Colorectal liver metastases: local therapy and molecular aspects

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Colorectal liver metastases: local therapy and molecular aspects

Colorectale levermetastasen: lokale therapie en moleculaire aspecten

(met een samenvatting in het Nederlands)

Proefschrift

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Co-promotoren:	Dr. R. van Hillegersberg Dr. O. Kranenburg

Aan mijn ouders

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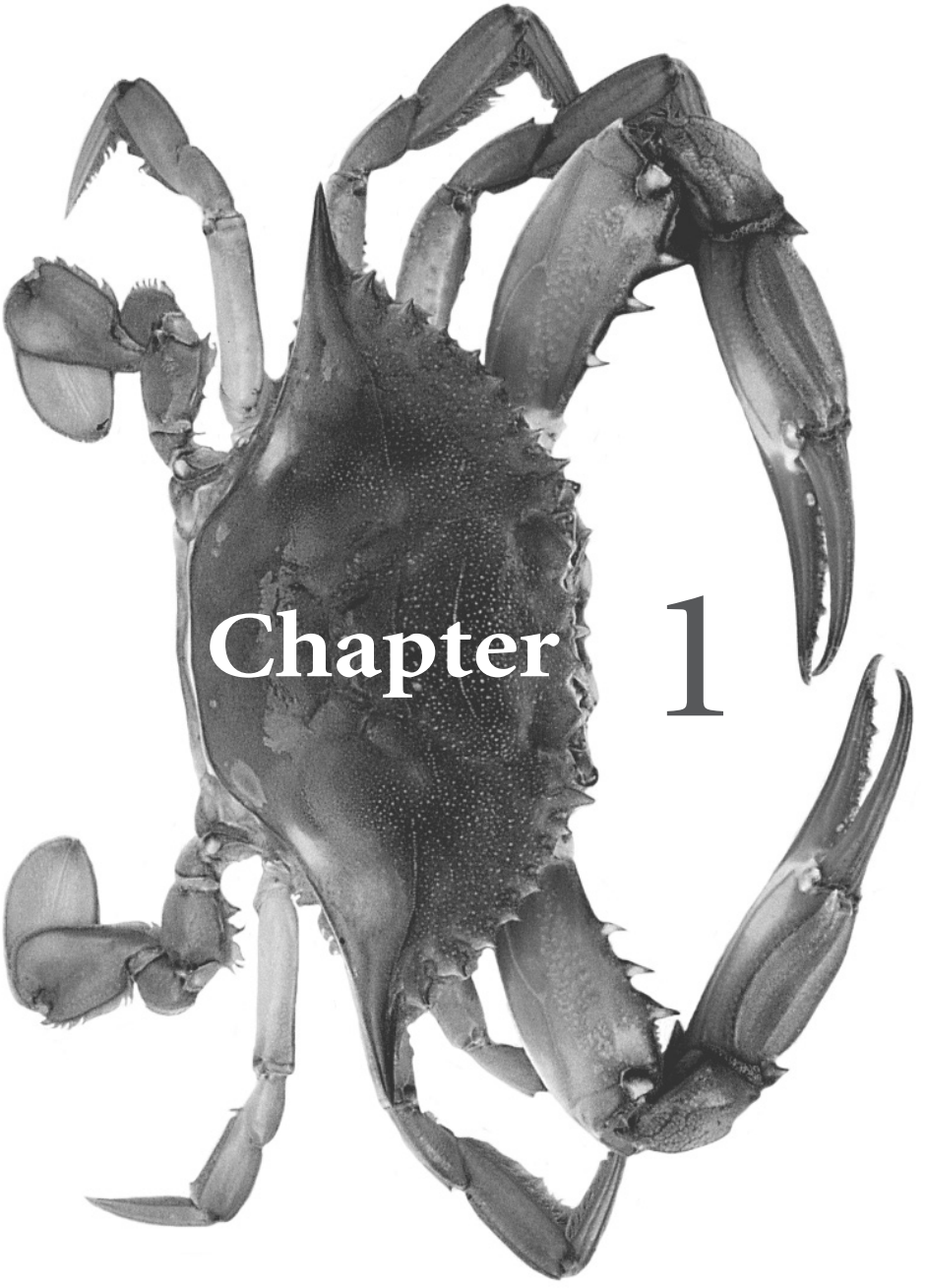

Chapter 1

General introduction and outline of the thesis

Introduction

Colorectal carcinoma (CRC) is the second most common cause of cancer related death in the Western World. In 2003, 9900 new patients with colorectal carcinoma were registered in the Netherlands (www.ikcnet.nl). In approximately 25% of patients liver metastases are detected at the time of presentation (synchronous metastases) and a further 25% of patients will develop metastases during the course of their disease (metachronous metastases).[1,2] Currently, surgical resection of liver metastases is the only form of treatment that offers a chance of long-term survival, with 5 and 10-year survival rates of 30-40% and 20-25% respectively.[3-5] However, due to extra-hepatic disease, poor general condition of the patients, number and location of metastases, or poor hepatic reserve, only 10-20% of liver metastases are respectable.[6,7] In the remaining patients the prognosis is poor and symptomatic treatment, chemotherapy or local ablation therapy are the only options available.

Chemotherapy

Systemic chemotherapy as a first-line treatment of metastatic colorectal cancer has greatly improved within the last decade. Response rates achieved with 5-fluorouracil (5FU) and leucovorin have been significantly increased to approximately 55% by combination with oxaliplatin or irinotecan, and changes in delivery regimes with a median survival of 22 months.[8-10] In addition to these novel cytotoxic agents new molecular target therapies have been developed. Both bevacizumab, a monoclonal antibody to vascular endothelial growth factor, and cetuximab, an anti-epidermal growth factor receptor antibody, have produced response rates approaching 70% when combined with cytotoxic agents.[11-13]

Local ablation therapy

Locally ablative techniques, such as cryoablation, laser-induced thermotherapy (LITT), and radiofrequency ablation (RFA), have recently been introduced as alternative treatment in patients not eligible for resection.
In cryoablation, a specially designed probe delivers liquid nitrogen to the tissue under ultrasound guidance.[14] During the rapid freezing process, ice crystals form and multiple freeze-thaw cycles destroy cellular structures.[15] However, cryoablation is associated with a relatively high rate of serious complications.[16,17]
Laser-induced thermotherapy, also named interstitial laser therapy (ILT) or interstitial laser coagulation (ILC), induces tissue damage and necrosis via heat photocoagulation by interstitially placed light guiding fibers.[18] The procedure is performed with a Nd:YAG laser, which has high tissue penetration and produces the greater volume of tissue destruction.[19] In literature this technique shows promising results.[20,21]
Radiofrequency ablation (RFA) induces temperature changes by using high-frequency alternating current applied via electrodes placed within the tissue to generate areas of coagulation necrosis and tissue desiccation. At a temperature above 50 °C cellular death becomes irreversible: intracellular proteins and collagen are denatured, and lipids are dissolved.[22] Locally ablative therapies can be performed percutaneously, via open surgery, or by laparoscopy.
For patients with nonresectable liver metastases, the above mentioned locally ablative treatments are gaining acceptance and studies show that overall survival may improve using these

techniques.[20,21,23-25] Vogl et al. demonstrated a mean overall survival of 4.4 years for patients after LITT (with a maximum of five liver metastases, none of which were more than 5 cm in diameter).[21] And Solbiati et al. calculated a median survival of 36 months in patients with colorectal liver metastases, although repeated treatments were performed in case of local recurrence.[23] Controversy still exists about the extent to which local ablation offers a better chance of survival when compared to medical treatment.

The main drawback of local ablation is the number, size and location of liver metastases. Although it is generally accepted that the prognosis of the patient after liver resection worsens as the number of the liver metastases increases, the number of lesions should not be considered an absolute contraindication to local ablation if successful destruction of all metastatic tumors can be accomplished. However, most studies on local ablation have excluded patients with more than five lesions or lesions larger than 5 cm in greatest diameter.[20,21,26] Furthermore, Elias et al. found that the total number of resected metastases retains a strong prognostic effect regardless of their location.[27]

Tumor size is of utmost importance for the outcome of local ablation. Several factors may influence the size of the area of induced necrosis, including tumor geometry and adjacent structures such as arteries, portal and hepatic veins and the biliary tree. Strategies to increase the volume of thermal necrosis include improving the ablation technique and applying the Pringle manoeuvre. The Pringle manoeuvre reduces dissipation of the generated heat, providing increased destruction volumes and greater tumor-free margins.[28-30] Vascular clamping used to increase lesion size during local thermal ablation, should be preferably applied to the portal vein only.[31,32]

Unfortunately, even after apparently complete tumor destruction, most patients will develop tumor recurrence in the liver within two years.[28,33] This thesis focuses on local ablation techniques to enlarge destruction volume and on the possibilities of additional chemotherapy after thermal ablation to overcome local recurrence.

Metastasis formation

Colorectal metastasis formation is a selective, non-random process which frequently occurs in the liver and is the major cause of death in patients with colorectal cancer. The biological properties of metastases can be quite different from those of the primary tumor. These can be defined partly by the genetic and phenotypic alterations that allowed survival following detachment from the primary tumor and partly by host-tumor interactions. The initial event of invasion occurs when the metastatic cells break out of the surrounding stroma and enter the circulation. Here they must evade immune defences in order to find a way to the metastatic site.[34,35]

One of the best characterized oncogenes is Kirsten Ras (KRAS). Mutations in this oncogene are acquired early in the progression from adenoma to carcinoma. KRAS is a Ras proteins which is associated with the inner face of the plasma membrane where the facilitate signalling initiated by diverse extracellular stimuli.[36] Ras activity is regulated by cycling between inactive GDP-bound and active GTP-bound forms.[37] Activating mutations in the oncogene KRAS are observed in approximately 35% of all sporadic CRC.[38-40] These mutations interfere with the intrinsic GTPase activity, leading to a constitutively active GTP-bound state which leads to growth factor-independent signalling.[36,41] Mutational activation of KRAS is in itself not sufficient to confer metastatic capacity to intestinal epithelial cells.[42-44] Nevertheless,

mutations in KRAS are associated with metastasis formation in colorectal cancer patients and signalling by activated RAS oncogenes contributes to the metastatic potential of tumor cells.[45,46] The exact underlying mechanism how and when KRAS is involved in metastases formation remains unclear. In this thesis we focused on the role of KRAS in the evasion of the immune system. Interleukin18 (IL18) is involved in promoting NK activity and inducing a Th1 response.[47] Even so, consistent with the notion that Th1 cells are involved in antitumor immunity, administration of IL18 confers significant antitumor activity. IL18 is also produced by colon epithelium, where it has been implicated in the host immune defense against tumor development.[48,49]

Furthermore, loss of epithelial morphology and the acquisition of mesenchymal characteristics may contribute to metastases formation during colorectal tumorigenesis.[50] Epithelial-mesenchymal transition (EMT) is a process in which cells lose epithelial polarity and acquire a mesenchymal phenotype with reduced intercellular interactions and increased migratory capacity. This process is regulated by signalling pathways, such as Wnt, Notch and TGFβ which normally control homeostasis in the gut. The Wnt signaling pathway plays an essential role in the development and homeostasis of the intestine.[51,52] The presence of activating Wnt pathway mutations leads to accumulation of β-catenin in the nucleus which finally leads to the uncontrolled transcription of Wnt target genes. Notch is a signaling system that regulates cell fate specification, stem cell maintenance and initiation of differentiation in embryonic and postnatal tissue.[53] The best-characterized Notch target genes are the hairy/enhancer of split (HES) transcripitional repressors, which in their turn regulate downstream genes.[54,55] The TGFβ superfamily of growth factors regulate a plethora of biological processes including embryonic development, wound healing, angiogenesis, proliferation, and cell differentiation.[56,57] Inactivating mutations of TGFβ signaling components have been identified at the adenoma-to-carcinoma transition.[58] Evidence is accumulating that EMT plays an important role in cancer progression and metastasis formation.[59-62] A better understanding of the signaling pathways and molecules involved in EMT can help to delineate more effective strategies for future therapeutic interventions.

In conclusion, the entire process of metastases formation is complex, in which phenotypic, transcriptional and genetic changes must occur either on a permanent or transient basis.[63,64] Interfering with this process could lead to failure at some stages and might halt the progression of metastasis formation.

Outline of the thesis

The aims of this thesis were first to investigate new strategies to more effectively ablate non-resectable liver metastases and to evaluate the effect of additional chemotherapy after ablation in experimental models on local recurrence and second to asses the contribution of mutant KRAS and different signaling pathways during late stages of colorectal cancer and liver metastasis formation.

To reduce local tumor recurrence we investigated different ablation techniques for unresectable liver metastases. In **chapter 2** we describe the use of multiple fiber LITT together with vascular clamping in an attempt to increase lesions size in patients with large colorectal liver metastases. The use of multipolar radiofrequency ablation in similar patients is addressed in **chapter 3**.
In **chapter 4**, a literature review is presented to create an overview of the management of neuroendocrine hepatic metastasis, focused on locally ablative treatment. Local ablation may be particularly suited for treatment of such metastases as they tend to progress slowly but symptomatically.
To study the effect of local ablation treatment combined with chemotherapy, we established a mouse model for solitary colorectal liver metastases. In **chapter 5** we investigated the combined treatment of LITT plus doxorubicin in this mouse model.

The second part of this thesis is focused on the molecular aspects of colorectal metastases formation. Mutational activation of KRAS is frequently observed in early stages of colorectal cancer formation but its activity is also essential for metastasis formation. In **chapter 6** we have assessed the role of KRAS in an aggressive murine colorectal cancer cell line and investigate its role in evasion of the immune system. **Chapter 7** addresses signaling pathways involved in metastases formation of colorectal cancer. In this chapter, we have investigated whether the Wnt, Notch, TGFβ and KRAS pathways are differentially active in primary tumors versus regional and distant metastases and whether this is accompanied by changes in the expression of different epithelial and mesenchymal markers.
Chapter 8 contains the general discussion and conclusions.

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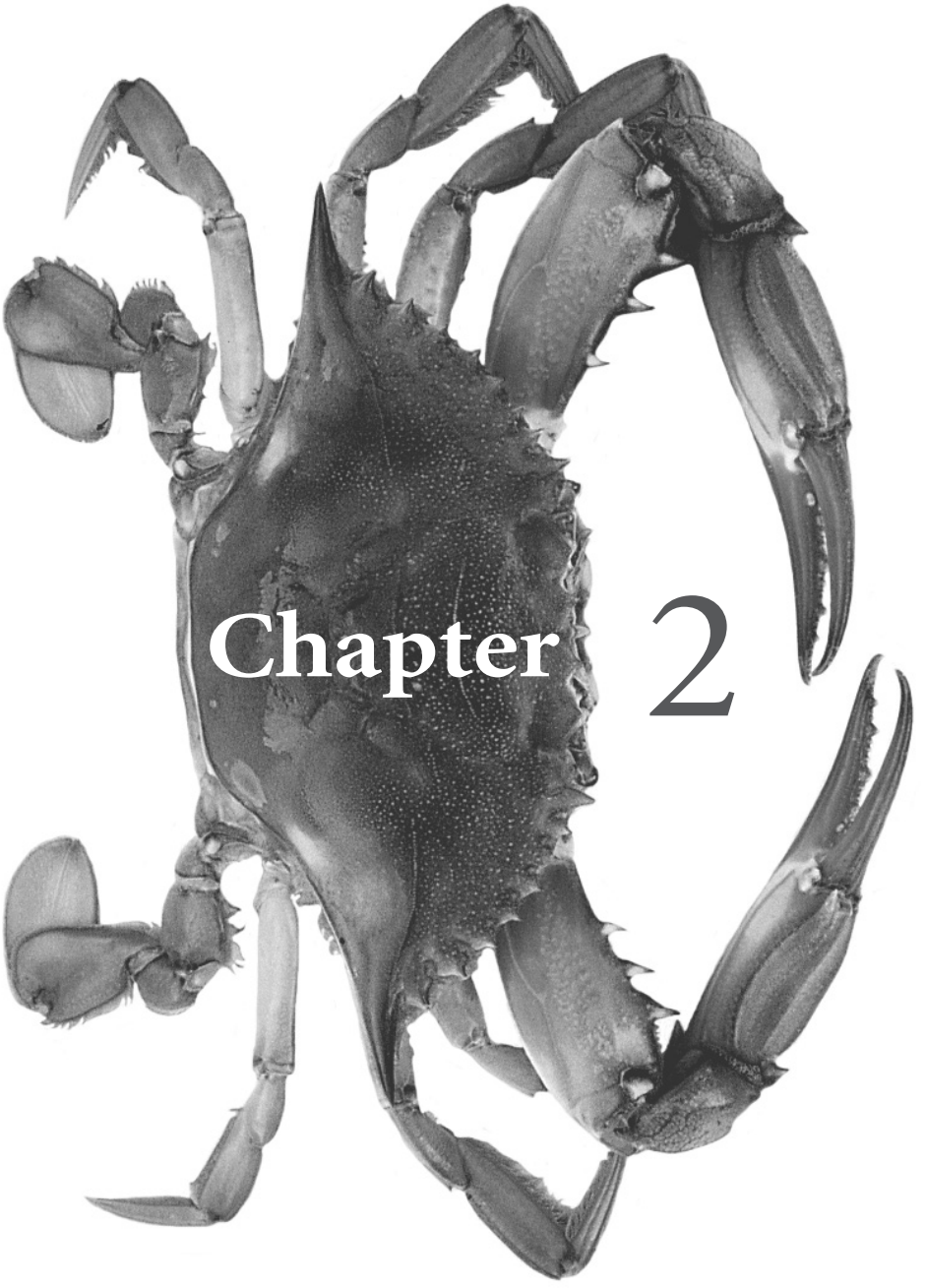
Part

I

Local therapy of liver metastases



Chapter 2

Multiple Fibre Laser-Induced Thermotherapy (LITT) for ablation of Large Intrahepatic Tumors

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Abstract

Objective: The aim of this study was to test three techniques used simultaneously to increase lesion size.
Background Data: Laser-induced thermotherapy (LITT) is a method of local tumor ablation, which may prolong survival in patients with unresectable liver metastases. The main limitation has been the production of lesions with sufficient tumor free margin. Three techniques to increase lesion size were used simultaneously.
Methods: LITT treatment was performed with water-cooled, multiple fibre application and hepatic blood flow occlusion in 6 patients with unresectable intrahepatic metastases. Response was measured by CT scan.
Results: In all patients tumors were effectively ablated. In 2 patients with colorectal metastases, lesions up to 8.6 cm could be created.
Conclusion: The use of water-cooled multiple fibre application and hepatic inflow occlusion makes LITT an effective ablative method expanding the treatment options for patients with large intrahepatic masses.

Introduction

Hepatic metastases are a major cause of morbidity and mortality in patients with malignant tumors of different origins. The treatment options for patients with liver metastases are limited. Laser-induced thermotherapy (LITT) also named interstitial laser therapy (ILT) is a local ablative technique presently in clinical use for the treatment of patients with liver tumors, in particular colorectal liver metastases.[1] LITT destroys solid tumors by heat coagulative necrosis produced by interstitially placed light guiding fibers. Lasers operating in the near-infrared spectrum, such as neodynium:yttrium-alluminium-garnet (Nd:YAG) are most suitable due to their high penetration depth in tissue. In selected patients with liver tumors, LITT may achieve long-term survival that is comparable to surgical resection.[2]
One factor that limits the wider application of LITT is the maximum volume of tumor necrosis that can reliably be achieved with a single optical fibre. In lesions exceeding 3 cm, a sufficient margin is not reached and often incomplete necrosis leads to tumor recurrence.[3-5] Preferably, tumors should be ablated with a margin of 1 cm surrounding normal tissue. The size of the heated region generally depends on the optical wavelength, the applicator system, power and time protocol, thermal tissue properties, and the blood perfusion rate. By using beam-splitting devices, which simultaneously deliver power to multiple fibres, an increased heat distribution between fibres can be achieved. This multiple fibre application has a synergistic effect owing to reduced heat dissipation between fibres. This increases the volume of tissue necrosis by four- to sixfold, combined with a reduced application time.[6,7] Four-fibre systems may produce a maximal lesion diameter of approximately 5 cm.[6] Hepatic blood flow impedes the expansion of thermal injury through its heat dissipating effect. Hepatic inflow occlusion has been shown to cause a four fold increase of the thermal tissue injury.[8,9] In addition, specially designed fibres, equipped with diffusing endings, emitting light over its entire length of 10-40 mm may further increase the lesion size. These fibres can be externally liquid cooled to avoid carbonization of the surrounding tissue, thereby increasing the maximal input power.[10,11] By combining of all of these modalities we have used LITT to ablate large intrahepatic lesions. We report two cases with liver metastases with a maximal diameter of 6.0 cm that were successfully ablated with this new treatment modality.

Materials And Methods

From July 2003 through April 2005, 6 patients (3 males; mean age 70 years; range 54-80) were admitted with unresectable intrahepatic metastases and treated with LITT during open abdominal surgery. Three patients had liver metastases from colorectal carcinoma (CLM), one from hepatocellular carcinoma (HCC) and two patients from a carcinoid tumor. Four patients had single metastases to the liver with a mean diameter of 5.8 cm (range 5.3-6.2). The two patients with carcinoid tumor had multiple metastases throughout the liver. LITT was performed with a Nd:YAG (TT Yag-80, Trumpf Medizine Systeme, Umkirch, Germany) with a wavelength of 1064 nm. The laser light was delivered in the continuous wave mode through a 1 mm fibre, in a water-cooled hand piece (Power applicator, Trumpf Medizine Systeme, Umkirch, Germany) (outer diameter 4 mm, diffuser length 3 cm). LITT was performed with multiple fibres using a beam-splitter at various power settings and time periods directed by size and location of the tumor (**Figure 1**). Two representative case reports follow. Plasma aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) of all patients were measured at different time points.

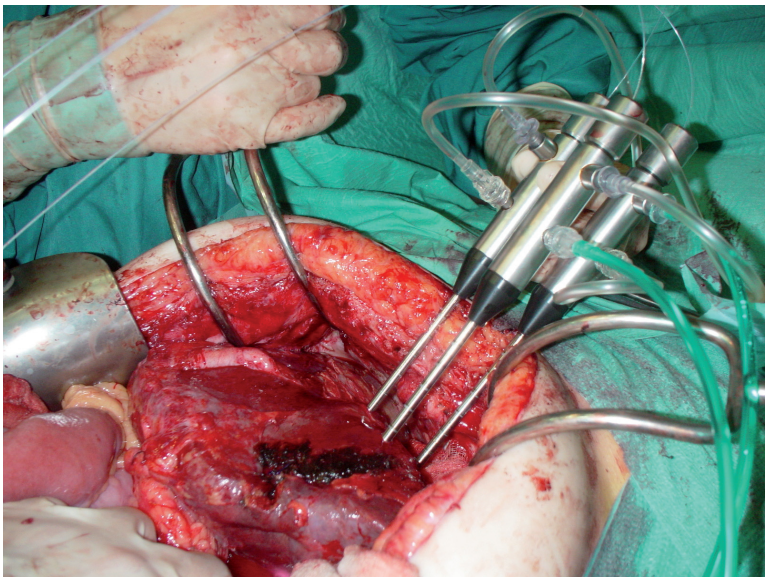

Figure 1. Photograph demonstrating the placement of three fibres in water-cooled hand pieces, 2.5 cm distance between fibres, into the tumor in the liver of the patient described in case report 2.

Results

Five patients were treated in a single session LITT, and one patient (**case report 1**) in two sessions. One patient died 12 days postoperatively from the inferior caval vein syndrome and one patient died one month postoperatively from pneumonia with multiple organ failure caused by hematemesis and aspiration of blood from a gastric ulcer (**Table 1**). No complications occurred in the remaining patients. Follow-up of patients ranged from 1 to 22 months (mean 9 months). **Figure 2** represents serum ASAT and ALAT levels of all patients at different time points as indicators of liver tissue damage. Directly post LITT, ASAT and ALAT levels were increased in all patients. Within two weeks these levels were normalized.

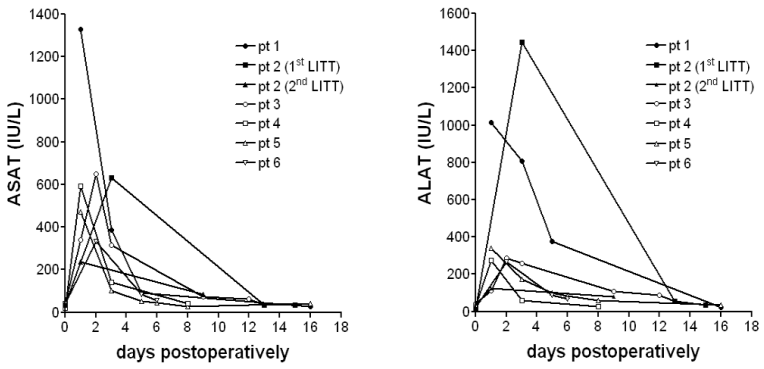

Figure 2. (**A**) Serum ASAT levels of all patients at different time points, (**B**) Serum ALAT levels of all patients at different time points.

TABLE 1. PATIENT CHARACTERISTICS AND OUTCOME AFTER LITT

pt	age	sex	Tumor type	Tumor size (cm)	Number of tumors	Lesion size after LITT (cm)	Hepatic location	Occlusion time (min)**	Complications	Outcome or DF survival (months)	Time to discharge (days)
1	80	F	CLM	5.3	1	6.8	RHL	45	none	22	12
2	76	F	CLM	6.0	1	8.6	LHL	35	Local recurrence	6 months after 2nd LITT	34 (1st LITT) 10 (2nd LITT)
3	63	F	C	4.2* 8.1 9.0	>10	6.2 8.4 10.8	diffuse	0	ICV syndrome	death	na
4	54	M	CLM	5.5	1	9.3	RHL	45	none	New lesions after 4 months	10
5	70	M	C	3.1* 4.2 8.5	>10	4.1 6.0 7.5	diffuse	45	none	9	8
6	78	M	HCC	6.2	1	7.1	RHL	45	Pneumonia, multi organ failure	death	na

*CLM = colorectal liver metastases; C = carcinoid tumor; HCC = hepatocellular carcinoma; * patient with multiple lesions, only 3 lesions were ablated; RHL = right hemiliver; LHL = left hemiliver; ** occlusion time is the total time of hepatic artery and portal vein occlusion applied in 15 minutes cycles, and separated by 5 minutes of liver reperfusion between cycles; ICV syndrome = inferior caval vein syndrome; DF survival = disease free survival; na = not applicable*

Case Report

Case Report 1

An 80-year-old woman was referred to our institution with a colorectal metastasis of 5.3 cm in diameter in the right hemiliver (**Figure 3A**). The patient had a history of a T4N0 sigmoid carcinoma 4 years before, which was resected. Because of severe cardiopulmonary comorbidity, resection was considered too high of risk. She was treated during open abdominal surgery with LITT under ultrasound (US) guidance. LITT was performed in three cycles of 15 minutes each using three fibres simultaneously, 20 W per fibre, 2 cm distance between probes and with temporary occlusion of the hepatic artery and portal vein (Pringle Manoeuvre). After each cycle the fibres were withdrawn 1 cm. Between every cycle a 5 minutes liver reperfusion interval was applied. The patient withstood the surgery well, had a smooth postoperative recovery and was discharged on the 12th postoperative day. A CT scan one week postoperatively showed a coagulation lesion of 6.8 cm in diameter (**Figure 3B**). Follow up with CT scans 15 months later demonstrated no recurrence of tumor in the liver, the scar lesion was reduced to 4.1 cm in diameter (**Figure 3C**).

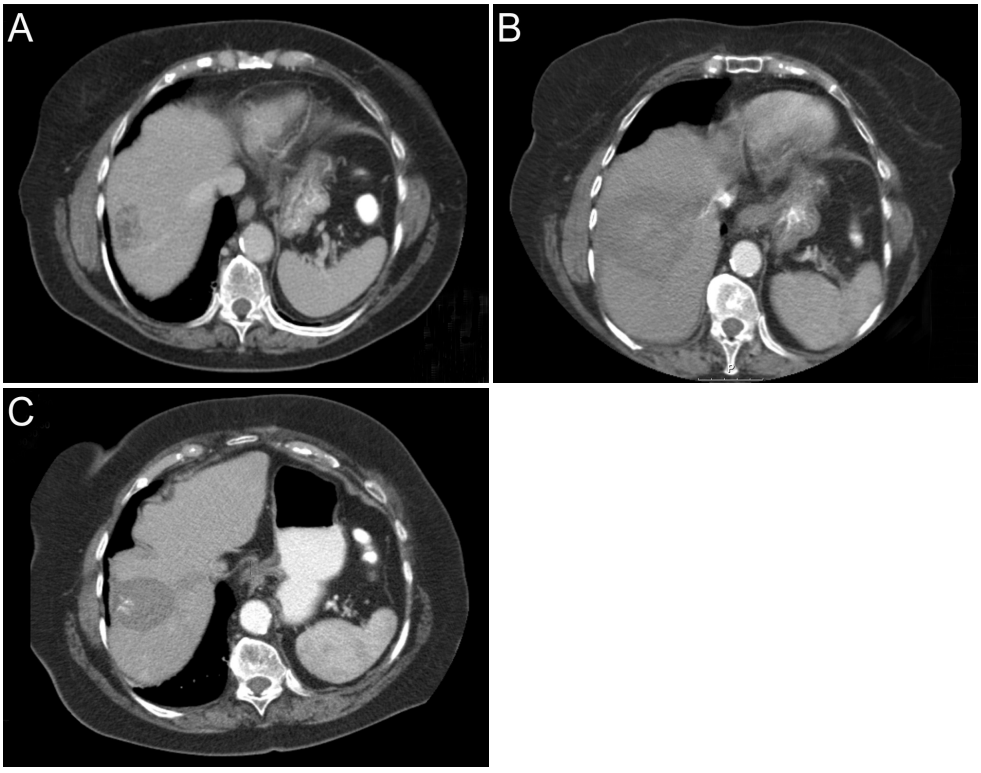

Figure 3. (**A**) CT scan of a 80-year-old woman demonstrating a hepatic metastasis from colorectal cancer in liver segment VIII with a diameter of 5.3 cm, (**B**) CT scan one week post LITT demonstrating complete ablation with a coagulation lesion of 6.8 cm in diameter, (**C**) CT scan 15 months after LITT treatment showing a scar lesion of 4.1 cm in diameter.

