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Die Signalwegskomponenten LEF-1 und EGFR als Biomarker in der Karzinogenese des kolorektalen Karzinoms

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Summary

Since colorectal cancer is the second most common tumor entity in Europe there is a great need for research regarding the disease and its cure. The understanding of the origin and development of cancer has increased with the exploration of certain hallmarks of this disease over the last decades. The deregulation of signaling pathways such as the Wnt pathway and the epidermal growth factor receptor (EGFR) signaling pathways leads to properties that cause cancer and let it expand. Components of these pathways can be used in order to make predictions about the course of the disease.

The presence of the transcription factors LEF-1 and TCF4 of the Wnt pathway was analyzed with immunohistochemical methods in tumor tissue from patients with colorectal cancers to correlate them with the overall survival of patients. Univariate analysis showed that the expression of TCF4 constitutes a negative prognostic factor with shorter overall survival. In contrast the expression of LEF-1 as well as a LEF-1/TCF4 ratio were positive prognostic factors and correlated with longer overall survival. This work takes a closer look on the in vitro characteristics of those two transcription factors to get an insight into the different roles and functionalities. Cell cultures with reduced and enhanced TCF4 or LEF-1 expression were studied and analyzed. Several assays analyzing the cells characteristics like proliferation and migration showed no differences between the two transcription factors.

There are also drugs that are being developed and used to interact with these signaling pathways to reduce the progression of the disease. A prediction of their efficacy is important since side effects often occur and not all patients respond to these drugs. For this purpose, suitable predictive biomarkers can be used to assign patients to groups and introduce them to the most suitable therapies. However, not all patients respond to the selected therapy because predictive biomarkers which would allow assigning the best therapy with 100 % certainty have not been found yet. In case of the therapy of metastatic colorectal carcinomas with the monoclonal antibody cetuximab there is already an

established predictive biomarker being used in diagnostics – the *KRAS* gene. When mutated, the *KRAS* gene leads to an exclusion of the therapy. Yet only 40 % of patients with wild type *KRAS* respond to the treatment with cetuximab. Thus a more convincing predictive biomarker is needed. Another marker that is already known to indicate a good response to therapy is the development of skin toxicity. However this rash only occurs after patients have been treated with the antibody and it is therefore not a suitable predictive biomarker.

To find a predictive biomarker for the response, a marker that correlates with the skin toxicity was being looked for. For this purpose the EGFR was analyzed further because it is the primary target for the antibody. For this work the coding region of the *EGFR* from samples of patients with and without skin toxicity was sequenced. Both groups were then correlated. In the analyzed samples there were no polymorphisms in the coding region of the *EGFR* gene that were associated with skin toxicity induced by the targeted anti-EGFR therapy in metastatic colorectal cancer using cetuximab.

Zusammenfassung

Da Kolorektale Karzinome die zweithäufigste Tumorentität in Europa darstellen besteht ein großer Bedarf an Forschung in Bezug auf die Krankheit und ihre Heilung. Das Verständnis über die Entstehung und Entwicklung von Krebs hat in den letzten Jahrzehnten mit der Erforschung bestimmter Eigenschaften dieser Krankheit zugenommen. Die Deregulierung von Signalwegen wie dem Wnt-Signalweg und den EGFR-Signalwegen führt zu den Eigenschaften, die Krebs entstehen und sich ausbreiten lassen. Komponenten dieser Signalwege lassen sich nutzen, um Prognosen über den Verlauf der Krankheit zu stellen.

Das Vorliegen der Transkriptionsfaktoren LEF-1 und TCF4 des Wnt-Signalwegs wurde mittels immunhistochemischer Methoden in Tumorgewebe von Patienten mit Darmkrebs analysiert, um sie mit deren Überleben zu korrelieren. Univariate Analysen zeigten, dass die Expression von TCF4 als negativ prognostischer Faktor mit kürzerem Gesamtüberleben gesehen werden kann. Im Gegenteil hierzu ist die LEF-1-Expression und das LEF-1/TCF4-Verhältnis mit einem längeren Gesamtüberleben assoziiert und hat somit einen positiv prognostischen Wert. Diese Arbeit betrachtet die in-vitro-Eigenschaften der beiden Transkriptionsfaktoren, um einen genaueren Einblick in ihre verschiedenen Rollen und Funktionen zu erhalten.

Es werden auch Medikamente entwickelt und genutzt, die in diese Signalwege eingreifen, um das Voranschreiten der Krankheit einzudämmen. Da häufig Nebenwirkungen auftreten und nicht alle Patienten auf diese Medikamente ansprechen ist eine Vorhersage über ihre Wirksamkeit wichtig. Hierfür eignen sich prädiktive Biomarker, die genutzt werden können, um Patienten im Vornherein in Gruppen einzuteilen und diese den optimalen Therapien zuzuführen. Dennoch reagieren meist nicht alle Patienten auf die für sie ausgewählte Therapie, da die optimalen prädiktiven Biomarker, die es zulassen, jeden Patienten der passenden Therapie zuzuführen, noch nicht gefunden wurden. Im Falle der Durchführung einer Behandlung von metastasierenden kolorektalen Karzinomen mit dem monoklonalen Antikörper Cetuximab wird in der Diagnostik bereits der prädiktive

Biomarker *KRAS* genutzt, welcher beim Vorliegen einer Mutation zum Ausschluss der Therapie mit Cetuximab führt. Dennoch reagieren nur 40 Prozent der Patienten mit dem wildtypischen *KRAS* auf die Therapie mit Cetuximab. Somit wird ein aussagekräftigerer prädiktiver Biomarker benötigt. Ein weiterer bereits bekannter Marker für ein gutes Ansprechen auf die Therapie ist die Entwicklung eines Hautauschlags. Dieser setzt allerdings erst nach Start der Behandlung mit dem Antikörper ein und eignet sich somit nicht als prädiktiver Biomarker.

Um einen prädiktiven Biomarker für das Ansprechen zu identifizieren, wurde in dieser Arbeit nach einem Marker gesucht, der mit diesem Hautausschlag korreliert. Hierfür wurde der EGF-Rezeptor analysiert, da dieser den primären Angriffspunkt für den Antikörper darstellt. Der kodierende Bereich des *EGFR* von Proben von Patienten mit und ohne Hautausschlag wurde sequenziert und diese beiden Gruppen korreliert. Es wurden in den untersuchten Proben keine Polymorphismen gefunden, die mit einer der beiden Gruppen in Zusammenhang stehen. Somit korrelieren Polymorphismen in der kodierenden Region des *EGFR* nicht mit dem Hautauschlag, der durch die anti-EGFR Therapie mit dem monoklonalen Antikörper Cetuximab hervorgerufen wird.

1 Introduction

1.1 Colorectal cancer

Colorectal cancer (CRC) is the second most common cancer in Europe, and with more than 200,000 deaths per year in 2012 also the second most common cancer cause of death [28] (Figure 1).

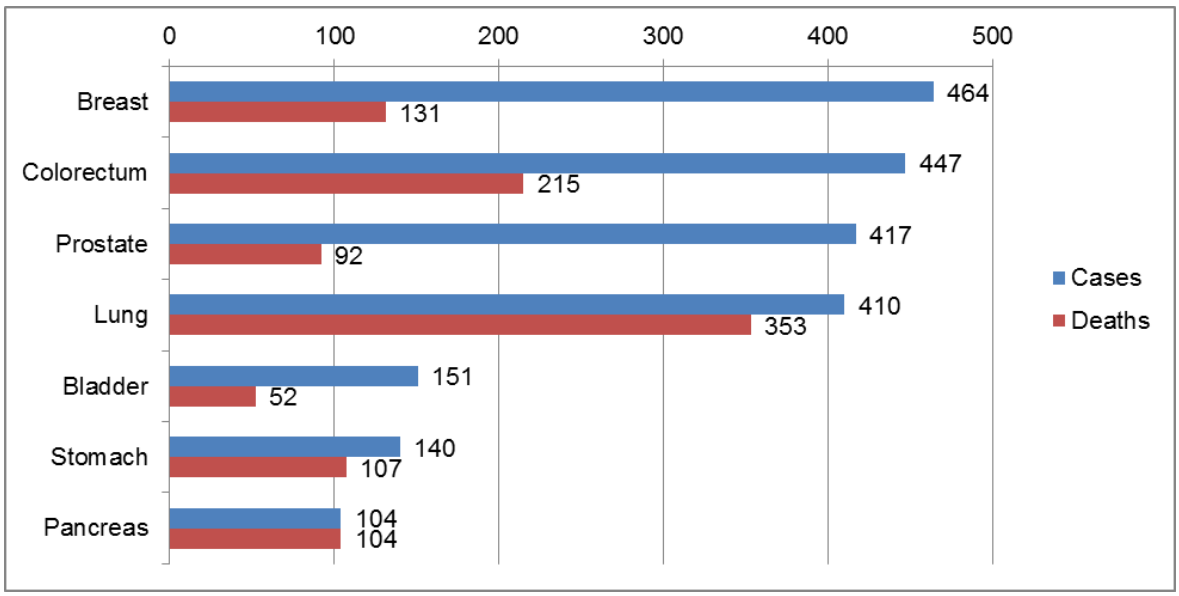

Figure 1. Estimated numbers of cancer cases and cancer deaths of the most common cancers in the 40 European countries (in thousands) (*numbers from Ferlay J et al.* [28])

Most cases of CRC arise sporadically and there are many known risk factors favoring this disease: increasing age, male sex, previous colonic polyps, environmental factors and others [66]. Inflammatory bowel disease (ulcerative colitis and Crohn's disease) also accounts for about two-thirds of the cases [23, 106]. It is assumed that colorectal carcinogenesis is a multistep process and takes years to decades to evolve. Vogelstein *et al.* postulated the development via a stepwise acquisition of changes in gatekeeper and caretaker molecular pathways during the adenoma-carcinoma sequence [105] (Figure 2).

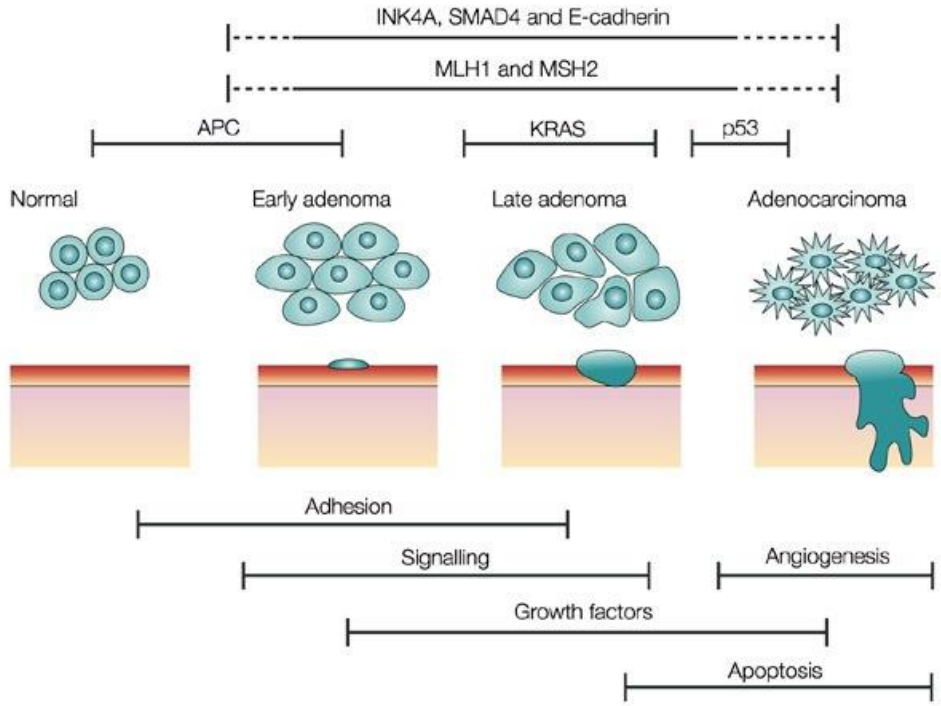

Figure 2. Basic outline of the transition from normal colon epithelium to adenoma and then to carcinoma with affected genes (top) and associated phenotypic changes (bottom) (*from Kerr D* [50])

In CRC some pathways like the Wnt/β-catenin pathway and pathways downstream of the epidermal growth factor receptor (EGFR) are often affected by mutations [19, 22, 32]. A deficiency of the DNA mismatch repair (MMR) also drives tumor manifestation [15]. CRC can be classified into distinct subtypes based on those affected mechanisms. One postulated classification system is shown in Figure 3 [94].

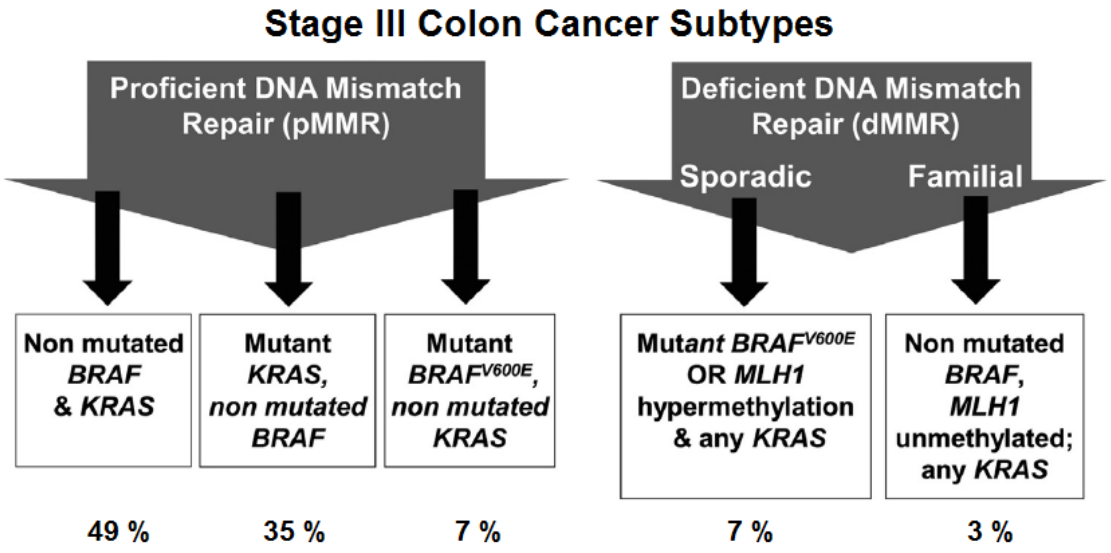

Figure 3. Categorization of stage III colon cancer into five subtypes based on MMR status and mutations in KRAS (exon2) and BRAF (V600E) (*modified from Sinicrope FA et al.* [94])

Effects of those subtypes on prognosis and therapy are mentioned in the following chapters.

Intensified screening for the disease has increased survival rates. Nevertheless the five year relative survival rate in most European countries is still less than 60% [82, 104]. A lot of research focuses on the understanding and treatment of CRC and cancer in general due to the high threat of this disease.

1.2 Hallmarks of cancer

Cancer is a disease during which several changes in the genome occur. Some mutations can result in a dominant gain of function in oncogenes or a recessive loss of function in tumor suppressor genes. Cancer needs to accumulate certain traits to evolve into the serious stages of the disease. It has been shown that tumorigenesis is a multistep process, each step reflecting genetic alterations that drive the progressive transformation. Normal cells evolve through several stages to become neoplastic and eventually tumorigenic and malignant. During that course they acquire certain universal hallmarks. Six hallmarks have been postulated by Weinberg et al. more than 10 years ago and two more have been added recently showing the still ongoing research and the complexity of this disease [39]. These hallmarks include sustaining proliferative signaling, evading

growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis. The recently added hallmarks are the reprogramming of energy metabolism and evading immune destruction [40] (Figure 4).

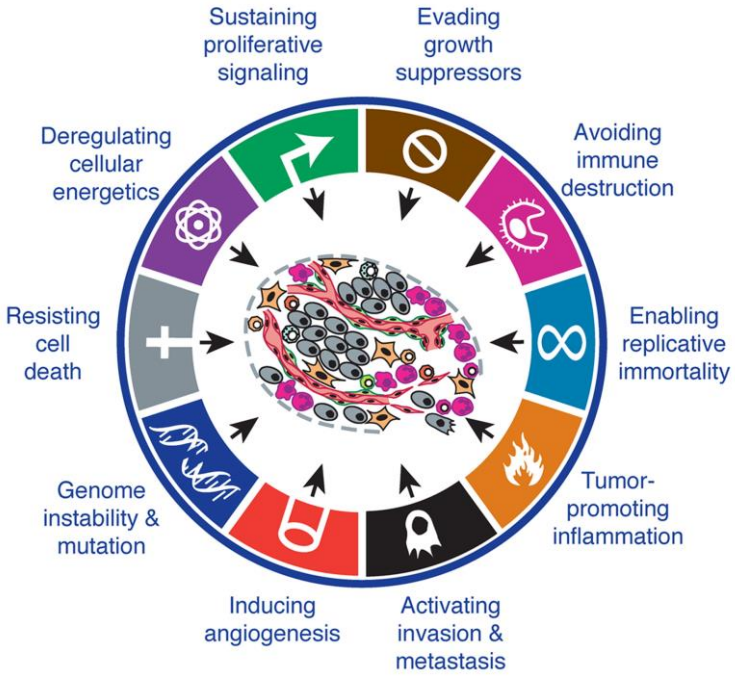

Figure 4. Hallmarks and characteristics acquired by cancers during the multistep development of tumors (*modified from Hanahan D et al.* [40])

Cancerous cells have acquired most, if not all of these hallmarks [40]. Additionally tumors create their own supportive microenvironment which adds even more complexity. Underlying this development are changes in the cells signaling machineries. Two of the most prominent pathways that are deregulated in cancer will be discussed in the following chapters.

1.2.1 Self-sufficiency in growth signals in cancer

One of the hallmarks of cancer is the ability of cells to generate their own mitogenic signals endogenously [109]. Normal cells need mitogenic signals to proliferate. These are transduced into the cell via transmembrane receptors and conveyed via signaling pathways. Those principles are also being used by many cancers to mimic those proliferative mechanisms. One way is to produce their own growth factor ligands which enable an autocrine proliferative stimulation [27]. Cancer cells can also send paracrine signals to surrounding cells and cause them to produce growth stimulating factors [11]. To maintain high proliferative signaling, cancer cells can also elevate their receptor levels. This leads to a hyper responsiveness of the cells to growth factor levels that would normally not trigger proliferation [27]. Examples are the epidermal growth factor receptor (EGFR/*erbB*) in stomach, brain and breast tumors and the HER2/*neu* receptor in stomach and mammary carcinomas [95]. And finally, proliferative signaling can be maintained by ligand-independent pathway activation. For example, truncated versions of the EGFR lacking most of their cytoplasmic domain remain ubiquitously active [27]. Independent activation can also result from activating changes downstream in the pathway of the receptor. The aforementioned growth factor activated receptors act via the important mitogenic SOS-Ras-Raf-MAP kinase pathway [67] (Figure 5).

