporadic CRC patients, who were admitted and underwent surgery in the time between (01/98 - 07/01) at the Municipal Hospital in Nürnberg (Department of Abdominal-, Thorax-, and Endocrine Surgery) were selected.

The samples included in this study (for IHC and real-time PCR) were used based on the patients informed consent and approved by the Ethics Committee of the Universities of Heidelberg and Erlangen.

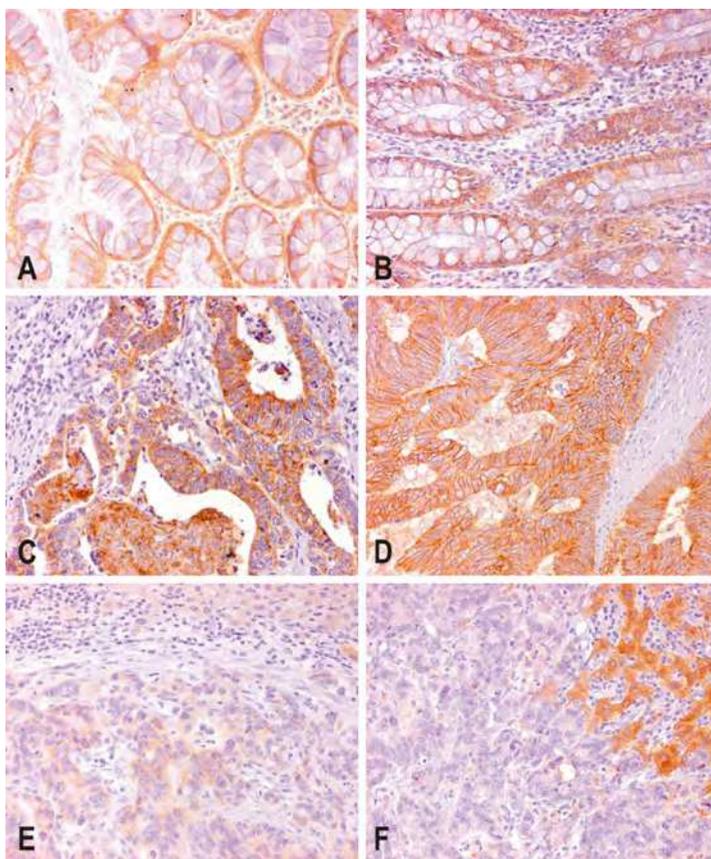


Fig. 15. Expression of CLDN1 and CLDN4 proteins in human CRC and liver metastasis tissues compared to normal mucosa as shown by immunohistochemistry. (A), (C) and (E) Expression of CLDN1 in normal mucosa, cancerous tissue and liver metastasis, respectively. (B), (D) and (F) expression of CLDN4 in normal mucosa, cancerous tissue and liver metastasis, respectively. Magnification x64

The 67 CRC patients (42 men and 25 women) had an average age of 67 years and were classified into 4 UICC stages (I, n=11; II, n=25; III, n=20; IV, n=11). The expression levels of *CLDN1* and *CLDN4* were significantly correlated ($P < 0.05$; Fig. 16A). No correlation between *CLDN1* expression and age ($p = 0.19$), tumor stage ($p = 0.88$), or overall survival ($p = 0.2$) was seen. With respect to *CLDN4*, also no correlation with age ($p = 0.69$) or tumor stage ($p = 0.38$) was noticed. However, the overall survival of CRC patients with high or low risk in relation to the median split of *CLDN4* expression levels differed significantly according to the log-rank test ($p = 0.018$; Fig. 16B). Similarly, using a Cox model, there was not significant difference ($p = 0.07$) between the low and high risk groups taking all stages together, whereas in patients with tumor stages I-III, an elevated *CLDN4* level was clearly associated with a less favorable prognosis ($p = 0.05$; Fig. 16C).