Case report 2

A 76-year-old woman presented with recurrent colorectal metastases of 6.0 cm in diameter in the left hemiliver 2 years after a right hemihepatectomy for metachroneous metastases of a T3N0 rectal carcinoma (**Figure 4A**). Resection of the recurrent liver metastases was not an option because of the limited size of the remnant liver. LITT was performed at open abdominal surgery under ultrasound guidance. Two cycles of 15 minutes and one cycle of 10 minutes were given with 3 fibres simultaneously, 20 W per fibre, 2.5 cm distance between fibres and 5 minutes interval between procedures (**Figure 1**). After the first and second cycle the fibres were withdrawn 2 cm. The Pringle Manoeuvre was applied in all three cycles. The operation was combined with a correction of a large parastomal hernia with mesh. The patient experienced a slow postoperative recovery due to temporary intestinal pseudo-obstruction but no serious complications occurred. A CT scan two weeks post LITT showed a coagulation lesion of 8.1 cm in diameter (**Figure 4B**). Follow up with CT scan six months later revealed a local recurrence, confirmed by positron emission tomography (PET)-scan (**Figure 4C and D**). Therefore the patient was treated again with open LITT under ultrasound guidance. The first cycle of 15 minutes was given with 3 fibres simultaneously, 20 W per fibre, 2 cm distance between probes. Hereafter two more cycles were given with one fibre each, 35 W for 15 minutes. The Pringle Manoeuvre was not applied as adhesions prevented access to the hepatoduodenal ligament. After the second course of ILC, the postoperative period was uncomplicated and the patient was discharged on the 10th postoperative day. A CT scan in the first post-operative week showed a large ablative zone of 8.6 cm in diameter (**Figure 4E**). Follow up demonstrated no recurrence so far.

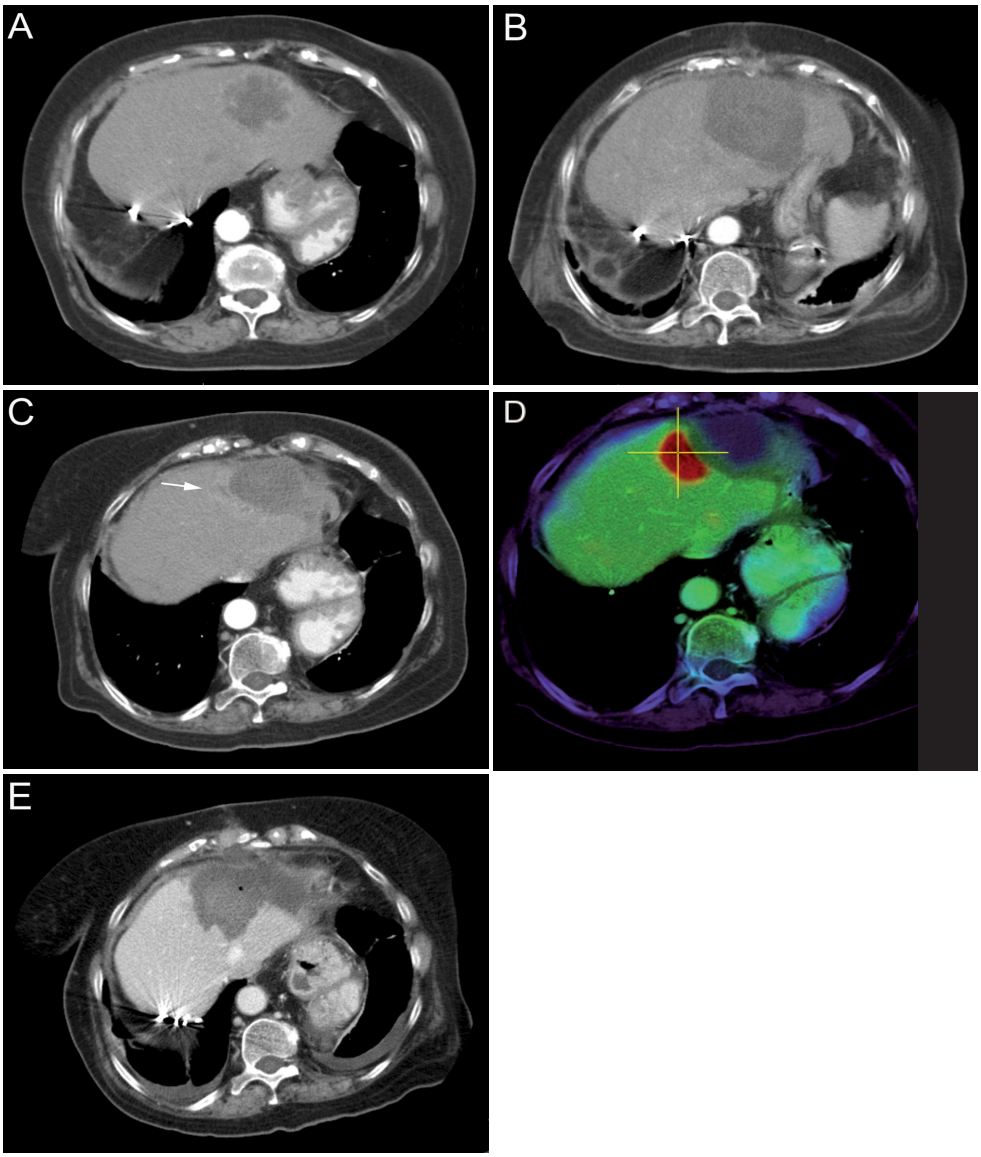

Figure 4. (**A**) Abdominal CT scan of a 76-year-old woman showing a colorectal liver metastases of 6.0 cm in diameter in segment IV, (**B**) CT scan two weeks post LITT demonstrating complete ablation with a coagulation lesion of 8.1 cm in diameter, (**C**) CT scan 6 months after LITT treatment demonstrating a rim of 0.9 cm recurrent tumor tissue on the medial site of the necrotic lesion (arrow), (**D**) Fusion scan of PET and CT scan 6 months after LITT demonstrating recurrent tumor tissue in the liver, (**E**) CT scan 5 days after second LITT treatment demonstrating complete ablation with a coagulation lesion of 8.6 cm in diameter.

Discussion

Although local tumor ablation is currently used worldwide for unresectable liver metastases, tumor size has been shown to be a limiting factor. The cases described here demonstrate that LITT with the use of multiple water-cooled fibres applied simultaneously, together with hepatic inflow occlusion, can produce large lesions up to 8.6 cm in diameter. Number and lobar distribution of the tumors have not been found independent factors for survival as long as a radical (R0) resection has been achieved.[12] Therefore, the aim of the ablative treatment should be to clear the liver of all tumor tissue to obtain disease control.

Although we produced large lesions, tumor lysis syndrome was not observed. The tumor lysis syndrome describes the metabolic derangements that occur with tumor breakdown and will finally lead to acute renal failure.[13] LITT causes heat fixation of tissue[14] hence no circulating metabolites will develop. Furthermore, none of our patients showed any sign of acute renal failure that was directly related to LITT.

Presently, it appears that the best way to evaluate the clinical benefit of local ablation methods is to examine the rate of local recurrence at the treated site. The results of several studies indicate that complete tumor ablation is important and directly related to survival, just as a free resection margin is important for prognosis in hepatic resection.[15,16] Consequently, with large lesions there is a risk of residual tumor tissue after ablation, which gives rise to tumor recurrence. Contrast-enhanced CT performed after LITT identifies tissue necrosis as non-perfused areas, and correlates closely with histological findings. Residual tumor appears as a region of contrast enhancement adjacent to non-perfused tissue, as demonstrated in our second case. This case also demonstrates that fibre positioning remains crucial. A large ablative lesion was produced in the first LITT treatment, but a small rim of viable tumor tissue remained causing tumor recurrence. This tumor was not identified on the early CT scan. An important advantage of the ablative method is that treatment can be repeated with minimal morbidity, because normal liver tissue is preserved.

LITT of liver metastases has been performed mainly via the percutaneous route. In the two cases presented here, treatment was performed by laparotomy. We prefer the open approach for several reasons (**Table 2**). Apart from enabling accurate tumor localization, previously unrecognized disease may be detected. Furthermore hepatic inflow can be occluded temporarily to maximize LITT-induced tumor necrosis.[1]

Reported local recurrence rates after LITT vary up to 63% of cases (**Table 3**).[17-19] Serious complications have been rare, with intrahepatic abscess formation being the most common (0.37%) severe event in 6 cases reported in the literature.[20] One of the largest series comes from Vogl et al. and involves 603 patients with colorectal liver metastases.[21] Local tumor control was achieved in 96.3% to 98.8% after 3 months and between 95.6% and 98.8% after 6 months using a MR guided percutaneous approach with one to five cooled diffusing fibres but without hepatic inflow occlusion. Only patients with less than five tumors, each smaller than 5 cm, and no extrahepatic disease were included. These good results should be interpreted with caution as follow up data are six months. As expected, the highest recurrence rate of 63% was found in the group with the largest lesions (**Table 3**).[22]

Other alternatives to surgical resection are cryotherapy and radiofrequency ablation (RFA). Cryotherapy is based on cooling the tissue to –190 °C. Usually, the treatment consists of two successive freeze and thaw cycles that kill tumor cells by mechanical disruption of cell membranes and intracellular structures with ice crystals. Local recurrence rates are often reported

TABLE 2. ADVANTAGES OF OPEN VERSUS PERCUTANEUS LITT

Open	Percutaneous
Accurate localization of tumor and extent of intrahepatic disease (intraoperative US)	Less traumatic
Precise positioning of fibres (US and tactile feedback, mobilizing the liver)	
Oncologic staging of the abdomen	
Free surrounding structures (bowel, stomach, diaphragm)	
Visual control of ablation size	Real time treatment monitory (MRI)
Hemostatic control	
Hepatic inflow occlusion	Inflow occlusion only in highly specialized centers
Multiple fibre application with Cooled hand piece high laser power	

US = ultrasonography; MRI = magnetic resonance imaging

TABLE 3. RESULTS AFTER LITT

Authors	Number of tumor/ patients	Tumor type	Tumor size (cm)	Local recurrence rate (%)	Compli-cation rate (%)	Mortality (%)
Gillams and Lees	-/69	CLM	3.9 (1-8)	63	3.2	1.4
Giorgio	85/77	HCC	3.2 (1.0-6.6)	18	3.9	?
et al.	31/27	LM	4.2 (3.0-9.0)	23	3.7	0
Vogl et al.	1914/676	LM + HCC	<5	5	5.1	0.4
Vogl et al.	1801/603	CLM	<2	1.9	1.5*	0.3**
			2.1-3.0	2.4		
			3.1-4.0	1.2		
			4.0-5.0	4.4		

*CLM = colorectal liver metastases; HCC = hepatocellular carcinoma; LM = liver metastases; * overall complication rate; ** overall mortality*

to be quit high and range from 8.7 to 14% after intraoperative treatments to 52-100% after percutaneous therapy.[23-26] Complications are relatively common (26-41%) and include hepatic cracking, direct vascular injury from probe insertion, intrahepatic abscess formation, thrombocytopenia, disseminated intravascular coagulation and multiorgan failure, and pure procedure-related mortality varies from 0 to 3.3%. With RFA, the reported local recurrence rate is between 21.6% for tumors up to 2.5 cm and 68.4% for lesions larger than 4 cm in diameter.[27] Once more, lesion size proves to be a critical factor to the success of ablative therapy.

Conclusion

LITT with simultaneously multiple fibres and hepatic blood flow occlusion offers a unique novel technique for producing large lesions within a short time frame, thereby expanding the possibilities for patients with intrahepatic large metastases.

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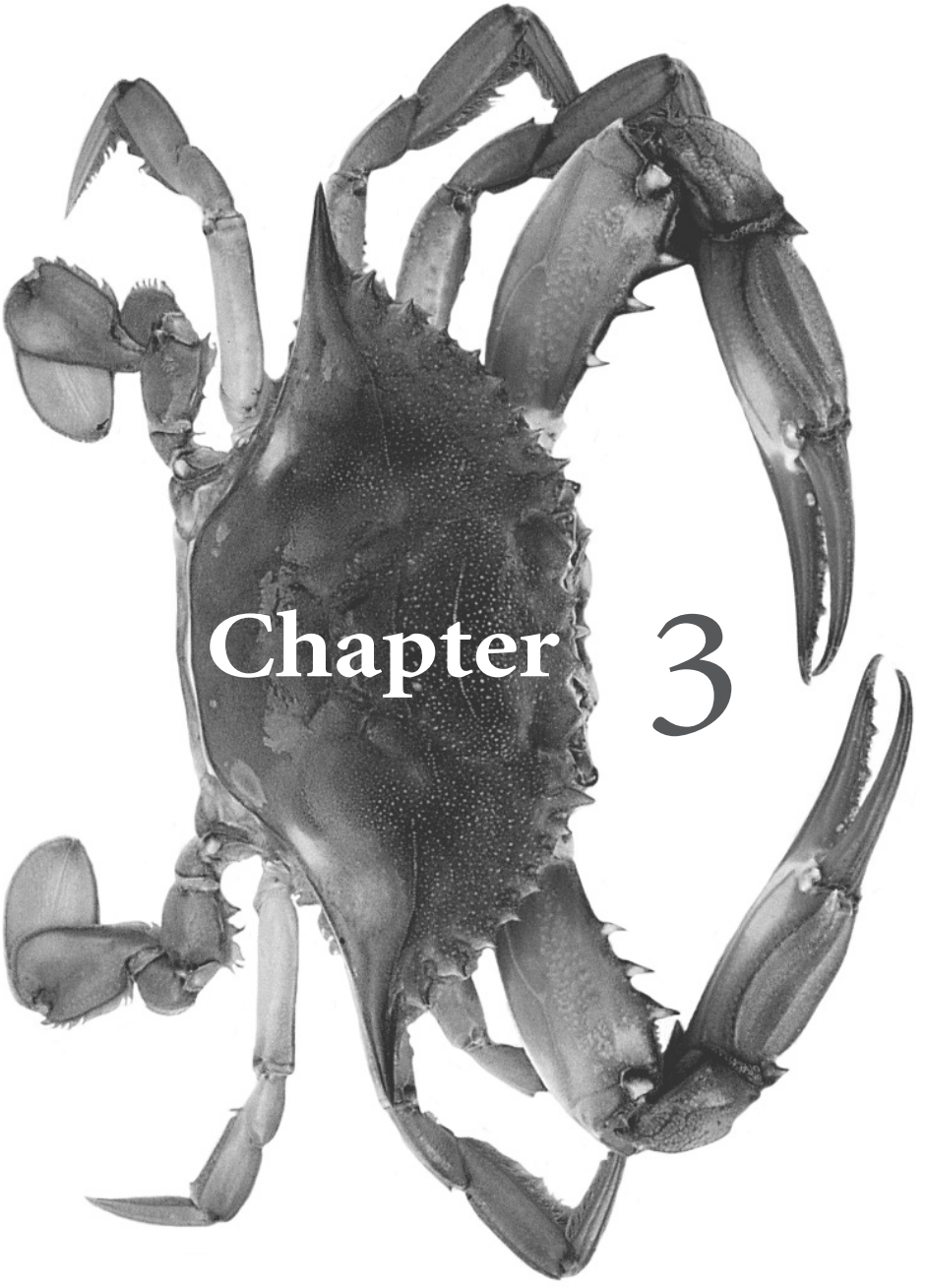

Chapter 3

Multipolar Radiofrequency Ablation of Large Hepatic Metastases of Endocrine Tumors

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Abstract

Radiofrequency ablation (RFA) is a reliable method of creating thermally-induced coagulation necrosis. Local recurrence after RFA of hepatic metastases is directly dependent on tumor size related to the free margin of ablation. To produce larger coagulation volumes a bipolar radiofrequency device was developed that allows simultaneous activation of 3 active needles. This technique was used at laparotomy in a patient with liver metastases of an endocrine tumor. Coagulation size up to 12 cm in diameter could be created. The postoperative recovery of the patient was uncomplicated. No local recurrence was seen after 13 months of follow up with CT scan. The use of simultaneously operated multiple radiofrequency electrodes in a multipolar mode expands the treatment options for patients with large and unresectable intrahepatic metastases.

Introduction

Despite advances in cancer therapy the treatment of liver metastases remains a challenge. Most patients are poor candidates for surgical resection, only 20% are suitable for surgery.[1-3] New minimally invasive techniques for ablation of unresectable tumors have gained increased attention as effective treatment alternatives.[4,5] Radiofrequency ablation (RFA) is a promising method to focally ablate tumors. This technology involves delivery of radiofrequency current with a frequency of 375-500 kHz direct to tissue to cause resistive heating as a result of the movement and vibration of the ions. Once cells are heated above 50 °C, cellular proteins become denaturised, lipid bilayers melt, DNA and RNA are destroyed, and irreversible cell death occurs.[6] The volume of the heated region depends on different factors such as applied energy, probe geometry, duration of heat exposure, fluid content of tissue, blood perfusion rate, blood vessel density, and others.[7] Several strategies have been developed to improve tissue-energy interactions for thermal ablation therapy with the goal of increasing the target lesion. The first RFA systems with monopolar devices, which require grounding pads on the thighs in order to close the electrical circuit, resulted in lesion sizes up to 1.6 cm in diameter.[8] Technical developments with electrode design, optimized ablation algorithms, more powerful generators and bipolar techniques have resulted in more effective ablation with lesions up to 4 cm in diameter.

Bipolar technologies in which the radiofrequency current flows between the two electrodes at the tip of the probe are safe and gentle on the patient in contrast to monopolar RF applications in which current flows through the patient's body. A new concept has recently been developed applying bipolar devices in a multipolar mode, i.e., all the possible electrode pairs are activated automatically one after the other for a short period of time (**Figure 1**).[9] Additionally, larger coagulation volumes can be achieved with internally liquid-cooled bipolar applicators. Both strategies were used in combination to achieve even larger coagulation volumes. We report a case in which liver metastases were successfully ablated with the use of RFA in a multipolar mode.

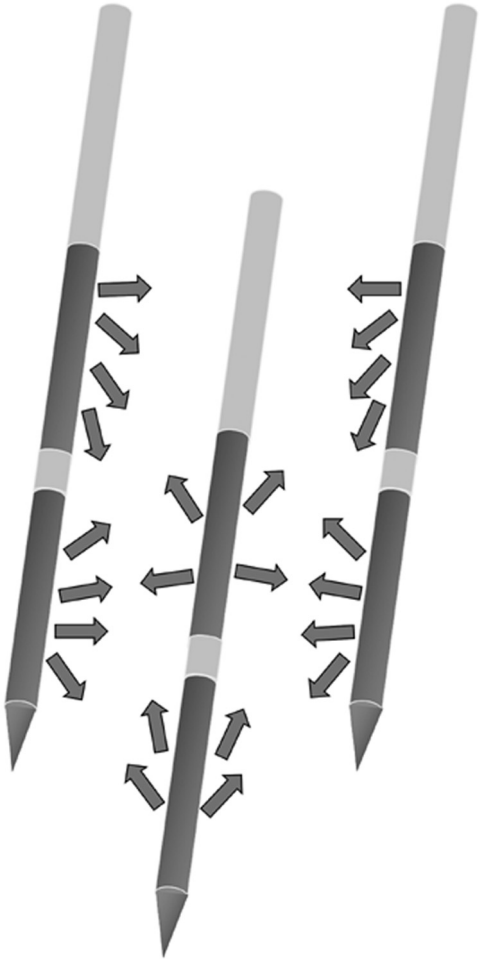

Figure 1. Schematic illustration of RFA probes. Positive and negative electrodes are located on each probe. In the multipolar mode, all electrodes are activated in pairs one after the other.

Case report

A 63-year-old man with multiple endocrine neoplasia syndrome type 1 (MEN1) was referred to our hospital with asymptomatic unresectable liver metastases from a locally-advanced endocrine pancreatic tumor. A CT scan of the liver showed a metastasis in segment V of 3.6 cm in diameter with a satellite lesion of 1.0 cm in diameter, a metastasis in segment VII of 7.4 cm in diameter and seven small lesions of 1-2 cm in diameter diffusely distributed throughout the liver (**Figure 2A**). RFA was performed at open abdominal surgery under ultrasound guidance. Ultrasound analysis during surgery revealed larger metastases; the metastasis in segment V was 4.0 cm in diameter and the metastasis in segment VII was 8.8 cm in diameter. The metastasis in segment V was ablated using 3 bipolar coagulation elec-

trodes simultaneously, 4 cm active length, 3 cm distance between probes in a triangular formation with 140 W power setting and an applied energy of 150 kJ (34 minutes of exposure) (ProSurge electrodes and LabPower generator, Celon AG medical instruments, Germany). At the beginning of the RFA procedure, portal and hepatic artery inflow occlusion (Pringle manoeuvre) was applied for 15 minutes. Track ablation was performed in the bipolar mode for each probe separately with 40 W of power and disabled resistance control.
Second, the metastasis in segment VII was ablated using 4 bipolar coagulation electrodes simultaneously, 4 cm active length, 3.5-4 cm distance between probes in a rectangular formation with 180 W power setting and an applied energy of 180 kJ (33 minutes exposure). Pringle manoeuvre was applied for 15 minutes during this application. Then all four electrodes were withdrawn 2 cm and another 130 kJ were administered at the same power setting and during another 15 minutes of Pringle manoeuvre (23 minutes exposure). All four bipolar coagulation electrodes were removed using track ablation. Finally two lesions in segment IV (both 2 cm in diameter) were ablated simultaneously with a single bipolar coagulation electrode in each lesion, 4 cm active length with 25 kJ each without the Pringle manoeuvre.
The patient withstood surgery well and was discharged on the 8th postoperative day. CT scan one week after RFA demonstrated a sharply demarcated zone of coagulation necrosis of 7.3 cm in diameter in segment V and in segment VII a coagulation necrosis of 12 cm in diameter (**Figure 2B**). A CT scan 13 months postoperatively demonstrated no local recurrence despite the appearance of several new small metastases (0-1 cm) that were seen throughout the liver.

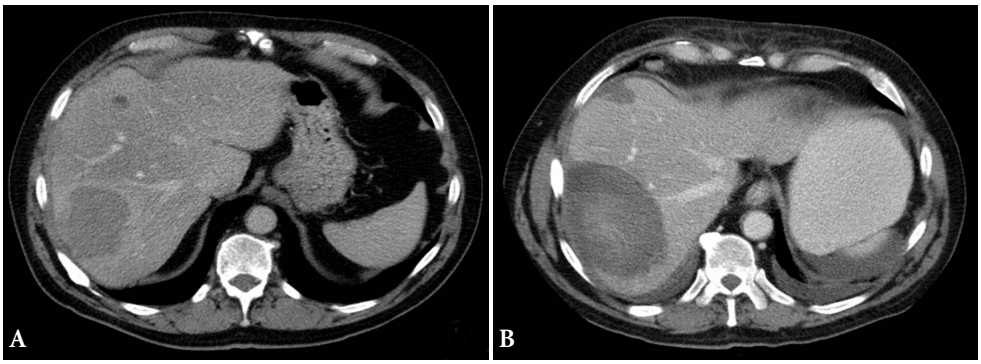

Figure 2. CT scan of a 63-year-old man with multiple hepatic metastases from an endocrine tumor, (**A**) CT scan showing a metastasis of 7.4 cm in diameter in segment VII, (**B**) CT scan one week after multipolar RFA showing a coagulation lesion of 12.0 cm in diameter in segment VII.

Discussion

In recent years, local tumor ablation by radiofrequency ablation (RFA) has become the most frequently used technique for local tumor destruction. Analysis of local recurrence after RFA of hepatic metastases revealed tumor size to be a key factor.[10,11] For tumors < 3 cm in diameter the post RFA- recurrence rate is 6.6%, while for tumors > 3 cm in diameter ~56% appear to recur.[12] As alternative to RFA, other local ablation techniques such as interstitial laser coagulation (ILC) are used to clear the liver from metastatic tumor lesions.[13,14] Recently we have demonstrated that ILC with the use of multiple water-cooled fibres applies simultaneously, together with hepatic inflow occlusion, can produce large lesions of up to 8.6 cm in diameter. In the case report presented here we demonstrate that next generation bipolar RFA is able to produce even larger coagulation lesions up to 12.0 cm in diameter.

Local ablation is a well-established treatment for unresectable hepatocellular carcinomas[15] and liver metastases from colorectal carcinomas.[16] A few series have also shown good local tumor control with a satisfactory duration effect on symptoms after RFA in hepatic metastases of endocrine tumors.[17-19] In metastases of endocrine tumors, the goal is to reduce the tumor mass with preservation of surrounding normal hepatic tissue. There is a tendency to destroy metastases early in the course of disease, thereby postponing or eliminating the surgically untreatable stage. Patients with hepatic metastases of endocrine tumors treated with RFA showed symptom relief in 95% with significant or complete symptom control in 80% for a mean of 10 months.[17] Even in patients with extrahepatic disease liver metastases ablation may provide symptom relief.[17] The complication rate is 5-10% and the mortality rate is low.[5,20] Although we produced large lesions, tumor lysis syndrome was not observed. The tumor lysis syndrome describes the metabolic derangements that occur upon cellular disruption leading to acute renal failure.[21] RFA as well as laser-induced thermotherapy (LITT) cause heat fixation of the tissue[22] without release of circulating cellular compounds. Therefore, local ablation techniques are especially suitable for repeated treatment in patients with hepatic metastases of endocrine tumors in which new metastases develop during follow up.

Possible contraindications for this new RFA system are comparable to those of other ablative therapies, depending on the location of the tumor. Tumors adjacent to large vessels are difficult to treat, because the blood flow in the vessels cools the heating process, potentially leading to residual viable tumor cells against the blood vessel. Tumor adjacent to major branches of the portal vein are particularly problematic, because the ablation is likely to cause obstruction of the associated bile duct. Careful selection of patients is obviously required to avoid complications.

RFA may be applied through a percutaneous, laparotomic or laparoscopic route.[23] Mulier et al. found in their meta-analysis that a surgical open approach yielded statistically significantly superior results compared to a percutaneous approach, independent of the size of the tumor.[24] The surgical approach provides for bipolar RFA in a multipolar mode the best degree of freedom for inserting the electrodes.

The newly developed RFA system consisted of electrodes that were constructed using bipolar technology and energy applied in a multipolar mode. As energy is concentrated in the point of interest, the bipolar electrode produces larger and more homogeneous thermal lesions than monopolar saline-enhanced electrode method.[25,26] In addition, relative contraindications for monopolar RFA such as pacemaker, surgical clips or other metallic structures are not valid any more, because the electrical circuit does not involve the whole body. Besides, skin

burns due to undersized grounding pads cannot occur.
Previous investigators have shown that simultaneous operation of multiple applicator needles for tissue coagulation is significantly more effective compared to the same number of sequentially applied applicators with the same amount of energy.[27] Consequently, larger coagulation volumes can be achieved with multiple simultaneous electrodes. In the patient of our case report we used four bipolar electrodes simultaneously and withdrawn these two times which resulted in a coagulation lesion of 12 cm in diameter. Hence, even larger coagulation lesions can be achieved by repositioning of the electrodes along their axis.
The size of the coagulation volume does not only depend on the set power, but also on the delivered energy and therefore on the duration of the application. Higher power levels quickly induce evaporation of the cellular water content in the immediate vicinity of the probe and thus leads to desiccation of this region of tissue. The rise in tissue resistance impedes the further input of energy and any increase in the coagulation volume. If lower power is applied, the desiccation process takes place later. A greater amount of energy can be applied to the tissue and a larger coagulation volume achieved. This process is supported by the additional effect of thermal conductivity.
In conclusion, bipolar RFA in a multipolar mode is an exclusive technique able to produce large coagulation lesions in patients with intrahepatic metastases.