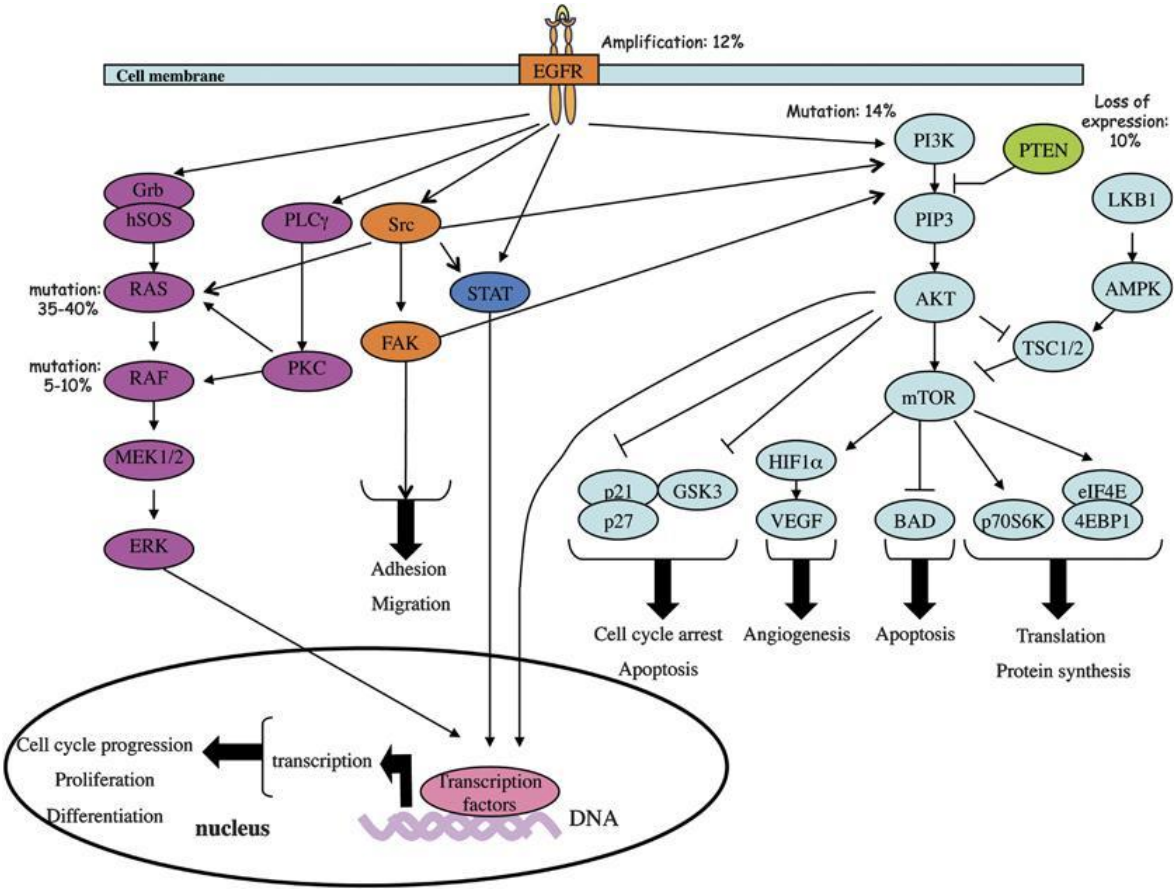

Figure 5. The EGFR and its downstream pathways. The frequencies of mutations observed in CRC are depicted. Additionally the roles of these pathways are given. (*modified from Lievre A et al.* [64])

In about 25 % of human tumors there are structurally altered Ras proteins that enable an ongoing flux of mitogenic signals without an external stimulation by the upstream regulators [71]. In colon carcinomas about half of the tumors express mutant *ras* oncogenes [53]. But there are many more effects in signaling pathways and networks that support the cancer promoting mitogenic signaling.

1.2.2 The Wnt/β-catenin pathway in cancer

Another pathway that is regularly altered in human carcinomas is the Wnt/β-catenin pathway (Figure 6). It influences several of the hallmarks mentioned above. This pathway which is normally active during embryogenesis can lead to effects on transcription and cell migration [85].

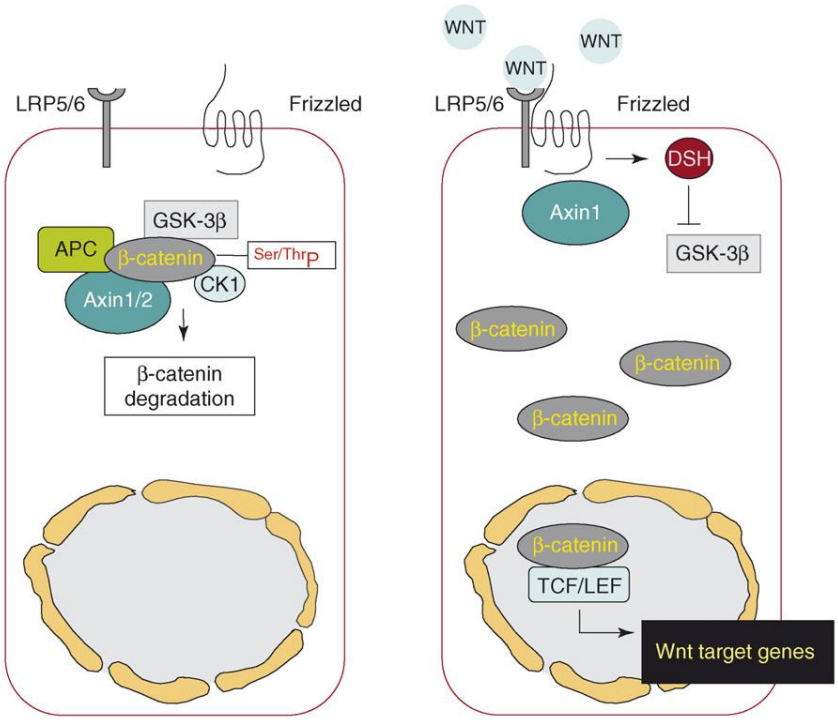

Figure 6. The Wnt/β-catenin signaling pathway. Left: β-catenin is marked for degradation in the absence of Wnt ligands. Right: in the presence of Wnt ligands the destruction complex is inhibited and β-catenin can accumulate and translocate to the nucleus where it can activate target genes (*modified from Fodde R et al.* [30])

The spectrum of target genes of the activated pathway that are controlled by β-catenin/TCF is seen to be the key for understanding the initial and following steps of transformation of intestinal and other cells. When β-catenin translocates to the nucleus it transactivates TCF/LEF target genes and promotes cellular growth and represses differentiation programs [43, 93, 98, 103, 108]. The functional roles of LEF-1

and TCF4 are not so clear. Different roles have been described. In melanomas the differential expression of LEF-1 and TCF4 is involved in melanoma cell phenotype switching. Expression of LEF-1 is primarily found in differentiated / proliferative phenotype cells whereas TCF4 is expressed preferentially by dedifferentiated / invasive phenotype cells [24]. In contrast experiments performed by Nguyen et al. showed that LEF-1 mediates lung adenocarcinoma metastasis [81]. In an immunohistochemical analysis of colorectal carcinomas it was found that LEF-1 and TCF4 expression are independent predictors of longer and shorter overall survival, respectively [57].

Over 90 % of colorectal carcinomas show mutations that activate the Wnt/β-catenin pathway leading to the stabilization and accumulation of β-catenin [32]. After acquiring this growth advantage, mutations inactivating tumor suppressor genes are required for tumor progression. The additional mutations may be facilitated by mutations in the adenomatous polyposis coli (*APC*) gene which is also a member of the pathway. By this acquisition of mutations it can lose one of its roles: stabilizing microtubules. These mutations can therefore result in chromosomal instability [29, 48]. Then changes in cell adhesion and migration that are influenced by the Wnt pathway further promote development of tumors [32, 38, 42, 77]. Even invasion and inhibition of death receptor-mediated apoptosis are driven by the deregulation of this pathway [79, 110].

In conclusion it can be said that the target genes of the deregulated Wnt pathway lead to tumor supporting traits including the hallmarks of cancer. Understanding its role in carcinogenesis is important, as some components have been shown to correlate with clinical stages of some tumors and may therefore be useful prognostic aids [22].

1.3 Signaling pathway components as prognostic biomarkers in CRC

Understanding the Wnt and EGFR pathways has clinical relevance because of their severe influence on the progression of cancer. Components of those pathways might serve as prognostic biomarkers (measurable indicators for how the disease develops regardless of the type of treatment).

The aberrant expression of Wnt pathway components correlates with advanced tumor stages, the probability of metastasis and the survival rate [22]. So the analysis of the pathway components can offer important prognostic information and might help to direct an appropriate treatment.

The protein β-catenin, one of the components of the pathway, has been well investigated. It has been shown that the β-catenin gene (*CTNNB1*) is an indispensable oncogene in some CRC cell lines [52]. A reduced colorectal tumor growth can be observed when targeting β-catenin in mice [60]. Another experiment showing its direct influence on cancer progression was the knockdown of β-catenin mRNA leading to a dose-dependent inhibition of tumor growth rates in colon carcinoma xenografts [36]. There is a reduction of the localization of β-catenin at the cell membrane in 70-84 % of established CRCs. The transcriptionally active nuclear and the cytoplasmic localization is increased in 66-79 % [41, 69]. Immunohistochemical studies have shown that there is a progressive increase in nuclear β-catenin staining while the epithelium changes from normal to dysplastic to cancerous [41]. Therefore, assessing the nuclear localization levels of β-catenin, could be used as a prognostic marker for CRCs. But not only β-catenin plays an important role in prognostic significance, also its target genes - the target genes of the Wnt pathway - are of interest. It is known, that the down-regulation of E-cadherin which is important for epithelial cell-cell adhesion correlates with an invasive potential and a poor prognosis in CRC [74, 100]. c-Myc, a prominent and important target gene is overexpressed in nearly half of all CRC while matrix metalloproteinase-7 (MMP7) is even overexpressed in 90 % [8, 84].

As already mentioned above, the EGFR plays an important role in CRC [19]. But the prognostic value of this prominent receptor is still under dispute. Several studies have looked into EGFR as a prognostic marker. These studies showed a correlation between its expression and advanced stage, worse histological grade and lymphovascular invasion [54, 56, 96]. In contrast, other more recent studies have found no relationship between the EGFR expression and histological type, tumor grade, stage or survival [21, 33, 70, 83]. Looking further downstream into the SOS-Ras-Raf-MAP kinase pathway, there are the Ras proteins KRAS and NRAS which are

frequently mutated in CRC (especially in the large group of CRC with proficient DNA MMR). The prognostic relevance for *KRAS* is still under dispute. Some studies showed evidence for it being a prognostic marker [3, 4], while others found no association [91, 107]. There is no convincing evidence that suggests that *KRAS* mutations are independent prognostic biomarkers. In contrast it has been shown that the *BRAF* V600E mutation in mCRC with proficient DNA MMR correlates with poor prognosis [86]. But despite the lack of an association of EGFR with clinical outcome, there have been successful pharmacological approaches inhibiting the EGFR which have a beneficial effect on CRC patients [80].

1.4 Signaling pathways as targets for therapy in CRC

The single agent 5-fluorouracil (5FU) therapy had been the treatment for mCRC since the 1950s. During the last 15 years the therapy evolved into combination chemotherapy and more recently even to a targeted therapy with the arrival of monoclonal antibodies. Targeted therapeutics are substances or drugs interfering with specific molecules that are involved in cancer cell growth and survival [78]. The promise of those therapies lies in the more specific inhibition of altered molecular pathways in cancer [17]. The use of monoclonal antibodies like cetuximab and panitumumab for the treatment of mCRC is the most prominent example of specifically directed therapies [14, 101]. They target the EGFR and therefore inhibit the downstream SOS-Ras-Raf-MAP kinase pathway which is known to promote tumor progression [14, 31, 35, 111].

The therapy of interest for this work is the therapeutic use of the monoclonal antibody cetuximab. There is evidence that a first-line treatment with cetuximab plus FOLFIRI (5-fluorouracil, leucovorin and irinotecan) compared to FOLFIRI treatment alone reduces the risk of progression of metastatic CRC [102]. Another study showed the same results for a treatment of cetuximab with FOLFOX-4 (5-fluorouracil, leucovorin and oxaliplatin) [7]. Although there have been advances using the combination of chemotherapy with EGFR inhibitors, the use of two antibodies has not proven to be successful. There have even been studies demonstrating a worse toxicity and efficacy when combining antibodies [44, 99].

The use of small EGFR-targeted tyrosine kinase inhibitors is another example for a targeted therapy. Gefitinib and erlotinib are small molecules that reversibly inhibit the EGFR tyrosine kinase [1, 76]. There have been studies showing an increased overall survival time using those small molecules in combination with chemotherapy for treatment but the side effects were severe [58, 72].

1.5 Predictive biomarkers in CRC

Targeted therapies are typically only applicable to a smaller subset of CRC. Consequently, there is a demand for better biomarkers to predict the response. This is essential because otherwise patients might not benefit from the therapy or show side effects. The approval of new therapies is associated with the availability of biomarkers for that therapy. Once again the most prominent example and already existing biomarker is connected to the EGFR pathway. The receptor itself plays an important role in CRC and is being used as a target for therapeutic antibodies [14, 101]. Important exceptions are CRC patients with mutated *KRAS/NRAS* genes which cause resistance to the therapy [16, 68]. Lying downstream of the EGFR, activating mutations in *KRAS/NRAS* cause an activation of the pathway even when the receptor is blocked for example by cetuximab. This makes mutated *KRAS/NRAS* a very meaningful negative predictive marker for anti-EGFR treatment (predictive markers can identify patients who benefit most likely from a given therapy). It has been shown, that patients with mutated KRAS/NRAS do not benefit from anti-EGFR treatment. They do not respond to the therapy and do not show prolonged survival or quality of life benefits [2, 49]. There are about 40 % of mCRC patients that have somatic activating mutations in *KRAS*. Far less patients have activating mutations in *NRAS* (~ 2 %) [46]. All those patients are therefore excluded from a therapy with monoclonal antibodies targeting the EGFR [2, 49].

1.6 Need for predictive biomarkers in CRC

Despite the appealing character of *KRAS* being a potent predictive biomarker, only about 60 % of mCRC patients with wild type *KRAS* do respond to the therapy with cetuximab or panitumumab [7, 102]. This lack of response can result from additional factors like absence of amphiregulin and epiregulin (EGFR ligands), activating mutations of *BRAF* or *NRAS*, loss of *PTEN* or *PI3K* activation [5]. Looking closer at

those factors, none could be confirmed as a convincing predictive biomarker [15, 55]. So there is still a need for further predictive biomarkers to be identified. One clue could lie in the fact that patients treated with anti-EGFR targeted drugs usually show a good response when they develop skin toxicity or acneiform rash [7, 14, 63, 102]. Thus, skin toxicity is a biomarker indicating response but unfortunately only after starting the treatment and therefore lacks predictive value.

1.7 Aims of this work

Predictive biomarker for anti-EGFR treatment

Skin toxicity is a biomarker that correlates with good response to anti-EGFR treatment. As this can only be seen after treatment of patients the aim of this work was to analyze whether skin toxicity and therefore good response are associated with a factor that can be measured before treatment. Therefore, the coding region of the *EGFR* gene was screened for the presence of genetic alterations: especially single nucleotide polymorphisms (SNPs) and mutations which might be associated with skin toxicity and thus indicate a response to anti-EGFR targeted therapy. So the 28 exons of the coding region of the *EGFR* gene of patients with metastatic colorectal cancer (mCRC) treated with cetuximab displaying either high grade (grade 3) or absence (grade 0) of skin toxicity that correlated with clinical response were analyzed with bidirectional Sanger sequencing. The aim was to find alterations that could therefore act as a new predictive biomarker for anti-EGFR treatment in mCRC.

β-catenin/LEF-1 and β-catenin/TCF4-regulated programs in CRC

Although some prognostic biomarkers for CRC have been established, there is still a need for further understanding the development of CRC to detect additional prognostic biomarkers. Therefore, the role of LEF-1 and TCF4 which are components of the Wnt pathway needs to be examined further. Consequently it was analyzed whether the change of β-catenin/TCF4 to β-catenin/LEF-1 mediated Wnt/β-catenin pathway activity induces migration, invasion and epithelial-mesenchymal transition (EMT) in the context of the invasion front of human CRC and therefore causes the malignant progression. This theory was investigated further by characterizing and comparing the β-catenin/TCF4 and β-catenin/LEF-1 regulated cellular processes. Knockdown and overexpression of the transcription factors LEF-1 and TCF4 and subsequent investigation of effects using assays for cell analysis were the main methods used. The aim was to find differences between the two transcription factors LEF-1 and TCF4 to therefore provide a new prognostic biomarker for CRC.

2 Material and methods

2.1 Molecular methods

Isolating genomic DNA from eukaryotic cells

The isolation of genomic DNA from eukaryotic cell cultures was performed using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol.

Isolating genomic DNA from CRC tissue (FFPE sections)

FFPE (formalin-fixed paraffin-embedded) tissue from the primary site of CRC of 19 patients with skin toxicity grade 0 and 26 patients with skin toxicity grade 3 was available from the CIOX study (AIO KRK-0104), a clinical study comparing the effects of capecitabine therapy with either irinotecan (CAPIRI) or oxaliplatin (CAPOX) in combination with cetuximab (Table 7) [75]. Skin toxicity was scored according to the common terminology criteria for adverse events [12]. The study protocol was approved by ethics committees of all participating centers. All patients provided written informed consent before entry into the study. Two whole FFPE sections (3 µm) of the tumors from each patient were used for DNA isolation. Tissue from tumor or normal tissue was not discriminated due to the low mutation rate in the *EGFR* gene in CRC [62] and the strict statistical conditions chosen (see 2.5 Statistical analysis page 31). DNA was isolated using QIAamp FFPE Tissue kits together with a QIAcube device (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Isolating genomic RNA from eukaryotic cells

RNeasy Mini Kits in combination with QIAshredder and the QIAcube device (all Qiagen, Hilden, Germany) were used for the purification of RNA from eukaryotic cell cultures following the manufacturer's instructions.

Determining DNA and RNA concentration

Concentrations of DNA and RNA were determined with the Nano Drop Spectrophotometer ND1000 (peqlab Biotechnologie GmbH, Erlangen, Germany). The nucleic acid solutions were diluted in water. Measurement was compared to a reference without nucleic acids.

Restriction enzyme hydrolysis of DNA

All restriction enzyme hydrolyses were performed with Fermentas FastDigest Enzymes strictly following the manufacturer's manual (Fermentas, St.Leon-Rot, Germany).

Polymerase chain reaction (PCR)

Amplification of DNA fragments was performed by PCR. Primers were designed flanking the DNA of interest using Primer 3 software [89]. All primers were obtained from biomers (Ulm, Germany). Primers were also used to add desired flanks to 3' or 5' ends of the DNA (e.g. tags, restriction sites). Standard PCR was performed using the HotStarTaq DNA Polymerase Kit (Qiagen, Hilden, Germany) using the following PCR set up (Table 1).