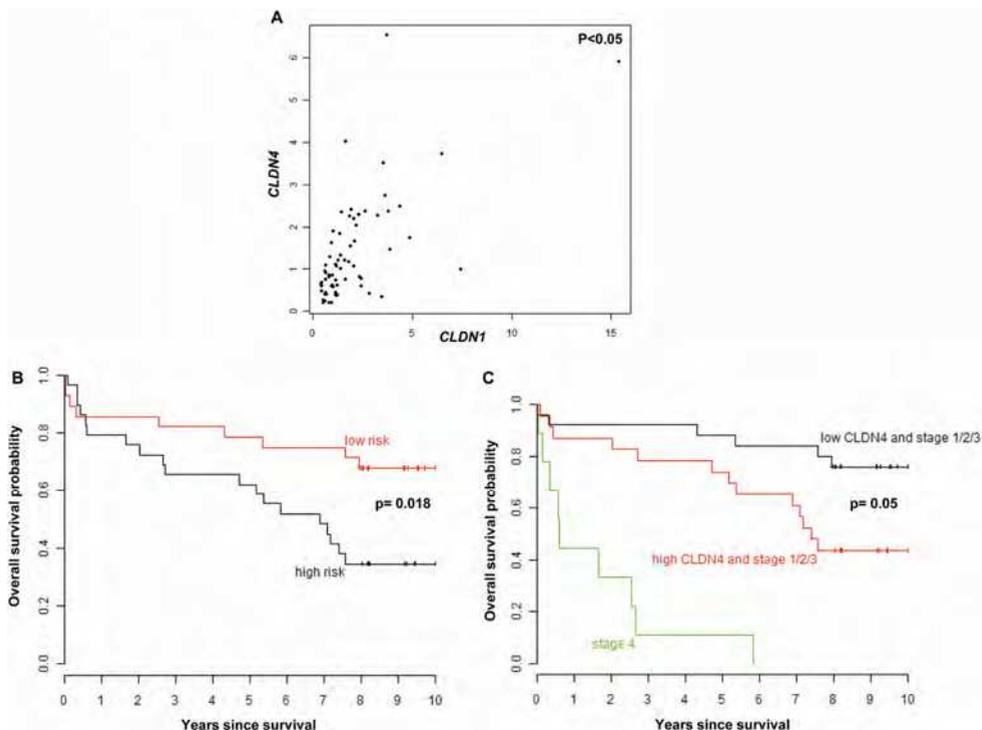


Fig. 16. Correlation of *CLDN1* or *CLDN4* expression levels with prognosis in 67 CRC patients and with each other. **(A)** Scatterplot of the correlation between *CLDN1* and *CLDN4* expression levels assessed with the non-parametric correlation coefficient from Spearman **(B)** The Kaplan-Meier plot represents the overall survival probability of CRC patients with high or low risk according to *CLDN4* median split for overall survival. The log-rank test shows a significant difference ($p=0.018$) between the two groups. **(C)** The Kaplan-Meier plot demonstrates the overall survival probability of CRC patients with high or low *CLDN4* expression, dichotomized into high and low risk groups by the *CLDN4* median split for overall survival and after separating the stages into I-III and IV stages. The Cox model shows a significant association between *CLDN4* elevated levels and less overall survival ($p=0.05$)

3.7 OPN expression profile in CC531 cells ex-vivo

After explanting the liver, a piece of liver containing tumor cells was resected and used for re-culturing CC531 cells (Georges, et al., 2010). These cells were cultured for three weeks and within this period passaged five times corresponding to 11, 13, 15, 18 and 21 days; respectively (passages 1-5). At these intervals the cells were investigated for their mRNA- and protein expression levels of OPN.

At the mRNA level, *Opn* was up-regulated in CC531 cells explanted from the tumor. Over time, this *Opn* mRNA level gradually decreased till it disappeared after the third passage; i.e. after two weeks (corresponding to a reduction by 88%; Fig. 17, left).

The OPN protein level, as shown by Western blot (Fig. 17, right), was first highly expressed but then down-regulated to minimally 5% within the next 15 days. A slight increase to 20% of the initial level was seen at the final passages 4 and 5.

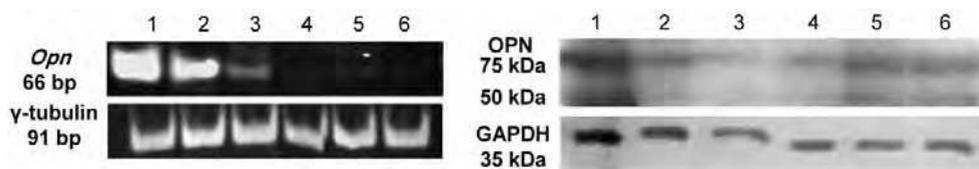


Fig. 17. Expression of OPN on mRNA (left; RT-PCR) and protein (right; Western blot) levels. Lane 1: CC531 cells explanted from the tumor, lanes (2-6): CC531 cells after 11, 13, 15, 18, and 21 days; respectively

4. Conclusion

In conclusion, the model described in this chapter evolved in two main steps. First, for quantitation of tumor cell load, the CC531 cells had been marked with GLB1. This marker proved useful for the purposes of therapeutic studies with various anti-cancer agents. Using the GLB1-based chemoluminescence assay, it could be shown that the efficacy of hepatic artery chemoembolization with gemcitabine-microspheres was significantly higher than with 5-FU in reducing the tumor cell load.

Also it was shown, that HACE with irinotecan-microspheres had a greater effect than that of HACE with 5-FU, setting the basis for further investigations in clinical trials.