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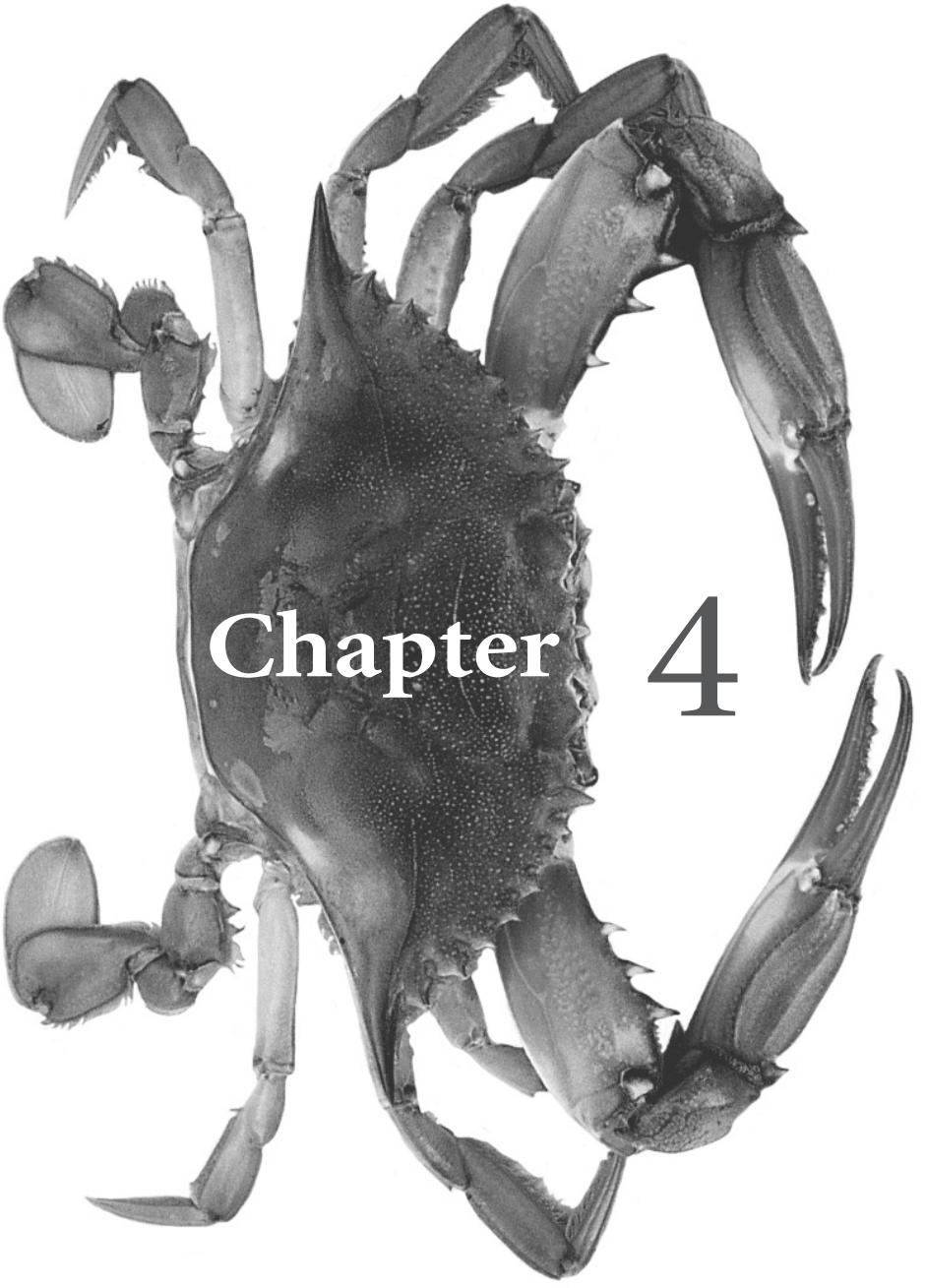

Chapter 4

Liver metastases of neuroendocrine tumours; early reduction of tumour load to improve life expectancy

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Abstract

Background: Neuroendocrine tumours frequently metastasize to the liver. Although generally slowly progressing, hepatic metastases are the major cause of carcinoid syndrome and ultimately lead to liver dysfunction, cardiac insufficiency and finally death.
Methods: A literature review was performed to define the optimal treatment strategy and work-up in patients with neuroendocrine hepatic metastases. Based on this, an algorithm for the management of these patients was established.
Results: Platelet serotonin and chromogranin A are useful biomarkers for detection and follow-up of neuroendocrine tumour. Helical computed tomography and somatostatin receptor scintigraphy are the most sensitive diagnostic modalities. Surgical debulking is an accepted approach for reducing hormonal symptoms and to establish better conditions for medical treatment, but is frequently impossible due to the extent of disease. A novel approach is the local ablation of tumour by thermal coagulation using therapies such as radiofrequency ablation (RFA) or laser induced thermotherapy (LITT). These techniques preserve normal liver tissue. There is a tendency to destroy metastases early in the course of disease, thereby postponing or eliminating the surgically untreatable stage. This can be combined with postoperative radioactive octreotide to eliminate small multiple metastases. In patients with extensive metastases who are not suitable for local destruction, systemic therapy by octreotide, ^{131}I-MIBG treatment or targeted chemo- and radiotherapy should be attempted. A final option for selective patients is orthotopic liver transplantation.
Conclusion: Treatment for patients with neuroendocrine hepatic metastases must be tailored for each individual patient. When local ablative therapies are used early in the course of the disease, the occurrence of carcinoid syndrome with end stage hepatic disease can be postponed or prevented.

Introduction

Carcinoids are neuroendocrine tumours that arise from neoplastic proliferation of enterochromaffin or Kulchitsky cells.[1] In 1963, carcinoids were classified according to their embryologic site of origin as foregut carcinoids (respiratory tract, stomach, duodenum, biliary system and pancreas), midgut carcinoids (small intestine, appendix, cecum, and proximal colon), and hindgut carcinoids (distal colon and rectum).[2] According to the WHO classification in 2000, distinction was made between well-differentiated neuroendocrine tumours (benign behaviour or uncertain malignant potential, <2% Ki67 positive cells), well-differentiated neuroendocrine carcinomas (low grade malignancy, presence of metastasis and/or invasiveness), and poorly differentiated neuroendocrine carcinomas of high-grade malignancy (usually small cell, >15% Ki67 positive cells).[3] Ki67 is an immunohistochemical biomarker for cell proliferation.
Neuroendocrine hepatic metastases represent about 10% of all hepatic metastatic neoplasm's.[4] These metastases occur in about 25-90% of patients with neuroendocrine tumours. Although these tumours run a rather indolent course, the 5-year survival of patients with neuroendocrine tumours and liver metastases is 40% compared with 75-99% in those free of liver metastases.[5-7] Neuroendocrine liver metastases often progress slowly but may cause significant symptoms due to their size and/or hormone production. Ultimately the hepatic tissue is replaced by tumour, causing mechanical pressure to surrounding tissues, liver dysfunction, cardiac failure and finally death. Manifestations of the carcinoid syndrome usually occur in patients with liver metastases due to production and release of serotonin directly in the blood stream. Classically, the carcinoid syndrome is characterised by episodic flushing, tachycardia, diarrhoea and bronchospasm.[8] Treatment of neuroendocrine hepatic metastases is aiming at symptomatic improvement and reduction of hormonal hypersecretion by elimination of the tumour. However, the most effective management and timing of treatment remains unclear.[9,10] Here, we have reviewed the literature and used our own experience to provide a balanced guideline for imaging and management of patients with neuroendocrine hepatic metastases.

Biochemical diagnosis

Neuroendocrine tumours of the small intestine produce large quantities of serotonin (5-hydroxytryptamine), reflected in raised levels of platelet serotonin and a high urinary excretion of 5-hydroxyindoleacetic acid (5-HIAA).[11,12] The platelet serotonin concentration is more sensitive in the detection of carcinoid tumours than urinary 5-HIAA, particularly in tumours with relatively low serotonin production.[13,14] Circulating free serotonin is removed very rapidly and effectively by the liver. In contrast to urinary 5-HIAA, platelet serotonin is not effected by serotonin-containing diet.[15] Hence platelet serotonin is the most discriminating marker for detection of most neuroendocrine tumours. However, in hindgut carcinoids, hydroxylase and decarboxylase are absent and no serotonin is produced.
Plasma chromogranin A (CgA) has been claimed the most sensitive and specific marker of tumour volume.[16] CgA is a precursor for several peptides and is stored in secretory granules of neuroendocrine tissue.[17] Circulating CgA allows early detection of persistent or recurrent neuroendocrine tumours.[18] The highest CgA levels were noted in metastatic midgut lesions.[19] Both tumour markers, platelet serotonin and CgA, can be reliably used for diagnosis of neuroendocrine tumour and for monitoring the outcome of treatment in individual patients.

Work-up of patients with neuroendocrine hepatic metastases

Several imaging modalities are available to detect hepatic metastases and their primary neuroendocrine tumours. Conventional ultrasonography (US), computed tomography (CT), magnetic resonance imaging (MRI) and somatostatin receptor scintigraphy (SRS) are the cornerstones for the localisation of neuroendocrine tumours with sensitivities of respectively 46%, 42%, 43% and 90%.[20-23] The use of helical computed tomography (hCT) has increased the diagnostic sensitivity in the localisation of both primary (94%) and metastatic tumour (lymph node 69%, liver 94%).[24] As somatostatin receptor subtype 2 is present in almost 80% of neuroendocrine tumours, binding 111Indium-labelled octreotide can be used for both disease staging and to indicate whether or not somatostatin analogues can be used in the treatment of these tumours.[25] SRS is very helpful in detecting bone and lung metastases and thereby aids in confirming or refuting the presence of extrahepatic disease. Based on these considerations, both hCT and SRS should be performed in all patients prior to treatment.

Treatment modalities

Surgical resection

Surgical resection is to be considered when no extrahepatic disease is present. Hemihepatectomy or segmental resection is feasible when metastases are solitary and resection can be radical with enough functional liver tissue remaining. Symptomatic response rates have been reported to be 90% for a mean duration of 19.3 months after surgical resection.[26] Unfortunately, neuroendocrine metastases are usually multiple and diffuse and therefore resection is often impossible. Furthermore, in most patients treated by surgical resection with curative intent, additional metastases develop that presumably were occult at the time of surgery.[26] Therefore even in resectable cases, liver tissue sparing therapies should be considered, allowing future repeated treatment.

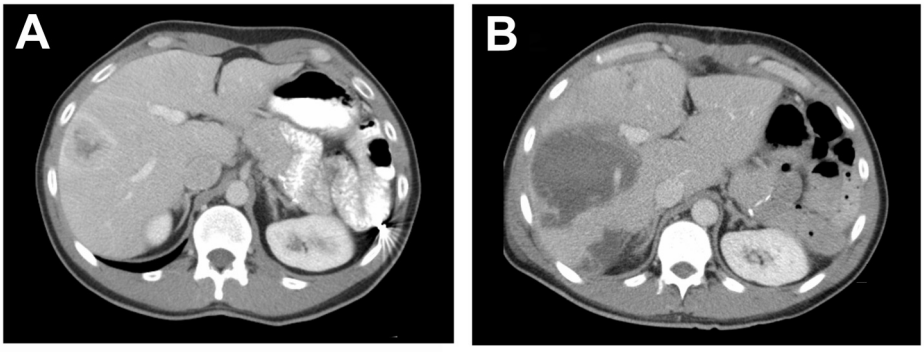

Figure 1. CT scan of the liver of a 34-year old man with metastases of a neuroendocrine tumour of the pancreas. Before LITT the CT scan shows a metastases of 4.7 cm in diameter in segment VII and a second metastases of 2.0 cm in diameter in segment VII subcapsular (not visible) of the liver (**A**). Control CT scan one week after LITT showing a coagulation lesion in segment VII of 9.0 cm in diameter and subcapsular in segment VII of 4.8 cm in diameter (**B**).

Local ablative therapy

Local therapy using radiofrequency ablation (RFA) or laser induced thermotherapy (LITT) is a well-established treatment for unresectable hepatocellular carcinomas and liver metastases from colorectal carcinomas.[27-28] A few small series and case reports have also shown good response in neuroendocrine hepatic metastases.[29-33] Up until now, a disadvantage of these therapies has been the relatively small volume of tissue that can be coagulated. Clinical trials with RFA have shown that complete tumour eradication is more likely to occur with small tumours, i.e. diameter ≤ 4 cm, than with large tumours.[34] With the use of simultaneous multiple fiber LITT or next generation bipolar RFA, we have been able to ablate tumours as large as 7 cm in diameter (**Figure 1**).[35] Furthermore, up to 7 lesions at one time may be ablated using specialized techniques to increase lesion size.[36] It has been reported that cytoreduction of ≥ 90% is adequate for durable symptomatic relief.[9] In our most recent strategy, we aim at complete destruction of the intrahepatic tumour to prevent the occurrence of surgically untreatable disease. The largest reported study of 34 patients with neuroendocrine hepatic metastases treated with RFA showed symptom relief in 95% of these patients with significant or complete symptom control in 80%, for a mean of 10 months.[29] Even in patients with extrahepatic disease and liver metastases ablation may also provide symptom relief.[29] The complication rate is 5-10% and the mortality rate is about 0.5%.[37-39] Therefore these techniques are especially suitable for repeated treatment in patients in which local recurrence or new metastases developing during follow up.

All invasive procedures during surgery such as liver resection and ablation and even anaesthesia can induce hormone release and even provoke a life-threatening carcinoid crisis.[40] In the severe crisis of carcinoid syndrome the flush is usually accompanied by hypotension and occasionally shock. Injection of octreotide, the long-acting analog of somatostatin, usually prevents or aborts this vasomotor reaction.[41] Studies have shown that the use of octreotide intraoperatively for patients with metastatic carcinoid tumours undergoing surgery with manipulation of tumour is associated with a decreased frequency of intraoperative complications.[42,43]

Arterial embolisation

Hepatic arterial embolisation with or without chemotherapy is a palliative option for those unresectable lesions in which the predominant mass of tumour is localised in one of the liver lobes. In the past, more radical blunt techniques to occlude the main hepatic artery were used. However, recently, superselective techniques have become available with the advantage of leaving the main segmental arteries open. Contraindications of hepatic arterial embolisation include complete portal vein occlusion, hepatic failure and previous biliary anastomoses.[44] Symptomatic improvement after hepatic arterial embolisation is reported to occur in 64-90%.[45,46] Reports on chemoembolisation show a slight better biochemical response and tumour response than hepatic artery embolisation.[47] Embolisation techniques are associated with mortality rates of about 5% and almost all patients develop the 'postembolic syndrome' (elevated liver function tests and fever) although mostly transient and in different grades of severity.[48-50] In addition, serious complications have occurred in about 10% of patients treated with hepatic embolisation for neuroendocrine tumours.[51] Complications can be reduced by prophylactic octreotide infusion during the procedure and the use of forced diuresis during and after the embolisation. In case of partial or no response, supplementary embolisation or additional RFA or LITT could be an option. In selected cases with good response to embolisation a partial hepatic resection may be considered.

Pharmacological therapy

Pharmacological therapy consists of long-lasting octreotide injections, Iodine-131 metaiodobenzylguanidine (^{131}I-MIBG), interferon-α (IFN-α) or targeted chemo- and radiotherapy. Octreotide is a somatostatin analogue and appears to be an efficacious treatment for carcinoid syndrome, reducing symptoms in more than 70% of patients.[52,53] Some patients with partial response after local ablation have relief of symptoms by additional treatment with octreotide.[37] Prolonged symptomatic relief can be provided by ^{131}I-MIBG therapy. In individual cases, improved quality of life may be obtained.[54] Even improved survival was seen by symptomatic response to ^{131}I-MIBG treatment.[55] The clinical benefit of IFN-α treatment has been limited by their modest anti-tumour effect as well as serious side-effects.[56,57] In addition, combination treatment with octreotide and IFN-α showed little advantage. Biochemical responses were observed in 72-77%, however no objective tumour regression was observed.[57,58] A promising approach is the concept of somatostatin receptor (SSTR)-mediated chemo-or radiotherapy of SSTR-expressing metastatic carcinoid. Currently, clinical trials with cytotoxic compounds, such as methotrexate and doxorubicin, linked to an analog of somotostatin are under way.[59,60] Also promising is targeted SSRT-mediated radiotherapy using radionuclides such as ^{90}Y and ^{177}Lu. Experimental studies in patients who have somatostatin-positive tumours show complete remission by the use of tetra-azacyclododecane tetra-acetic acid Tyr^3-octreotide.[61] After surgical reduction of tumour load, repeated intermediate-dosage ^{90}Y, Tyr-octreotide, ^{177}Lu or ^{131}I-MIBG treatment appears to be a reliable and well-tolerated radionuclide therapy and might be a useful adjunct in patients with malignant neuroendocrine carcinoma, providing long-lasting palliation and prolonged survival.[62]

Liver transplantation

Young patients with surgically unresectable tumours, hepatomegaly and uncontrollable symptoms, in whom all other therapies have been unsuccessful, may benefit from liver transplantation.[63] However, liver transplantation for metastatic disease is controversial and in most cases even contraindicated, as the results have been poor due to complex operative procedures.[64-66] Well differentiated tumours and a low proliferation rate (Ki67<10%) are important selection criteria.[67] Overall, post-operative mortality of 19% is reported in a group of 31 patients undergoing orthotopic liver transplantation for metastatic neuroendocrine tumours.[65] In the same study, 50% of the carcinoid patients suffered from one or more major complications i.e. peritoneal bleeding, acute/chronic rejection and acute pancreatitis.[65]

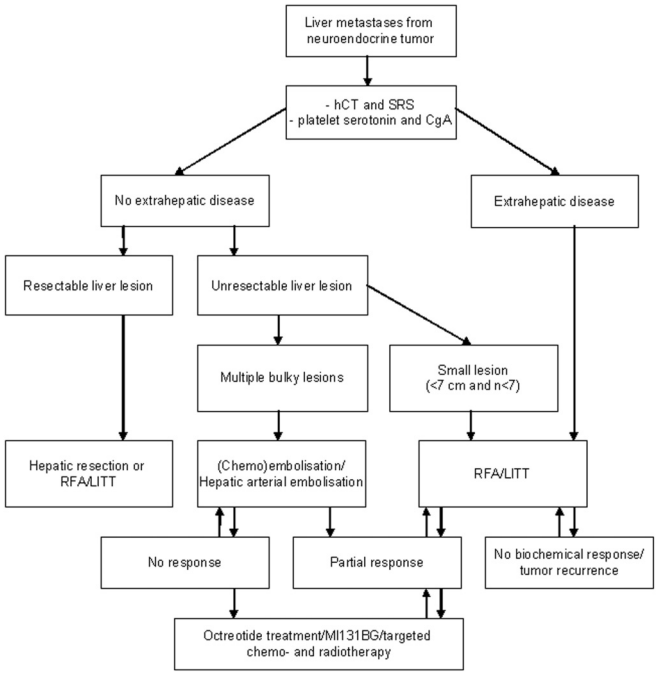

Figure 2. Protocol for management of patients with neuroendocrine hepatic metastases. CT, computed tomography; MRI, magnetic resonance imaging; SRS, somatostatin receptor scintigraphy; RFA, radiofrequency ablation; LITT, laser induced thermotherapy; ^{131}I-MIBG, Iodine-131 metaiodobenzylguanidine.

Conclusions

Hepatic metastases are frequently encountered in patients with digestive endocrine tumours and their presence plays an important role in quality of life and overall prognosis. Tailored multimodality treatment is the key to increase survival and achieve good palliation in patients with hepatic metastases from neuroendocrine tumours. A flow sheet such as presented in **figure 2** can be helpful in the decision of choice of treatment. Determination of platelet serotonin and plasma CgA is useful for detection of neuroendocrine tumour and to evaluate therapy efficiency. Visualisation of neuroendocrine hepatic metastases should be performed by hCT/MRI and SRS. Determination of platelet serotonin and plasma CgA is useful for detection of neuroendocrine tumour and to evaluate therapy efficiency. The proliferation marker Ki67 is a very important tool in guiding the type of treatment. Surgery is the treatment of choice for hepatic metastases however cure is frequently impossible due to the extent of disease. Treatment aimed at cytoreduction of hepatic metastasis and diminished secretion of bioactive amines may achieve good palliation. Tumour destruction by RFA or LITT provides a novel liver preserving option. These techniques will now be used more often as liver preserving option to treat patients early in the course of their disease postponing drug intervention and preventing the end stage carcinoid syndrome and thereby improving life expectancy.

Competing interests

The author(s) declare that they have no competing interests

Authors' contributions

LV reviewed the literature and drafted the manuscript. IBR and CL critically reviewed the paper and were involved in the preparation of the final manuscript. RH was involved in the conception of the work and manuscript preparation. All authors read and approved final version for publication.

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Written consent was obtained from the patient for publication of his case record.

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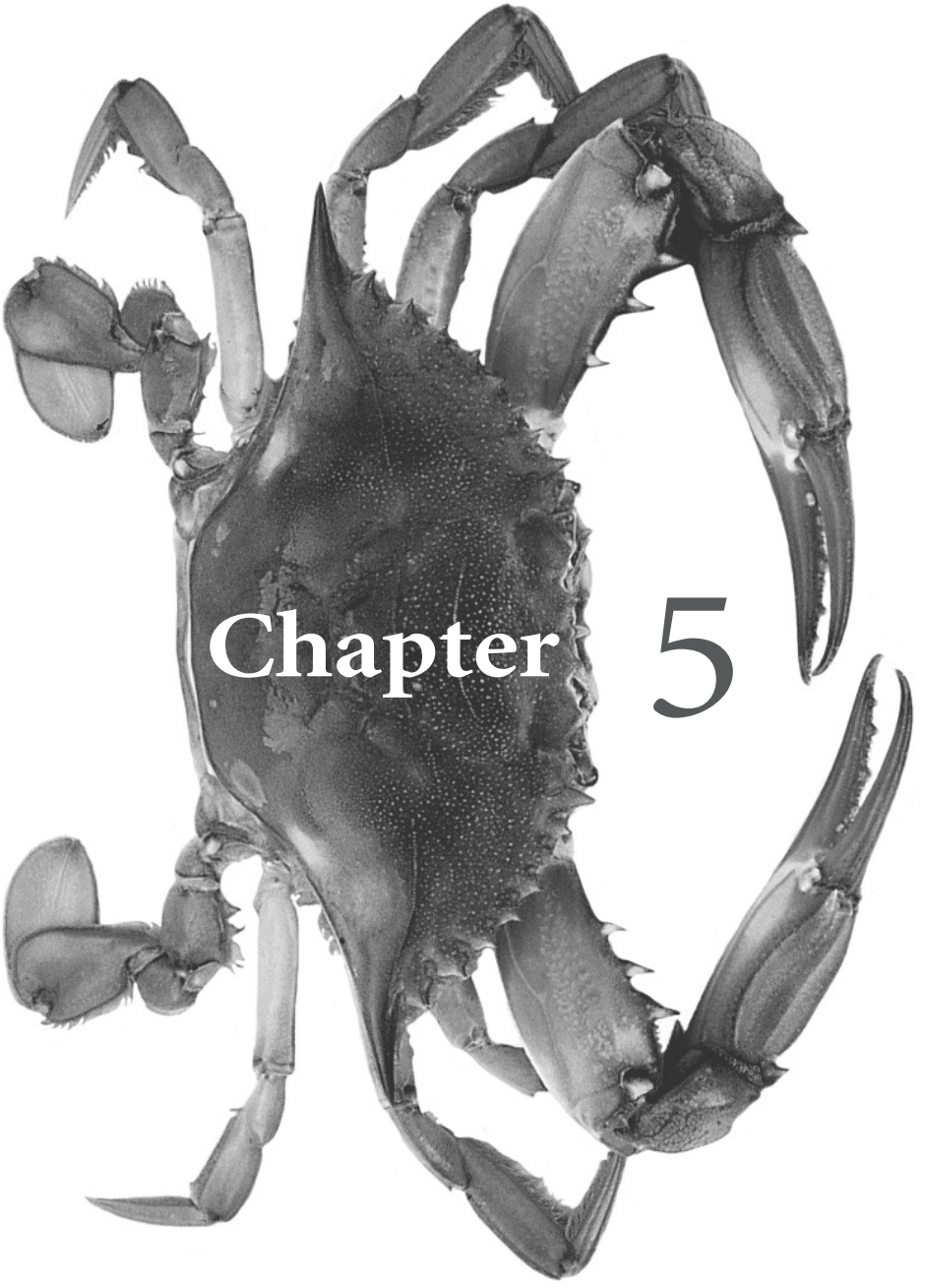

Chapter 5

Synergistic effect of Interstitial Laser Coagulation and Doxorubicin in a Murine Tumor Recurrence Model of Solitary Colorectal Liver Metastasis

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Abstract

Background: Interstitial laser coagulation (ILC) is gaining acceptance for treatment of unresectable colorectal liver metastases. However, local recurrence rates are still high. To overcome this problem we investigated the potential of additional systemic therapy after ILC in a murine model.
Methods: Single C26 colon carcinoma nodules (~1 mm^3) expressing firefly luciferase were implanted in the left liver lobe of 32 Balb/c mice. Seven days post implantation, tumors were treated with either ILC alone (Nd:YAG, 6 W/cm, 800 J/cm) or ILC followed by 1 mg/kg doxorubicin i.v. Controls received either doxorubicin alone or sham treatment. Tumor load was measured by in vivo bioluminescent imaging.
Results: Solitary colorectal liver metastases developed over 7 days following tumor implantation in the liver. Extrahepatic disease was not observed. ILC dose was set to ablate the liver metastases with recurrent tumor growth in 9/16 mice after 7 days. After ILC plus doxorubicin complete tumor destruction occurred without recurrence (0/14). Sham treatment or treatment with doxorubicin alone showed an exponential increase in tumor load.
Conclusion: A murine tumor recurrence model after local ablative treatment of solitary liver metastasis was developed. Combination of ILC and doxorubicin had a strong synergistic effect leading to complete tumor remission in all animals treated.

Introduction

The liver is the most common site of metastatic disease of colorectal carcinoma.[1] The treatment options for patients with colorectal metastases are limited. If possible, surgical resection has been the standard of care, leading to 5 year survival rates of up to 40%.[2-4] However, following radical resection about 30-50% of patients will develop intrahepatic recurrence.[5] In addition, resection is applicable to only a small number of patients due to high tumor stage, unfavorable intrahepatic tumor distribution, comorbidity, or limited residual liver function. For unresectable metastases, radiofrequency ablation (RFA) or interstitial laser coagulation (ILC) may be an alternative approach.[6] These therapies use interstitial application of energy transmitting sources that result in local destruction of the tumor by heat coagulation. However, a key limitation of these techniques is incomplete ablation particularly of larger lesions. Foci of viable tumor at the tumor border can persist even after apparently adequate thermal ablation.[7] Studies on ILC report local recurrence rates of up to 63%.[8-10] Systemic treatment in conjunction with ILC could destroy these remaining tumor foci, thereby leading to a reduction of intrahepatic tumor recurrence. The aim of this study was to investigate this combined strategy in a murine model for solitary liver metastasis. For this purpose a murine tumor recurrence model after ILC was established and evaluated.

Materials And Methods

Animals

Male Balb/c mice (Charles River, The Netherlands), 12 weeks old, were housed under aseptic conditions using filter paper-topped cages and were given standard diet and water ad libitum. Experiments were carried out according to the guidelines of the Animal Welfare Committee of the University Medical Center Utrecht, The Netherlands. Mice were monitored daily using the scoring system provided by the animal facility as described before.[11]

Cells and cell culture conditions

The murine colon carcinoma cell line C26 was transduced with a lentiviral construct containing the firefly luciferase gene under control of the CMV promoter as described earlier.[12] C26-luciferase cells were routinely cultured in Dulbecco's modified Eagle Medium (DMEM; Dulbeco, ICN Pharmaceuticals, Costa Mesa, CA, USA) supplemented with 5% heat-inactivated fetal calf serum, 2mM glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin in a 5% CO_2 environment. The cells were washed once with PBS and then harvested after brief trypsinization (0.05% trypsin in 0.02% EDTA). The single cell suspension was then washed and suspended at a density of 1.0 x10^6 cells/100 µl in PBS and then kept on ice before injecting the cells. Cells were injected in the subcutaneous space of the left and right flank of one mouse in a total volume of 100 µl within 60 min of harvesting.

Intrahepatic tumor implantation

One mouse with flank tumors was sacrificed, after which the tumors were dissected. Necrotic tissue and noncancerous tissue of the specimen was removed and the remaining tumor tissue was divided into small pieces of ~1 mm in diameter with the help of a template and kept in phosphate-buffered saline solution. Mice were anaesthetized with the induction of 4% isoflurane and maintained by ventilation with a mixture of 1.5-2% isoflurane and O_2.

Buprenorfine (3 μg/mouse) was given intramuscularly prior to surgery to provide sufficient analgesia. A midline incision was made, and the left lobe of the liver was exteriorized. A small incision was made through the liver capsule and a piece of tumor was implanted into the liver. The liver was repositioned, and the peritoneum and skin were closed in two layers with 5.0 vicryl sutures.

Measurements of intrahepatic tumor growth

After laparotomy on day 7 after subcapsular implantation, tumor size was measured using a caliper. In addition, tumor growth was assessed on days 6, 9, 14, 19 and 22 after subcapsular implantation by in vivo bioluminescent imaging with a highly sensitive, cooled charge-coupled device camera (VersArray 1300B, Roper Scientific inc., Vianen, The Netherlands) mounted in a light-tight imaging chamber (Roper Science Inc., Vianen, The Netherlands). Imaging and quantification of signals were controlled by the acquisition software MetaVue (Universal Imaging Corporation, Downingtown, USA). Prior to imaging, mice were anaesthetized with an intramuscular injection of KXA (60 mg Ketamine, 2 mg Xylazine, 0.4 mg Atropine per ml, Aescoket Plus, Aesculaap bv, Boxtel, The Netherlands). The substrate D-luciferin sodium salt (Synchem Laborgemeinschaft OHG, Kassel, Germany) dissolved in phosphate-buffered saline (PBS) was injected i.p. at a dose of 125 mg/kg.[13] Mice were then placed onto the stage inside the light-tight camera box. Approximately 5 minutes after the intraperitoneal injection of D-luciferin, the bioluminescent signal had reached maximum intensity and remained fairly constant for over 15 minutes.[13] Therefore, all mice were imaged with an integration time of 5 minutes, exactly 10 minutes after the i.p. injection of D-luciferin. Eight mice were imaged simultaneously. Total photon counts were quantified with MetaMorph software measuring the same delineated abdominal region in each mouse, large enough to fit the largest tumor-bearing liver.