Table 1. Set up for PCR

Component	Final concentration
10x PCR Buffer	1x
dNTP mix (10 mM of each)	200 µM of each dNTP
Primer Mix	400 nM
HotStarTaq DNA Polymerase	2.5 units/reaction
Distilled water	variable
Template DNA	~150 ng genomic DNA / ~50 ng cDNA

The reaction was carried out in standard or gradient thermal cycler blocks (Thermo Hybaid, Ulm, Germany) following the protocol in Table 2. The annealing temperature was optimized with a gradient of temperatures before final PCR.

Table 2. PCR parameters

PCR cycle		Duration	Temperature
Stage 1		15 min	95 °C
Stage 2 (35 cycles)	Denaturation	30 s	94 °C
	Annealing	30 s	Tested for each primer pair
	Elongation/kb	1 min	72 °C
Stage 3		10 min	72 °C

PCR of the DNA isolated from FFPE material for subsequent sequencing was performed differently. Due to the low quality and concentration of that DNA a nested PCR approach was chosen to reduce non-specific binding of primers and therefore DNA amplification. Nested PCR involved two sets of primers that were used in two successive runs of PCR. The second set amplified a secondary target within the product of the first run. The second run was also used for adding tails to the PCR product to simplify subsequent sequencing. Therefore, exon spanning primer pairs for the 28 exons of the *EGFR* gene (biomers, Ulm, Germany) were designed using the Primer 3 software [89]. At the 5' end of each of the nested primers a tail was added which represented either the M13 universal- (GTAAAACGACGGCCAGT) or T7 RNA polymerase binding site sequences (TAATACGACTCACTATAGG) (see 7.2, Primers used for sequencing of the *EGFR* gene, page 83). This approach resulted in tailed PCR products which could be uniformly sequenced by using either M13 universal- or T7 primers. PCR conditions were optimized using HotStarTaq polymerase (Qiagen, Hilden, Germany) in the presence of 400 nM of each of the respective primers, 200 µM dNTP (Fermentas, St.Leon-Rot, Germany), 1x PCR reaction buffer (Qiagen, Hilden, Germany) containing 1.5 mM $MgCl_2$. Exon 1 to 27 were amplified using the protocol from Table 2 with slight adjustments: elongation for 30 seconds and 50 cycles altogether. The PCR protocol for exon 28 differed only in that the extension step was 1 minute. As the template either 2 µl of the DNA isolate or 1 µl of the first PCR product were taken for the first or nested PCR respectively.

Proof-reading PCR was used for reactions whose products were used for cloning of plasmids. KOD Hot Start DNA Polymerase (Novagen, Merck, Darmstadt, Germany) was used according to the manufacturer's protocol.

Agarose gel electrophoresis

Agarose gel electrophoresis was used for DNA separation and sizing. For a 1% agarose gel 1 g of agarose (Biozym, Hessisch Oldendorf, Germany) was dissolved in 100 ml 0.5xTBE (Thermo Fisher Scientific, Waltham, USA) and heated by microwave. The mixture was then cooled down to approximately 60 °C and 3 µl of ethidium bromide (Sigma Aldrich, St. Louis, USA) were added. Liquid gel was poured into gel sledges and cooled down at room temperature. For DNA analysis, gels were loaded with 5 µl of DNA standard (O'GeneRuler Ultra Low Range DNA Ladder, GeneRuler 100 bp DNA Ladder or GeneRuler 1 kb DNA Ladder, Thermo Fisher Scientific, Waltham, USA) and DNA samples that were mixed with loading dye (5 volumes of DNA sample and 1 volume of loading dye solution which is supplied with the DNA ladder).

Purification of DNA from agarose gel

The purification of DNA from agarose gels was performed using QIAquick Gel Extraction Kits (Qiagen, Hilden, Germany) following the manufacturer's protocol.

Cloning of plasmids

The plasmid overexpressing LEF-1 (pLNCX2-Lef1_3xmyc) was obtained using the In-Fusion 2.0 Dry-Down PCR Cloning Kit. To obtain the pLNCX2 backbone a pLNCX2 plasmid (Clontech, Mountain View, USA) was digested with *NotI* and *XhoI* and purified applying DyeEx 2.0 Spin Kits (Qiagen, Hilden, Germany). Preparing the backbone for the In-Fusion reaction was performed by proof reading PCR (KOD Hot Start DNA Polymerase, primers: pLNCX2_bb_fw, pLNCX2_bb_rev see Table 3, 40 cycles, annealing at 60 °C, extension for 5 minutes) of the digested pLNCX2 plasmid. The product was verified using an agarose gel and the band was cut out and purified (QIAquick Gel Extraction Kits; Qiagen, Hilden, Germany). As PCR products are not methylated but the plasmid originating from bacteria was, the purified PCR product was digested with methylation-dependent

DpnI to eliminate remains of the original plasmid and purified again with the DyeEx 2.0 Spin Kit (Qiagen, Hilden, Germany). The second component for the In-Fusion reaction (the LEF-1 insert) was also prepared by proof reading PCR (KOD Hot Start DNA Polymerase, primers: LEF1_fw, LEF1_rev see Table 3, 40 cycles, annealing at 60 °C, extension for 1 minute) of a pcDNA-LEF-1 plasmid introducing enzyme digestion sites and a triple myc-tag. The PCR product was purified with QIAquick PCR Purification Kit, digested with *DpnI* (FastDigest, Fermentas, St.Leon-Rot, Germany) to eliminate remains of the original plasmid and purified again with the DyeEx 2.0 Spin Kit (Qiagen, Hilden, Germany). Both of the prepared components as well as appropriate controls were introduced into the In-Fusion reaction strictly following the manufacturer's manual. Single clones were picked and screening PCRs flanking the insert (HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany) primers: pLNCX2_screen_fw, pLNCX2_screen_rev see Table 3) were applied to identify positive clones.

Plasmids overexpressing TCF4 (pLNCX2-TCF4_3xmyc), and EGFP (pLNCX2-EGFP_3xmyc) were obtained by Gateway cloning [6, 51, 61, 73]. attB-PCR products were obtained by proof reading PCR (KOD Hot Start DNA Polymerase, 40 cycles, annealing at 60 °C, extension for 1 minute):

- TCF4 (template: pLNCX2-TCF4-HA (Clontech, Mountain View, USA, modified by Silvio Scheel), primers: TCF4_attB_fw, TCF4_attB_rev see Table 3)
- EGFP (template: pEGFP-C1 (Clontech, Mountain View, USA), primers: EGFP_attB_fw, EGFP_attB_rev see Table 3)

The BP reaction of the Gateway cloning was performed following the user's manual (Invitrogen, Life Technololgies, Carlsbad, USA). For the BP reaction a PCR product with flanking attB sites is combined with a Donor Vector containing attP sites by BP Clonase Enzyme Mix to receive an Entry Clone containing attL sites that are flanking the gene of interest. In this case the Donor Vector (pDONR 201, Invitrogen, Life Technololgies, Carlsbad, USA) was mixed with the respective

attB-PCR product (see above) and incubated with the BP Clonase Enzyme Mix according to the manufacturer's instructions to receive the Entry Clones.

The LR reaction of the Gateway cloning was performed following the user's manual (Invitrogen, Life Technololgies, Carlsbad, USA). For the LR reaction the Entry Clone containing attL sites is combined with the Destination Vector containing attR sites, promotor and tags by BP Clonase Enzyme Mix to receive the Expression Clone containing attB sites that are flanking the gene of interest. The expression vector is ready for gene expression. In this case the respective Entry Clones and the Destination Vector (pDEST-LNCX2-3xMYC-pA, modified from pLNCX2-Lef1_3xmyc) were mixed and incubated with the LR Clonase Enzyme Mix according to the manufacturer's instructions to receive the expression plasmids (pLNCX2-TCF4_3xmyc, pLNCX2-EGFP_3xmyc).

Table 3. Primers used for cloning of plasmids

Primer	**Sequence 5' → 3'**
pLNCX2_bb_fw	TGAGTCCGGTAGCGCTAGC
pLNCX2_bb_rev	ATCATAATCAGCCATACCACATTTG
LEF1_fw	GCGCTACCGGACTCAGATCTGCCACCATGCCCCAACTCTC
LEF1_3xmyc_rev	ATGGCTGATTATGATCTACAGGTCCTCCTCGGAGATCAGCTTCTGCTCC ATCAGGTCCTCCTCGGAGATCAGCTTCTGCTCCATCAGGTCCTCCTCG GAGATCAGCTTCTGCTCCATGGATCCGATGTAGGCA
pLNCX2_screen_fw	ACCTACAGGTGGGGTCTTTCATTCCC
pLNCX2_screen_rev	CGTGTACGGTGGGAGGTCTA
TCF4_attB_fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTAGATCTGCCACCATGGA GCAGAAGCTGATCAG
TCF4_attB_rev	GGGGACCACTTTGTACAAGAAAGCTGGGTATTCTAAAGACTTGGTGAC GA
EGFP_attB_fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTAGATCTGCCACCATGGT GAGCAAGGGCGAGGAGCTG
EGFP_attB_rev	GGGGACCACTTTGTACAAGAAAGCTGGGTATCTAGATCCGGTGGATCC CGG

Sequencing of DNA

Sequencing was performed using the Sanger sequencing approach. For the sequencing PCR BigDye Terminator v1.1 (for PCR products) or v.3.1 (for plasmids) Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany) was used following the manufacturer's instructions. PCR products of the sequencing reaction were purified applying DyeEx 2.0 Spin Kits (Qiagen, Hilden, Germany), denatured in the presence of highly deionized formamide (HiDi, Applied Biosystems, Darmstadt, Germany) and finally analyzed with the help of a 3130 Genetic Analyzer (Applied Biosystems, Darmstadt, Germany) following the respective user's instructions.

The resulting sequences were analyzed by alignment to the respective known mRNA/cDNA sequence as the reference (i.e. NM_005228 for EGFR). Alignment of sequences and comparisons was done applying the software Geneious Pro 4.7.4 (biomatters, Auckland, NZ).

Isolation of RNA from eukaryotic cells

Isolation of total RNA from eukaryotic cells was performed using RNeasy Mini Kits (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Reverse transcription

For the reverse transcription of RNA to cDNA the RevertAid Reverse Transcriptase Kit (Fermentas, St.Leon-Rot, Germany) was used according to the manufacturer's instructions.

Quantitative real time PCR (qPCR)

qPCR was performed using the ready-to-use hot start reaction mix for probe-based real-time PCR from Roche (LightCycler 480 Probes Master, Roche Applied Science Penzberg) in combination with Universal Probes using the Light-Cycler 480 (Roche Applied Science, Penzberg, Germany). For each gene the online Universal ProbeLibrary Assay Design Center was used to design primer pairs and to find the corresponding Universal Probe. The following Table 4 shows the analyzed genes and associated primers and probes.

Table 4. Primers and probes (UPL) used for qPCR

Gene	Acc#	forward primer	reverse primer	UPL	Length [bp]
HPRT	NM_000194.2	tgaccttgatttattttgcatacc	cgagcaagacgttcagtcct	73	102
LEF-1	NM_016269.2	cgacacttccatgtccaggt	tcctgtttgacctgaggtgtt	42	108
TCF4	NM_030756	acgtacagcaatgaacacttcac	ggcgatagtgggtaatacgg	10	128
E-cadherin	NM_004360	cccgggacaacgtttattac	gctggctcaagtcaaagtcc	35	72
vimentin	NM_003380	tacaggaagctgctggaagg	accagagggagtgaatccag	13	104
fibronectin	NM_005434	ctttggtgcagcacaacttc	tcctcctcgagtctgaacca	15	90

qPCRs were performed in triplicates and following the manufacturer's instructions. Primer concentrations were optimized in test measurements before use in final measurements. To determine the absolute concentration of cDNA, standard series of known cDNA concentrations were analyzed in parallel.

2.2 Proteinchemical methods

Protein isolation

Protein lysates were prepared with protein lysis buffer (triple-detergent lysis buffer: 50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.02 % (w/v) NaN_3; 0.5 % (w/v) sodium deoxycholate; 0.1 % (w/v) SDS; 1 % (v/v) Nonidet^{TM} P-40) supplemented with 0.7 mM PMSF and 1x Complete Protease Inhibitor (Roche Applied Science, Penzberg, Germany). Protein concentrations were determined using DC^{TM} Protein Assay following the protocol (BioRad, Hercules, USA).

Western blot

20 µg of the protein were used for polyacrylamide gel electrophoresis (minigel system, BioRad, Hercules, USA). The size standard used was the PageRuler Plus Prestained Protein Ladder (Thermo Fisher Scientific, Waltham, USA). The denaturing gels were prepared as follows.

Table 5. Polyacrylamide gel composition for electrophoresis of proteins

	separating gel (10 %)	**stacking gel (3.9 %)**
acrylamide rotiphoresis gel (Roth)	5 ml	650 µl
4xTris-HCl, pH 8.8	3.75 ml	-
4xTris-HCl, pH 6.8	-	1.25 ml
H_2O	6.25 ml	3.05 ml
APS	200 µl	100 µl
TEMED	40 µl	20 µl

After blotting onto PVDF membranes (Millipore, Merck, Darmstadt, Germany), primary antibodies were added. HRP conjugated secondary antibodies and subsequent incubation with ECL Western Blotting Detection Kit (Amersham, GE healthcare, UK) was used for the visualization of bands. The antibodies are summarized in Table 6.

Table 6. Primary and secondary antibodies used in the studies

Antibody	**Dilution**	**Source**	**Company**	**Molecular weight**
Mouse anti-TCF4	1:2000	Mouse	Upstate	66 kDa
Rabbit anti-LEF-1	1:1000	Rabbit	Cell signaling	25 – 58 kDa
Mouse anti-myc-tag	1:1000	Mouse	Upstate	n.a.
Mouse anti-β-actin	1:2000	Mouse	Sigma-Aldrich	43 kDa
Mouse anti-rabbit	1:10000	Mouse	Pierce	n.a.
Rabbit anti-mouse	1:20000	Rabbit	Pierce	n.a.

2.3 Microbiological methods

Transformation

Transformation of plasmids was performed using Subcloning Efficiency DH5α Competent Cells (Invitrogen, Life Technololgies, Carlsbad, USA) according to the manufacturer's instructions.

Transformation of ligation reactions were performed using Library Efficiency DH5α Competent Cells (Invitrogen, Life Technololgies, Carlsbad, USA) according to the manufacturer's instructions.

Transformation of InFusion products were performed using Fusion-Blue Competent Cells (Clontech, Mountain View, USA) according to the manufacturer's instructions.

Isolation of single cell clones

Single clones were picked with pipette tips, transferred to LB Medium with appropriate antibiotics and incubated at 37 °C overnight.

Plasmid isolation from *E. coli* suspension cultures

Isolation of plasmids was performed using either GeneJet Plasmid Miniprep Kits (Fermentas, St.Leon-Rot, Germany) or QIAfilter Plasmid Maxi kits (Qiagen, Hilden, Germany) following the respective manufacturer's instructions.

2.4 Cell culture methods

Culturing of eukaryotic cells

Cell lines used for experiments were: HEK293, HCT116, HT29, SW480, DLD1 (ATCC, USA). The cells were cultivated in an incubator at 37 °C under an atmosphere with 100 % humidity and 5 % CO_2. The cultures were checked for mycoplasma in regular intervals using the PCR Mycoplasma Test Kit (AppliChem, Darmstadt, Germany). All cell lines were maintained in DMEM/Ham's F-12 (1:1) (Biochrom, Berlin, Germany) with 7.5 % fetal bovine serum (Biochrom, Berlin, Germany) and 50 µM 2-mercaptoethanol (Invitrogen, Life Technololgies, Carlsbad, USA). Cultivation of colorectal cancer stem cells (coCSCs) and spheroid derived adherent cells (SDACs) was performed by Achim J. Schäffauer as described in his PhD Thesis (Die Bedeutung von BMI1 beim Cancer-Stem-Cell-Phänotyp kolorektaler Krebszellen). coCSC were kept in StemPro® hESC SFM Medium (Life Technologies, Carlsbad, USA) supplemented with EGF (Life Technologies, Carlsbad, USA) and FGFb (Life Technologies, Carlsbad, USA) or also with Matrigel® (Corning Incorporated, Corning, USA). Differentiation of coCSCs to

SDACs was done with DMEM/F-12 (1:1) (1x) + GlutaMAX™ medium supplemented with FBS (Biochrom, Berlin, Germany) or also with collagen. Harvesting of coCSCs and SDACs for RNA isolation was done after 7 or 14 days after induction of differentiation.

Passaging of cells

Cells were passaged when reaching ~ 90 % confluence into larger culture volumes or proportionately kept in similar sized cultures. The medium was aspirated and the cells washed with PBS. Trypsin/EDTA (Biochrom, Berlin, Germany) was added and the cells were incubated at 37 °C for 5 minutes or until cell detachment could be seen under a microscope. The detached cells were then partly or in total transferred to the consecutive culture vessels filled with pre-warmed culture medium.

Determining the cell count

The cell count was determined using a hemocytometer. Counting of cells was necessary to determine defined cell seeding densities, e.g. for transfection.

Transfection of eukaryotic cells

Lipofection

Transient transfections of plasmids were conducted in 6-well plates (Corning Incorporated, Corning, USA) with FuGene® 6 Transfection Reagent (Roche Applied Science, Penzberg, Germany) following the manufacturer's instructions. $3x10^5$ cells were plated per well. FuGene® 6 : plasmid DNA ratio was 6 µl : 2 µg. Successful transfection was visualized by transfecting pEGFP-C1 (Clontech, Mountain View, USA). For RNA isolation or protein lysate preparation cells were harvested after 48 hours.

Transfection of siRNA

Lipofectamine™ RNAiMAX (Invitrogen, Life Technololgies, Carlsbad, USA) was used for transfection of siRNA. The manufacturer's protocol for forward transfection was used (cells are being plated before the transfection mix is added).

$3x10^5$ cells were plated per well of 6-well plates (Corning Incorporated, Corning, USA). Lipofectamine™ concentrations and siRNA concentrations were tested for each cell line. The following concentrations were the optimum in both transfected cell lines (SW480 and DLD1):

- si LEF-1 (Dharmacon OnTargetplus Smartpool): 33.3 nM + 7.5 µl Lipofectamine per 6-well.
- si TCF4 (Dharmacon OnTargetplus Smartpool): 50 nM + 7.5 µl Lipofectamine per 6-well.

For RNA isolation or protein lysate preparation cells were harvested after 48 hours.

Electroporation

Stable transfections of circular plasmids were conducted with the Amaxa™ Nucleofector™ (amaxa, lonza, Basel, Switzerland) and cell line specific Nucleofector Kits (HCT116: Cell Line Nucleofector™ Kit V, HT29: Cell Line Nucleofector™ Kit R) following the manufacturer's instructions. For one nucleofection sample $1x10^6$ cells and 2 µg plasmid DNA were used. Successful transfection was visualized by transfecting 2 µg pmaxGFP™ (amaxa, lonza, Basel, Switzerland). Selection of transfected cells was performed with 0.4 mg/ml G418 (Calbiochem, Merck, Darmstadt, Germany).