The same assay allowed us to evaluate the combination effect of pemetrexed disodium and gemcitabine administered by hepatic artery and portal vein chemoembolization in our rat liver metastasis model. These experiments showed that HACE/gemcitabine was superior to systemic intravenous bolus treatment, while PVCE/pemetrexed disodium was ineffective. Interestingly, a maximum additive tumor growth inhibition *in vivo* was noticed using a regimen of (intravenous or PVCE)/pemetrexed disodium preceding HACE/gemcitabine indicating that these two agents can successfully be combined and favor further evaluation in patients.

The third set of therapeutic experiments, using HACE with drug eluting beads (DEBs) loaded with either doxorubicin hydrochloride or irinotecan hydrochloride showed that irinotecan is more advantageous because of its significant activity and excellent tolerability. In addition, HACE with DEBs with either agent may have potential for treating patients with colorectal liver metastasis.

The CC531 tumor cells marked with GLB1 allowed a determination of the tumor cell load only after termination of the experiment. To further develop the model for determining the tumor cell load in living animals, other markers had to be introduced. These were eGFP and RFP, which enabled the next series of experiments that were related to liver metastasis genes.

Realizing the fact that the most common cause of CRC-related death is the development of metastasis, especially into the liver, denotes the importance of identifying the metastasis-related changes in tumor cells, which probably differ from those related to the primary tumor. Therefore, temporal changes in gene expression of CRC cells homing into the liver have been investigated using our *in vivo* model, which had been improved by eGFP and RFP markers. The intraportal inoculation of CC531 cells defines the onset of metastatic proliferation in rat liver. This, in turn, permits a close following of the time-dependent modulation of gene expression, as the tumor cells home into the liver and then grow to a lethal size. The technique of re-isolating these tumor cells from rat liver permits monitoring for the first time the expression of several candidate genes in a time-dependent manner. The

following cDNA microarray analysis showed that the initial phase of rat CRC cells homing into the liver involves a transient down-regulation of *Cldn1* and in particular of *Cldn4*. The transcription repressor Snail, which regulates both claudins, was concomitantly up-regulated during the early stages of metastasis before returning to normal expression levels. Silencing of *Cldn1* and *Cldn4* by siRNA increased migration and reduced colony formation, with these phenotypes consistent with metastatic homing. These experimental results were paralleled in human CRC tumor samples, which show increased *CLDN1* and *CLDN4* expression in UICC stages I–III, and significantly reduced expression in stage IV and in liver metastasis. The results obtained with human specimens give first evidence of a modulated claudin expression similar to those in the rat model. However, a prospective study is needed to corroborate these results, taking into account separately the entities, colonic and rectal cancers. That research could be driven by our hypothesis that primary CRC tumors have an initial growth advantage from increased claudin expression, whereas metastasizing cells require a transient reduction in claudin expression to be liberated from the primary tumor and to then initiate metastatic growth in the liver.

Based on these experiments we conclude that the CC531 colorectal cancer liver metastasis model in rats is attractive for translational research: it is suited for therapeutic studies as well as for identifying genes involved in colorectal cancer liver metastasis.

5. References

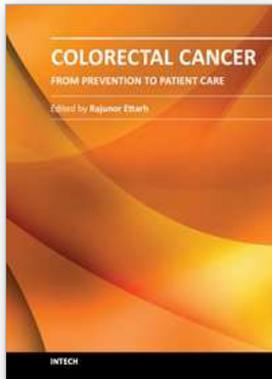
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Colorectal Cancer - From Prevention to Patient Care

Edited by Dr. Rajunor Ettarh

ISBN 978-953-51-0028-7

Hard cover, 538 pages

Publisher InTech

Published online 17, February, 2012

Published in print edition February, 2012

The projections for future growth in the number of new patients with colorectal cancer in most parts of the world remain unfavorable. When we consider the substantial morbidity and mortality that accompanies the disease, the acute need for improvements and better solutions in patient care becomes evident. This volume, organized in five sections, represents a synopsis of the significant efforts from scientists, clinicians and investigators towards finding improvements in different patient care aspects including nutrition, diagnostic approaches, treatment strategies with the addition of some novel therapeutic approaches, and prevention. For scientists involved in investigations that explore fundamental cellular events in colorectal cancer, this volume provides a framework for translational integration of cell biological and clinical information. Clinicians as well as other healthcare professionals involved in patient management for colorectal cancer will find this volume useful.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Rania B. Georges, Hassan Adwan and Martin R. Berger (2012). Experimental Colorectal Cancer Liver Metastasis, *Colorectal Cancer - From Prevention to Patient Care*, Dr. Rajunor Ettarh (Ed.), ISBN: 978-953-51-0028-7, InTech, Available from: <http://www.intechopen.com/books/colorectal-cancer-from-prevention-to-patient-care/experimental-colorectal-cancer-liver-metastasis>

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