Interstitial Laser Coagulation

A Nd:YAG laser (Medilas 4060 N, MBB, Medizin Technik, München, Germany) was used in all experiments with a wavelength of 1064 nm. The laser light was delivered in the continuous wave mode through a 400 μm fiber, which had a diffuser tip applicator (outer diameter 2.1 mm, active length 5.0 mm) (Trumpf Medizine Systeme, Umkirch, Germany).

Experimental protocol

A laser dose-effect relation was established in ex vivo porcine livers. Porcine livers were heated to 37 °C with warm water sacs and divided into two separate parts. The diffuser tip of the laser was positioned between the two repositioned parts of the porcine liver. ILC was applied at a power setting of 6 or 8 W per cm diffuser length for different time periods ranging from 75 to 225 seconds, corresponding to a total energy output of respectively 600 to 1800 J/cm. Diameter of coagulation lesion was measured using a caliper. Each measurement was repeated three times.

To develop a murine intrahepatic tumor recurrence model, laser treatment was performed on day 7 after tumor implantation (6 W/cm with 800 J/cm). At different time points post treatment mice were examined for liver metastases outgrowth and for possible extrahepatic disease. Mice were sacrificed and the liver was removed and fixed in 4% neutral buffered formalin for 24 h and embedded in paraffin for histological examination.

In the experimental protocol, solitary liver metastases were established in 31 mice. Six days

after tumor implantation tumor load was measured by in vivo bioluminescent imaging, and animals with established liver metastases were randomized into 4 groups. In the treatment groups ILC (n=8) and ILC plus doxorubicin (n=8), laser treatment was performed on day 7 after tumor implantation at a power setting of 6 W per cm diffuser length for 133 seconds, corresponding to a total energy output of 800 J/cm. Doxorubicin treatment consisted of i.v. injections of doxorubicin into the lateral tail vein (Pharmacia and Upjohn, Woerden, The Netherlands) in a dose of 10 mg/kg body-weight on days 9 and 14 after implantation. This is in accordance with the experimental protocols in metastatic murine colorectal cancer used by other groups and by us.[11,14] In the control group (sham, n=7) sham ILC and sham treatment with NaCl i.v. was given. In the doxorubicin group (doxorubicin, n=8) the drug was administered i.v. combined with sham ILC. Sham ILC consisted of laser fibre placement into the tumor. Tumor load was analyzed on day 9, 14, 19 and 22 after tumor implantation by in vivo bioluminescent imaging. On day 22, all animals were sacrificed and livers were harvested and either fixed in 4% neutral buffered formalin for 24 h and embedded in paraffin or snap frozen in liquid nitrogen.

Histological analyses

Formalin-fixed pieces of liver were sectioned into 4 μm slices and embedded completely in paraffin and processed for routine haematoxylin and eosin (H&E) staining. Additional liver sections were frozen in liquid nitrogen for the detection of reduced nicotinamide adenine dinucleotide diaphorase (NADHd), as a marker of mitochondrial and thereby cell viability.[15] For NADHd activity analysis, 8 μm cryostat-cut unfixed sections were placed on glass slides. Incubation media consisted of 1.3-1.5 mM NADH.Na2 in a NBT solution consisting of 1.5 mM Nitroblue tetrazolium, 5 mM MgCl, 5 mM Sodium Azide and 1.7 mM PVP in a 0.2 M phosphate buffer. Each slide was covered with 1 ml of incubation media for 25 minutes at 37 ^{o}C. Each slide was then rinsed with distilled water and post-fixed with 4% buffered formaldehyde, rinsed with water and dehydrated in ethanol, cleared in xylene and coverslipped.

Statistical analysis

Statistical analysis was performed with GraphPad PrismTM version 3.0 for Windows (GraphPad Software, San Diego, California, USA). Statistical differences between groups were analyzed by Mann-Whitney U test and $p<0.05$ was used to denote statistical significance. Values are expressed as mean ± SEM.

Results

Reference dosimetry ILC in ex vivo liver

To establish reproducible dose-response curves for hepatic ILC, porcine livers were used ex vivo. The relationship between diameter of coagulation lesion and energy applied is illustrated in **Figure 1**. Lines with different power output showed a similar slope for increasing energy levels. The lesion diameter enlarged with increasing power output. The coagulation size reached a plateau at 6 W/cm with a total energy output of 1400 J/cm and at 8 W/cm with 1400 J/cm energy output.

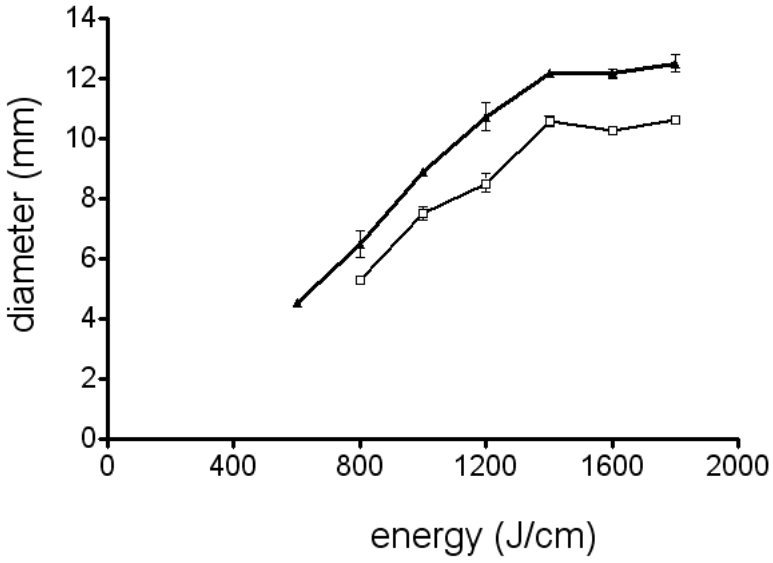

Figure 1. Reference dosimetry curves describing diameter of coagulation lesion versus energy applied by ILC in the porcine liver ex vivo. Each line represents a fixed laser power setting: 6 W/cm (❐), 8 W/cm (▲). Each point represents the mean ± SEM of three experimental results.

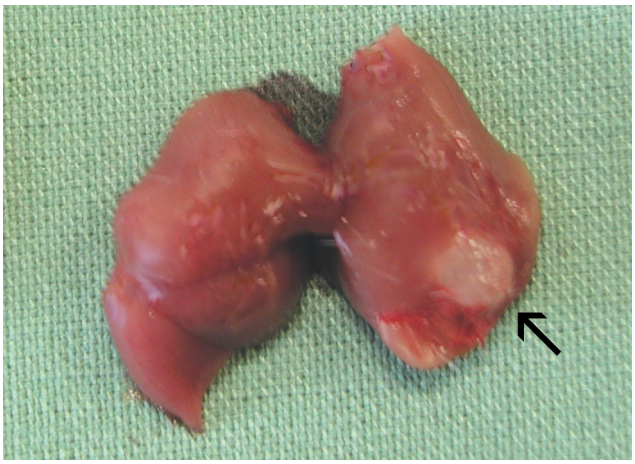

Figure 2. Macroscopic appearance of solitary C26-luciferase colorectal metastasis in the left lobe of the mouse liver, 7 days after implantation (arrow).

Tumor induction and recurrence after ILC

Tumor diameter on day 7 after implantation ranged from 5.3 to 5.8 mm (**Figure 2**). We did not observe any intrahepatic or extrahepatic (peritoneum, lungs) tumor spread. These studies show that these solitary liver metastases are induced in a reproducible way with a remarkably constant size one week post-implantation. Given that the average tumor diameter after day 7 was 5-6 mm, ILC was applied at 800 J/cm and 6 W/cm, representing the setting that could ablate ~5 mm pig liver tissue (**Figure 1**). With these settings tumor recurrence was established in 9/16 mice.

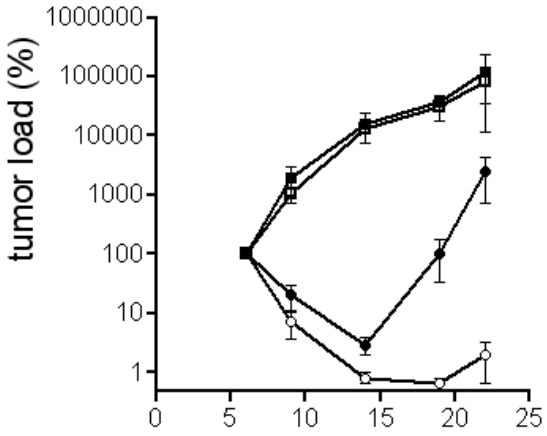

Figure 3. Relative tumor load (mean % ± SEM) at different time points after establishment of solitary C26-luciferase colorectal metastasis in the left lobe of the mouse liver. Bioluminescent imaging measurements on day 9, 14, 19 and 22. ILC or sham treatment was applied on day 7, doxorubicin or vehicle was given on day 9 and 14 by i.v. injections. Sham (■), doxorubicin (❒), ILC (●), ILC plus doxorubicin (❍), n=8 per group.

Effect of ILC plus doxorubicin

Next we investigated the efficacy of tumor ablation by ILC alone, by ILC plus doxorubicin or by doxorubicin alone. Seven days after tumor implantation in the liver, tumor load, as measured by bioluminescent imaging was set at 100% in each animal in all groups. One day later, mice were treated with either ILC or sham and tumor load was analyzed on day 9, 14, 19 and 22 after tumor implantation. As expected, mice treated with ILC alone showed a massive decrease in tumor load, but tumor recurrence was observed from day 14 onwards (**Figure 3**). Mice treated with ILC plus doxorubicin showed complete remission of tumor load (**Figure 3**). The graph in **Figure 3** seems to suggest that tumor load in the ILC plus doxorubicin group increases after 22 days. However, the increase in detected luminescence is very low (from 0.7 to 2%) and is not significant. It should be noted that the y-axis of this graph is in log scale. Additionally, following termination of the experiment all livers were examined for microscopic tumor residue by H&E-stained liver tissue sections. In the ILC plus doxorubicin group we did not find a single lesion in any of the livers examined. Possibly, the apparent increase in bioluminescence is due to the scattered background signal. In the sham group, all animals showed exponential increase in tumor load and a similar pattern was observed after treatment with doxorubicin alone (**Figure 3**). This experiment was repeated with similar results. The pooled data from both experiments showed that tumor recurrence occurred in 9/16 mice treated with ILC alone. We have observed a clear distinction between tumor-related mortality and mortality directly related to the ILC and doxorubicin procedure. In the ILC group tumor recurrence was observed in 6/16 mice (1 on day 19; 1 on day 20; 4 on day 22) (**Table 1**). Autopsy showed that mortality was associated with massive tumor growth in the liver, frequently accompanied by the formation of abscesses and large abdominal lymph nodes. In the ILC plus doxorubicin group 2 mice died (day 16 and 22), possibly due to the combined adverse effects of ILC, doxorubicin and anesthesia. Autopsy did not reveal a clear cause of death but did show that (macroscopic) tumor recurrence had not occurred in these mice. Therefore, we concluded that mortality in these mice was not related to tumor growth. In general, we observed that ILC therapy caused morbidity in some of the animals as shown by decreased activity, untreated fur and loss of body weight. Mortality was seen in approximately 50% of all control mice (9/16) (1 on day 9; 3 on day 19; 4 on day 20; 1 on day 22) and doxorubicin treated mice (8/16) (1 on day 15; 3 on day 19; 2 on day 20; 2 on day 22) (**Table 1**), which was invariably attributed to excessive tumor growth.

TABLE 1. Tumor-related mortality and recurrence.

	Tumor-related mortality	Recurrence
ILC plus doxorubicin	0/14*	0/14
ILC	6/16	9/16
Doxorubicin	8/16	n.a.
Sham	9/16	n.a.

** two mice died due to ILC related morbidity*
n.a.: not applicable

Immunohistochemical analyses

On the first day after ILC treatment, H&E staining showed tumor cells with acidophilic cytoplasm and dark elongated nuclei (**Figure 4A and B**). Large cavities surrounded by densely coagulated cells with pyknotic nuclei were observed. These cavities are caused by boiled tissue water which creates bubbles of steam within the tissue. Around the tumor, a sharply demarcated concentric rim of necrotic liver tissue was visible, which consisted of two zones. Zone 1 showed hepatocytes with vacuolated acidophilic cytoplasm (**Figure 4C**). Zone 2 consisted of acidophilic necrosis containing deliquesced cells without nuclei and areas of massive inflammatory cells (**Figure 4D**).

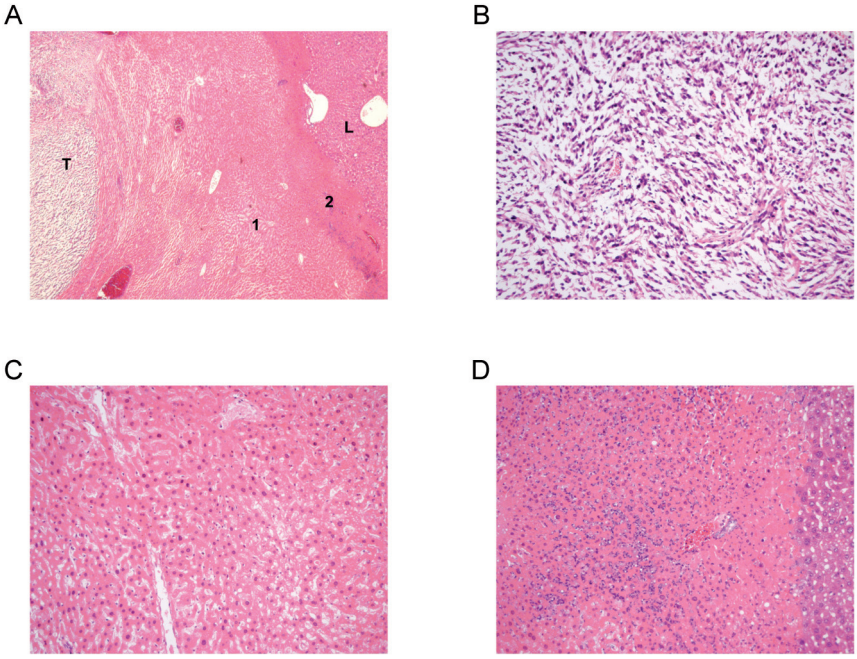

Figure 4. H&E stained sections of a solitary metastasis in the liver one day following ILC treatment, (**A**) Coagulated tumor tissue surrounded by two zones of necrotic liver tissue sharply demarcated from normal tissue. T: tumor tissue, 1: Zone 1, 2: Zone 2, L: normal liver, magnification 20x, (**B**) Tumor cells show characteristically dark elongated nuclei and acidophilic cytoplasm surrounded by large cavities (boiling effect), magnification 100x, (**C**) Zone 1: hepatocytes with vacuolated cytoplasm, magnification 100x, (**D**) Zone 2: deliquesced hepatocytes infiltrated by inflammatory cells, magnification 100x.

In the experimental protocol, tumor tissue in the sham group, the doxorubicin group and the group treated with ILC showed no morphological differences on day 28 as judged by conventional histology (H&E). H&E sections of tumor tissue demonstrated poorly differentiated tumor cells with vascular and lymphatic invasion. All tumors in sham-operated mice showed massive outgrowth with infiltration in adjacent liver tissue. H&E sections on day 28 of the liver of mice treated with ILC plus doxorubicin showed sharply demarcated lesions around the area of laser application (**Figure 5A**). The border between normal and necrotic tissue consisted of a practically straight line. The coagulation area was clearly divided into three zones

(**Figure 5A**). Zone I consisted of necrotic liver tissue. Zone II was a rim of necrotic tumor tissue. Zone III, the inner centre, showed morphologically intact tumor cells (**Figure 5B**). However, enzyme histochemical analysis of cell viability by NADH-diaphorase of this area showed no staining of cells in either of these ILC-ablated zones, consistent with complete loss of cell viability (**Figure 5C**). This was seen in all mice treated with ILC plus doxorubicin. Adjacent liver tissue demonstrated normal liver architecture with strong NADH activity. In contrast, H&E sections on day 28 of the liver of mice in the group treated with ILC showed tumor recurrence at the border of the coagulation area in 9/16 mice. Cell viability analysis by NADH-diaphorase showed a border of blue stained viable tumor cells around the ILC-ablated region (**Figure 5D**).

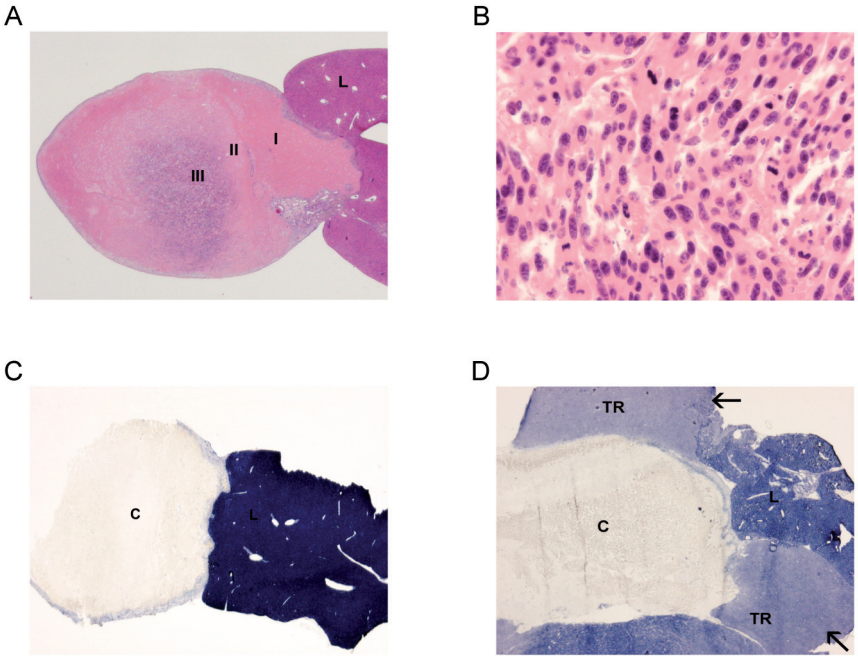

Figure 5. H&E stained sections of a solitary liver metastasis 22 days following treatment with ILC plus doxorubicin, (**A**) The coagulation area divided into three different zones. I: zone I, II: zone 2, III: zone 3, L: normal liver, magnification 10x, (**B**) Morphologically intact tumor cells in zone III which are in a in a frozen state caused by heat fixation, magnification 400x, (**C**) NAHD staining on day 22 of tumor treated with the combination of ILC plus doxorubicin, magnification 10x. No tumor recurrence is encountered. Neither zone 1 nor zone 2 or 3 contain live tumor or liver tissue, (**D**) NAHD staining on day 22 of tumor treated with ILC alone showing tumor recurrence on the border (arrow). magnification 10x, L: normal liver, C: coagulation area, TR: tumor recurrence.

Discussion

As intrahepatic recurrence is common after local ablative therapies for colorectal hepatic metastases, novel strategies are needed to improve the antitumoral efficacy of such treatment modalities. We established a murine tumor model for solitary liver metastasis in which recurrent tumor growth occurs after ILC. Most mouse models for colorectal liver metastases have traditionally been based on intrasplenic or intraportal injection of tumor cell suspensions, thus rendering small, multifocal liver metastases.[11,16-18] There have been a few reports on experimental therapy with intrahepatic tumor implantation models. However, most of these were short-term experiments due to either lung metastases or peritoneal tumor seeding or both.[19,20] All other intrahepatic tumor implantation models were established in rats.[21,22] In our highly reproducible model for solitary liver metastasis in mice, tumor growth was assessed using non-invasive bioluminescence measurements. We have previously reported that luciferase imaging is a reliable method for measuring tumor growth in the liver without affecting tumor cell viability or the kinetics of tumor growth.[12] The use of C26-luciferase allowed us to asses the effect of ILC and chemotherapy on tumor growth over time in a non-invasive manner. The prognosis of patients with liver metastases from colorectal cancer is poor.[23-25] Surgical resection provides the only hope for cure, but only 10 to 20% are eligible for surgery.[2,4] In patients not eligible for surgery, locally ablative techniques, such as radiofrequency ablation (RFA) or ILC may still offer a survival benefit.[6,26] ILC has a number of advantages over surgical resection including a minimally invasive approach associated with significantly lower morbidity and mortality rates.[2,27-29] However, reported recurrence rates vary from 0 to 63%, probably related to either the failure to achieve adequate coagulation of the lesion, or the presence of micrometastases.[8,9,29] These micrometastases may be seeded from primary lesions or originate from the macrometastatic lesions as satellite lesions.[5] Therefore, there is a need for an efficient, additional treatment that destroys any residual disease following tumor ablation. We postulate that complete remission in the combined treatment group results from synergy between sublethal thermal tissue damage at the rim of the tumor induced by ILC and the doxorubicin chemotherapy. In our experimental protocol, microscopic examination with H&E staining of tumor tissue treated by ILC plus doxorubicin demonstrated well-defined areas of characteristic cautery effects evidenced by acellular coagulum. The observed pathological features in the coagulated zone are in accordance with our previous work and that of others.[21,30] Enzyme histochemical analysis of cell viability by NADH-diaphorase showed no staining of tumor cells in the inner centre of the coagulation region, consistent with complete loss of tumor cell viability. However, morphologically these cells are only subvital and may not become necrotic due to "heat fixation". We found that additional treatment with doxorubicin could completely prevent intrahepatic tumor recurrence, whereas after ILC treatment without chemotherapy tumor recurrence occurred in almost all animals. The combination of ablation therapy with systemic antitumor therapy may be very attractive, as the remaining viable cells in the well perfused periphery of the tumor are particularly sensitive for chemotherapeutic agents.[31-33]

Tumor-related mortality was seen in ~50% of sham-operated mice irrespective of the use of doxorubicin. After ILC, tumor-related mortality was decreased and completely abrogated when ILC was combined with chemotherapy. However, we also observed morbidity and mortality which was not attributed to tumor growth. This is possibly due to the strong inflammatory response to tissue and necrosis induced by ILC.

In this model, doxorubicin alone had no effect on tumor growth, probably due to the fact that the initial tumor was too large for effective treatment. In contrast, in a mouse model of diffuse colorectal liver metastases induced by intrasplenic injection of C26 tumor cells, we have previously shown that doxorubicin (10 mg/kg body-weight) induced a significant decrease of tumor hepatic replacement area.[11] This might explain the successful antitumor effect of doxorubicin in minimal residual disease after ILC in this study. The use of an intravenous chemotherapeutic delivery route has several characteristics that would be potentially beneficial for its use in clinical practice. Most importantly, the known increased vascular permeability of tumors treated with hyperthermia is likely responsible for the maintenance of effectiveness for several days after administration.[34,35]

In conclusion, our study demonstrated a synergistic effect of doxorubicin after ILC treatment. Several mechanisms may underlie the observed synergy between ILC and doxorubicin treatment. First, a smaller tumor volume at the start of doxorubicin treatment (following ILC) is likely to be more effectively eradicated than a large tumor volume (no ILC). This notion is supported by our earlier findings that C26 micrometastases are effectively suppressed by doxorubicin.[11] Second, tumor cells exposed to hyperthermia (ILC) may have become sensitized to doxorubicin-induced cell death.[36] Third, an increase in vascular permeability following ILC may lead to more efficient targeting of tumor cells by doxorubicin.[34,35] Obviously, the above-mentioned possibilities are not mutually exclusive. Future experiments should reveal whether tumor cells in the transition zone surrounding the ablated area are better accessible and/or sensitized to chemotherapeutics.

Our results support the concept that combined ILC and adjuvant chemotherapeutic treatment can increase the extent of tumor destruction. The results of this study may provide a basis for further clinical investigation of combined treatment with local ablation plus chemotherapy in patients with colorectal liver metastases.

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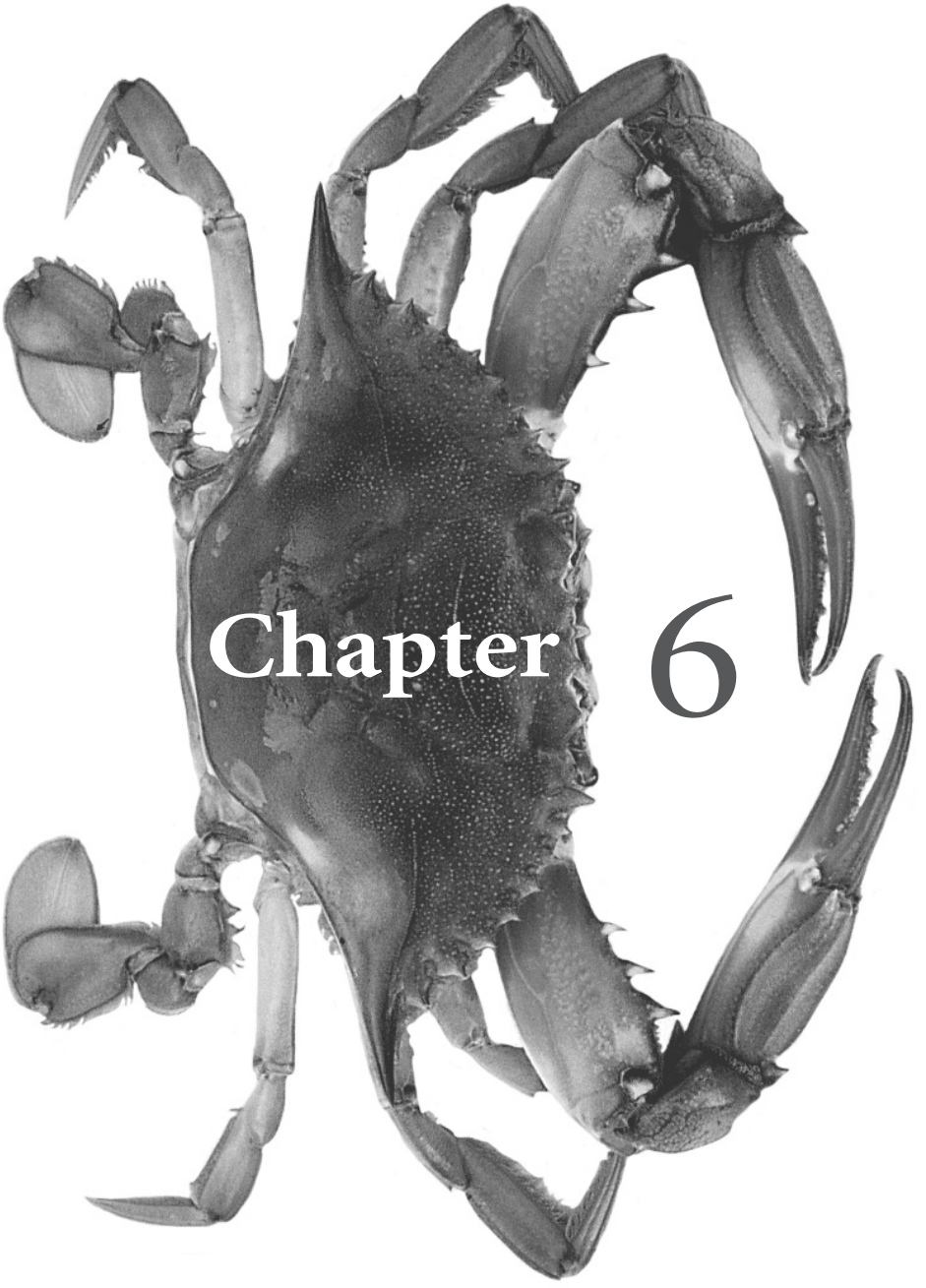
Part

II

Molecular characteristics of colorectal liver metastases



Chapter 6

Dual effect of Kras^{D12} knockdown on tumorigenesis: increased immune-mediated tumor clearance and abrogation of tumor malignancy

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Abstract

Activating mutations in the human KRAS proto-oncogene are acquired during the earliest stages of colorectal cancer development. If mutant KRAS is to be used as a target for therapy in colorectal cancer, tumor growth should depend on its continued presence. Here we report that stable knockdown of $Kras^{D12}$ in murine C26 colorectal cancer cells by RNA interference resulted in loss of transformed properties in vitro. The incidence of subcutaneous tumor formation was reduced by 60% and the lag time was increased 7-fold. $Kras^{D12}$-knockdown tumors grew non-invasively and did not cause morbidity. Remarkably, some of the $Kras^{D12}$-knockdown tumors regressed spontaneously, which rendered these mice resistant to parental C26 tumor growth. In immune-deficient hosts the incidence of tumor formation by $Kras^{D12}$-knockdown cells was 100%. None of these tumors regressed spontaneously. We conclude that the reduced incidence of tumor formation by $Kras^{D12}$-knockdown cells is due to tumor cell clearance by the host immune system, but not to an intrinsic inability of these cells to grow out as as tumors. Interestingly, $Kras^{D12}$ knockdown resulted in increased production of interleukin 18 (IL-18), an immune-stimulatory cytokine that has been implicated in limiting colorectal tumor formation. Thus, mutant $Kras^{D12}$ suppresses IL-18 production in colorectal tumor cells, which may contribute to evasion of the local immune system during tumor development.