Transduction of eukaryotic cells

Mission lentiviral particles (Sigma-Aldrich, St. Louis, USA) encoding shRNA specific for enhanced green fluorescent protein (SHC005V) as the control or the human LEF-1 (SHCLNV-NM_016269) or TCF4 (SHCLNV-NM_030756) were used for transduction of HCT116 or HT29 cells. Three different shRNAs against LEF-1 (TRCN0000020163, TRCN0000020162, TRCN0000020161) and six different shRNAs against TCF4 (TRCN0000061897, TRCN0000061895, TRCN0000061893, TRCN0000174115, TRCN0000061896, TRCN0000061894) were used. Ahead of transduction Polybrene® (Sigma-Aldrich, St. Louis, USA) was added to the culture medium (8 µg/ml). Transduction was performed with a MOI (multiplicity of infection) of 5 and selection for stable transfection started two

days later applying puromycin dihydrochloride (Calbiochem, Merck, Darmstadt, Germany) with the following concentrations: DLD1: 6 µg/ml, SW480: 2 µg/ml.

Isolating single cell clones

Single cell clones were picked with pipette tips from cell culture plates after seeding cells at low densitiy and visible colonies had grown. Picked colonies were transferred to new culture vessels.

Luciferase assay

Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, USA).

$5x10^4$ cells in 100 µl medium were seeded per well in 96-well culture plates (Corning Incorporated, Corning, USA). Three wells were seeded for each measurement so that triplicates could be analyzed. FuGene 6 Transfection Reagent (Roche Applied Science, Penzberg, Germany) was used in the following ratio: 0.4 µl FuGene : 0.1 µg DNA. Renilla Luciferase plasmid (see 7.5 Plasmids) level was constant at 10 % of the DNA amount for adjustment of wells. Reporter plasmids TopFlash firefly luciferase plasmid or FopFlash firefly luciferase plasmid level was 30 %. Some luciferase assays were performed with addition of a basic activator plasmid expressing β-catenin (pcI-neo-ßcatenin-D45, see 7.5 Plasmids). The plasmid that was to be tested in a luciferase assay was added in varying concentrations. The final amount of DNA was reached by addition of a fill up plasmid (pcDNA3-CAT, see 7.5 Plasmids). The addition of a fill up plasmid was performed to ensure the same final DNA concentration in each well and each experiment. The fill up plasmid was also used as the control. Preparation of cell lysates by passive lysis after 48 hours, preparation of Luciferase Assay Reagent ll and Stop & Glo Reagent as well as all following steps were performed following the manufacturer's instructions.

Proliferation assay

Proliferation of cells was determined using the Cell Proliferation ELISA, BrdU (colorimetric) kit (Roche, Mannheim, Germany) following the manufacturer's

instructions. Three wells of a 96-well culture plate (Greiner, Kremsmünster, Austria) were seeded with 4000 cells in 100 µl medium for each measurement so that triplicates could be analyzed. Cells were then transfected by lipofection. First measurements were taken after one day and then each following day. For this, BrdU labeling solution was added to the wells (final concentration: 10 µM BrdU) and cells were reincubated for another 20 hours. Then labeling medium was removed and 200 µl/well FixDenat were added and incubated for 30 minutes at room temperature. FixDenat was removed and 100 µl/well anti-BrdU-POD working solution were added and incubated for 90 minutes at room temperature. This solution was removed and the wells were rinsed three times with 200 µl Washing solution. The Washing solution was then removed and 100 µl/well Substrate solution were added. After an incubation of 30 minutes at room temperature and adding 25 µl stop solution (H_2SO_4) the absorbance was measured in an ELISA reader at a wavelength of 450 nm (reference wavelength: 690 nm).

Migration assay

The migration of cells was analyzed using ibidi chambers (ibidi, Planegg, Germany) following the manufacturer's instructions. In short, 70 µl of a $4x10^5$ cells/ml suspension were seeded per ibidi chamber well. Two chambers were used per experiment. After 48 hours chambers were removed, the cells carefully washed with PBS to clean the gaps and the gaps were then photographed. Migration was detected after 24 hours by photographing and evaluated by measuring the gaps at 5 to 10 randomly predefined spots.

2.5 Statistical analysis

Skin toxicity was correlated with a variety of clinico-pathological data (Table 7) applying Fisher's exact test. PFS and OS were compared between both groups using Kaplan-Meier estimation. For comparison of the differences the log-rank test was used. All statistical results were considered significant when the two-sided α-error was less than 0.05.

3 Results

3.1 Predictive biomarker for anti-EGFR treatment

To identify genetic alterations in the *EGFR* gene which associated with skin toxicity and thus response under anti-EGFR targeted therapy with a high predictive value, a teaching / validation set approach was chosen, employing FFPE tissue from CRCs of the clinical CIOX study (AIO KRK-0104) [75]. Only patients displaying the highest grade (3) versus lowest grade (0) skin toxicity were selected from the CIOX study as they represented the highest contrast which generally should ease the finding of genetic differences in those two groups. Importantly, skin toxicity and objective response rate (ORR) as well as disease control rate (DCR) correlated with high significance (Table 7) [7, 14, 63, 90, 102].

3.1.1 Teaching set analysis

When using boundaries of 5 and 95 % for the predictive values respectively, an effect size of 0.9 results (Δ=0.95 – 0.05). Together with a two-sided error of $\alpha \leq 0.05$ and $\beta \leq 0.05$ (power=0.95) in the context of exact test statistics a minimum sample size of 6 patients is needed for the analysis. To improve the quality of the results more patient samples were analysed in the teaching and validation set.

In a first step, the 28 exons of the *EGFR* gene from 20 patients of the teaching set with either grade 3 skin toxicity (10 patients) or absence of skin toxicity (grade 0, 10 patients) were analyzed using Sanger sequencing. A variety of genetic alterations was found (Figure 7, and 7.3 Genetic alterations, page 85, 7.4 Allelic frequencies, page 87) which contained the already known polymorphisms c.474C>T: rs2072454, c.1562G>A: rs2227983 (p.R521K), c.2361G>A: rs1050171, c.2709C>T: rs1140475, and c.2982C>T: rs2293347 [18].

Of the 25 different genetic alterations in the *EGFR* gene, 14 were silent alterations not resulting in an amino acid change while the remaining different 11 genetic alterations resulted in an amino acid change (Figure 7, Table 8).

Table 7. Baseline characteristics, pathological data and treatment efficacy of the 45 investigated patients. Reduced patient number for pathological data and treatment efficacy due to censored patients or missing data (e.g. follow up not possible, poor quality of tissue…). Significant differences are indicated by bold typing. CAPIRI: capecitabine and irinotecan; CAPOX: capecitabine and oxaliplatin; DCR: disease control rate (ORR plus stable disease); *KRAS* mut: mutations in codons 12 or 13 of the *KRAS* gene; NCI-CTCAE Grade: National Cancer Institute Adverse Event, version 3.0[12]; n.s.: not significant; ORR: objective response rate (complete remission plus partial remission); OS: overall survival; p: probability; PFS: progression free survival; yrs: years; ᵗ: logrank test; *: Fisher´s exact test (two sided); ♯:2 patients censored; ▲: 3 patients censored.

Baseline characteristics			
	NCI-CTCAE acneiform rash		
	grade 0 (n=19)	**grade 3 (n=26)**	**p**
age (mean) yrs range	63.2 (49 – 74)	61.4 (48 – 75)	n.s.
age > 65 yrs % (n)	47.9 (9)	42.3 (11)	0.770*
gender female % (n) male % (n)	 26.3 (5) 73.7 (14)	 15.4 (4) 84.6 (22)	 0.461*
treatment arm CAPIRI + cetuximab % (n) CAPOX + cetuximab % (n)	 42.1 (8) 57.9 (11)	 42.3 (11) 57.7 (15)	 > 0.99*

Pathological data			
KRAS mut % (n)	16.7 (3 of 18)	34.6 (9 of 26)	0.303*
liver limited disease	50.0 (9 of 18)	36.0 (9 of 25)	0.532*
Treatment efficacy			
ORR % (n)	0 (0 of 6)	58.3 (14 of 24)	**0.019***
DCR % (n)	50.0 (3 of 6)	100 (24 of 24)	**0.005***
PFS (months) (95 % CI)	2.8♯ (0.4 – 5.1)	8.2 (6.9 – 9.5)	0.305ᵗ
OS (months) (95 % CI)	17.0▲ (7.9 – 26.1)	23.5▲ (11.7 – 35.4)	0.255ᵗ

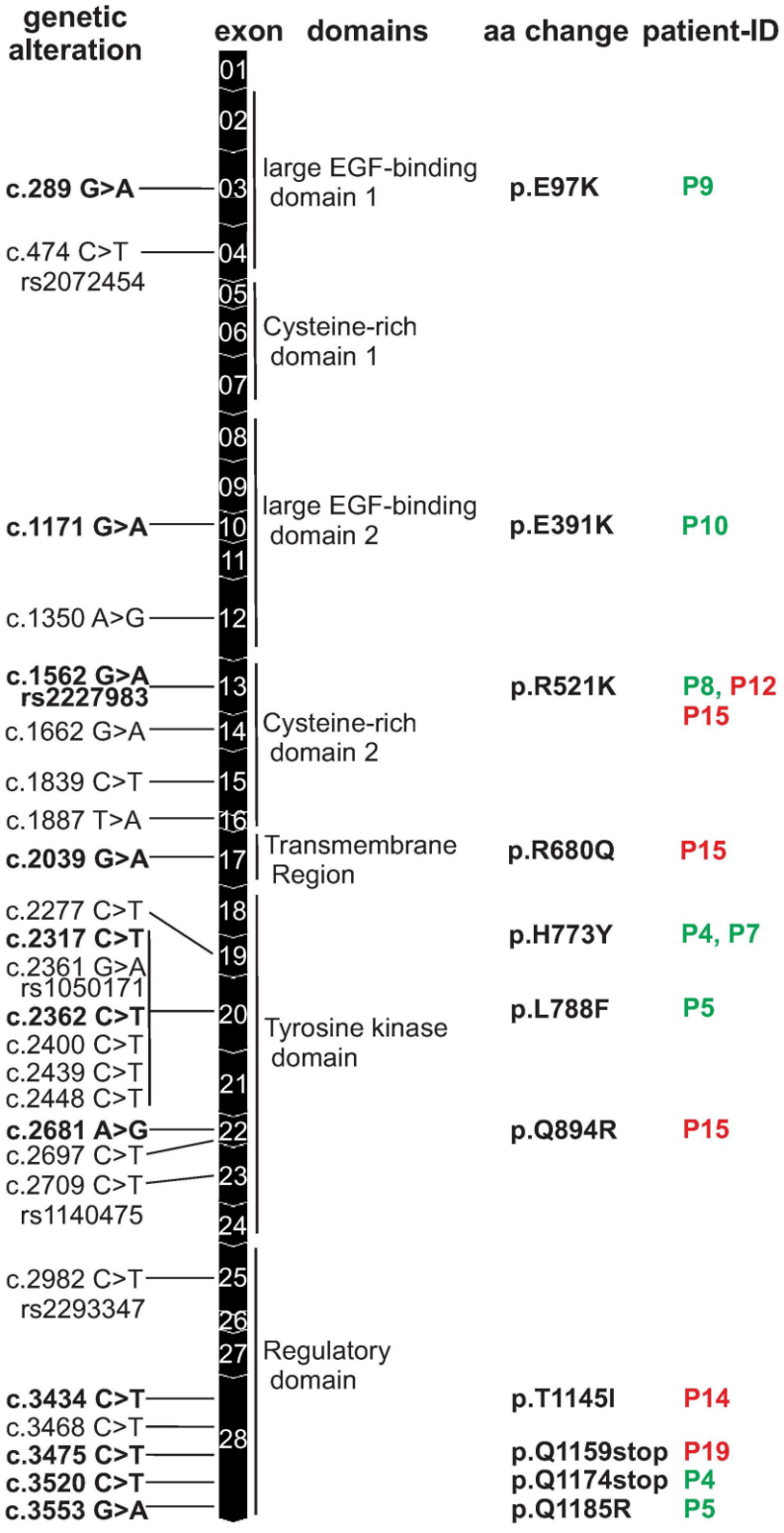

Figure 7. Schematic image of the 28 exons of the *EGFR* gene with its domains (column: domains) and all found alterations on the DNA level (column: genetic alteration). Genetic alterations in bold also result in amino acid changes (see also column: aa change). These were associated with the skin toxicity (column: patient ID, green: grade 0 (P1-P10); red: grade 3 (P11-P20)). Scheme is not drawn to scale.

Table 8. Amino acid (aa) exchanges in the EGFR of patients with skin toxicity grade 0 and 3.

skin toxicity	aa	teaching set	validation set
0	WT	P1, P2, P3, P6, P8	P21, P23, P24, P26, P27, P29
	p.E97K	P9	
	p.E102K		P28
	p.E391K	P10	
	p.P644S		P28
	p.H773Y	P4, P7	
	p.C775Y		P28
	p.L788F	P5	
	p.V1147I		P25
	p.Q1174stop	P4	
	p.P1178S		P22
	p.Q1185R	P5	
	p.P1202S		P28
3	WT	P11, P12, P13, P16, P17, P18, P20	P30, P32, P34, P35, P36, P41, P42, P44, P45
	p.E391G		P38
	p.H507Y		P40
	p.G514D		P38
	p.V659M		P33
	p.R680Q	P15	
	p.L815F		P37
	p.W880stop		P43
	p.I886T		P40
	p.Q894R	P15	
	p.P1098S		P39
	p.T1145I	P14	
	p.W1157stop		P31
	p.Q1159stop	P19	

Of these 11 genetic alterations, 6 were associated with high grade skin toxicity and 8 with the absence of skin toxicity. SNP rs1050171 was found twice in patients with absence of skin toxicity whereas SNP rs2227983 was found in the group without skin toxicity as well as in the group with high grade skin toxicity.

3.1.2 Validation set analysis

Next, the results were confirmed applying a validation set of 25 patients from the CIOX collection, again with either absence of, or high grade skin toxicity. Now only exons that had shown genetic alterations in the teaching set were analyzed. Again a variety of genetic alterations was found. Some had been described like SNPs c.1562G>A: rs2227983 or c.2361G>A: rs1050171 while others had not been identified before (Figure 8, Table 8, 7.3 Genetic alterations page 85, 7.4 Allelic frequencies page 87).

Importantly, the pattern of genetic alterations in the *EGFR* gene was heterogeneous and did not result in genetic alterations which could discriminate between high grade and absence of skin toxicity with a high predictive value.

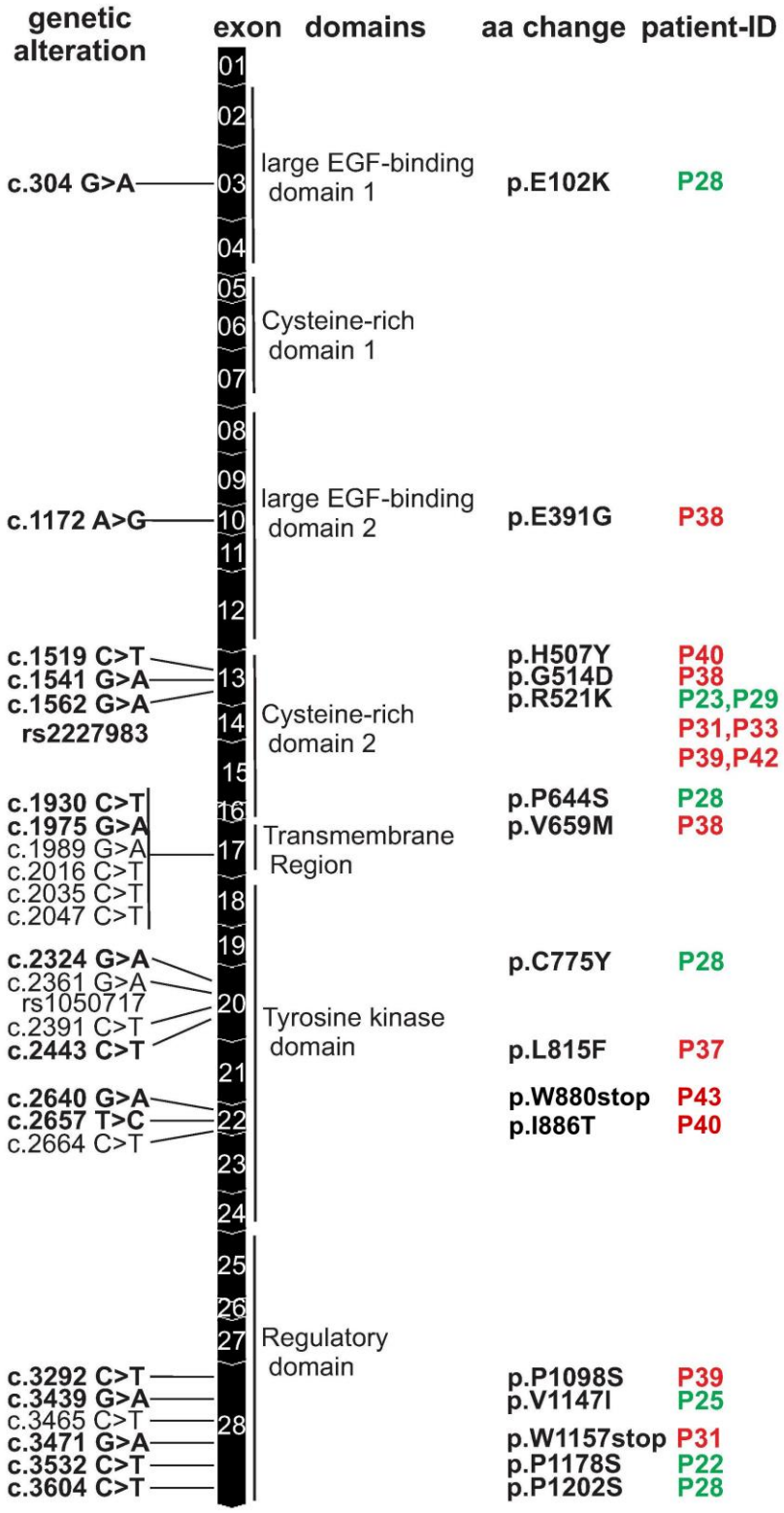

Figure 8. Scheme of the *EGFR* gene with allocated genetic alterations on DNA and protein level and their association with skin toxicity (validation set). Legend: see Figure 7, except skin toxicity (column patient ID, green grade 0 (P21-29); red grade 3 (P30-45))

3.2 Investigation, characterization and comparison of the programs regulated through β-catenin/LEF-1 and β-catenin/TCF4

3.2.1 Functional role of LEF-1 and TCF4 in CRC tissue

An indication of the functional role of LEF-1 and TCF4 in patients was examined immunohistochemically in CRC tissue [57]. The LEF-1 and TCF4 expression was analyzed with a tissue microarray of 214 colorectal carcinomas specimens. The expression patterns were compared with each other and the results were correlated with the clinico-pathological variables and overall survival in univariate and multivariate analysis [57].

This resulted in the following outcome: LEF-1 expression was found in 26 % and TCF4 expression in 46 % of tumors. Both proteins were heterogeneously distributed throughout the tumors. A subsequent comparison of the LEF-1, TCF4 and β-catenin expression showed no correlation. In contrast to that, a univariate analysis of the expression of TCF4 showed a correlation with a shorter overall survival. In addition, the correlation of LEF-1 as well as a LEF-1/TCF4 ratio was associated with a positive prognosis with longer overall survival. Summarized, the multivariate analysis with tumor stage, gender and age shows that LEF-1 and TCF4 expression are independent predictors of longer and shorter overall survival, respectively [57].

3.2.2 Expression of LEF-1 and TCF4 in cell lines

Several CRC cell lines were analyzed for their presence of the transcription factors LEF-1 and TCF4 and of the housekeeper β-actin (Figure 9). RNA of the shown cell lines was reverse transcribed (+RT) and PCR was performed. The lanes that are marked with –RT show the reverse transcription negative control where no reverse transcriptase was added to the reaction. The cell line HEK293 was used as a positive control for the PCR and water was used as a negative control. Of the cell lines used in this study, two didn't express LEF-1 but all of them expressed TCF4 (Figure 9). No cell line could be found that did not express TCF4. But the experimental setup of the analyses was chosen so that effects would still be seen. To examine, characterize and compare the programs regulated by β-catenin/LEF-

1 and β-catenin/TCF4 the two cell lines HCT116 and HT29 were converted to cell lines expressing LEF-1. As no cell line without TCF4 expression was available, TCF4 overexpression was used in those two cell lines to enhance the effects of TCF4 and analyze them.

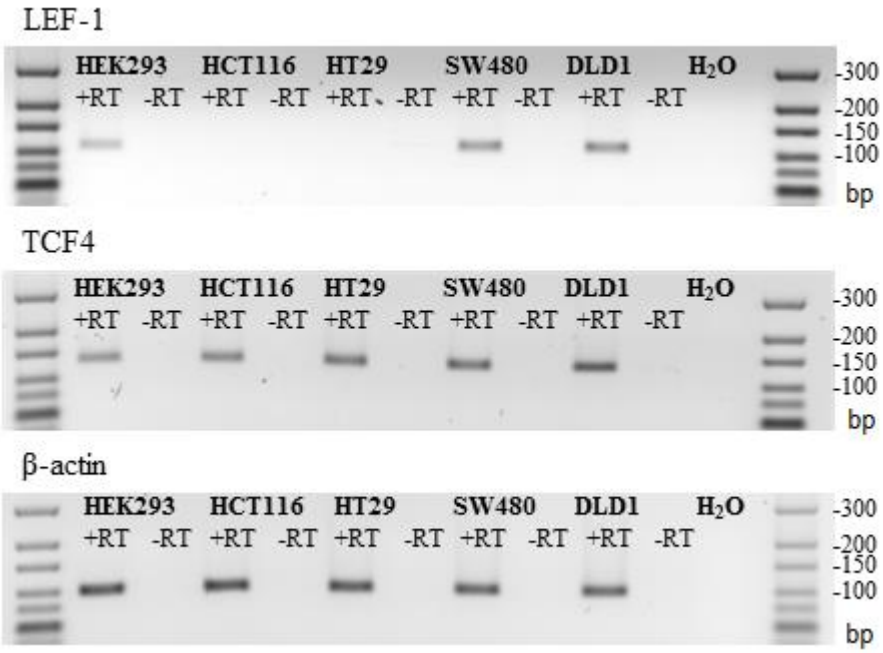

Figure 9. Expression of LEF-1, TCF4 and beta-actin in cell lines used in this work

PCR of LEF-1, TCF4 and β-actin on cDNA of the cell lines HEK293, HCT116, HT29, SW480 and DLD1, water (H_2O) used as control, RT = reverse transcriptase, -RT = no reverse transcriptase added

3.2.3 Plasmid preparation and testing

All the appropriate overexpression plasmids (expression plasmids for LEF-1, TCF4 and EGFP: pLNCX2-Lef1_3xmyc, pLNCX2-TCF4_3xmyc, pLNCX2-EGFP_3xmyc, see 7.5 Plasmids, page 88) were successfully cloned (see Material and Methods page, Cloning of plasmids, page 20) and tested as follows. Functionality of the plasmids (whether encoded protein is synthesized) was shown using Western blot (example see Figure 10) and functionality of the synthesized proteins was shown using luciferase assays (see Figure 11).

Protein presence in the transiently transfected HCT116 cells (by lipofection) was shown in a LEF-1, myc-tag and β-actin specific Western blot (Figure 10) after protein isolation.

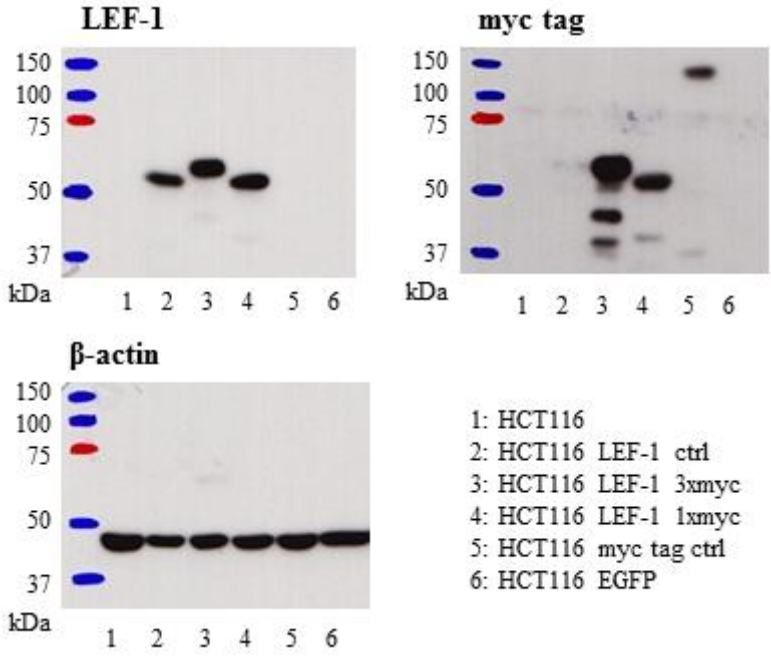

Figure 10. Western blot for testing of cloned LEF-1 plasmids

Western Blot specific for LEF-1, myc tag and β-actin of transiently transfected HCT116 cells; lane 1: HCT116; lane 2: HCT116 pCDNA LEF-1 (positive control for LEF-1), lane 3: HCT116 pLNCX2-Lef1_3xmyc; lane 4: HCT116 pLNCX2-Lef1_1xmyc; lane 5: pLNCX2-bCateninER-myc (positive control for myc tag), lane 6: HCT116 pEGFP-C1

The first lane shows the nontransfected HCT116 cell lysate with no detectable LEF-1 protein. The following lanes two to four show first of all a positive control lysate (HCT116 LEF-1 ctrl: HCT 116 transiently transfected with pcDNA-LEF-1) and next to that the lysates of the cells transfected with the cloned LEF-1 expression plasmids (lane 3: HCT116 pLNCX2-Lef1_3xmyc, lane 4: HCT116 pLNCX2-Lef1_1xmyc). Distinct bands can be seen in all three lanes in the blot showing the LEF-1 signals. The LEF-1 plasmid with a triple myc tag is slightly shifted towards a higher molecular weight. This is due to the prolonged length of the resulting protein as it carries three myc tags at its end. The blot showing the myc-tag signals only show bands in the lanes 3 and 4 where the myc-tagged LEF-1 lysates were applied. Lane 5 contains a lysate of cells that were transfected with

a myc-tag expressing protein as a positive control for the myc-tag. The last lane displays the negative control expressing EGFP. Functionality of the control-plasmid (expression of EGFP) was confirmed by checking for green fluorescence of the cells under the microscope.

Luciferase assay was performed to test the cloned plasmids for the functionality of their encoded proteins (Figure 11).

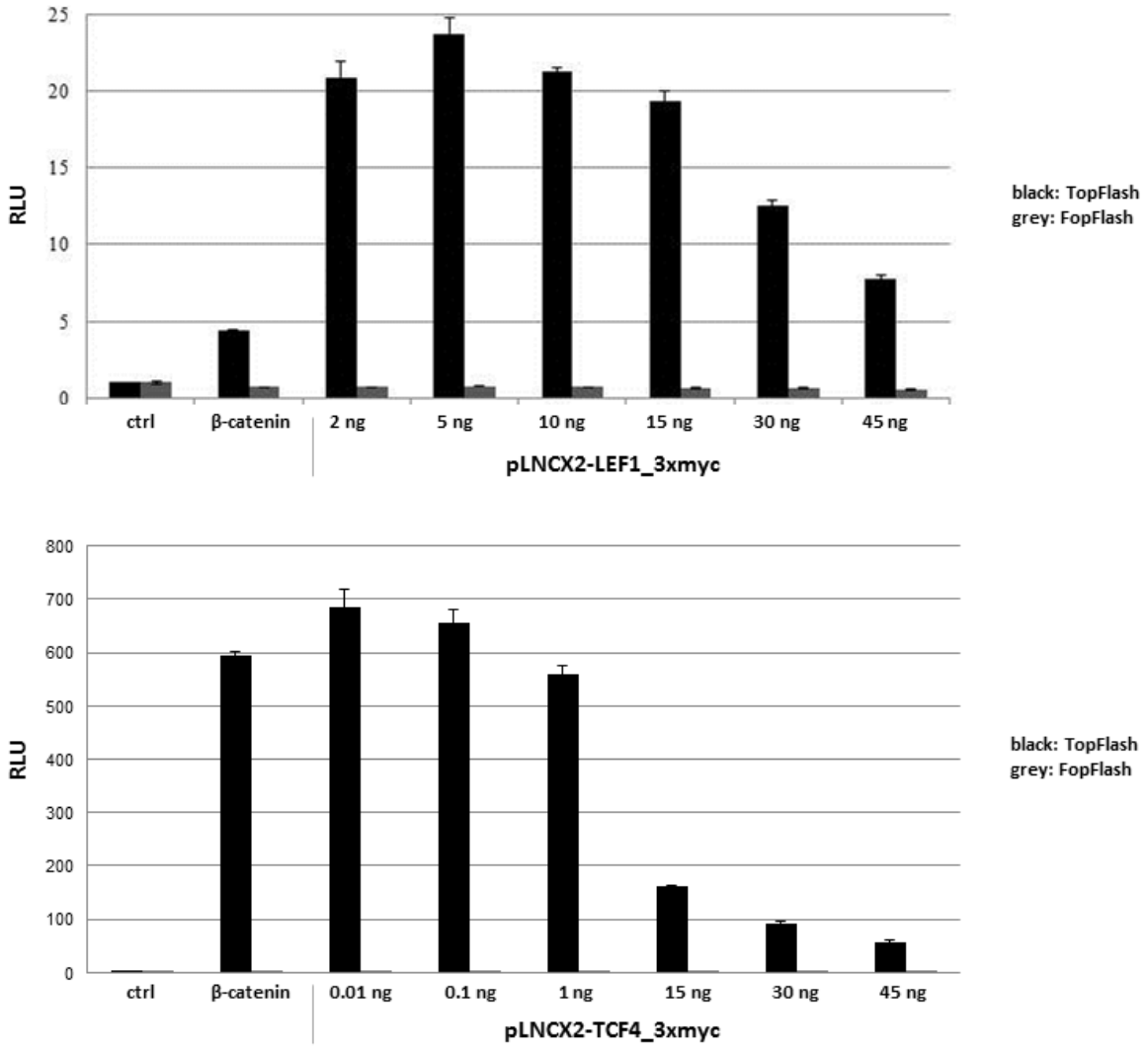

Figure 11. Luciferase assay for testing of LEF-1 and TCF4 plasmid functionality

top: HCT116 with transient expression of pLNCX2-LEF1_3xmyc; bottom: HEK293 with transient expression of pLNCX2-TCF4_3xmyc (ctrl column: transient expression of fill up plasmid; β-catenin column: transient expression of basic activator plasmid; following columns: transient expression with increasing concentrations of the two plasmids to be analyzed); RLU: relative light units

The luciferase test construct consists of a LEF-1 / TCF4 activatable luciferase gene downstream of LEF-1 and TCF4 binding sites. Functional transcription factors enable transcription of the luciferase and the result is measured in light units that result from an enzymatic reaction triggered by the luciferase. Figure 11 shows the luciferase assay of HCT116 cells with the transiently transfected LEF-1 plasmid. A dose dependent increase in luciferase activity can be seen. When transcription factor concentrations rise higher the activity decreases.

When the transcription factors bind (together with β-catenin) to specific binding sites (the β-catenin/Tcf/Lef-binding elements (TBE) in front of a luciferase gene), the transcription of the following gene starts. With increasing transcription factor concentrations, there is also an increase in the luciferase signal (relative light units). This increase in signal is a sign of functional transcription factors (Figure 11). With higher concentrations the luciferase signal decreases. This is due to the saturation of the transcription factor binding site. Both, transcription factor and β-catenin need to bind to the site to start transcription. When transcription factor concentrations rise too high, there is an oversupply in LEF-1 or TCF4 so they replace the β-catenin transcription factor pairs. Only LEF-1 or TCF4 without β-catenin cannot successfully start transcription. So the decrease in luciferase activity that can be seen is not a sign for missing functionality of the transcription factors but a sign for too high transcription factor concentrations.

Functionality of the overexpression constructs could be shown by luciferase assay. Thus the effects of the transcription factors on migration were analyzed next.

3.2.4 Functional characterization of transcription factor effects in transiently transfected cells

Migration assays were performed to check for altered behavior of the differently transfected cells (Figure 12). Migration was similar whether cells were transfected with LEF-1 or TCF4 or whether they were transfected with control plasmids (lacZ or EGFP) or no plasmids (transfection reagent (FuGene®) or untransfected).

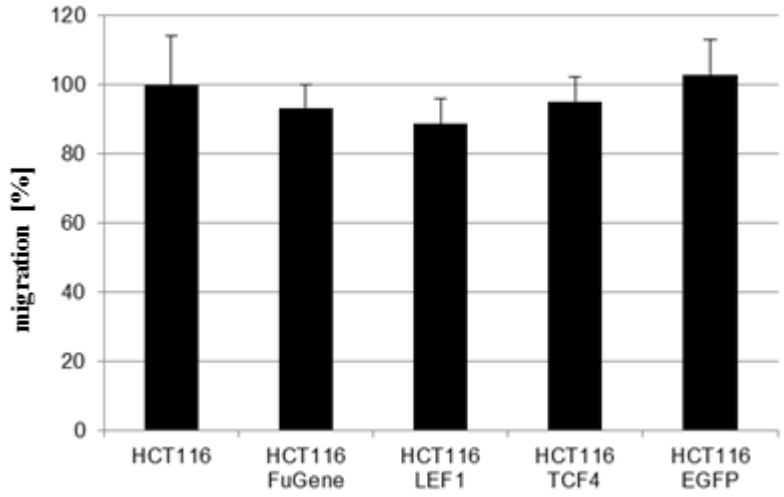

Figure 12. Migration assay of transiently transfected HCT116 cells

Migration of transiently with LEF-1 (pLNCX2-Lef1_3xmyc), TCF4 (pLNCX2-TCF4_3xmyc) and EGFP (pLNCX2-EGFP_3xmyc) transfected HCT116 cells after 24 hours; controls: untransfected HCT116 and HCT116 with transfection agent FuGene®

3.2.5 Preparation and testing of stable cell lines

Subsequently stable cell lines (HCT116 Figure 13, HT29 Figure 14) overexpressing LEF-1 or TCF4 were produced. Figure 13 shows the Western blot analysis of clones that have been stably transfected with the cloned LEF-1, TCF4 and EGFP plasmids. The positive controls (HEK293 cell line, transiently transfected with LEF-1 or TCF4) in lane 1 and 2 show distinct bands for LEF-1 and TCF4 expression. Additionally, it can be seen that only HCT116 LEF-1 clone 2 and clone 4 show detectable signals for LEF-1. Only HCT116 TCF4 clone 2 shows a second slightly heavier band for TCF4. Nevertheless, the HCT116 clones were additionally analyzed with qPCR of transcription factor target genes (Figure 15) and a luciferase assay (Figure 16).

On the other hand, the HT29 clones show no LEF-1 expression in Western blot analysis but TCF4 expression can be seen in the HT29 TCF4 clones (Figure 14).

Those stable cell lines were then functionally characterized.

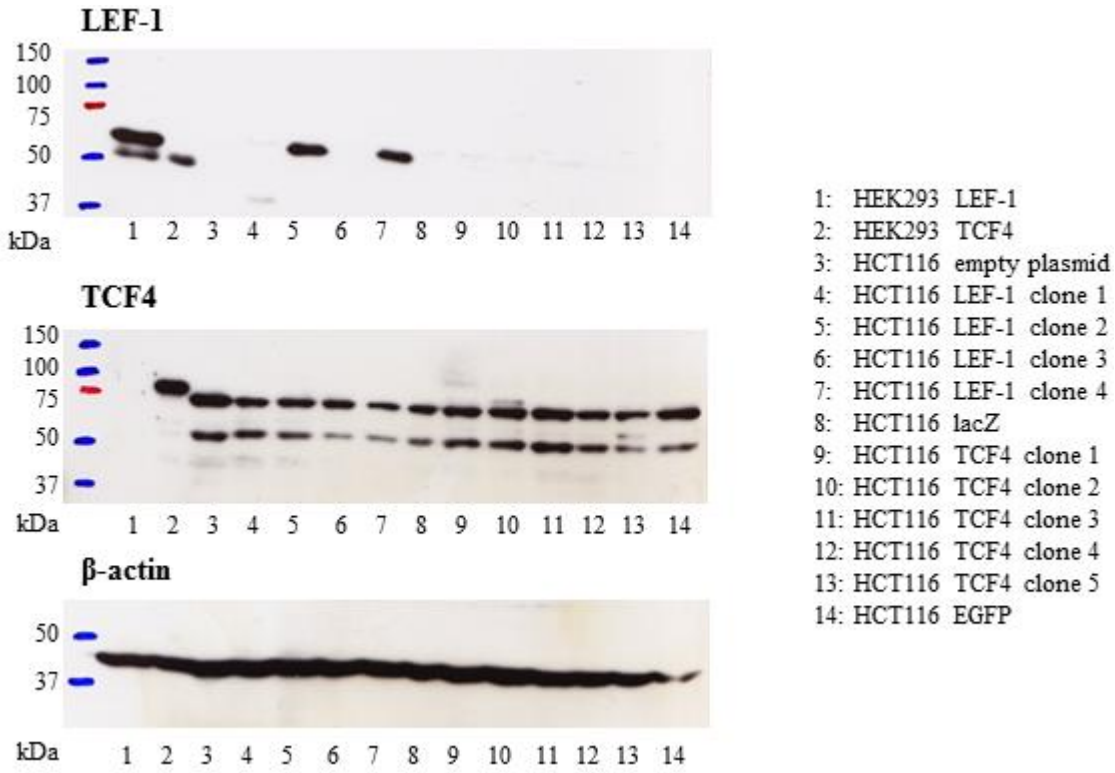

Figure 13. Western blot analysis of stably transfected HCT116 clones

Western blot specific for LEF-1, TCF4 or β-actin of HCT116 cells stably transfected with LEF-1 (pLNCX2-Lef1_3xmyc; lanes 4 - 7) or TCF4 (pLNCX2-TCF4_3xmyc; lanes 9 - 13). Lanes 1 and 2 show the positive controls for LEF-1 and TCF4 (HEK293 transiently transfected with pLNCX2-Lef1_3xmyc or pLNCX2-TCF4_3xmyc). The stable transfectants HCT116 empty plasmid (pLNCX2-3xmyc; lane 3), HCT116 lacZ (pLNCX2-lacZ_3xmyc; lane 8) and HCT116 EGFP (pLNCX2-EGFP_3xmyc; lane 14) are controls for further experiments.