Main Text

C26 is an aggressive murine colorectal cancer (CRC) cell line that is widely used for studying CRC growth and metastasis formation. The cell line was originally established from an N-nitroso-N-methylurea (NMU)-induced colorectal carcinoma in BALB/c mice.[1] The KRAS mutation status of many human but not murine CRC cell lines is known. Therefore, we analyzed the expression and activity of the three ras isoforms (H-, K- and N-ras) in C26 cells. Hras was not expressed in C26 cells, nor in any of the other human and mouse CRC cell lines analyzed. Nras was highly expressed but was not active, and Kras was expressed and constitutively active (**Figure 1A**). We next analyzed the C26 Kras gene for activating mutations by RT-PCR and sequence analysis. Whereas codons 13 and 61 showed the wild-type Kras sequence, codon 12 contained a point mutation (GGT>GAT) that results in a G12D amino acid substitution (**Figure 1B**). Alkylating N-nitroso compounds primarily cause G>A point mutations.[2,3] Since activating mutations in Kras contribute to tumor initiation, it seems likely that NMU-induced mutational activation of the Kras gene has been a major causative event during development of the original C26 tumor. G12D is also the most frequently found activating KRAS mutation in human CRC.[4-6]

To test the contribution of mutant $Kras^{D12}$ to the transformed and tumorigenic properties of C26 cells, we targeted the $Kras^{D12}$ allele by RNA interference using a lentiviral vector. We isolated a set of cell lines in which Kras, but not Nras, was stably suppressed (C26-KrasKD). As a control, we established cell lines using lentiviruses that were produced using the empty lentiviral pLL3.7 vector (C26-pLL) (**Figure 1C**). C26 cells have a spindle-shaped morphology, characteristic for many transformed cells (**Figure 1D**). Knockdown of $Kras^{D12}$ resulted in loss of the spindle shape and cells appeared flattened and enlarged when compared to parental control cells or to cells transduced with control lentivirus (**Figure 1D**). In the set of stable cell lines that were isolated we noted that the morphological reversion strictly correlated with successful Kras knockdown. The in vitro proliferation rate of C26-KrasKD cells was approximately 4-fold lower than that of the C26-pLL control cells (**Figure 1E**). Analysis of the cell cycle profile showed that $Kras^{D12}$ knockdown prolonged G1 relative to the S and G2/M phases of the cell cycle (**Figure 1F**). Taken together, the results show that $Kras^{D12}$ knockdown produced a dramatic reversion of the transformed phenotype in these aggressively growing CRC cells *in vitro*.

Next, we analyzed the effect of $Kras^{D12}$ knockdown on the tumorigenic potential of C26 cells *in vivo*. To this end, we injected C26-pLL and C26-KrasKD cells subcutaneously into the flanks of syngenic BALB/c mice. C26-pLL control cells rapidly produced visible tumors with a lag time of about 6-7 days. Within 21 days the tumors had reached a volume of more than 1000 mm^3 with necrosis of the overlying skin and signs of local inflammation. Over time, the health of the mice deteriorated and eventually they had to be sacrificed within 24 days following tumor cell injection. In contrast, 60% of mice (15 of 25) that were injected with C26-KrasKD cells failed to develop macroscopic tumors throughout the course of the experiment which was ended 100 days after tumor cell injection (**Table 1**). The remaining 40% of the mice (10/25) did develop tumors, but the mean lag time was extended to 44.4 days (**Table 1**). In contrast to the aggressively growing C26-pLL tumors, the tumors formed by C26-KrasKD cells grew slowly and indolently over time with the overlying skin remaining intact. Despite the fact that C26-KrasKD tumors grew to far larger volumes than C26-pLL control tumors, the mice remained in excellent condition and their health status remained unaffected (**Figure 2A, Table 1**). On cross sectioning and microscopic examination of Haematoxylin and Eosin

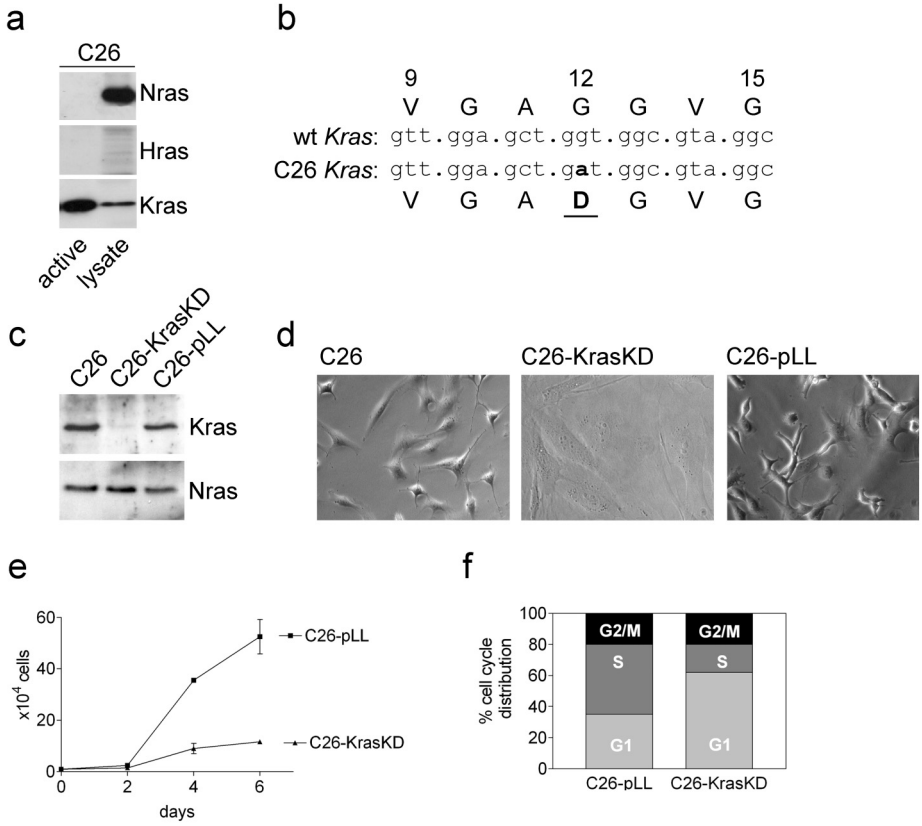


Figure 1. C26 cells loose their transformed characteristics upon Kras^{D12} knockdown. (**A**) Exponentially growing C26 cells were serum-starved overnight and were lysed. The expression and activity of Hras, Nras and Kras was then assessed by the Ras activity assay and subsequent Western blotting, using isoform-specific antibodies (Santa Cruz Biotechnology, F235, F155 and F234) exactly as described.[17] Nras is expressed but inactive. Hras is not expressed. Kras is expressed and constitutively active. (**B**) Total RNA was isolated from C26 cells using RNAzol B. 1 μg total RNA was reverse transcribed with random hexamers to obtain cDNA for PCR with forward (5'-atgactgagtataaacttgtg) and reverse (5'-tcacataactgtacaccttgtcc) primers, yielding a 541 bp product. DNA was isolated from agarose gels (Zymoclean Gel DNA Recovery Kit; Zymo Research, Orange, CA) and was sequenced using primer 5'-gtattatttatggcaaatacac and the Big Dye terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Warrington UK), according to the manufacturer's instructions. Analysis of the products was performed on an ABI Prism 377 DNA Sequencer (PE Biosystems) and revealed an activating point-mutation in codon 12 (GGT>GAT), resulting in a G>D amino acid substitution. (**C**) pLentiLox 3.7 (pLL3.7) (kindly provided by Prof. van Parijs[18]), was digested with XhoI and HpaI and the annealed oligos 5'tGTTGGAGCTGATGGCGTAG-ttcaagaga-CTACGCCATCAGCTCCAAC-ttttttc3' and 5'-tcgagaaaaaa-GTTGGAGCTGATGGCGTAG-tctcttgaa-CTACGCCATCAGCTCCAACa-3' were ligated into pLL3.7 to yield a Kras^{D12}-directed shRNA-producing vector. The 19 nt Kras^{D12} target sequences are indicated in capitals in the oligonucleotide sequence; the G-A mutation that generates the Gly-Asp substitution in the twelfth amino acid of Kras is underlined. Lentiviruses were produced as described.[19] Exponentially growing C26 cells were transduced either by control lentivirus or by lentivi-

rus targeting $Kras^{D12}$ using plasmids encoding the lentiviral gag and pol elements (pMDLgpRRE), the rev protein (pRSV-Rev) and the viral envelope (pMD2G), all kindly provided by Prof. D. Trono. Following isolation of stable clonal cell lines, knockdown of mutant Kras (C26-KrasKD) was tested by Western blot analysis for Kras and Nras. Parental (C26) and control (C26-pLL) cells are shown as a reference. (**D**) Light microscopic images (10x40) of parental, control (C26-pLL) and Kras knockdown (C26-KrasKD) cells. C26-KrasKD cells are larger and flatter and have lost the characteristic spindle-shaped appearance of Ras-transformed cells. (**E**) Control (C26-pLL) and Kras knockdown (C26-KrasKD) cells were plated at a 10^4 cells/9.6 cm^2 density in 6-well plates, in medium containing 5% fetal calf serum. Cells were trypsinized and counted in a blinded manner 2, 4, and 6 days after plating. Results shown are from 2 independent experiments, performed in triplicate. (**F**) Exponentially growing C26-pLL and C26-KrasKD cells were labeled with BrdU (50 μM) for 10 min. and were subsequently processed for FACScan analysis using FITC-labeled anti-BrdU (Boehringer, Mannheim) and propidium iodide. Fluorescence was measured on a FACSCalibur (Becton Dickinson) and the data were analyzed using CellQuest software (Becton Dickinson).

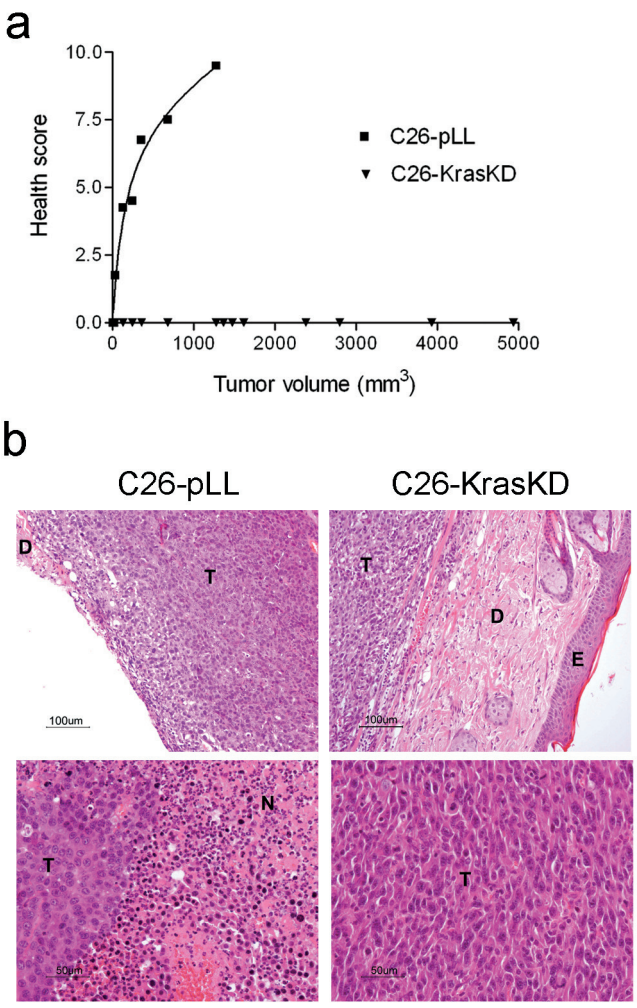
(H&E)-stained tissue sections, C26-pLL tumors showed massive epidermal invasion, whereas the skin overlying C26-KrasKD tumors remained intact and was not invaded (**Figure 2B, upper panel, Table 1**). Towards the centre of C26-pLL tumors large necrotic areas were observed. In contrast, C26-KrasKD tumor tissue was healthy throughout the entire tumor (**Figure 2B, lower panel**). C26-pLL tumors are characterized as poorly differentiated carcinomas with no signs of tubule formation or mucus production[7] (**Figure 2B**). We noted that Kras knockdown had no apparent effect on the histological differentiation grade (**Figure 2B, lower panel**). The effect of RAS deletion on the differentiation state, invasiveness, immunogenicity and morbidity of experimental tumors has so far not been investigated. It has previously been reported that deletion of the KRAS gene or mRNA from human tumor cells completely abolished tumor growth.[8,9] This is in apparent contrast to our results showing delayed non-malignant tumor growth of Kras-suppressed C26 cells. Our results are supported by another study showing that loss of NRAS from human HT1080 fibrosarcoma cells or deletion of KRAS from human DLD-1 colorectal carcinoma cells does not abrogate their tumorigenic potential in immune-deficient mice.[10] How may these discrepancies be reconciled? First, given the non-identical genetic background of the different cell lines they may differ in their dependency on endogenous mutant KRAS/Kras. A second explanation could be that tumor growth may have been missed in some of these studies due to termination of the experiments prior to a prolonged lag time.

C26 cells are poorly immunogenic and invariably produce tumors when injected into syngenic BALB/c mice without eliciting a detectable host CTL response.[11,12] During the course of the experiments 3 out of 10 C26-KrasKD tumors underwent spontaneous regression after having reached a volume of approximately 400 mm^3 (**Table 1**). We reasoned that, if tumor regression was due to an anti-tumor immune response, the mice may have become immunized against the parental C26 cells. Indeed, we found that the C26-KrasKD 'regressor' mice failed to develop tumors following subcutaneous injection of wild-type C26 cells, while control mice developed large subcutaneous tumors within 15 days (**Figure 3A**). Furthermore, depletion of CD8+ T-cells from the regressor mice by repeated injections of anti-CD8 antibody allowed subcutaneous C26 tumor growth following re-injection of tumor cells (**Figure 3B**). Although the number of mice in these experiments (n=3) is too low to draw firm conclusions, the results suggested that the failure of Kras knockdown cells to efficiently develop tumors could be due, at least in part, to tumor cell clearance by the host immune system.

To test this hypothesis, we injected C26-pLL control cells and C26-KrasKD cells into wild-type and immune-deficient BALB/c mice. C26 control cells formed aggressively growing tumors in 100% of the wild-type and in 100% of the immune-deficient mice with a similar mean lag time of 6.4 and 6.5 days respectively (**Table 1**). This result is consistent with the notion that the parental C26 cells are poorly immunogenic. In both groups of mice tumor growth had a strong adverse effect on animal health. In contrast to C26-pLL cells, C26-KrasKD cells formed tumors in only 40% of immune-competent mice, but the incidence in immune-deficient mice was 100% (**Table 1**). Furthermore, the lag time of 33.8 days in immune-deficient mice was significantly ($p<0.05$) shorter than the lag time in immune-competent mice (44.4 days). Partial clearance of the injected C26-KrasKD cells by immune-competent hosts may explain this difference. The slower kinetics with which Kras-suppressed tumors grow out allows more time for the generation of a CTL response and T helper cell production. In addition, a 4-5 fold increase in cell surface expression of H-2Kd MHC class I molecules in $Kras^{D12}$ knockdown cells (our unpublished results) may further facilitate tumor cell

clearance.[13] We conclude that the reduced incidence of tumor formation as a result of Kras^{D12} knockdown is most likely due to tumor cell clearance by the host immune system and not to an intrinsic inability of these cells to grow out as tumors.

Figure 2. Kras^{D12} knockdown delays subcutaneous tumor formation and prevents tumor-associated disease. (**A**) C26-pLL and C26-KrasKD cells (10^6) were injected subcutaneously and standard caliper measurements were performed thrice weekly. Tumor volumes were calculated using the following equation: volume (mm^3)= $AxB^2x0.5236$, where A is the largest dimension and B is the diameter perpendicular to A. The health status of all individual mice was assessed by using the 'clinical appearance scoring system' which analyses appearance, behavior, reactivity and body weight.[20] An increased score in this system signifies increased morbidity (i.e. 0=healthy). Health score is plotted as a function of C26-pLL or C26-KrasKD tumor volume. (**B**) C26-pLL tumors (day 21) and C26-KrasKD tumors (day 91) were excised, fixed in formalin and processed for standard H&E histochemistry. Excessive dermal invasion and large central necrotic areas were observed in C26-pLL tumors, but not in C26-KrasKD tumors. Kras knockdown had no overt effect on the differentiation grade. E: epidermis, D: dermis, T: tumor, N: necrotic area. Represen-tative pictures are shown. Bars upper panel=100μm; Bars lower panel=50μm. These experiments were performed in accordance with the guidelines of the Animal Experimental Committee, University Medical Center Utrecht, the Netherlands.



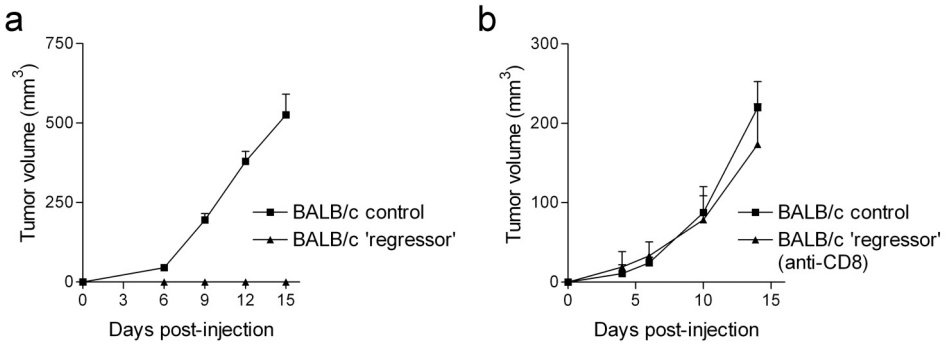


Figure 3. CD8-dependent anti-tumor immunity in C26-KrasKD regressor mice. (**A**) The regressor mice described above (n=3) and 2 control mice were challenged with 10^6 parental C26 cells by subcutaneous injection. Tumor growth was then followed over time by standard caliper measurements 6, 9, 12 and 15 days post injection. After 15 days the control mice had developed large subcutaneous tumors. The regressor mice did not develop tumors. (**B**) The regressor mice received intraperitoneal injections of anti-CD8 antibody (clone 2.43; 100 μg) on days –7, –3 and 4 hours prior to injection of 10^6 wt C26 tumor cells on day 0. The mice received two additional doses anti-CD8 antibody (100 μg) on days 3 and 7 after tumor cell injection. Tumor growth was measured by standard caliper measurements. These experiments were performed in accordance with the guidelines of the Animal Experimental Committee, University Medical Center Utrecht, the Netherlands.

TABLE 1. TUMOR INCIDENCE IN IMMUNE-COMPETENT AND IMMUNE-DEFICIENT MICE

Mouse strain	Cell line	Number of mice	Tumor incidence n (%)	Lag time (days ± SEM)	Regression n (%)	Host morbidity	Dermal invasion
Immune-competent	C26-pLL	9	9 (100)	6,4 ± 0,4	0 (0)	Yes	Yes
	C26-KrasKD	25	10 (40)	44,4 ± 5,0	3 (30)	No	No
Immune-deficient	C26-pLL	4	4 (100)	6,5 ± 0,5	0 (0)	Yes	Yes
	C26-KrasKD	12	12 (100)	33,8 ± 0,9	0 (0)	No	No

C26-pLL and C26-KrasKD cells (10^6) were injected subcutaneously into immune-competent male BALB/c mice and into athymic immune-deficient male BALB/cAnNCrl-NuBR mice, aged 10 weeks. Tumor growth was assessed thrice weekly until the end of the experiment on day 100 post-injection and the incidence of tumor formation was calculated. The lag time was defined as the time (in days) from the injection until the first signs of macroscopic tumor growth were detected. Regression of established macroscopic tumors was seen in 3 out of 10 mice. Host morbidity was classified 'Yes' when the health status of the host was compromised as described in **Figure 2A**. Dermal invasion was analyzed on H&E-stained tissue sections. These experiments were performed in accordance with the guidelines of the Animal Experimental Committee, University Medical Center Utrecht, the Netherlands.

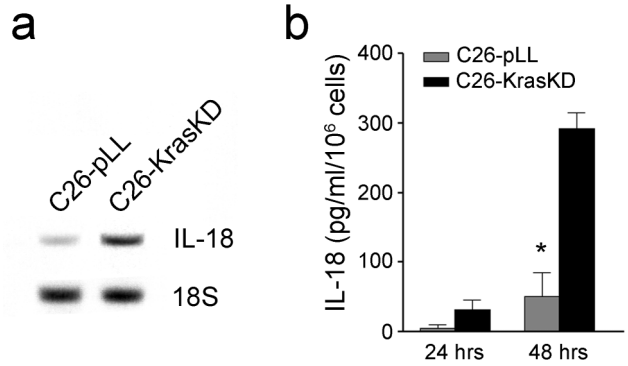


Figure 4. Kras^{D12} suppresses IL-18. (**A**) Total RNA was isolated (Trizol) and 10 μg aliquots were used for reverse transcription. PCR analysis (30 cycles) was performed using primer pairs designed to amplify mouse IL-18 (forward: 5'-actgtacaaccgcagtaatacg, reverse: 5'-agtgaacattacagatttatccc), and 18S rRNA (forward: 5'-agttggtggagcgatttgtc, reverse: 5'-tattgctcaatctcgggtgg) at 94 °C (30 sec), 53 °C (30 sec) and 72 °C (1 min.). (**B**) C26-pLL and C26-KrasKD cells were grown at 10^6 cells/200 μl complete DMEM for either 24 h or 48 h. The medium was harvested and IL-18 levels were measured using the IL-18 enzyme linked immunosorbent assay (ELISA) kit (MBL, Naka-ku Nagoya, Japan). The cells were harvested and counted. The bar diagram shows the production IL-18 per 10^6 cells after 24 h and 48 h. The asterisk indicated statistical significance ($p<0.05$).

To gain more insight into a possible role for Kras in modulating tumor immunity we performed a comparative cDNA microarray analysis of the gene expression profiles of parental C26 and Kras^{D12} knockdown cells. cDNA preparations of both cell types were labeled with either Cy-3 or Cy-5 and were competitively hybridized on 20K Agilent Mouse Development Chips. Technical variation was compensated for the performing dye swaps. We found that one of the most strongly upregulated genes (11.7 fold) following Kras knockdown was the gene encoding interleuking -18 (IL-18). This result was confirmed by RT-PCR analysis of IL-18 mRNA levels (**Figure 4A**). Furthermore, ELISA analysis of IL-18 levels in the conditioned media of both cell types indicated that Kras^{D12} knockdown resulted in a drastic increase in IL-18 secretion (**Figure 4B**).

IL-18 is produced and released by activated macrophages, Kupffer cells, dendritic cells, Langerhans cells and B-cells.[14,15] Importantly, it is also produced by epithelial cells of the gastrointestinal tract, the skin and the airway, where it has been implicated in the host immune defense against tumor development.[14,15] In line with this, IL-18 mRNA and protein are strongly reduced during colorectal tumor formation.[14,15] Interestingly, C26 tumor growth in the liver can be strongly suppressed by IL-18 producing hepatocytes, which was associated with increased cytolytic activity of splenic CTLs towards C26 cells.[16] This demonstrates the dramatic impact of local IL-18 production on C26 tumor development.

Our results indicate that endogenous mutant Kras, although acquired at the initial stage of C26 tumor development, remains essential for the tumorigenic properties of this highly aggressive CRC cell line, but not for tumor growth per se. Furthermore, the Kras oncogene controls initiation and maintenance of tumor growth by evasion of the host immune system. The strong suppression of IL-18 by mutant Kras in epithelial tumor cells may contribute to immune evasion.

Acknowledgements

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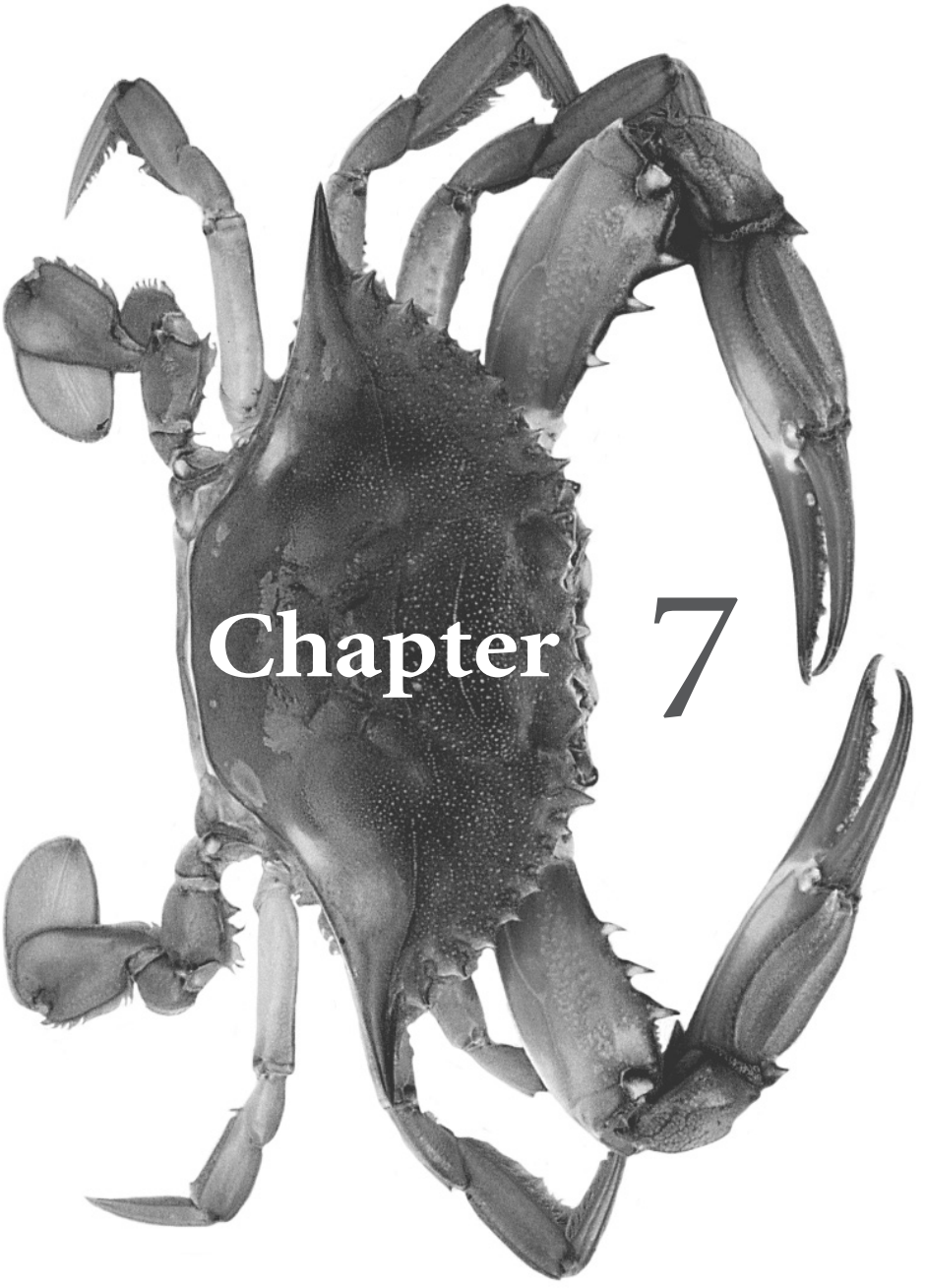

Chapter 7

Differential Notch and TGFβ signaling in primary colorectal tumors and their corresponding metastases

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Abstract

Background: Loss of epithelial morphology and the acquisition of mesenchymal characteristics may contribute to metastasis formation during colorectal tumorigenesis. The Wnt, Notch and TGFβ signaling pathways control tissue homeostasis and tumor development in the gut. The relationship between the activity of these pathways and the expression of epithelial and mesenchymal markers was investigated in a series of primary colorectal tumors and their corresponding metastases.
Methods: Tissue samples of primary colorectal tumors, normal colonic mucosa, and regional and systemic metastases were processed for immunohistochemistry in a tissue microarray format. The expression of mesenchymal (vimentin, fibronectin) and epithelial (E-cadherin) markers was related to markers of Wnt (β-catenin), Notch (HES1) and TGFβ (phospho-SMAD2) signalling. In addition, the KRAS mutation status was assessed.
Results: When compared to normal mucosa, primary colorectal tumors showed a marked increase in the levels of cytoplasmic vimentin and nuclear β-catenin, phospho-SMAD2 and HES1. Increased vimentin expression correlated with the presence of oncogenic KRAS and with nuclear β-catenin. The corresponding liver, lymph node, brain and lung metastases did not express vimentin and displayed significantly lower levels of nuclear phospho-SMAD2 and HES1, while retaining nuclear β-catenin.
Conclusions: Primary colorectal carcinomas display aberrant expression of vimentin, and have activated Notch and TGFβ signaling pathways. Surprisingly, many regional and distant metastases have lost nuclear HES1 and pSMAD2, suggesting that the activity of the Notch and TGFβ pathways is reduced in secondary colorectal tumors.