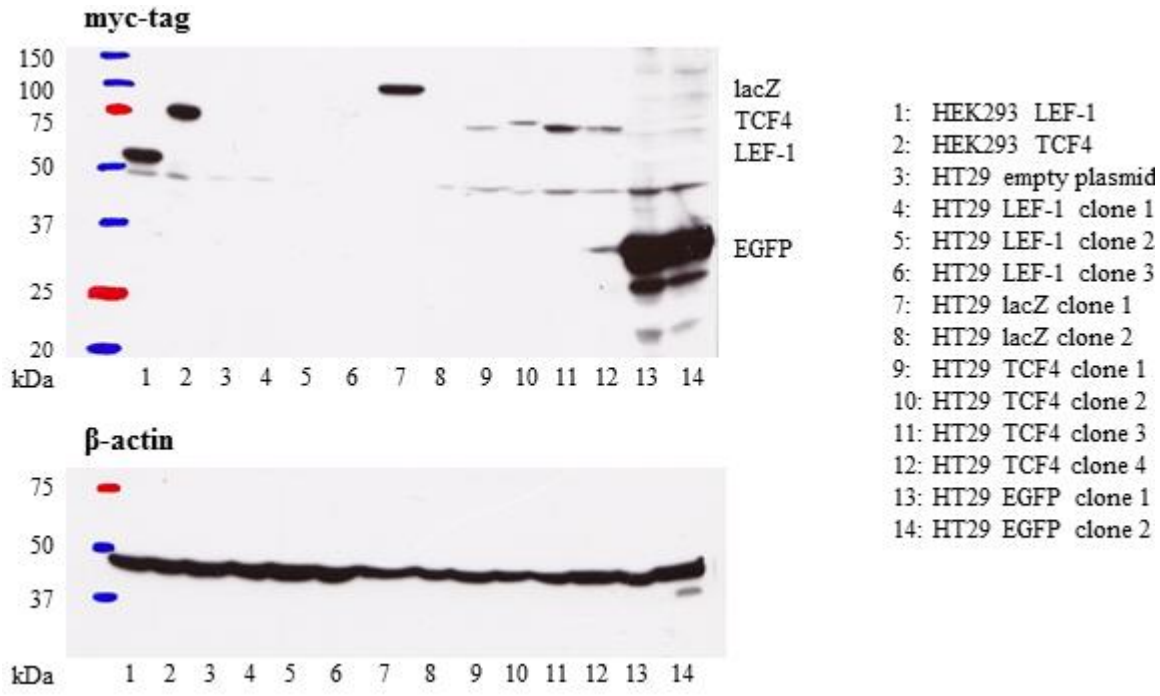

Figure 14. Western blot analysis of stably transfected HT29 clones

Western blot specific for myc-tag or β-actin of HT29 cells stably transfected with LEF-1 (pLNCX2-Lef1_3xmyc; lanes 4 - 6) or TCF4 (pLNCX2-TCF4_3xmyc; lanes 9 - 12). Lanes 1 and 2 show the positive controls for LEF-1 and TCF4 (HEK293 transiently transfected with pLNCX2-Lef1_3xmyc or pLNCX2-TCF4_3xmyc). The stable transfectants HT29 empty plasmid (pLNCX2-3xmyc; lane 3), HT29 lacZ (pLNCX2-lacZ_3xmyc; lane 7 - 8) and HT29 EGFP (pLNCX2-EGFP_3xmyc; lanes 13 - 14) are controls for further experiments.

3.2.6 Functional characterization of stable cell lines

A qPCR was performed to analyze the expression of target genes of the β-catenin/LEF-1 and β-catenin/TCF4 transcription factor complexes in the HCT116 clones (Figure 15). More precisely, the expression of the EMT markers E-cadherin and fibronectin was measured. Clones expressing LEF-1 and TCF4 all show a reduced E-cadherin expression. But when comparing the expression with the control cells that express EGFP, the expression of E-cadherin is higher. Looking at fibronectin mRNA, the expression patterns do not correlate with either LEF-1 or TCF4 overexpression. They fluctuate between the controls and exceed and undercut them. When looking at the luciferase assay the activity varies as well (Figure 16). The expression clones show luciferase activities that fluctuate between the values of the controls. The clones overexpressing LEF-1 or TCF4 cannot be distinguished from the controls.

As the overexpression of LEF-1 and TCF4 did not show differences in the expression of their target genes, another approach was chosen to analyze LEF-1 and TCF4: a siRNA knockdown of the two transcription factors.

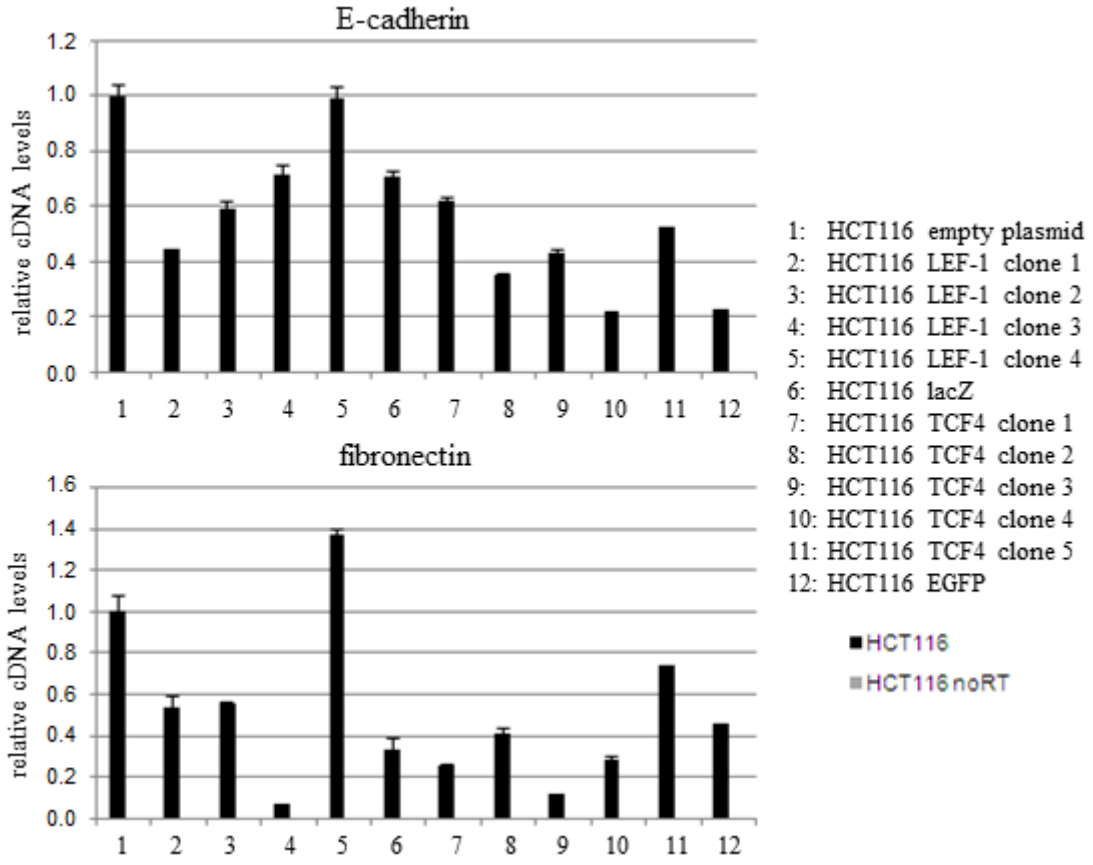

Figure 15. qPCR analysis of E-cadherin and fibronectin of stable HCT116 clones

pPCR analysis of E-cadherin and fibronectin on cDNA transcribed from RNA of stable HCT116 transfectants (empty plasmid: pLNCX2-3xmyc, LEF-1: pLNCX2-LEF1_3xmyc, TCF4: pLNCX2-TCF4_3xmyc, lacZ: pLNCX2-lacZ_3xmyc, EGFP: pLNCX2-EGFP_3xmyc). Relative expression is shown (compared to HCT116 stably transfected with pLNCX2-3xmyc). noRT = no reverse transcriptase added

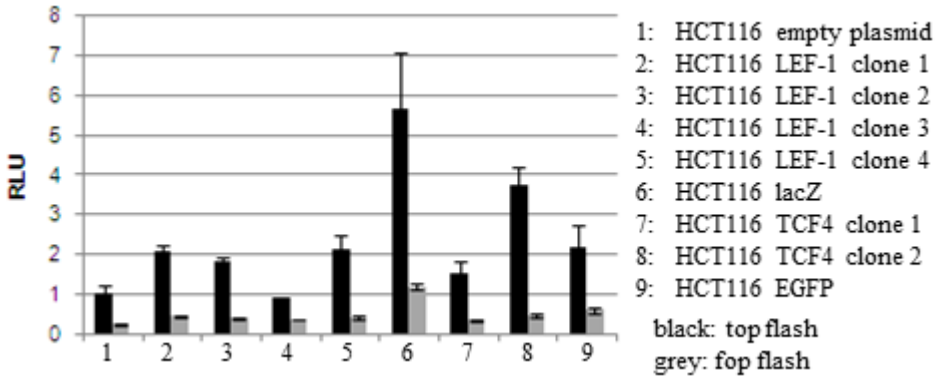

Figure 16. Luciferase assay of stably transfected HCT116 LEF-1 and HCT116 TCF4 clones

Measurement of luciferase activity in stably transfected HCT116 cells (transfected with empty plasmid: pLNCX2-3xmyc, LEF-1: pLNCX2-LEF1_3xmyc, TCF4: pLNCX2-TCF4_3xmyc, lacZ: pLNCX2-lacZ_3xmyc, EGFP: pLNCX2-EGFP_3xmyc). RLU: relative light units

3.2.7 Transient knockdown of LEF-1 and TCF4 and its effects on EMT gene expression and proliferation

Another way of investigating the characteristics of proteins is to knock down their expression and then analyze the effects. The two cell lines SW480 and DLD1 expressing LEF-1 and TCF4 (Figure 9) were treated with siRNA against either transcription factor. Successful knockdown of LEF-1 and TCF4 in both cell lines was shown by qPCR (Figure 17). When looking at the target genes vimentin and E-cadherin the knockdown of LEF-1 and TCF4 shows different results in either cell line. Vimentin expression goes up when knocking down LEF-1 and TCF4 in SW480 and goes down in DLD1. E-cadherin expression stays the same when knocking down LEF-1 in both cell lines. When knocking down TCF4 E-cadherin goes down in SW480 and up in DLD1. There is also an up regulation of LEF-1 when knocking down TCF4 in both cell lines. A trend for an up regulation of TCF4 can be seen when knocking down LEF-1.

Although no coherent effects could be seen with the knockdown of LEF-1 and TCF4, the siRNA treated cells were analyzed in a proliferation assay (Figure 18). In DLD1 cells transfected with siRNA against both transcription factors as well as controls there was no difference in the proliferation of cells. In SW480 cells a small effect showing slower proliferation can be seen after treatment with siTCF4.

Then stable knockdown cell lines were produced to maximize the effects of the knockdown for further analysis.

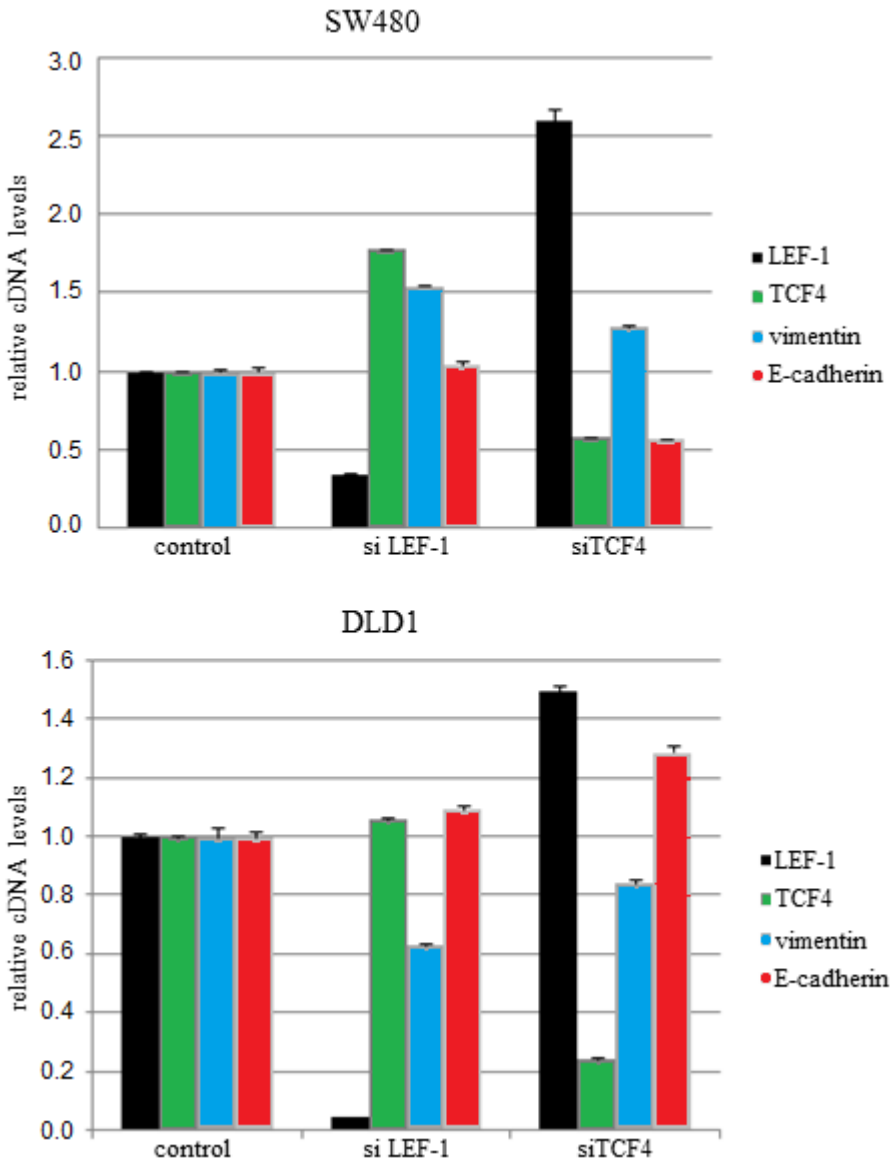

Figure 17. qPCR of SW480 and DLD1 transfected with siRNA against LEF-1 or TCF4.
qPCR of LEF-1, TCF4, vimentin and E-cadherin on cDNA transcribed from mRNA of the transiently with anti-LEF1 or anti-TCF4 siRNA transfected cell lines SW480 (top) and DLD1 (bottom). Relative expression is shown (compared to cDNA level of untransfected SW480 or DLD1 cells)

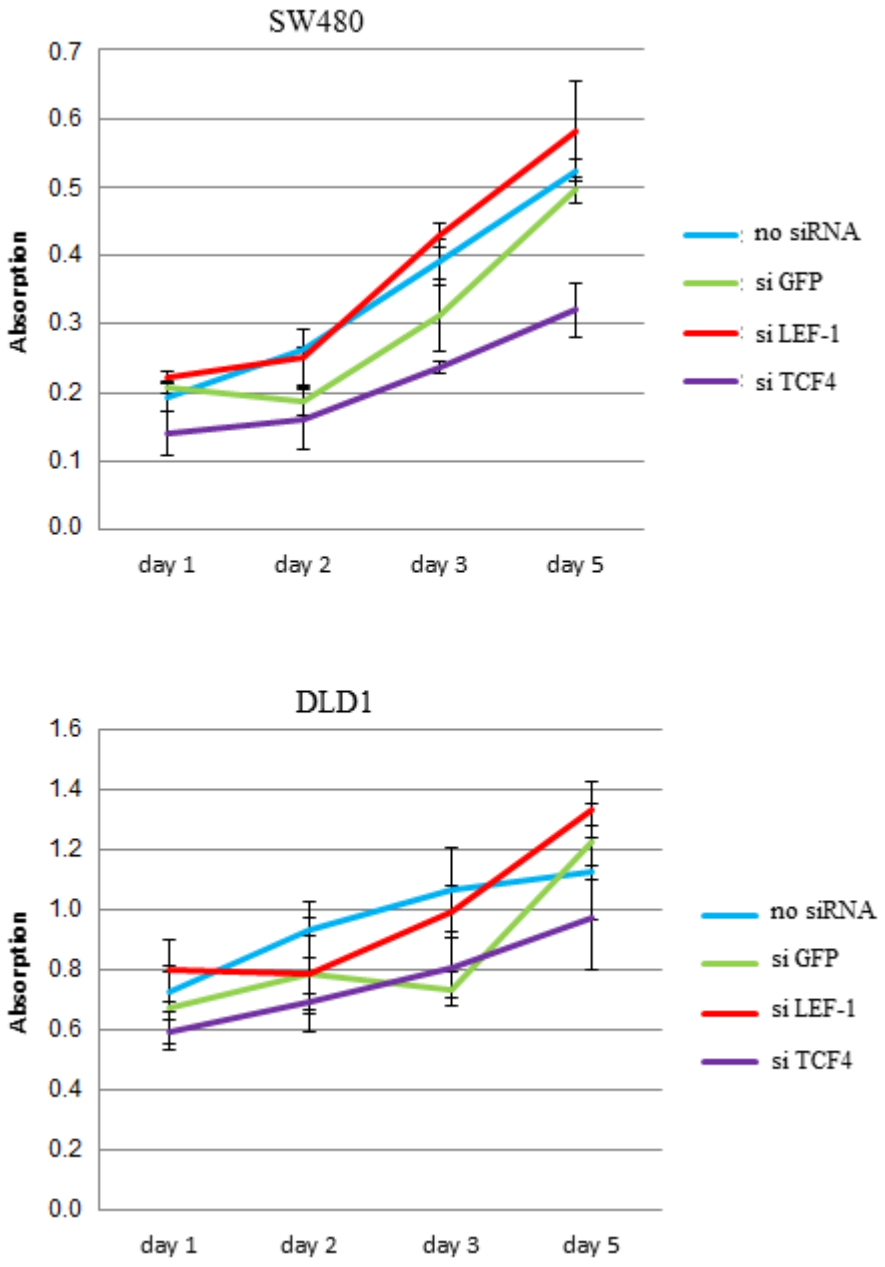

Figure 18. Proliferation assay of SW480 and DLD1 after LEF-1 or TCF4 knockdown

Proliferation of SW480 and DLD1 cells transiently transfected with anti-LEF1, anti-TCF4 siRNA or control transfections (anti-GFP si RNA and transfection reagent without si RNA) was measured by BrdU incorporation after day 1, 2, 3 and 5 (absorption at 450 nm)

3.2.8 Stable knockdown of LEF-1 and TCF4 and its effects on EMT gene expression

To analyze the effects further, stable knockdown clones were produced using lentiviral shRNA. Knockdown was once again tested by qPCR (Figure 19). Measuring the LEF-1 and TCF4 quantities after stable knockdown of each of the transcription factors in DLD1 cells shows an effective knockdown of LEF-1 (Figure 19 top). LEF-1 levels of DLD1 cells with anti-LEF-1 shRNA 1 are reduced to below 50 % compared to the controls. The anti-LEF-1 shRNA 2 did not result in a knockdown of LEF-1 (Figure 18 top: three furthermost columns to the right). The shEGFP controls and the clones with TCF4 knockdown (Figure 19 bottom) also show varying LEF-1 concentrations. Looking at TCF4 and its knockdown the expression goes down to a minimum of 75 %. Here the shEGFP controls show consistent TCF4 expression. Clones with LEF-1 knockdown show varying TCF4 expression.