Introduction

Colorectal cancer is one of the most common malignancies in the Western world. It is now well established that the development of colorectal cancer results from dysregulation of signaling pathways that normally control homeostasis in the gut, in particular those activated by the Wnt, Delta/Jagged and TGFβ ligands.[1] In addition, mutational activation of the KRAS oncogene contributes to the initiation and/or progression of colorectal cancer.[2] These pathways do not operate as single independent entities, but extensive cross-talk and cooperation is required for tumor formation. In addition to the specific mutations and deletions that cause activation of the above signaling pathways, the majority of colorectal tumors is characterized by gross chromosomal instability.[3] Furthermore, specific chromosomal aberrations and gene expression profiles are associated with the ability of colorectal tumors to metastasize.[4-6]
Approximately 50% of colorectal cancer patients develop metastatic disease. The mechanisms underlying metastasis formation are the subject of intense study. It has been proposed that the acquisition of mesenchymal properties allows epithelial tumor cells to detach from the primary tumor mass, invade and migrate to distant sites.[7,8] This process, which shares some features with the epithelial-mesenchymal transitions (EMT) that occur during embryogenesis, is controlled by the same signaling pathways that promote initial tumor formation.[9] However, metastases from colorectal tumors retain their epithelial phenotype, suggesting that metastatic tumor cells most likely do not undergo true EMT. Vimentin is an intermediate filament protein normally expressed in mesenchymal cells[10] and is the most well established marker of mesenchymal differentiation in human tumors. Accumulating evidence suggests that the aberrant expression of vimentin in epithelial cancer cells is related to local invasiveness and metastatic potential.[7,11-13]
The aim of this study was to investigate whether the Wnt, Notch, TGFβ and KRAS pathways are differentially active in primary tumors versus regional and distant metastases and whether this is accompanied by changes in the expression of established markers of epithelial and mesenchymal differentiation.

Material and Methods

Patients and materials

Patients with liver metastases of colorectal carcinoma treated at the University Medical Center Utrecht, the Antonius Hospital in Nieuwegein or the Diakonessenhuis in Utrecht between 1992 and 2004 were identified retrospectively. Samples of normal colon, primary colorectal carcinoma and their corresponding liver metastases were obtained during surgery from 61 patients. Paraffin blocks of metastatic lymph nodes from 4 patients were also available for study. There were 40 males and 21 females with a mean age of 62.7 years (range 40-77 years) at time of surgery for liver metastases.
A second group of 14 patients (10 male, 4 female, mean age 54.4 years (range 37-76 years) who underwent resection of their primary colorectal carcinoma at the University Medical Center Utrecht between 1995 and 1998 and who had remained disease-free for at least 5 years following resection were also identified. Samples of normal colon and colorectal carcinoma were obtained from all these patients.
Furthermore, samples of 5 colorectal lung metastases and 6 colorectal brain metastases were available for study.

Anonymous use of left over resection specimens for scientific purpose is part of the standard treatment agreement with patients in our hospital (opt-out system).[14] The research proposal was approved by the scientific committee of the Department of Pathology.

Preparation of Tissue Micro Arrays

A tissue microarray (TMA) was constructed as described before.[15] In brief, fresh 4 μm sections were obtained from the selected paraffin-embedded tumour blocks and stained with haematoxylin and eosin (H&E). Non-necrotic, representative areas of tumour specimens or normal tissue were marked on the H&E slide. Core needle biopsies were retrieved from the original tumor blocks (marked areas) using a manual tissue array instrument (Beecher Instruments, Sun Prairie, WI, USA) and positioned in a recipient paraffin array block. Three core biopsies from each sample were obtained.

Immunohistochemical analyses

Immunohistochemical staining was performed using standard methods. Sections of 4 μm were cut from the TMA, deparaffinized in xylene, followed by rinsing in graded ethanol and dehydrated in distilled water. After incubation with 3% hydrogen peroxide for 20 min, antigen retrieval was achieved by boiling in 10 mM citrate buffer pH 6.0 for 10 min or 1 mM EDTA buffer (pH 9.0) and sections were washed with TBS. For staining with E-cadherin, β-catenin and vimentin an automated immunostainer (MARK5, DPC, Breda, The Netherlands) was used. For staining with HES1 sections were blocked with 5% goat serum in TBS for 1 h, washed with TBS, and incubated at 4 °C overnight. All primary antibodies used were commercially available and well-validated: E-cadherin (1:200; Invitrogen Corporation, Carlbad, CA, USA), β-catenin (1:1600, Becton-Dickinson, Franklin Lakes, NJ, USA), vimentin (1:400, Dako, Glastrup, Denmark), HES1 (1:300, Chemicon, Temucula, CA, USA) fibronectin (1:200), p-SMAD2/4 (1:200, Cell Signaling Technology, Danvers, MA, USA). HRP-conjugated secondary antibodies (DPVM-55HRP or DPVM-110HRP, Immunologic, Duiven, The Netherlands) were detected with 3.3'-diaminobenzidine substrate (D4418, Sigma, Saint Louis, USA). Slides were counterstained with heamatoxylin and rinsed with water, dehydrated in graded ethanol, cleared in xylene and coverslipped. Appropriate positive and negative controls were used in all experiments.

DNA isolation

Genomic DNA was extracted from paraffin-embedded tumor specimens. Ten to twelve serial sections of 10 μm and one of 4 μm were cut from the tissue blocks. The 4 μm section was stained with haematoxylin and eosin for exact identification and delineation of tumor areas. The 10 μm sections were deparaffinized with xylene and rehydrated by washing with 100% ethanol, 95% ethanol, 70% ethanol and distilled water, and were dried. The indicated tumor areas were collected by microdissection. The dissected tissue was suspended in extraction buffer (1M Tris, 0.5M EDTA and 10% sodium dodecyl sulphate) containing proteinase K (1 mg/ml, Roche) and incubated at 56 °C for 48 hours. Proteinase K was freshly added every 12 hours. After heat inactivation of proteinase K, NaCl was added to a final concentration of 0.4 M and the solution was extracted twice with 25:24:1 mixture of phenol-chloroform-isoamylalcohol. Genomic DNA was precipitated with ethanol, pelleted and subsequently resuspended in TE buffer (Tris-HCL 10 mM, EDTA 1 mM). The concentration of DNA was measured by optical densitometry.

Detection of Kras mutations, PCR amplification and sequencing

Mutation in codon 12 and 13 of the Kras gene were detected by nested PCR. The first PCR was performed with gene-specific primers (forward 5'-TTCATTACGATACACGTCTGC; reverse 5'- GAAACCCAAGGTACATTTCAG) and carried out at 35 cycles each of 94 °C for 30s, 57 °C for 30s and 72 °C for 1 min, followed by a final elongation for 2 min at 72 °C. PCR reaction mixes contained 5 µl genomic DNA (10ng/µl), 0.2 µM forward primer, 0.2 µM reverse primer, 200 µM of each dNTP, 24 mM Tricine, 8.0% Glycerol (w/v), 1.6% DMSO (w/v), 2 mM $MgCl_2$, 85 mM Amonium acetate (pH 8.7), and 0.2 U Taq polymerase in a total volume of 10 µl. Of the first PCR reaction product, 1 µl was used as template for the nested PCR reaction. The second PCR reaction contained gene-specific primers (forward 5'- TGTAAAACGACGGCCAGTACTGGTGGAGTATTTGATAGTG; reverse 5'-AGGAAACAGCTATGACCATATCAAAGAATGGTCCTGCAC). The PCR reaction mixes contained 0.2 µM forward primer, 0.2 µM reverse primer, 200 µM of each dNTP, 24 mM Tricine, 8.0% Glycerol (w/v), 1.6% DMSO (w/v), 2 mM MgCl2, 85 mM Amonium acetate (pH8.7), and 0.2 U Taq polymerase in a total volume of 10 µl. Cycling conditions were the same as for the first PCR reactions. Nested PCR products were diluted with 20 µl distilled water and 1 µl was used as template for the sequencing reactions. Constructs were verified by automatic sequencing using BigDye Terminator v1.1 (Applied Biosystem, Warrington, UK). The samples were purified by Sephadex Superfine 75 column and analyzed on an ABI PRISM fluorescent automated DNA sequencer for point mutations.

Evaluation of immunostaining

Two investigators (LMV., PJvD.) simultaneously assessed the results of immunostaining without the knowledge of the patients' clinicopathological details. Single assessment of the intensity of staining only was performed for those markers showing very homogenous staining, for instance vimentin. In the few sections that showed non-homogeneous staining, the highest score was taken. For markers that did not show homogenous staining throughout the section (for instance HES1 and pSMAD2/4), we analyzed % positive nuclei.

Cytoplasmic, membraneus and nuclear staining were evaluated as absent, weak, strong, or very strong. Nuclear HES1 and pSmad 2/4 staining were evaluated as percentage. The mean scores of 3 needle biopsies per tumor were plotted. The inter-observer quality of evaluation was generally very good (80%). Sections that were evaluated differently were re-examined to reach a consensus. The criteria for evaluation of membrane and nuclear staining were: Membranous: absent, weak, strong. Nuclear: for β-catenin: absent, weak, strong (homogeneous staining intensity within the sections). Nuclear: for HES1 en Smad2/4: % positive cells (non-homogeneous staining intensity within the sections).

Statistical analysis

Statistical analysis was performed with GraphPad Prism™ version 3.0 for Windows (GraphPad Software, San Diego, California, USA). Statistical differences between groups were analyzed by Mann-Whitney U test and $p<0.05$ (two tailed) was used to denote statistical significance. Values are expressed as mean ± SEM.

Results

Expression of vimentin in primary colorectal tumors but not in metastases.
Normal mucosal epithelial cells did not express the mesenchymal marker vimentin, as expected. To our surprise, primary colorectal tumors expressed vimentin at varying levels (**Figure 1A and B**) but this expression was completely lost in the corresponding liver metastases ($p<0.001$). Similarly, metastases in lymph nodes (n=4), lung (n=5) and brain (n=6) were completely negative for vimentin (**Figure 1A and B**). Non-metastatic primary colorectal tumors expressed vimentin comparable to metastatic tumors (**Figure 1C**). Thus, vimentin was expressed in the majority of metastatic and non-metastatic primary colorectal tumors, but expression was lost in regional and distant metastases. The observed changes in vimentin expression were not associated with obvious EMT-like morphological changes of the tumor phenotypes (not shown). None of the primary tumors or their metastases expressed fibronectin (not shown).

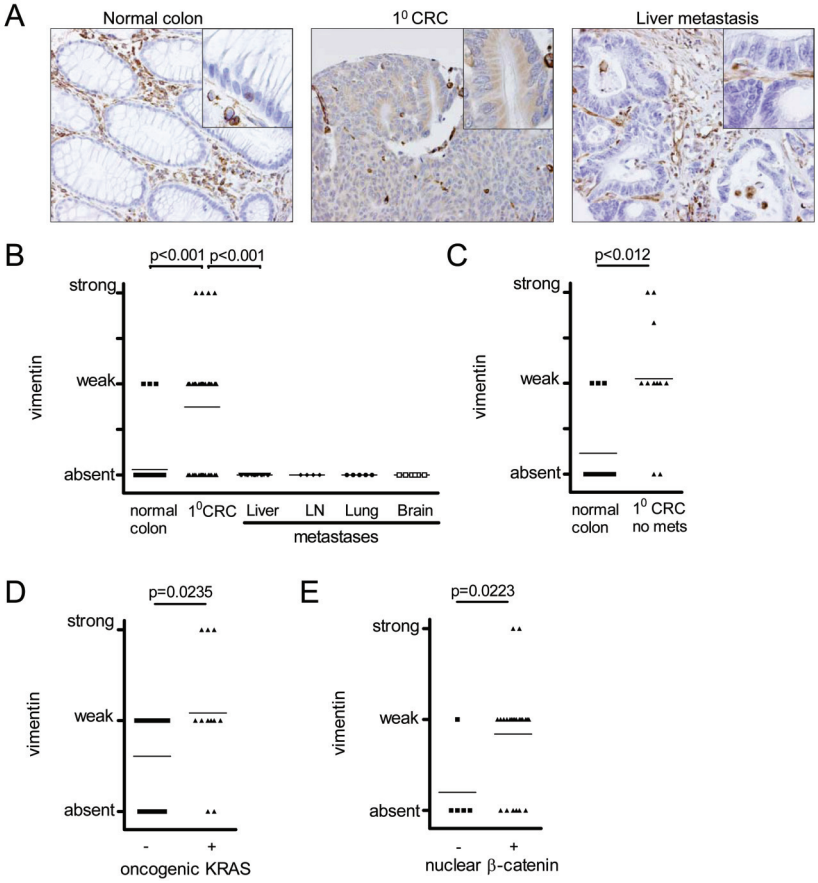

Figure 1. Expression of vimentin in primary CRC. (**A**) In normal colon epithelium vimentin is highly expressed in stromal fibroblasts but not in epithelial cells surrounding the lumen. In primary colorectal tumor samples the tumor cells express moderate levels of cytoplasmic vimentin in a homogenous

staining pattern. Also in these samples the stromal cells express high levels of vimentin. Vimentin was not expressed in the tumor cells of the corresponding liver and lymph node metastasis, whereas stromal cells in these tumors were highly positive. magnification 20x. Inset 40x. (**B**) Scatter diagram demonstrating expression of vimentin in primary colorectal carcinoma, normal colon epithelium and the corresponding metastases in liver lymph nodes (LN), lung and brain. (**C**) Vimentin expression in normal colon epithelium and in colorectal carcinoma of patients without liver metastasis. Vimentin expression was significantly higher in colorectal carcinoma tissue compared to normal colon epithelium, p=0.012. (**D**) Scatter diagrams demonstrating that the expression of vimentin in primary CRC correlates with the presence of oncogenic KRAS and (**E**) nuclear β-catenin.

Vimentin expression in primary CRC is associated with the presence of oncogenic KRAS and with nuclear β-catenin.

Mutational activation of the KRAS proto-oncogene is a critical event in the initiation and progression of colorectal cancer and metastasis formation.[2,16] Furthermore, Ras signaling promotes EMT in in vitro cell systems.[9] Therefore, we evaluated whether the presence of oncogenic KRAS in primary colorectal tumors was associated with vimentin expression. Mutations in the KRAS gene in primary colorectal tumors were found in 18 (30.5%) of 59 patients. Mutations in codon 12 and 13 were detected in 9 cases each. Mutation analysis failed in 2 patient samples. Tumors expressing oncogenic KRAS expressed significantly higher levels of vimentin (p=0.024, **Figure 1D**).

Activation of Wnt signalling leads to nuclear accumulation of β-catenin and is a key event in the development of the majority of colorectal tumors.[17] In our series the majority, but not all, of the primary colorectal tumors displayed accumulation of nuclear β-catenin (see below). Interestingly, the few tumors that did not show nuclear β-catenin (n=5) expressed significantly lower levels of vimentin (**Figure 1E**). Thus, vimentin expression in primary colorectal tumors is associated with the presence of oncogenic KRAS and with accumulation of nuclear β-catenin.

Membranous E-cadherin in primary CRC and liver metastases

E-cadherin is essential for maintaining proper cell-cell contacts and its loss contributes to tumor development and metastasis formation.[18] Therefore, we evaluated expression and localization of E-cadherin in normal mucosa, primary metastatic colorectal tumors and their liver metastases. A minority of primary tumors (13/57) and metastases (11/51) displayed reduced membranous localization of E-cadherin. Thus, the majority of the tumors retained strong membranous E-cadherin staining which was not significantly different from that observed in normal mucosa (**Figure 2**). Furthermore, the morphological differentiation status of primary tumors and their corresponding metastases was not significantly different (not shown). Taken together, the results show that primary colorectal cancers express the mesenchymal marker vimentin without undergoing gross loss of the epithelial phenotype.

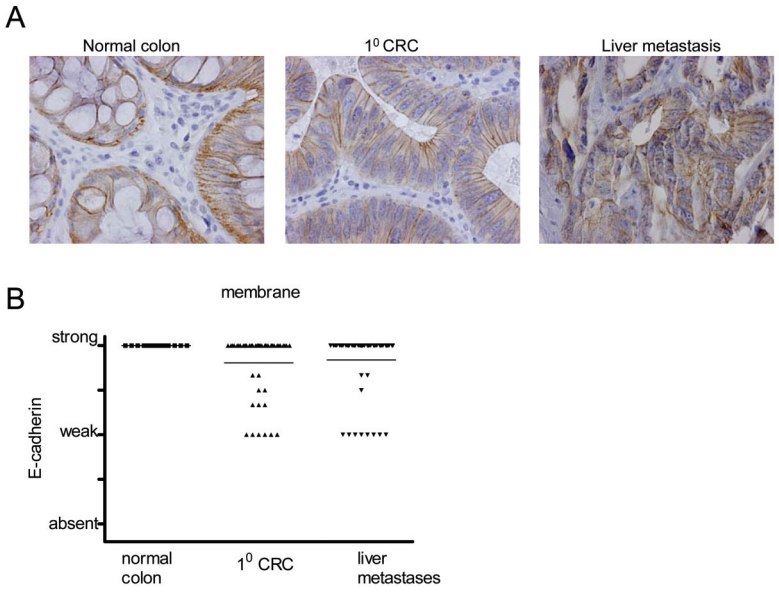

Figure 2. Membrane localization of E-cadherin in primary CRC tumors and liver metastases. (**A**) Immunohistochemical analysis of E-cadherin expression and localization in normal colon epithelium, primary colorectal carcinoma and corresponding liver metastases. E-cadherin was expressed in all samples examined. (**B**) Scatter diagram demonstrating that a minority of primary tumors (13/57) and liver metastases (11/51) displayed a clear reduction in the amount of staining at cell cell contacts.

Increased Wnt signaling in primary colorectal tumors and in metastases.
Next, we investigated whether signaling pathways known to control colorectal cancer development and epithelial (de-)differentiation, were associated with vimentin expression. Aberrant Wnt signaling leads to nuclear accumulation of β-catenin which contributes to initiation of colorectal tumor formation. Normal colonic epithelium showed localization of β-catenin at the membrane, but not in the cytoplasm or in the nucleus (**Figure 3A-D**). However, β-catenin was found in the cytoplasm and in the nucleus of the vast majority of primary metastatic colorectal tumors and the corresponding liver metastases, which indicates activation of the Wnt signaling pathway in the majority of these tumors (**Figure 3A-D**), as expected.

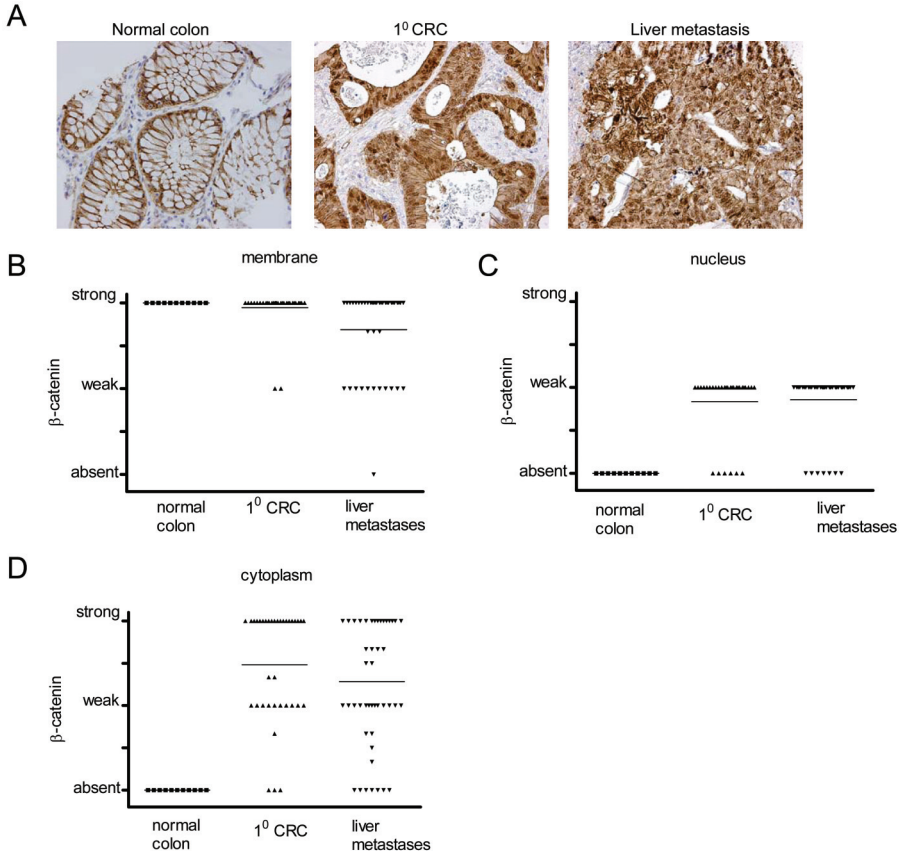

Figure 3. Wnt pathway activation in primary CRC and liver metastases. (**A**) Immunohistochemical analysis of β-catenin expression and localization in normal colon epithelium, primary colorectal carcinoma and corresponding liver metastases. (**B**) Scatter diagram showing that the localization of β-catenin to cell-cell contacts is markedly lower in a minority of primary CRC tumors (2/51) and liver metastases (15/57) when compared to that in normal colon. (**C**) Nuclear β-catenin was observed in the majority of primary CRC tumors (45/51) and liver metastases (50/57). In normal colon tissue β-catenin was absent from the nucleus, as expected. (**D**) Scatter diagram demonstrating the elevated expression of β-catenin in the cytosol of primary CRC and liver metastases.

Increased TGFβ signaling in primary colorectal tumors but not in metastases

TGFβ signaling suppresses the early development of colorectal tumors but can stimulate tumor progression at later stages. TGFβ signaling leads to the phosphorylation of SMAD proteins and translocation of these mediators into the nucleus to control gene expression. Normal colonic epithelial cells displayed undetectable or low levels of phospho-SMAD2 (p-SMAD2) in approximately 50% of the cells (**Figure 4A-C**). However, moderate to high levels of p-SMAD2, both in the cytoplasm and in the nucleus, were observed in primary metastatic colorectal carcinomas. Up to 100% (mean 77%) of the tumor cells showed strongly positive nuclear staining, which is indicative of active TGFβ signaling in the majority of primary CRC. These findings are in line with a previous report.[19] pSMAD2 staining in the primary CRC tumors was significantly higher than in normal colon (**Figure 4A-C**). Surprisingly, many of the corresponding liver metastases had lost p-SMAD2 ($p<0.0001$) (**Figure 4A-C**). The presence of nuclear p-SMAD2 was not associated with increased vimentin expression (not shown).

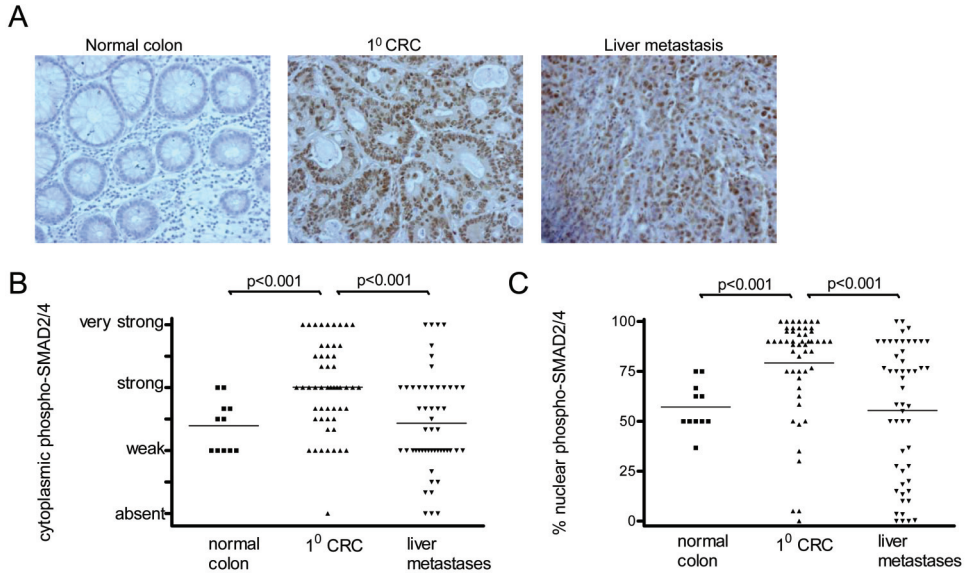

Figure 4. TGFβ pathway activation in primary CRC. (**A**) Immunohistochemical analysis of phospho-SMAD2 on TMAs of normal colon epithelium, primary colorectal carcinoma and corresponding colorectal liver metastases. Scatter diagrams demonstrating increased expression of phospho-SMAD2 in the cytoplasm (**B**) and in the nucleus (**C**) of primary colorectal carcinomas, when compared to normal mucosa ($p<0.001$). Many of the corresponding colorectal liver metastases show reduced levels of phospho-SMAD2 in the cytoplasm as well as in the nucleus ($p<0.001$).

Increased Notch signaling in primary colorectal tumors but not in metastases

Previous work has shown that Notch signaling is activated in intestinal crypt cells and in intestinal adenomas in APC^{min} mice.[20] To investigate Notch signaling in human colorectal cancer, we probed the TMAs for HES1, a well-established Notch target that acts as a transcriptional repressor. Normal colon tissue showed weak cytoplasmic HES1 staining but hardly any nuclear staining (2-3% of all cells) (**Figure 5**). In contrast, the majority of primary metastatic colorectal tumors showed strong to very strong expression of HES1 in the cytoplasm and up to 50% of colorectal tumor cells showed strong nuclear HES1 staining (mean 24%). Thus, elevated Notch signaling may contribute to the development of colorectal carcinoma. To our knowledge this is the first report showing activated Notch signaling in human colorectal cancer. Interestingly, many metastases in the regional lymph nodes, the liver, the lungs and the brain showed a strongly reduced expression of HES1 when compared to their paired primary tumors ($p<0.001$, **Figure 5**).

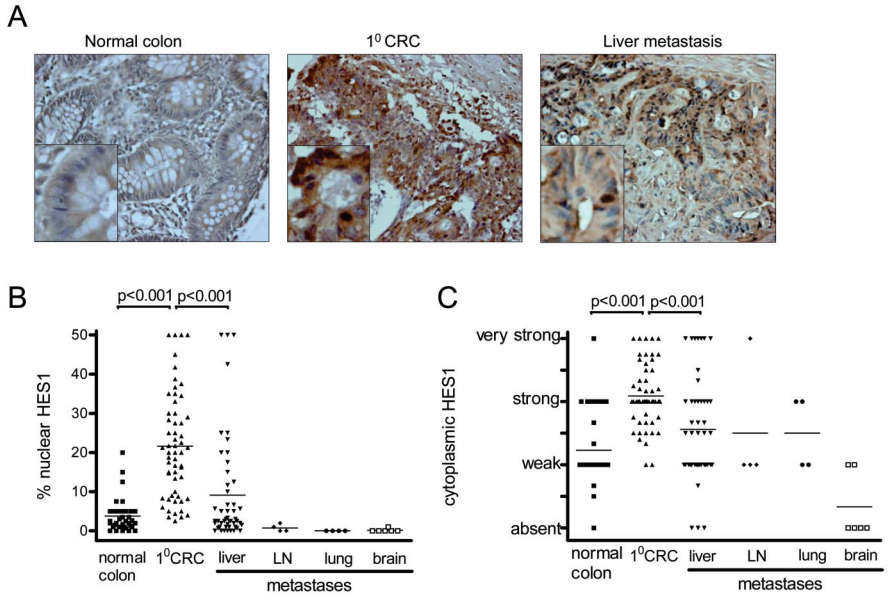

Figure 5. Notch pathway activation in primary CRC. (**A**) Immunohistochemical analysis of the Notch target HES1 on TMAs of normal colon epithelium, primary colorectal carcinoma and corresponding colorectal liver metastases. (**B,C**) Normal colon showed weak cytoplasmic staining in the majority of cells, but only 3% of the cells displayed positive nuclei. Primary colorectal carcinomas showed strong HES1 staining in the cytoplasm and up to 50% of the tumor cells displayed high HES1 levels in the nucleus. The intensity of HES1 staining in the cytosol and in the nucleus of the corresponding metastases in the liver, lymph nodes (LN), lungs and brain was markedly lower than that in the primary tumors.

Discussion

Our study shows that the activity of the Notch and TGFβ signaling pathways which regulate epithelial homeostasis in the gut and have been implicated in colorectal cancer development are differentially active in primary colorectal tumors and their corresponding metastases. Possibly, the activity of these pathways in primary and secondary colorectal tumors may depend on the local production of the ligands that activate these pathways. Alternatively, genetic differences between primary colorectal tumors and their corresponding metastases[21-26] may underlie the differential activation of the Notch and TGFβ signaling pathways in primary and secondary tumors. Further work is required to distinguish between these possibilities.
Vimentin, a type III intermediate filament protein, is normally only expressed in cells of mesenchymal origin. Thus, our finding that vimentin is expressed in primary colorectal tumors suggests at least some degree of epithelial de-differentiation to a more mesenchymal phenotype. Vimentin expression in carcinomas is associated with local invasion and metastasis formation. We found that vimentin was moderately expressed in our panels of metastasizing and non-metastasizing primary colorectal carcinoma samples. Thus, primary colorectal carcinomas are characterized by a general and moderate upregulation of vimentin which is not directly associated with metastatic potential (as both metastatic and non-metastatic primary tumors expressed vimentin). Vimentin expression was associated with mutations in the KRAS oncogene and with nuclear β-catenin. Ectopic expression of oncogenic H-Ras^{V12}, in concert with TGFβ signaling, efficiently establishes a stable mesenchymal phenotype in epithelial cells and this is accompanied by the expression of vimentin.[27] Our results show that endogenous mutant KRAS and nuclear β-catenin, but not p-SMAD2 is associated with the expression of vimentin in human colorectal tumors. Despite the expression of this mesenchymal marker, the tumors retained their epithelial morphology.
The trans-differentiation of epithelial tumor cells is a process that shares some features with the epithelial-mesenchymal transitions (EMT) that occur during embryogenesis. The mechanistic insights into the pathways that regulate the EMT-like process in cancer cells is mostly derived from in vitro cell culture models.[28] While metastasis formation clearly requires tumor cells to dislodge from the primary tumor mass, invade the surrounding tissue, and gain access to the vascular or lymphatic systems, it is far less clear whether they have to undergo EMT-like changes to do this.[29,30] Detached and invading tumor cells retain epithelial hallmarks like mucin production and cytokeratin expression. In addition, the morphological differentiation of distant metastases is often similar to that of the primary tumor. Also in the tumor series examined here we found no differences in morphological differentiation between primary colorectal tumors and their corresponding metastases (LMV PJvD, unpublished observations). Taken together, the concept that invading tumor cells undergo true trans-differentiation is disputable.
In addition to KRAS and TGFβ signaling, Notch signaling has also been implicated in the control of EMT.[31,32] Notch signaling is active in intestinal crypts and in adenomas in APCmin mice[20] and Notch pathway inhibitors are attractive therapeutics in the treatment of intestinal neoplasia[33] by virtue of their ability to turn proliferative adenoma cells into goblet cells.[20] Our data show that Notch signaling is also strongly activated in primary human colorectal cancer. At present it remains unclear whether this is due to increased expression of Notch ligands, or to (epi)genetic changes in the tumor cells themselves.
Future work should establish whether metastases from colorectal cancer are dependent on Notch and TGFβ signaling pathways for their proliferation and survival.