Additionally to the analysis of the proteins LEF-1 and TCF4 that were directly affected by the knockdown, the clones were also analyzed for their expression of the EMT target genes E-cadherin and vimentin to measure the effects of the knockdown of LEF-1 and TCF4. qPCR was used to examine the mRNA expression of those two genes (Figure 20).

The E-cadherin controls in themselves vary in a big interval. When then looking at the LEF-1 and TCF4 knockdown clones two of the shTCF4 and one of the shLEF-1 clones show a reduced expression. The vimentin controls vary within an interval of a relative expression between one and five. Two of the TCF4 knockdown clones show a slightly higher vimentin expression. The shLEF-1 clones lie in the range of the controls.

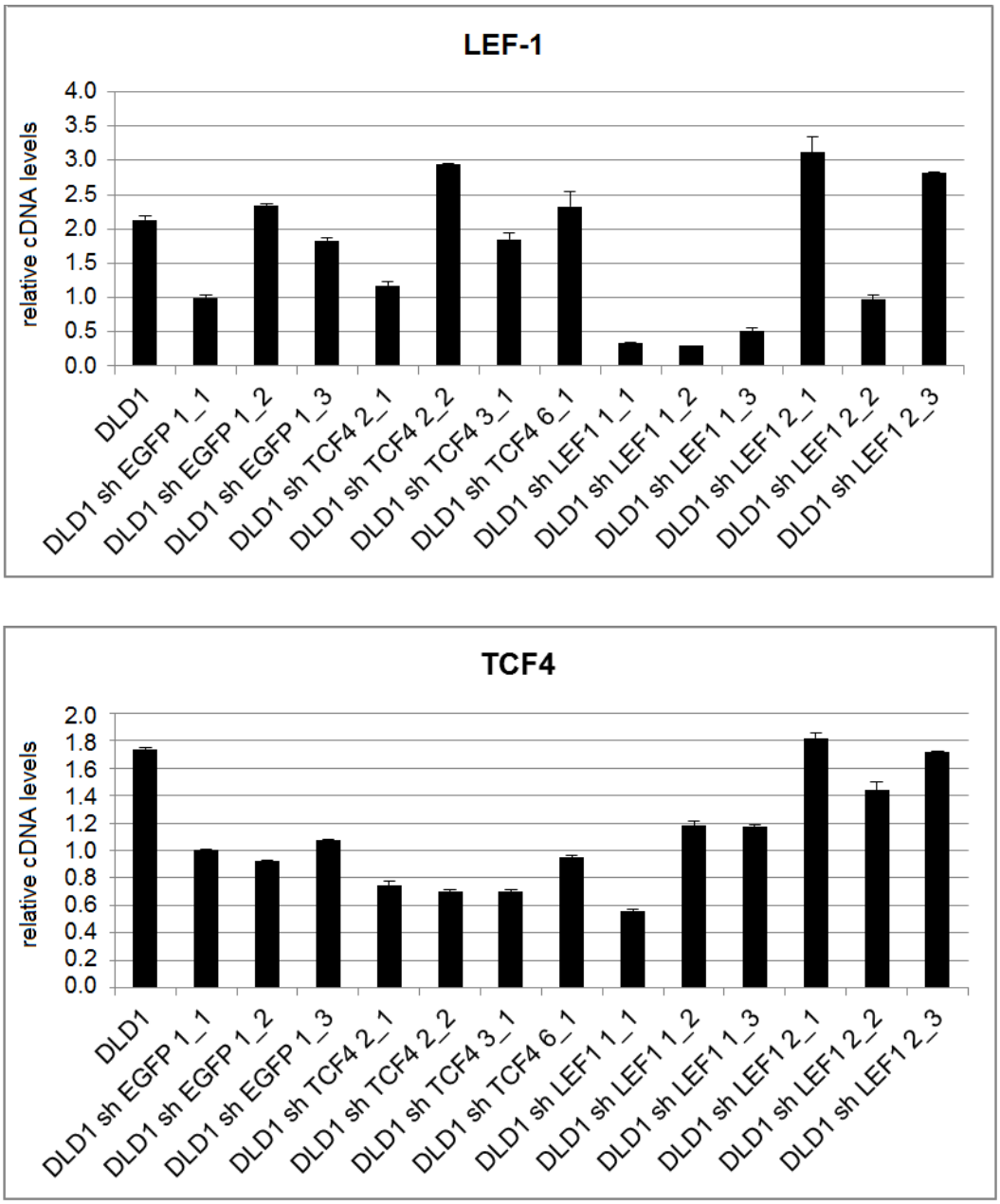

Figure 19. qPCR of LEF-1 (top) and TCF4 (bottom) of stable DLD1 knockdown clones

qPCR of LEF-1 and TCF4 on cDNA transcribed from mRNA of stably transfected DLD1 knockdown clones (shRNA against EGFP, TCF4 and LEF-1). Relative expression is shown (compared to cDNA level of a DLD1 clone transfected with shRNA against EGFP)

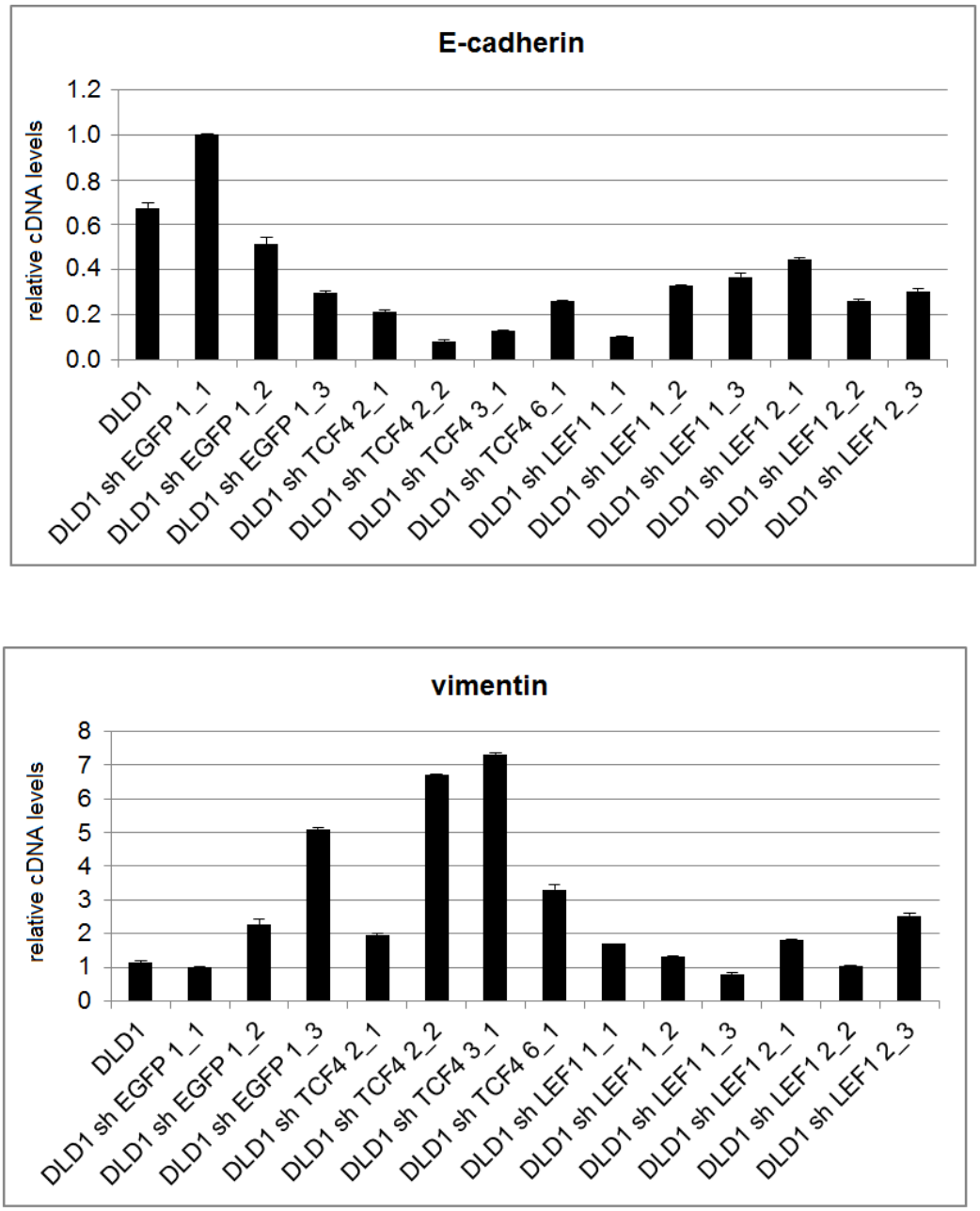

Figure 20. qPCR of E-cadherin (top) and vimentin (bottom) of stable DLD1 knockdown clones

qPCR of E-cadherin and vimentin on cDNA transcribed from mRNA of stably transfected DLD1 knockdown clones (shRNA against EGFP, TCF4 and LEF-1). Relative expression is shown (compared to cDNA level of a DLD1 clone transfected with shRNA against EGFP)

No uniform effects could be seen that explain specific effects of either TCF4 or LEF-1 knockdown.

3.2.9 LEF-1 and TCF4 in tumor cells

The observation of differences between LEF-1 and TCF4 in paraffin embedded tumor tissue was the basis for the following investigation of LEF-1 and TCF4 expression in primary colorectal tumor tissue. Due to the successful establishment of colorectal cancer stem cell (coCSC) cultures and spheroid derived adherent cells (SDAC) from primary colorectal tumors by Achim Schäffauer in our group, a measurement of LEF-1 and TCF4 expression from these cells was conducted. The associated spheroids and SDACs of two tumors were analyzed by qPCR (Figure 21). In both tumors the LEF-1 and TCF4 expression seem to be higher in the differentiated cell population (SDAC) compared to the coCSC populations.

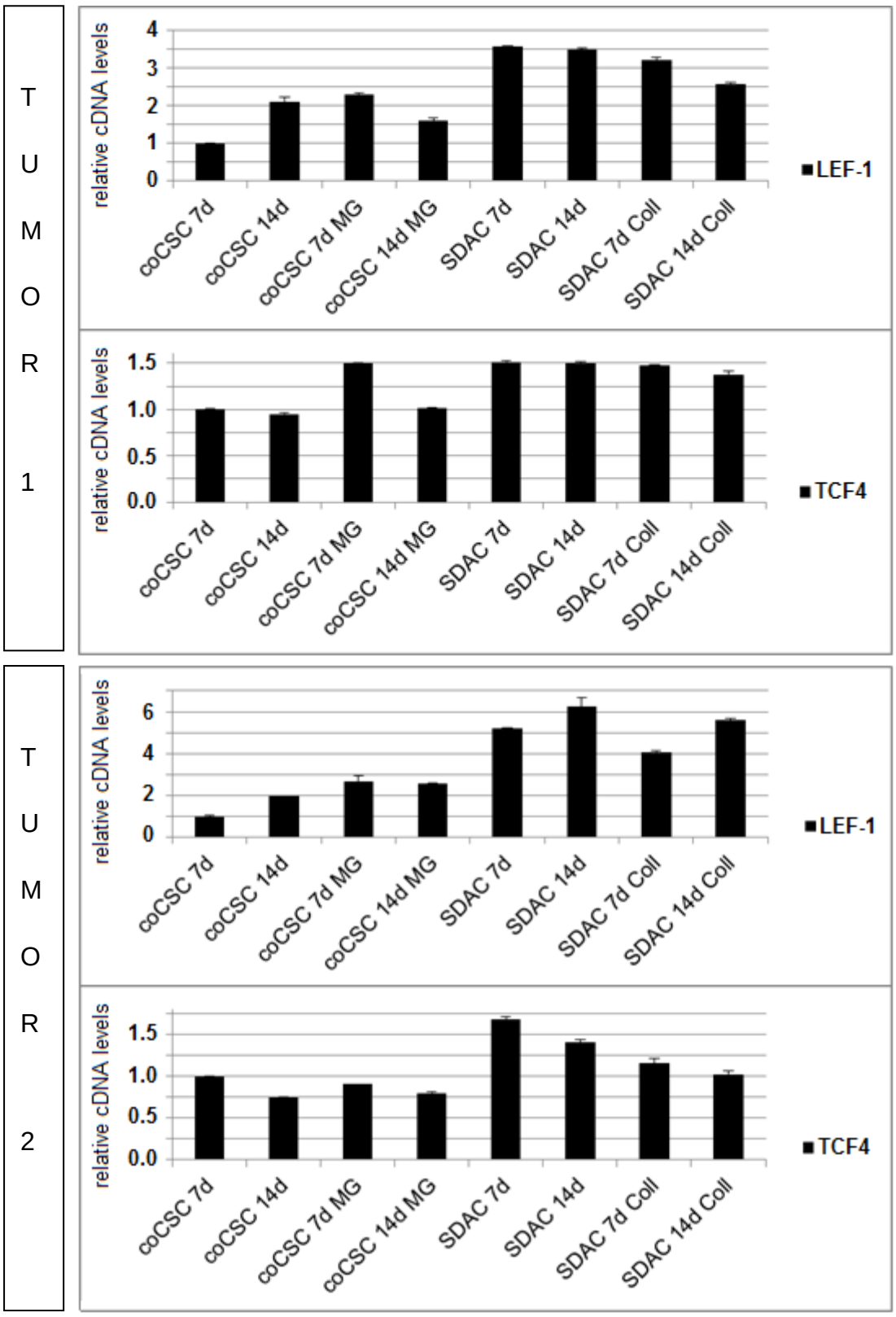

Figure 21. qPCR for LEF-1 and TCF4 in primary CRC material

qPCR of LEF-1 and TCF4 on cDNA transcribed from mRNA of coCSCs and SDACs of two tumors (coCSC: colorectal cancer stem cell; SDAC: spheroid derived adherent cell; MG: Matrigel®; Coll: collagen), mRNA was harvested 7 or 14 days after induction of differentiation

4 Discussion

4.1 Predictive biomarker for anti-EGFR treatment

Several approaches are being used to understand and treat colorectal cancers (CRC). This leads to a better knowledge of the molecular and cellular traits of cancer and can therefore help to further apprehend and embed the prognosis of this disease. Furthermore, more specific treatments are being investigated and introduced. This also leads to a need for better predictive biomarkers to ensure an appropriate therapy.

Anti-EGFR targeted therapy employing cetuximab is a common treatment option for mCRC [7, 102]. One adverse effect of this therapy is the development of skin toxicity in some patients. These patients tend to show a better response to anti-EGFR treatment, turning the skin toxicity into a retrospect positive predictive value [7, 14, 63, 102]. The underlying mechanism of this correlation is not yet fully understood. However, EGFR is a central regulator of multiple epidermal functions [47]. As it is also the primary target of the treatment, it was standing to reason that genetic alterations in the *EGFR* gene might play a role in this correlation. Therefore, this study searched the exon sequences of this gene for a suitable biomarker with high negative as well as positive predictive value for the occurrence of skin toxicity that can be evaluated before the treatment instead of in retrospect.

The correlation between skin toxicity and response can clearly be seen in the disease control rate (DCR) and objective response rate (ORR) of analyzed patients (Table 7). Although differences of mean values in survival times between both cohorts (grade 0 and 3) were large, statistical significance was not reached due to large confidence intervals probably due to comparably small numbers of patients. As the overall survival (OS) was not a result that was concentrated on in this study it did not influence the analysis of the *EGFR* gene. In a further analysis of the CIOX study the OS was analyzed between two different groups: patients with skin toxicity grade 0-1 and patients with skin toxicity grade 2-3 [97]. Here a

strong trend for longer PFS and OS could also be seen in the group with high skin toxicity, but statistical significance was not reached [97].

Finally, the objective of this analysis was to identify genetic alterations in the *EGFR* gene as a predictive biomarker that were superior to *KRAS*. *KRAS* has a very good negative predictive value implicating that CRC with mutant *KRAS* will not respond to the therapy. Here, the false negative rate predicting shrinkage of the tumor lesion is less than 5 % which is outstanding [2]. However, the positive predictive value of *KRAS* is weak being only 61 % [2]. This weakness can be seen especially when this value is compared to other predictive biomarkers like the EML4-ALK (echinoderm microtubule associated protein like 4 - anaplastic lymphoma kinase) inversion in lung cancer. Here, a compelling positive predictive rate of higher than 95 % was reached [59]. Therefore, when using boundaries of 5 and 95 % for the predictive values respectively, an effect size of 0.9 results (Δ=0.95 – 0.05). The aim of this study was to find a biomarker that is as compelling as the two markers mentioned above. So together with a two-sided error of $\alpha \leq 0.05$ and $\beta \leq 0.05$ (power=0.95) in the context of exact test statistics a minimum sample size of 6 patients is needed for the analysis [25, 26]. Therefore a setup with 20 patient samples for the teaching set and another 25 patient samples for the validation set (N=45) was chosen as an approach that would produce a clear result. This higher amount of sample sizes was used to improve the quality of the results. With the amount of patient samples used in this study it would be highly probable to identify genetic alterations in the *EGFR* gene as a predictive biomarker superior to the *KRAS* biomarker.

In a first step, the 28 exons of the *EGFR* gene from 20 patients of the teaching set with either grade 3 skin toxicity (10 patients) or absence of skin toxicity (grade 0, 10 patients) were analyzed by Sanger sequencing. A variety of genetic alterations was found (Figure 7, Table 8, 7.3 Genetic alterations in *EGFR* page 85) which contained already known polymorphisms, thereby validating the analysis system for the detection of genetic alterations. Together, the results indicated that the distribution of genetic alterations in the *EGFR* gene is frequent but quite heterogeneous and complex.

The analysis of the validation set also resulted in the finding of known and new genetic alterations (Figure 8, Table 8, 7.3 Genetic alterations in *EGFR* page 85). Again, the pattern of genetic alterations in the *EGFR* gene was heterogeneous and did not result in genetic alteration which could discriminate a biomarker with a high predictive value between high grade and absence of skin toxicity and thus a responsiveness of the patient to anti-EGFR targeted therapy.