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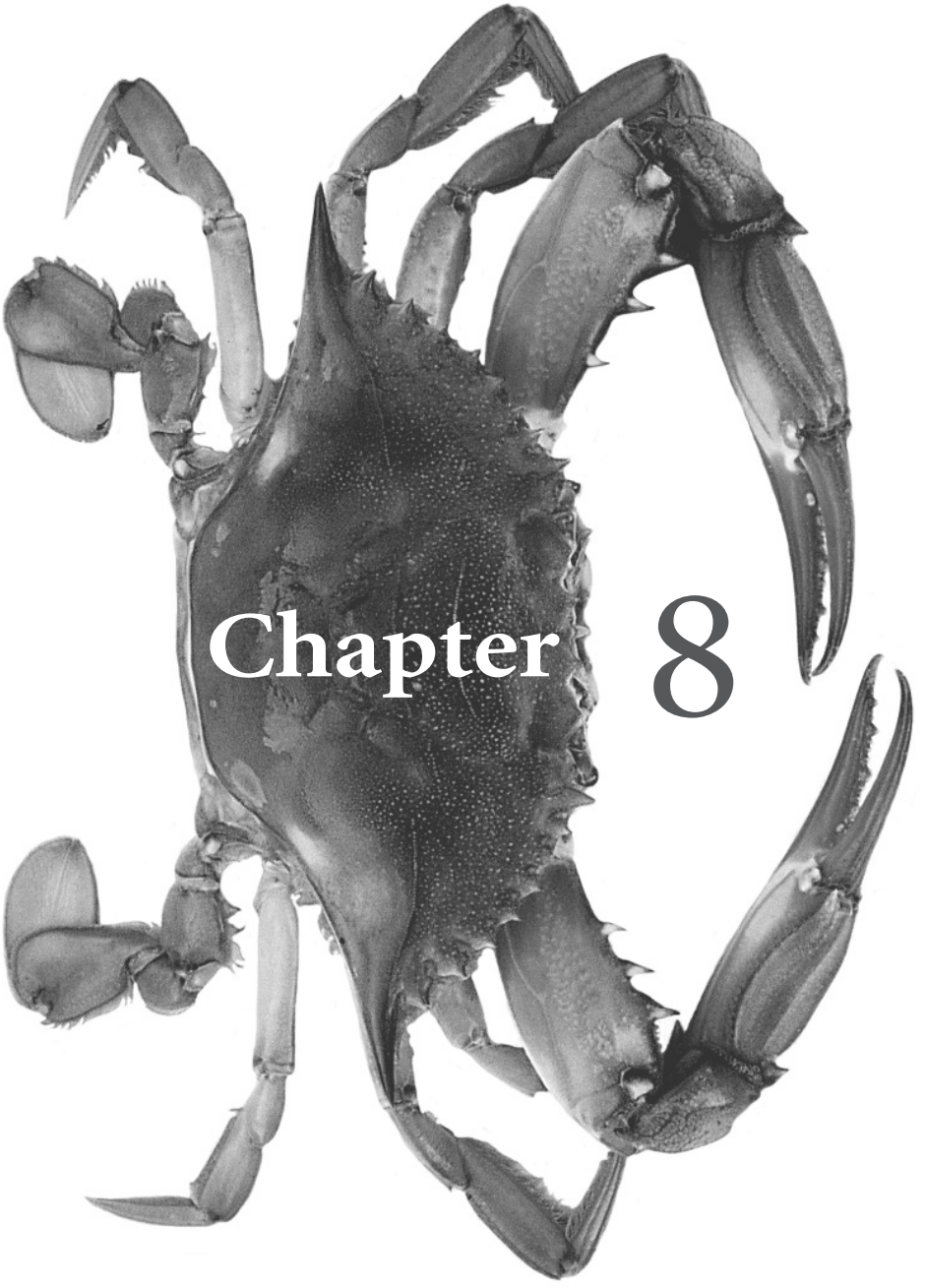

Chapter 8

General discussion and conclusions

Colorectal cancer is one of the most prevalent malignancies in the western world. Mortality is strongly associated with the formation of liver metastases, which eventually occurs in about 50% of patients. Once liver metastases have developed, the natural course of the disease is associated with poor survival rates. In this thesis we have focused on local ablation for unresectable liver metastases and on some of the molecular processes involved in colorectal cancer and metastases formation.

Part 1 Local therapy of liver metastases

Local ablation therapy

Locally ablative therapies are gaining acceptance in the treatment of unresectable liver metastases. A major limitation of these therapies is the production of lesions with sufficient tumor-free margin. In **chapter 2** we applied three techniques of LITT to increase lesion size. In LITT, as in other ablative techniques, the size of tissue ablation achieved by a single optical fibre is limited. The maximum volume of tissue necrosis achieved is dictated by the balance between the conduction of thermal energy and its removal by conduction and convection, mostly through hepatic blood flow. During thermal ablation, hepatic inflow occlusion by clamping the portal vein is advised to reduce dissipation of the generated heat, and to increase the extent of tissue injury.[1] The patients described in this chapter demonstrate that LITT with the use of multiple water-cooled fibers applied simultaneously, together with hepatic inflow occlusion, can produce large lesions (up to 8.6 cm in diameter). Although we produced large lesions with LITT, tumor lysis syndrome was not observed in our five patients. The tumor lysis syndrome is a constellation of metabolic derangements secondary to tumor cell breakdown and in the end will lead to acute renal failure.[2] As described in **chapter 5**, LITT causes heat fixation of tissue consequently, no circulating metabolites will develop.

In literature, recurrence rates after LITT vary in up to 63% of cases.[3-5] For lesions up to 3 cm, local ablation is effective and can result in definite local tumor control in > 90% of the lesions treated[6]. For lesions > 3 cm, local recurrence rates at the site treated are still high and have been reported to be over 50%.[6] Complete tumor ablation is very important and directly related to survival, just as a free resection margin is important for prognosis in hepatic resection.[7,8] In our second patient, a large ablative lesion was created, however after 6 months recurrent tumor tissue was seen on the CT- and PET scan. A great advantage of the ablative method is that the treatment can be repeated with minimal morbidity.

Thermal ablation can be performed percutaneously,[9,10] or during an open surgical procedure.[1] The percutaneous approach has the advantage of a minimal invasive procedure and can be monitored real time by magnetic resonance imaging (MRI). Our group prefers the open approach, for the reason that more accurate tumor localization is possible and previously undetected disease may be recognized. Furthermore, hepatic inflow occlusion is applicable and the use of multiple water-cooled fibers is feasible.

Taken together, lesion size is a critical factor to the success of ablative treatment. With the latest technical improvements, including the use of multiple water-cooled fibers applied simultaneously, together with hepatic inflow occlusion, we may expect to overcome lesion size limitations.

RFA has received increasing attention as a promising technique for treatment of different hepatic metastases. In **chapter 3** we demonstrate that large coagulation lesions of up to 12.0 cm in diameter can be produced with bipolar RFA in a multipolar mode for patients with hepatic metastases of a neuroendocrine tumor. Local ablation by RFA in hepatic metastases of neuroendocrine tumors has shown encouraging results with good local tumor control and a satisfactory duration of symptom relief.[11-13] The aim of treatment in metastases of endocrine tumors is to reduce the tumor mass while preserving normal hepatic tissue, thereby causing symptoms to improve. Patients with hepatic metastases of endocrine tumors treated with RFA showed symptom relief in 95% with significant or complete symptom control in 80% of cases for a mean period of 10 months.[11] Even in patients with extrahepatic disease, ablation of liver metastases may provide symptom relief.[11] Morbidity is minimal and the anticipated mortality rate associated with this procedure is low.[14,15] As with LITT, RFA causes heat fixation (**chapter 5**) without the release of circulating metabolites, thus no tumor lysis syndrome will occur. Therefore, RFA can be applied repeatedly for maintaining tumor control in the liver without increasing morbidity during follow-up.
Possible contraindications to such ablative technique depends on the location of the tumor. Tumors neighbouring large vessels or major branches of the portal vein are difficult to treat. The blood flow in these vessels cools the heating process, potentially leading to residual tumor cells. In addition, coagulation adjacent to bile duct can lead to obstruction of bile with the associated consequences. Careful selection of patients is therefore required to avoid complications. As described earlier we prefer the open procedure above the percutaneous method for local ablation (**chapter 2**). The open surgical procedure provides for bipolar RFA in a multipolar mode the best degree of freedom for inserting the probes. Furthermore radiofrequency ablation by laparotomy results in superior local control, independent of tumor size.[16]
In literature, one-year overall survival rates after local ablation for unresectable colorectal liver metastases varies between 80 and 93%, 2-year overall survival is reported between 50-75%.[10,17-25] However, in most studies the mean number of metastases is < 2, while most lesions treated are < 3 cm. Such criteria justifies the question whether such lesions could, and should not have been treated by surgical resection. Notably, there are vast differences between the studies regarding study design, inclusion/exclusion criteria, and surgical approach (open or percutaneous). In our study we used four bipolar electrodes simultaneously in the patient, withdrew them two times, and applied hepatic inflow occlusion during an open procedure to achieve a coagulation lesion of 12 cm in diameter.
As a result, bipolar RFA in a multipolar mode is an exclusive technique to produce large coagulation lesions in patients with unresectable liver metastases.

Local ablation in liver metastases of neuroendocrine tumors

Neuroendocrine malignancies of the gastrointestinal tract encompass a unique group of neoplasms associated with an indolent yet relentless growth pattern and with frequent development of liver metastases. They are often characterized by unique hormonal syndromes, which are proportional to tumor burden. In **chapter 4** we reviewed the literature to define the optimal treatment strategy and work-up in patients with liver metastases of neuroendocrine tumors. Liver metastases of neuroendocrine tumors are best managed with a multidisciplinary approach and treatment must be tailored for each individual patient.
Chromogranin A, together with platelet serotonin is an important marker both for diagnosis and follow-up of patients to evaluate therapy efficiency. Several imaging modalities are avai-

lable to detect liver metastases, though helical-CT and somatostatin receptor scintigraphy (SRS) are the most sensitive imaging tools and should be performed prior to treatment. The Ki-67 proliferation index is often used to predict clinical aggressiveness and is a very important tool guiding the type of treatment. Treatment should be offered, depending on the number and localisation of liver metastases and possible extrahepatic disease. Surgical management is the gold standard for treatment of hepatic metastasis, providing extended survival and symptom improvement. Unfortunately, most liver metastases of neuroendocrine tumors are multiple and diffuse and therefore unresectable. Locally ablative therapy is a novel approach in the treatment of unresectable liver metastases of neuroendocrine tumors, a few small series have shown good results particularly regarding symptom relief.[11,12,26,27] In patients with extensive metastases who are not suitable for local ablation, systemic therapy by octreotide, ^{131}I-MIBG treatment or targeted chemo-and radiotherapy should be attempted.
From now, tumor destruction by locally ablative techniques, such as RFA or LITT, should be used more often as liver preserving option to treat patients early in the course of their disease, postponing drug intervention and preventing end stage carcinoid syndrome and thereby improving life expectancy.

Local ablation therapy combined with chemotherapy
For unresectable liver metastases, local ablative therapies, such as LITT and RFA have emerged as effective strategies to achieve tumor clearance and to potentially improve life-expectancy.[28-30] Nevertheless local intrahepatic recurrence is common after local ablative treatment. Local recurrence may develop from microscopic tumor deposits or satellite metastases that reside at the periphery of the lesion.[9,31] Hence, there is a need for an effective additional treatment with aim to destroys any residual tumor after ablation.
In **chapter 5** we established a murine tumor model for solitary liver metastasis in which recurrent tumor growth occurs after LITT. The use of C26-luciferase allowed us to asses the effect of LITT and chemotherapy on tumor growth over time in a non-invasive manner. The findings in this study show that additional treatment with doxorubicin after LITT could completely prevent intrahepatic tumor recurrence, whereas after LITT without doxorubicin, tumor recurrence occurred in almost all mice. This is most likely the result from synergy between sublethal thermal tissue damage at the rim of the tumor by LITT and the doxorubicin treatment. Microscopic examination of tumor tissue treated with LITT plus doxorubicin demonstrated sharply demarcated lesions around the area of laser application. In the inner center of the coagulation lesion, tumor cells are morphological intact. However, enzyme histochemical analysis of cell viability of this area showed no staining of tumor cells, consistent with a complete loss of tumor cell viability. This phenomenon, heat fixation, is described for the first time. Various potential mechanisms have been postulated to explain the synergy between LITT and doxorubicin. First, a smaller tumor volume at the start of doxorubicin treatment (after LITT) is likely to be more effectively eradicated than a large tumor volume (no LITT). Second, hyperthermia (LITT) does not modify intracellular chemosensitivity of either cell line, but acts on membrane permeability, thereby facilitating attainment of the intracellular drug concentrations needed to cause the cytotoxic effect.[32] Third, hyperthermia (LITT) induces nonspecific vascular changes that increases vessel permeability leading to more efficient targeting of tumor cells by doxorubicin.[33,34] Future investigation should be focused on perilesional microcirculatory disturbances to find attractive approaches to reduce local recurrence after thermal ablation of liver metastases.

Our results support the concept that combined LITT and adjuvant chemotherapeutic treatment can increase the extent of tumor destruction. The local recurrence rates obtained by locally ablative treatment are encouraging in case of unresectable colorectal metastases. Nevertheless, no data from randomized trials are available evaluating the effect of LITT or RFA on overall survival when compared with chemotherapy alone. Local ablative treatment for unresectable colorectal liver metastases seems justified only when such trials demonstrate a clear benefit on overall survival of LITT or RFC over chemotherapy. The results of the EORTC-CLOCC trial have to be awaited for to determine the benefit on survival of RFA over chemotherapy. Unfortunately, this trial was recently closed prematurely due to insufficient accrual. As such, randomised control trials evaluating the value of local ablation combined with systemic chemotherapy are still urgently needed.

Part 2 Molecular characteristics of colorectal liver metastases

Evasion of the immune system by mutant KRAS in colorectal cancer

Activating mutations in the KRAS oncogene are frequently observed during the early stages of CRC. If mutant KRAS is to be used as a target for therapy in CRC, tumor growth should depend on the continued presence of mutant KRAS. In **chapter 6** we demonstrated that stable knockdown of the mutant KRAS allele, by RNA interference, resulted in loss of transformed properties in vitro. The incidence of subcutaneous tumor formation by C26-KRASKD cells was dramatically reduced, lag time was enlarged and the C26-KRASKD cells grew noninvasively and did hardly cause morbidity. Previously, it has been reported that deletion of KRAS from human tumor cells completely abolished tumor growth.[35,36] This discrepancy with our results could be due to the nonidentical genetic background of the different cell lines or by the fact that they missed tumor growth in the KRAS knockdown cells due to termination of experiments prior to a prolonged lag time. Furthermore, we demonstrated that C26-KRASKD cells are rapidly cleared by the immune system. Upon KRAS knockdown, cells showed an increase in cell surface expression of H-2Kd MHC class 1 molecules and was also accompanied by increased IL18 production. How these molecular changes interact and how the stimulation of the anti-tumor immune response is induced remains unclear and must be investigated further. The antitumor effect of IL18 indicate the possibility of applying this cytokine to tumor therapy and this warrants further studies on the role of IL18 in immunity. In conclusion, the reduced incidence of tumor formation by C26-KRASKD cells is at least in part a result of tumor cell clearance by the host immune system and not to an intrinsic inability of these cells to grow out as a tumor.

Signaling pathways involved in colorectal cancer

The Wnt, Notch, and TGFβ signaling pathways control tissue homeostasis and tumor development in the intestine. These same pathways are also involved in the trans-differentiation of epithelial tumor cells into cells with mesenchymal characteristics, a process that shares some features with the epithelial-mesenchymal transition (EMT). Vimentin is an intermediate filament protein normally expressed in mesenchymal cells and is the most well established marker of mesenchymal differentiation in human tumors.[37] In **chapter 7** we have demonstrated that primary colorectal carcinomas are characterized by a general and moderate upregulation of vimentin, which is not directly associated with metastatic potential as both metastatic and non-metastatic primary tumors expressed vimentin, nor with obvious EMT-like

changes in tumor histology. Furthermore, endogenous mutant KRAS and nuclear β-catenin (Wnt signalling pathway), is associated with the expression of vimentin in human CRC. Notch signalling is strongly activated in primary CRC, whether this is due to increased expression of Notch ligands or to changes in the tumor cells themselves remains unclear. The signal-transduction pathways that contribute to the invasive and metastatic properties of cancer cells present a great opportunity to block tumor progression and prevent metastasis. For example, the RAS pathway has provided several targets for drug therapy. Inhibitors of farnesyltransferase, which is thought to be necessary for anchoring RAS to membranes, have been tested in several cancers.[38] Our finding that CRC metastases display relatively low levels of activated Notch and TGFβ signaling components may have significant consequences for the use of inhibitors that target these pathways in the treatment against metastatic CRC.

Conclusions

Locally ablative therapy, such as LITT and RFA, plays an important role in the treatment of unresectable liver metastases. We found that with the latest technique of LITT and RFA we are able to produce large coagulation lesions. Consequently, LITT and RFA are good alternatives for patients who are not suitable for surgical resection, aiming at curation with less complications.

Furthermore, additional chemotherapy after LITT may induce maximal efficacy and improve survival in patients with colorectal liver metastases. Ongoing randomised trials are assessing the role of (neo)adjuvant chemotherapy in patients with resectable liver metastases, and RFA in patients with unresectable disease as an adjunct to chemotherapy. A future national guideline will contribute to an optimal and evidence-based use of the therapeutic options for patients with colorectal liver metastases.

Mutant KRAS in CRC cells enhances their metastatic potential and invasiveness, and facilitates immune evasion. This makes mutant KRAS an interesting therapeutic target for treatment against metastases of CRC.

Liver metastases of CRC demonstrated low levels of activated Notch en TGFβ signaling components, suggesting these signaling pathways are less involved in metastases growth. These findings have important consequences for the use of inhibitors that target these pathways for treatment of metastatic CRC.

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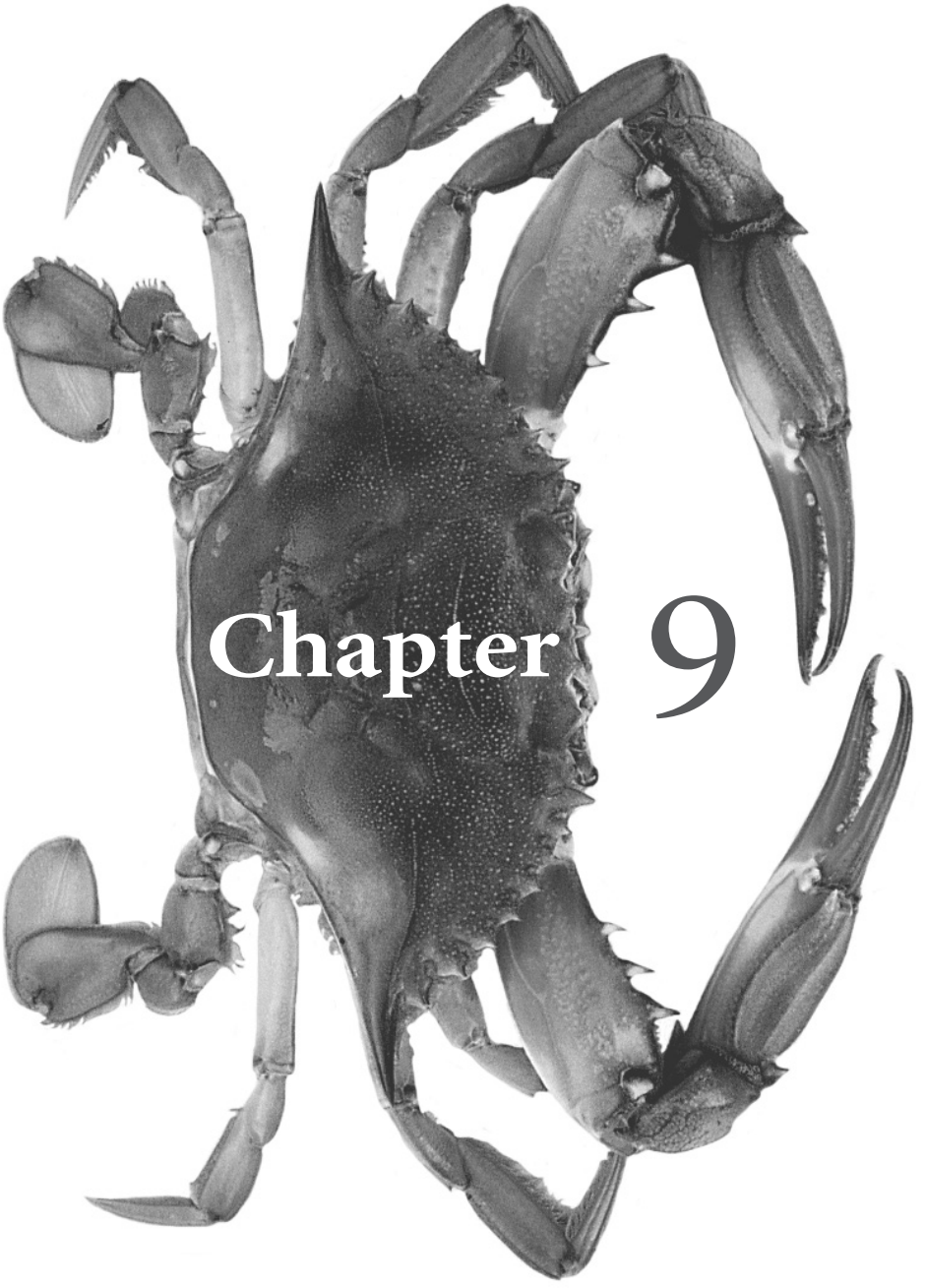


Chapter 9

Summary

Chapter 1 gives a short introduction on liver metastases of colorectal cancer, the treatment of nonresectable liver metastases and the mechanisms involved in metastases formation. It discusses the aim of this thesis, which was to evaluate local therapy techniques for nonresectable liver metastases and to asses the contribution of mutant KRAS and different signalling pathways during late stages of colorectal cancer and liver metastases formation.

Currently, surgical resection offers the best chance of cure for colorectal liver metastases, but is applicable to only a small number of patients. Methods of local tumor destruction have thus been proposed and among these, LITT is considered a promising treatment for patients with nonresectable liver metastases. A major limitation of this therapy is the production of lesions with sufficient tumor-free margin. In **chapter 2** we have analyzed different techniques of LITT to increase lesion size in nonresectable colorectal liver metastases. Patients described in this chapter demonstrate that LITT with the use of multiple water-cooled fibres applied simultaneously, together with hepatic inflow occlusion, can produced lesions up to 8.6 cm in diameter. We have performed all LITT procedures by laparotomy. This open surgical procedure is preferred because apart from enabling accurate tumor localization and detection of previously unrecognized tumor we are able to perform hepatic inflow occlusion to maximize tumor destruction. Thus, LITT with simultaneously multiple fibres and hepatic inflow occlusion offers a unique technique for producing large lesions, thereby expending the possibilities for patients with nonresectable large metastases.

RFA is a well established method of local tumor ablation. RFA in hepatic metastases of neuroendocrine tumors has shown encouraging results with a good local tumor control and a satisfactory duration of symptom relief. An important limitation of local tumor ablation is the extent of coagulation necrosis that could be produced. **Chapter 3** addresses the use of multipolar RFA in neuroendocrine hepatic metastases. In the patient described here, we have used four bipolar electrodes simultaneously and applied hepatic inflow occlusion to finally produce a coagulation lesion of 12 cm in diameter. Thus, the use of simultaneously operated multiple radiofrequency electrodes in a multipolar mode expands the treatment options for patients with large and nonresectable intrahepatic metastases.

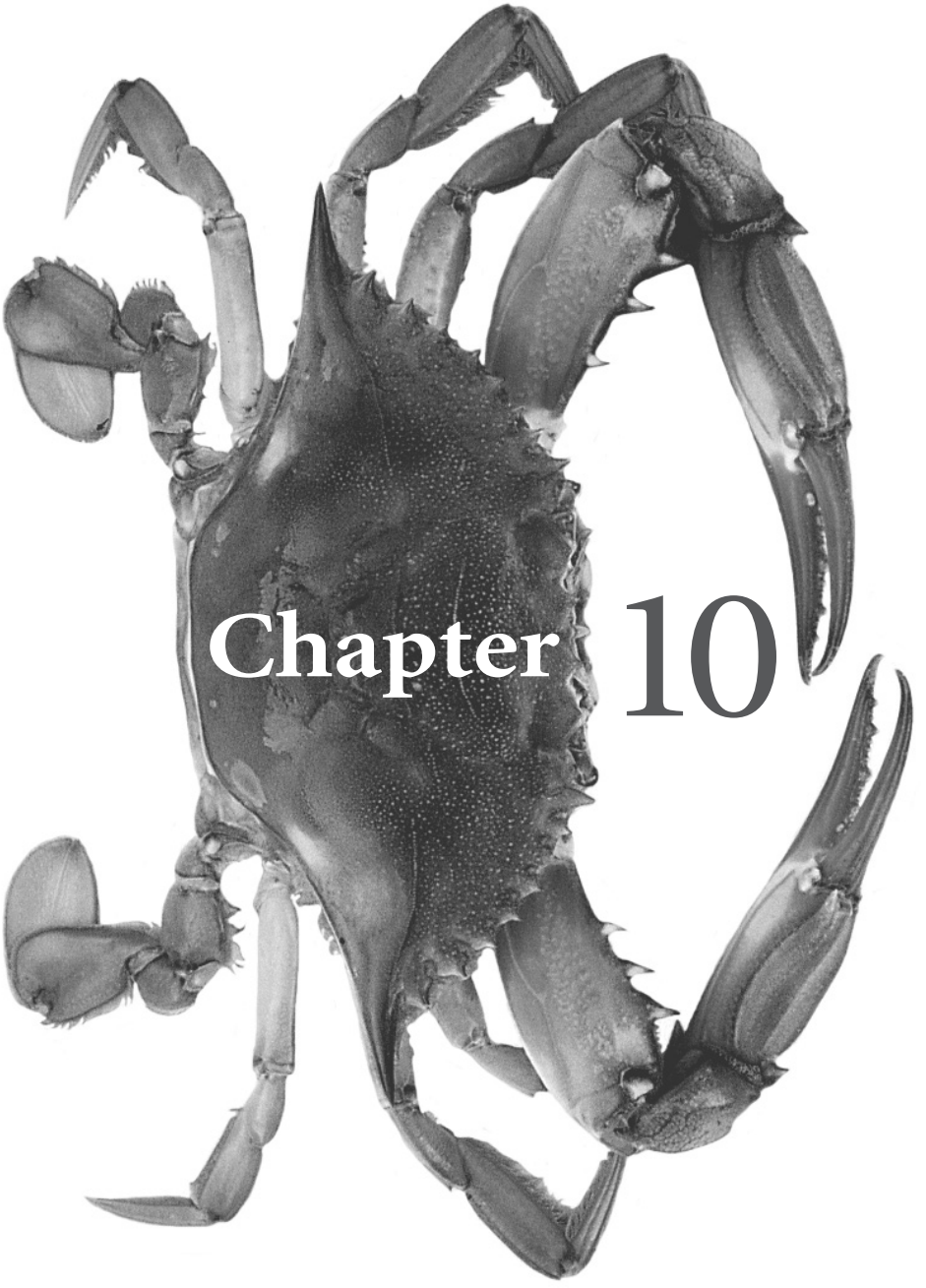
Chapter 4 gives an overview of the management of neuroendocrine hepatic metastases. Hepatic metastases are frequently encountered in patients with neuroendocrine tumors and their presence plays an important role in quality of life and overall prognosis. For diagnosis, helicalCT and somatostatin receptor scintigraphy are the most sensitive modalities. Platelet serotonin and plasma chromogranin A can be reliably used for diagnosis as well as for monitoring the outcome of treatment in individual patients. Surgery is the treatment method of choice for hepatic metastases but is frequently impossible due to the extent of the disease. In neuorendocrine tumors treatment is aimed at cytoreduction of hepatic metastasis to decrease secretion of bioactive amines. Tumor ablation by RFA or LITT provides a novel liver preserving option. These ablative techniques should be used early in the course of the disease in order to postpone drug intervention and prevent end stage carcinoid syndrome, thereby improving life expectancy.

Local recurrence after LITT or RFA is still high. To overcome this problem we have investigated in **chapter 5** the potential of additional chemotherapy after LITT in an animal model. We

established a murine tumor model in which recurrent tumor growth occurs after locally ablative treatment of solitary liver metastases. We have shown that combination of LITT and doxorubicin had a strong synergistic effect leading to complete tumor remission in all animals treated. The result of this study may provide a basis for further clinical investigation of combined treatment with local ablation together with chemotherapy in patients with colorectal liver metastases.

In the second part we have assessed the contribution of mutant KRAS and different signalling pathways during late stages of colorectal cancer en liver metastasis formation.
Oncogenic mutations in the KRAS gene are found in approximately 40% of human colorectal carcinoma and are acquired during the earliest stages of colorectal cancer development. Mutant KRAS might haven potential as a target for therapy in colorectal carcinoma, but tumor growth should then be still dependent on its continued presence in a background of many genetic mutations. In **chapter 6**, we have analyzed the effect of suppression of the mutant KRAS allele by RNA interference in a highly aggressive mouse CRC cell line (C26). We have found that stable knockdown of the mutant KRAS allele resulted in loss of transformed properties in vitro. The incidence of subcutaneous tumor formation was spectacularly reduced, the lag time was increased and KRAS-knockdown tumors grew noninvasively and did not cause morbidity. Surprisingly, KRAS-knockdown tumors elicited an anti-tumor immune response that resulted in spontaneous regression in some KRAS-knockdown tumors. In addition, KRAS-knockdown resulted in increased production of interleukin18 (IL18), a cytokine that has strong immune-stimulatory functions. Thus mutant KRAS suppresses IL18 production in colorectal tumor cells. This may contribute to evasion of the local immune system during tumor development.

Loss of epithelial morphology and the acquisition of mesenchymal characteristics may contribute to metastases formation during colorectal tumor development. Different signaling pathways, such as Wnt, Notch and TGFβ regulate epithelial homeostasis and tumor development in the gut. In **chapter 7** signaling pathways involved in metastases formation of colorectal cancer are investigated. We have analyzed the expression of epithelial and mesenchymal markers in a series of primary colorectal carcinoma and their corresponding metastases. We have found that primary colorectal tumor displayed an increased expression of cytoplasmic vimentin, nuclear β-catenin (Wnt), p-SMAD2 (TGFβ) and HES1(Notch). The corresponding metastases did not express vimentin and displayed significant lower levels of p-SMAD2 and HES1, while retaining nuclear β-catenin. Furthermore, endogenous mutant KRAS and nuclear β-catenin, but not p-SMAD2 was associated with the expression of vimentin in human colorectal tumors. These data suggests that the activity of the Notch and TGFβ pathways is reduced in secondary colorectal tumors compared to primary colorectal carcinomas. Whether metastases from colorectal cancer are dependent on Notch and TGFβ signaling pathways for their proliferation and survival needs further investigation.



Chapter 10

Nederlandse samenvatting

Dikke darmkanker (colorectaal carcinoom) is in Nederland en wereldwijd de tweede meest voorkomende vorm van kanker. Ieder jaar komen er in Nederland 10.000 nieuwe patiënten met een kwaadaardige tumor in de dikke darm bij en dit aantal neemt nog steeds toe. Ongeveer de helft van deze patiënten krijgt vroeg of laat één of meer uitzaaiingen naar andere organen, vooral naar de lever (levermetastasen). Een kwart van de patiënten met levermetastasen kan worden behandeld met een operatie, waarbij het aangedane deel van de lever wordt verwijderd (leverresectie). Deze operatie is momenteel de gouden standaard omdat het de beste kans op genezing en langdurige overleving biedt. Vijf jaar na deze operatie is 30-40% van de patiënten nog in leven. Niet iedere patiënt kan deze ingreep ondergaan omdat ofwel te weinig gezond leverweefsel na de operatie overblijft ofwel ook uitzaaiingen buiten de lever zijn geconstateerd. Tevens is naast de conditie van de patiënt, het aantal en de locatie van de metastasen in de lever van belang. Chemotherapie kan voor patiënten met zeer uitgebreide metastasering een optie zijn. Hierdoor is, dankzij recente ontwikkelingen, de gemiddelde overleving van deze patiënten de afgelopen jaren toegenomen van 10 naar 20 maanden.
Voor patiënten met niet-operabele levermetastasen zijn tevens technieken ontwikkeld waarbij met behulp van hitte de tumor vernietigd kan worden (lokale ablatie). Door de hoge temperatuur valt het DNA van de kankercellen uit elkaar en coaguleren de eiwitten, waardoor de cellen het niet overleven. Er bestaan verschillende technieken voor de behandeling met hitte. Bij lokale ablatie met laser-geïnduceerde thermotherapie (LITT) wordt tijdens de operatie een glasvezel (fiber) rechtstreeks in de tumor gebracht, waarna laserlicht via de fiber het weefsel lokaal verhit tot meer dan 60 graden Celsius. Ook kan gebruik worden gemaakt van radiofrequente ablatie (RFA). Bij RFA wordt een elektrode in de tumor gebracht die een hoogfrequente wisselstroom veroorzaakt. Door de wisselstroom ontstaat een beweging van ionen rondom de elektrode, waardoor het weefsel wordt verhit. Inmiddels worden er goede resultaten behaald met deze lokale ablatie technieken.

Ondanks deze veelbelovende behandelingsmethode komen levermetastasen bij een groot deel van de patiënten terug, er is dan sprake van een recidief. Een oorzaak hiervan is meteen ook één van de belangrijke beperkingen van deze technieken, namelijk dat het behandelde gebied, ook wel coagulatie laesie genoemd, vaak niet groot genoeg is om een coagulatie laesie met voldoende tumor-vrije marge te bewerkstelligen. In **hoofdstuk 2** hebben wij verschillende technieken van LITT geanalyseerd om de grootte van de coagulatie laesie te maximaliseren. LITT kan op twee manieren worden uitgevoerd: door de huid (percutaan) of open, waarbij de lever wordt blootgelegd. Alle in dit hoofdstuk beschreven LITT behandelingen worden via de open procedure toegepast. Deze open chirurgische procedure heeft de voorkeur omdat, behalve dat het mogelijk is de tumor nauwkeurig te lokaliseren en een eventueel eerder niet erkende tumor op te sporen, het mogelijk is om de bloedtoevoer naar de lever af te klemmen. De patiënten die in dit hoofdstuk worden beschreven tonen aan dat door het gebruik van meerdere met water gekoelde fibers tegelijk een grotere coagulatie laesie gemaakt kan worden. Tevens kan de laesie nog worden uitgebreid door de circulatie naar de lever af te klemmen, waardoor voorkomen wordt dat de hitte wordt weggevoerd met de bloedstroom. Uiteindelijk konden coagulatie laesies geproduceerd worden tot 8,6 cm in diameter.
Dus met deze unieke techniek, door het gebruik van meerdere water gekoelde fibers tegelijk en het afklemmen van de bloedtoevoer, wordt de tumorvernietiging gemaximaliseerd. Dit vergroot de behandelingsmogelijkheden voor patiënten met niet-operabele grote levermetastasen.

Lokale ablatie kan worden toegepast voor de behandeling van levermetastasen van meerdere soorten kanker. In **hoofdstuk 3** wordt RFA toegepast in patiënten met levermetastasen van neuroendocriene tumoren. Neuroendocriene tumoren komen onder andere voor in het maag-, darm- en longstelsel en in de alvleesklier en kunnen uitzaaien naar andere organen, met name naar de lever. Bij het stellen van de diagnose zijn vaak al metastasen aanwezig. Deze tumoren produceren hormonen die veel en uiteenlopende symptomen kunnen geven. Bij uitzaaiingen in de lever komen deze stoffen direct vrij in de bloedcirculatie en kunnen zo aanleiding geven tot klachten als opvliegers, diarree en aanvallen van kortademigheid. RFA van neuroendocriene levermetastasen bleek zeer bemoedigende resultaten te geven met een goede lokale tumorcontrole en een bevredigende duur van symptoomverlichting. Echter, een belangrijke beperking van lokale tumorablatie met RFA bleek ook weer de omvang van de coagulatie laesie die geproduceerd kan worden. **Hoofdstuk 3** is toegespitst op het gebruik van multipolair RFA (meerdere bipolaire elektrodes tegelijk) in neuroendocriene levermetastasen om een zo groot mogelijke coagulatie laesie te verkrijgen. In de beschreven patiënt, werden vier bipolaire elektroden gelijktijdig gebruikt en werd de bloedtoevoer naar de lever afgeklemd. Hierbij werd een coagulatie laesie bereikt met een diameter van 12 cm. Ook de RFA procedures werden via de open methode toegepast. Deze resultaten laten zien dat door het gebruik van meerdere bipolaire elektrodes tegelijk (multipolair RFA) in combinatie met het afsluiten van de bloedtoevoer naar de lever, een zeer grote coagulatie laesie kan worden geproduceerd. Deze vernieuwde techniek vergroot de behandelingsmogelijkheden voor patiënten met zeer grote levermetastasen.

In **hoofdstuk 4** werd een literatuurstudie verricht om te bepalen hoe het beste de diagnose kan worden gesteld en wat de beste behandelingsstrategie is voor patiënten met levermetastasen van neuroendocriene tumoren. Serotonine is het meest voorkomende en bekendste product van deze tumorcellen. Om de diagnose te stellen kan in het bloed het serotonine gehalte worden bepaald. Daarbij is ook Chromogranin A in het bloed een zeer sensitieve tumormarker. Beide tumormarkers kunnen ook worden gebruikt om de effectiviteit van de behandeling te bepalen en om eventueel een recidief tumor te diagnosticeren. CT-scan en somatostatine receptor scintigrafie zijn onderzoeken die zeer sensitief zijn voor het lokaliseren van neuroendocriene tumoren en eventuele uitzaaiingen. Chirurgische resectie is de behandeling van eerste keuze voor deze levermetastasen. Helaas komen veel patiënten niet in aanmerking voor een resectie omdat de levermetastasen vaak te uitgebreid zijn. De behandeling van neuroendocirene levermetastasen is er op gericht om tumoren te reduceren en de uitscheiding van actieve hormonen te verminderen. Met de nieuwe technieken van RFA en LITT kan de tumor lokaal worden vernietigd en gezond leverweefsel worden gespaard. RFA of LITT moet in een zo vroeg mogelijk stadium van de ziekte worden toegepast zodat behandeling met medicijnen zo lang mogelijk kan worden uitgesteld en de levensverwachting wordt verbeterd.

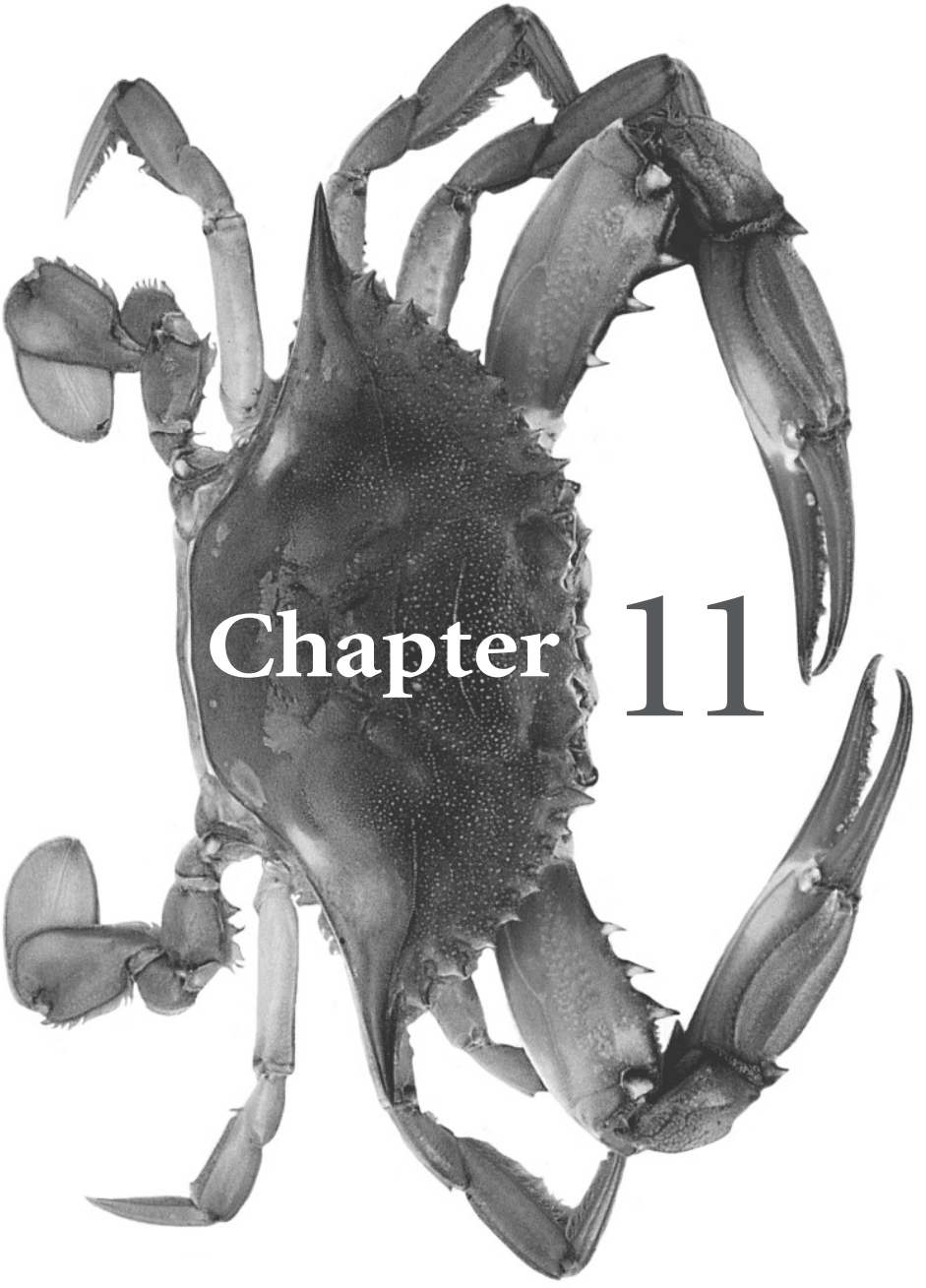
Voor patiënten met niet-operabele levermetastasen van colorectaal carcinoom zijn locale behandelingen met LITT of RFA sterk in opkomst. Helaas zijn de resultaten niet subliem en worden er recidief percentages gerapporteerd tot 63%. In **hoofdstuk 5** werd onderzocht of doxorubicine als aanvullende chemotherapie na LITT recidief tumor groei kan voorkomen. Hiervoor werd een tumormodel in muizen ontwikkeld waarin recidief tumorgroei werd gezien na LITT van een solitaire levermetastase. In dit levermetastasen model in de muis leidt

aanvankelijke behandeling met LITT tot een aanzienlijke afname van de grootte van de levermetastasen. Lokaal recidief trad op in 25 % van de muizen na LITT. Behandeling met alleen chemotherapie (doxorubicine) zonder LITT had geen effect op tumor groei. De combinatie van LITT en doxorubicine had een sterk synergistisch effect en leidde tot volledige tumor remissie. Het resultaat van deze studie vormt een basis voor verder klinisch onderzoek van gecombineerde behandeling van chemotherapie en LITT in patiënten met levermetastasen van colorectaal carcinoom.

In het tweede deel van dit proefschrift werd de rol van het gemuteerde KRAS gen en verschillende signaal routes in de ontwikkeling van colorectaal carcinoom bestudeerd.
Bij ongeveer 40% van de patiënten met darmkanker wordt een fout (mutatie) in het KRAS gen gevonden. Dit gen maakt het KRAS eiwit, dat de cel aan en uit kan zetten. Bij een mutatie blijft de cel aan staan. Dit houdt in dat de cel zich blijft delen en dit kan leiden tot darmkanker. Deze mutatie wordt verworven tijdens de vroegste stadia van ontwikkeling van colorectaal carcinoom. Veelvoudige additionele veranderingen in de genen zijn daarna vereist voordat uiteindelijk darmkanker en eventuele metastasen kunnen ontstaan. In **hoofdstuk 6** werd gekeken naar de rol van het gemuteerde KRAS gen in het ontstaan van dikke darmkanker. Hiervoor hebben we gebruik gemaakt van een zeer agressieve dikke darmkanker cellijn (C26 cellen) uit muizen. Na genetisch onderzoek van deze C26 cellijn bleek dat deze cellen een mutatie in het KRAS gen hebben. Indien deze C26 cellen vlak onder de huid van muizen worden geïnjecteerd, ontstaan er zeer snel, binnen 12 dagen, grote onderhuidse tumoren. Met een nieuwe moleculaire methode werd het gemuteerde KRAS gen uitgeschakeld in deze C26 cellen. Deze cellen werden wederom onder de huid van muizen geïnjecteerd. Na het uitschakelen van het KRAS gen konden deze cellen nog in slechts 40% van de ingespoten muizen uitgroeien tot een waarneembare tumor. Daarnaast duurde het vele malen langer voordat deze tumoren gingen groeien en ondervonden de muizen totaal geen hinder van de tumoren. Opmerkelijk verdween na enige tijd de ontstane tumor bij 30% van de muizen. Dit bleek te berusten op het feit dat na het uitschakelen van het KRAS gen het immuunsysteem van de muis deze tumorcellen wist op te ruimen. Bovendien toonden de tumorcellen waarin het KRAS gen was uitgeschakeld een verhoogde productie van interleukine 18 (IL18). IL18 is een eiwit dat betrokken is bij de afweer tegen kankercellen. Door het uitschakelen van het KRAS gen zijn de tumorcellen in staat om het immuunsysteem te activeren. Het KRAS gen zou daarom een goed aangrijpingspunt kunnen zijn om een therapie tegen dikke darmkanker te ontwikkelen.

De ontwikkeling van dikke darmkanker en het uitzaaien hiervan naar andere organen is een zeer ingewikkeld proces waarbij verschillende signaal routes op moleculair niveau zijn betrokken. In **hoofdstuk 7** werden verschillende signaal routes die betrokken zijn bij de vorming van metastasen van colorectaal carcinoom onderzocht. Hiervoor werd gekeken naar de expressie van de adhesiemolekulen, E-cadherine (epitheliale marker) en vimentine (mesenchymale marker), in tumorweefsel van de dikke darm en de metastasen. Daarnaast werd in dit tumorweefsel gekeken naar markers van verschillende signaal routes, te weten β-catenine voor Wnt signalering, HES1 voor Notch signalering en pSMAD2 voor TGFβ signalering. Bovendien werd bij elke patiënt gekeken of een KRAS genmutatie aanwezig was in het primaire tumor weefsel en in de bijbehorende levermetastase. In de primaire colorectale tumor werd een verhoogde expressie gezien van vimentine, β-catenine, HES1 en pSMAD2. De ver-

hoogde expressie van vimentine in de primair colorectale tumor correleerde met de aanwezigheid van een mutatie in het KRAS gen en met de verhoogde expressie van β-catenine. Heel opmerkelijk vertoonden de metastasen van het colorectaal carcinoom geen expressie van vimentine en daarbij een verlaagde expressie van HES1 en pSMAD2. Dit suggereert dat de activiteit van de Notch en TGFβ routes in colorectale metastasen verlaagd is ten opzichte van de primaire tumor. Verandering in expressie en functie van adhesiemolekulen aan de oppervlakte van kankercellen en veranderingen in signaal routes zijn belangrijke kenmerken in het ontstaan van kanker en kunnen mogelijk in de toekomst fungeren als prognostische factoren of nieuwe targets voor diagnostische en therapeutische doeleinden. De bevindingen in dit hoofdstuk kunnen belangrijke consequenties hebben voor de ontwikkeling van nieuwe therapieën.



Chapter 11

Dankwoord

Een proefschrift maken is een niet-geregistreerde vorm van team sport. Ook dit proefschrift kwam tot stand door hulp van velen die ik graag persoonlijk wil bedanken.

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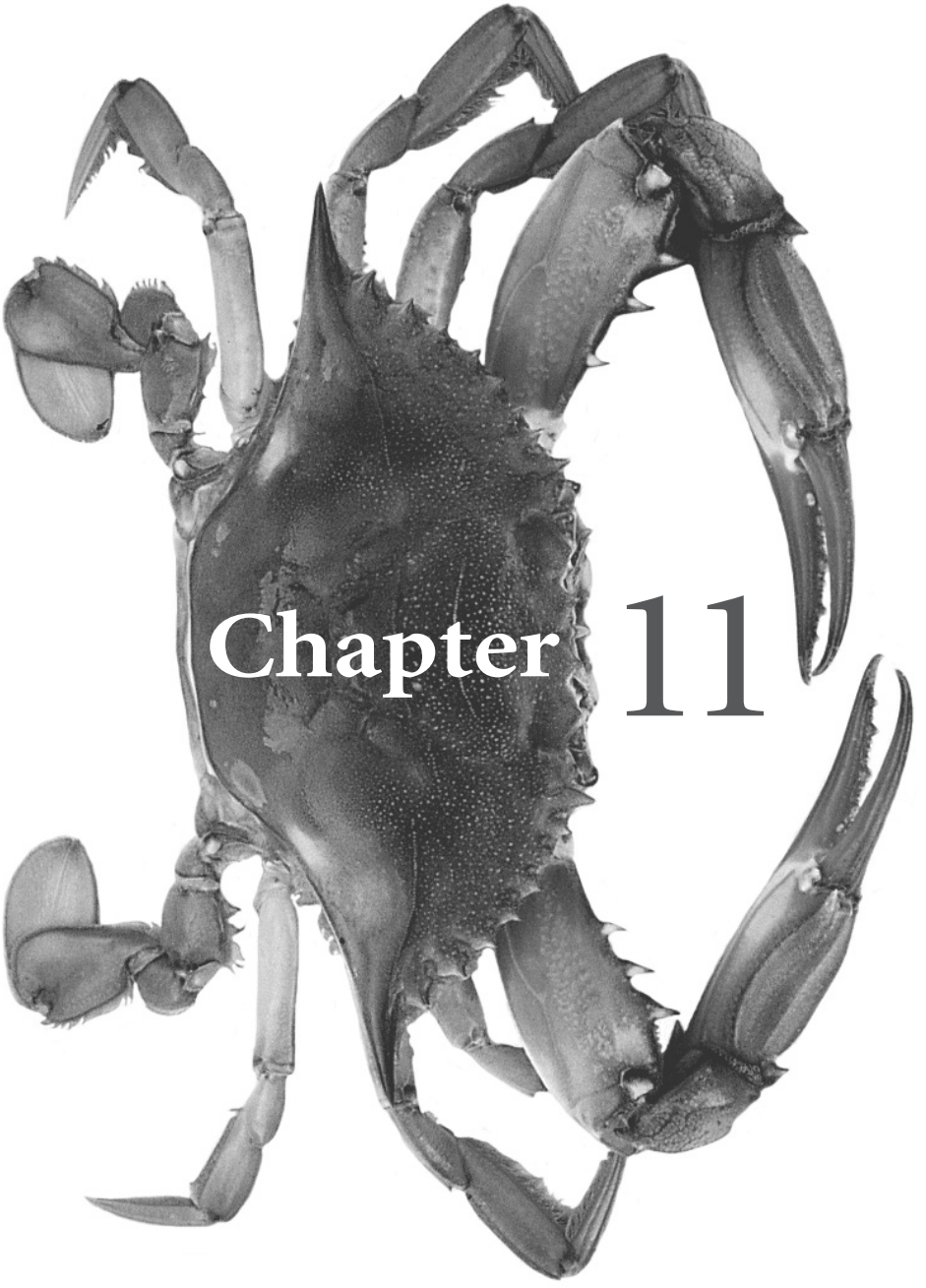
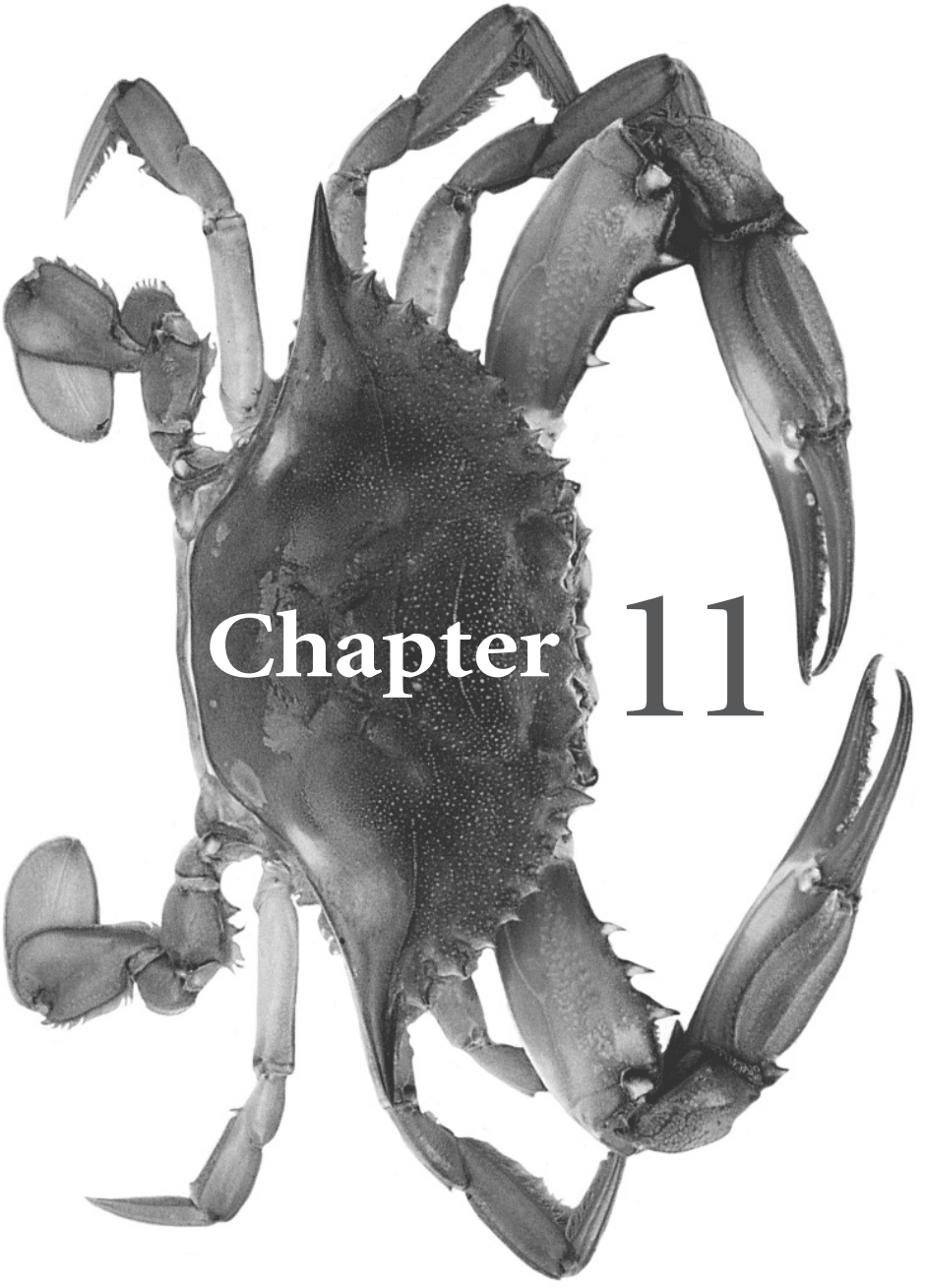


Chapter 11

Curriculum vitae

Curriculum vitae

Liesbeth Maaike Veenendaal was born on October 22nd, 1972 in Waalre, The Netherlands, where she grew up and graduated from the Eindhovens Protestants Lyceum, in 1992. From there she went on to study Medical Biology at the University of Utrecht, and in 1995 she entered the Faculty of Medicine, combining both studies at the University of Utrecht.
During her studies she completed a number of important research projects, both in the Netherlands and the United States. In 1997, under the supervision of Dr. G.J.J.C. Boonen at the department of Haematology, University of Utrecht, she conducted a research project focussing on intracellular cytokine production of T cells in AML patients. At the M.D. Anderson Cancer Center in Houston, Texas, under Prof. dr. M.G. Rosenblum and Prof. dr. A.P.M. Heintz, she conducted in vitro and in vivo studies of a VEGF 121-rGelonin chimeric fusion toxin targeting the neovasculature of solid tumours. This research project was made possible by grants from the Dutch Cancer Society (KWF) and the University of Utrecht (Trajectum beurs), and in 1998 she was awarded the Nijbakker-Morra Foundation prize for her work.
Liesbeth received her M.Sc. in Medical Biology in 1999 and her M.Sc. in Medicine in 2001, after which she began her surgical residency at the Diakonessenhuis Hospital in Utrecht under Dr. G.J. Clevers. During this time she studied the long-term recurrence rate and perioperative complications after posterior inguinal mesh hernia repair (Ugahary). In 2006 she began her residency program in general surgery, region Utrecht (head: Prof. dr. I.H.M. Borel Rinkes) at the Diakonessenhuis Hospital in Utrecht (headed by Dr. G.J. Clevers).
The research described in this thesis was conducted between 2003 and 2006 at the department of Surgery under Prof. dr. I.H.M. Borel Rinkes, and the laboratory of Experimental Oncology under Prof. dr. E.E. Voest and Prof. dr. R. Medema. It was made possible by an AGIKO stipend (Assistent Geneeskundige in Opleiding tot Klinisch Onderzoeker) from The Netherlands Organization for Health Research and Development (NWO). Her work described in this thesis was awarded the best abstract at the 6th Congress of the European Hepato-Pancreato-Biliary Association in Heidelberg, Germany.



Chapter 11

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List of publications

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