The aim of the study was to find a biomarker with a high positive predictive value for anti-EGFR therapy of CRC. The skin rash reaction of responsive patients is such a biomarker, however it can only be retrospectively assessed.

As the amount of samples used for the study suffices to find frequently occurring genetic alterations correlating with skin rash there might be infrequent genetic alterations or alterations with a low minor allele frequency that correlate with skin rash. Those would be undetectable with the chosen approach but were also without interest in this study.

As it turned out that no polymorphisms in the *EGFR* gene correlated with the extreme forms of skin toxicity (either high grade (grade 3) or absence of skin toxicity (grade 0)) more complex combinations of genetic alterations might be the basis for finding biomarkers. The situation might be more complex in the sense that combinations of genetic alterations might have an applicable predictive value. Large patient collections are needed for analyses like this especially when the results should also be validated.

Moreover, other components of the EGFR signaling pathway might be further reasonable targets for investigations. Additionally, their regulatory regions like promoter/enhancers, introns, 5’ UTR (untranslated region) as well as 3’ UTR where mostly binding sites for miRNAs are located. A more complex analysis employing next generation sequencing seems to be a rational approach to solve the problem. Alternatively, post transcriptional or post translational modifications of the EGFR or its associated factors in the different signaling pathways might be altered (splicing variants / regulation via miRNAs / ubiquitination) [10, 13, 37, 88]. New high throughput techniques can facilitate the search for better prognostic and

predictive biomarkers [34]. Due to the complex signaling pathways that are connected with the EGFR, there are numerous other components that need to be analyzed to eventually find the optimal predictive biomarker or set of biomarkers for the response to the therapy. The markers already in use are just the tip of the iceberg and further research will help finding more specific and significant biomarkers.

4.2 β-catenin/LEF-1 and β-catenin/TCF4 regulated programs in CRC

To further support the research on prognostic biomarkers the two transcription factors LEF-1 and TCF4 that are part of the Wnt pathway which plays a central role in the carcinogenesis of colorectal cancer were analyzed for their effect in CRC cells.

To analyze their prognostic values, the expression in samples from patients with colorectal cancer were determined and the results were correlated with the overall survival of those patients [57]. TCF4 was correlated with a shorter overall survival which identifies TCF4 as a negative prognostic marker. Contrary to TCF4 the expression of LEF-1 as well as a LEF-1/TCF4 ratio were found to correlate with a longer overall survival [57]. This finding might suggest that TCF4 is the main binding partner for β-catenin during the development and progression of CRC. TCF4 expression might indicate cells that show traits of cancer stem cells (CSC). Contrary, LEF-1 expression was more often found in central tumor areas and correlated with a better survival which might indicate differentiated tumor cells without invasive or metastatic potential [57].

Due to the different prognostic values of these two transcription factors, a closer look was taken at the molecular background. CRC cell lines were used for this analysis. Of the cell lines used in this study, two didn't express LEF-1 but all of them expressed TCF4 (Figure 9). No cell line could be found that did not express TCF4. But the experimental setup of the analyses was chosen so that effects would still be seen. To examine, characterize and compare the programs regulated by β-catenin/LEF-1 and β-catenin/TCF4 those two cell lines (HCT116, HT29) were converted to cell lines expressing LEF-1. As no cell line without TCF4 expression was available, overexpression was used in those two cell lines to analyze the effects of TCF4. Overexpression of the transcription factors was used to make sure that the respective other transcription factor did not compete for the β-catenin binding site (LEF-1 with TCF4 overexpression and TCF4 with LEF-1 overexpression).

Several cells with varying LEF-1 and TCF4 expression patterns could then be analyzed for their characteristics.

First of all, the overexpression plasmids were prepared. They were then tested by Western blot to check for protein expression (shown for LEF-1 in Figure 10), and luciferase assay for checking the functionality of the expressed transcription factors (shown for LEF-1 and TCF4 in Figure 11). Importantly, it could be seen that there is a dose dependent rise in assay signal. But concentrations that are too high inhibit functionality due to binding site blocking. Therefor, for optimal transcription there needs to be an optimum of LEF-1/TCF4 and β-catenin concentration. Overall the functionality of the overexpression constructs could be shown on protein level and protein functionality.

Consequently, the effects of the transcription factors could be analyzed next.

It has been shown, that the Wnt/β-catenin pathway has an influence on migration [85]. To analyze whether the expression of LEF-1 and TCF4 shows effects on the cells, a functional characterization was performed using a migration assay. The differences between the transiently transfected cells were marginal and the standard deviation was bigger than the differences in migration between the differently transfected cells. Therefore, no clear assertion about differences could be made. Reasons for this could be inherent in the setup of the experiment as only a subset of cells is transfected by transient transfection [45]. Not all cells express the transcription factors and can show newly accomplished traits. So differences could be diluted in the background.

To maximize effects that could be seen after transfection, stable cell lines were produced, tested and characterized.

After transduction of cells and raising clones, they were tested and characterized. Protein expression was shown by Western blot and mRNA expression of target genes was analyzed using qPCR, as this was the suitable method to screen the large amount of clones obtained. The target gene mRNAs of the analyzed clones showed varying concentrations. No correlation could be found between LEF-1 or TCF4 and E-cadherin or vimentin mRNA levels. Reasons for this could be inherent in the system used to change the cells. Overexpression of a gene could saturate the cells with unnaturally high amounts of protein as physiological protein

concentrations are far exceeded (especially when using a CMV promoter) [87]. The reaction of the cells could result in promoter silencing [9]. In this particular setting, the overexpression of the transcription factors would also catch all free β-catenin and might, in higher concentrations, block the TBEs. So in a next step LEF-1 and TCF4 were down regulated to simulate and analyze more natural conditions.

The transient knockdown of LEF-1 and TCF4 in SW480 and DLD1 cells worked, as shown in Figure 17. The resulting effects were measured by qPCR of the pathway target genes E-cadherin and vimentin. The results were not consistent between the two cell lines as well as with the knockdown of each transcription factor. The observed changes were not significant. A possibility for this can once again be incomplete transfections with the siRNA. But as the knockdown worked very well it was more likely, that the cells responded by trying to regain a pathway equilibrium through compensatory mechanisms. Knockdown of LEF-1 did not exert a marked effect whereas knockdown of TCF4 lead to an upregulation of LEF-1 which could be a compensation for the missing transcription factor TCF4. Therefore, effects of a TCF4 knockdown on the cells characteristics might also be effects from an upregulation of LEF-1. Considering that the aim of this work was to find a difference between the two transcription factors on cellular behavior, this mutual regulation does not generally prevent an analysis of the results. If effects of a knockdown are not directly related with that knockdown but with the upregulation of the other transcription factor, this would also show that the transcription factors have different impacts on the cells.

When looking at the Proliferation Assay (Figure 18), the knockdown only leads to a slight difference in SW480 cells with TCF4 knockdown. The amount of cells at day one had been below the amount of the other transfected cells and considering the standard deviation the final difference at the end of the experiment was not convincing. But a slight difference that was there indicates that knockdown of TCF4 in SW480 cells lead to reduced proliferation.

To go into more detail and elevate the effects, the cell lines were transfected stably with shRNA. The two cell lines DLD1 and SW480 were stably transfected with shRNA against LEF-1 and TCF4 in order to rule out low transfection efficiency

and associated poorly visible effects. Looking at LEF-1, variable effects could be seen: The LEF-1 knockdown clones showed a LEF-1 reduction (LEF-1 shRNA 1). All other cells, including the shEGFP controls showed varying expression. The TCF4 expression was more consistent but the reduction of TCF4 expression in the knockdown clones was very small. The expression of the target genes E-cadherin and vimentin was also very variable. The expression patterns in the analyzed clones vary so much, that possible effects are lost in this variety of signals and are therefore not visible. Again the effects might be too small to be seen in the analysis system used in this work. Additionally, differences between the clones could result from single cell cloning. It has been shown that in one cell line there can be several subpopulations which complicate finding specific effects[20].

As the above mentioned assays showed inconclusive results, primary tumor cells were studied to gain a better understanding. The observation of differences between LEF-1 and TCF4 in paraffin embedded tumor material was the basis for the investigation of LEF-1 and TCF4 expression in primary tumor cells. From these samples, two contrasting cell populations were analyzed: tumor stem cells would resemble a more aggressive phenotype compared to their differentiated form, spheroid derived adherent cells (SDACs).

The advantage of this system is the proximity to humans. The cells analyzed were derived from human colorectal carcinomas just weeks before the measurement of RNA levels.

When comparing the tumor stem cells with corresponding SDACs the expression of the two transcription factors seemed higher in the differentiated cells. The difference to the measured expression in the cell culture experiments could lie in the small amount of stem cells that can be found in cell culture. The effects of LEF-1 or TCF4 knockdown may only affect a small subgroup of cells (the stem cells) in cell culture. This result could explain the small and varying effects seen in cell culture.

Based on the slightly different LEF-1/TCF4 expression patterns in tumor stem cells and differentiated cells, other interesting questions arise. Do LEF-1 or TCF4 drive

differentiation or do they favor stemness? A method for testing this would be LEF-1 or TCF4 overexpression. The transfected cells can then be further analyzed for the resulting characteristics. Effects of the absence of the transcription factors on stem cells could be measured by knockdown experiments.

As no consistent or prominent differences could be seen between LEF-1 and TCF4 the question arises whether, in a complex setting like colorectal carcinomas the effects are similar in each analyzed cell line or carcinoma.

Looking at other organs, varying effects of the transcription factors can be seen. In melanomas the differential expression of LEF-1 and TCF4 is involved in melanoma cell phenotype switching. Expression of LEF-1 is primarily found in differentiated / proliferative phenotype cells whereas TCF4 is expressed preferentially by dedifferentiated / invasive phenotype cells [24]. In contrast, experiments performed by Nguyen *et al.* showed that LEF-1 mediates lung adenocarcinoma metastasis [81]. So depending on the characteristics of the cells or the tumor, the transcription factors can show opposite effects on tumor features.

Those results complicate the use of LEF-1 or TCF4 as biomarkers. Especially for colorectal carcinomas, analyses with contrasting results have been performed. Once again there have been analyses showing that TCF4 induces EMT and therefore tumor invasiveness [92]. Additionally, analyses have shown that LEF-1 could be identified as a prognostic biomarker for liver metastases from primary colorectal carcinomas [65].

Further research is needed to examine the possible functional role and the prognostic and predictive capability of LEF-1 and TCF4.

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7 Appendix

7.1 Abbreviations

5FU	5-fluorouracil
A	adenine
aa	aminoacid
ACC#	accession number
APC	adenomatous polyposis coli
APS	ammonium persulfate
attB	attachment site B
bp	base pair
BrdU	bromodeoxyuridine
C	cytosine
CAPIRI	capecitabine, irinotecan
CAPOX	capecitabine, oxaliplatin
CAT	chloramphenicol acetyltransferase
cDNA	complementary DNA
CMV	cytomegalovirus
coCSC	colorectal cancer stem cell
coll	collagen
CSC	cancer stem cell

CRC	colorectal cancer
ctrl	control
d	days
DC	detergent compatible
DCR	disease control rate
DNA	deoxyribonucleic acid
DMEM	Dulbecco’s Modified Eagle Medium
dNTP	deoxynucleotide
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacedic acid
e.g.	exempli gratia (for example)
EGFP	green fuorescent protein
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EML4-ALK	echinoderm microtubule associated protein like 4 - anaplastic lymphoma kinase
EMT	epithelial-mesenchymal transition
FFPE	formalin-fixed, paraffin-embedded
FOLFIRI	5-fluorouracil, leucovorin, irinotecan
FOLFOX	5-fluorouracil, leucovorin, oxaliplatin
fw	forward
G	guanine

HA	human influenza hemagglutinin
HER2	human epidermal growth factor receptor 2
HPRT	hypoxanthine-guanine phosphoribosyltransferase
HRP	horseradisch peroxidase
JNK	c-Jun N-terminal kinase
kb	kilobase
kDa	kilodalton
KOD	thermococcus kodakaraensis
KRAS	Kirsten rat sarcoma viral oncogene homolog
LB	lysogeny broth
LEF-1	lymphoid enhancer-binding factor 1
MAP	mitogen-activated protein
mCRC	metastatic colorectal cancer
MG	Matrigel®
$MgCl_2$	magnesium chloride
min	minute
miRNA	microRNA
MLH1	MutL homolog 1
MMP7	matrix metalloproteinase-7
MMR	mismatch repair
MOI	multiplicity of infection
mRNA	messenger RNA

MOI	multiplicity of infection
n.a.	not applicable
NCI-CTCAE	national cancer institute - common terminology criteria adverse events
NRAS	neuroblastoma RAS viral oncogene homolog
n.s.	not significant
NSAIDs	nonsteroidal anti-inflammatory drugs
ORR	objective response rate
OS	overall survival
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFS	progression free survival
PI3K	phosphatidylinositol 3-kinase
PMSF	phenylmethanesulfonyl fluoride
PTEN	phosphatase and tensin homolog
PVDF	polyvinylidene fluoride
qPCR	quantitative PCR
RAS	rat sarcoma
Rev	reverse
RL	Renilla luciferase
RNA	ribonucleic acid
RLU	relative light units

rs	Reference SNP cluster ID
RT	reverse transcriptase
SDAC	spheroid-derived adherent cell
s	second
shRNA	small hairpin RNA
siRNA	small interfering RNA
SNP	single nucleotide polymorphism
SOS	son of sevenless
T	thymine
TBE	β-catenin/Tcf/Lef-binding element
TCF	T-cell factor
TEMED	tetramethylethylenediamine
UPL	universal probe library
UTR	untranslated region
Wnt	int/wingless
WT	wildtype

7.2 Primers used for sequencing of the *EGFR* gene

Primer	nucleotide sequence	
	outer forward	outer reverse
Exon 01	CCCTGACTCCGTCCAGTATT	GCAGCTGATCTCAAGGAAACA
Exon 02	TCATGTGATATCTGTCTTTTTCTTCC	CCCAGGCCTTTCTCCACTTA
Exon 03	GCTCCCTGGACCCATTTTAG	TAATACGACTCACTATAGGTTATGAACCCCCAGCCTTG
Exon 04	AGCTGGAAAGAGTGCTCACC	TAGAGCTGTCCCCCATAGGA
Exon 05	GAAAGGGCGTCATCAGTTTC	ACATGGGTCTGAGGCTGTTC
Exon 06	TTCAGCTCACAGGGAACCTT	ACTTTGTCACCAGGCAGAGG
Exon 07	GCTTTCTGACGGGAGTCAAC	GACAAGGATGCCTGACCAGT
Exon 08	CTTTCCATCACCCCTCAAGA	TTTGGAGGTGGCATGAGAG
Exon 09	CTTCCCTCTGCCTGTGGAT	TAATACGACTCACTATAGGCAAGCAACTGAACCTGTGACTC
Exon 10	GTAAAACGACGGCCAGTTTTAGGGGGGTGAGTCACAGG	TCCAAGGGAACAGGAAATATG
Exon 11	TGCGCATGTACACTCAGAGA	TAATACGACTCACTATAGGGCTTTGGCTGTGGTCAACTT
Exon 12	TCACCACATGATTTTTCTTCTCTC	TAATACGACTCACTATAGGGGACCCATTAGAACCAACTCC
Exon 13	GCATGTCTGTGTCACCCAAG	TCAACGCAAGGGGATTAAAG
Exon 14	TGGAGTCCCAACTCCTTGAC	TCATCACTGTTCGGCTTCTG
Exon 15	GCATGAACATTTTTCTCCACCT	ACAAACCTCGGCAATTTGTT
Exon 16	AACCACCAATCCAACATCCA	CCACAGCAGTGTGGTCATTC
Exon 17	ACCTCACCCTTCCTTGTTCC	TAATACGACTCACTATAGGAACTGCTAATGGCCCGTTC
Exon 18	CAAATGAGCTGGCAAGTGCCGTGTC	GAGTTTCCCAAACACTCAGTGAAAC
Exon 19	GCAATATCAGCCTTAGGTGCGGCTC	CATAGAAAGTGAACATTTAGGATGTG
Exon 20	CCATGAGTACGTATTTTGAAACTC	CATATCCCCATGGCAAACTCTTGC
Exon 21	CTAACGTTCGCCAGCCATAAGTCC	GCTGCGAGCTCACCCAGAATGTCTGG
Exon 22	GTAAAACGACGGCCAGTCACTGCCTCATCTCTCACCA	CTGGACTCGATTTCCTCTGC
Exon 23	GTAAAACGACGGCCAGTCCACTGCCTTCTTTTCTTGC	GCCTCAGCTGTTTGGCTAAG
Exon 24	GAAGTGTCGCATCACCAATG	TAATACGACTCACTATAGGAATGGAAGCACAGACTGCAA
Exon 25	AGACCCCTGCTCCTATAGCC	TAATACGACTCACTATAGGCACAGCTTGAGAGAGAGAGAGA
Exon 26	ATACCCTCCATGAGGCACAC	TAATACGACTCACTATAGGGGAAAAACCCACACAGGAAG
Exon 27	GTAAAACGACGGCCAGTCCGGAGTAACCTTCCCTCAT	GAGGAGCAGGACTGTTTCCA
Exon 28	ATCCTGCATGGGATGGTG	TAATACGACTCACTATAGGTTGGTCCTGGGTATCGAAAG

Primer	nested forward	nested reverse
Exon 01	GTAAAACGACGGCCAGTCCAGTATTGATCGGGAGAGC	TAATACGACTCACTATAGGGCTGATCTCAAGGAAACAGGA
Exon 02	GTAAAACGACGGCCAGTTGTGATATCTGTCTTTTCTTCCA	TAATACGACTCACTATAGGCCAGGCCTTTCTCCACTTAG
Exon 03	GTAAAACGACGGCCAGTCCCTGGACCCATTTTAGACC	TAATACGACTCACTATAGGTTATGAACCCCCAGCCTTG
Exon 04	GTAAAACGACGGCCAGTAAAGAGTGCTCACCGCAGTT	TAATACGACTCACTATAGGCATAGGAGCTGGAGGCAGAG
Exon 05	GTAAAACGACGGCCAGTGGGCGTCATCAGTTTCTCAT	TAATACGACTCACTATAGGATGGGTCTGAGGCTGTTCAC
Exon 06	GTAAAACGACGGCCAGTCTCACAGGGAACCTTTGCTC	TAATACGACTCACTATAGGGCAGAGGGCAATATCCTGTC
Exon 07	GTAAAACGACGGCCAGTGTGTGGCGCTGAGTGTACTT	TAATACGACTCACTATAGGCAGTTAGAGGGCCCACAGAG
Exon 08	GTAAAACGACGGCCAGTCACCGTCATCACCTTCCTTT	TAATACGACTCACTATAGGCTCAGCAGCCGAGAACAAG
Exon 09	GTAAAACGACGGCCAGTTCCAACAAATGTGAACGGAAT	TAATACGACTCACTATAGGCAAGCAACTGAACCTGTGACTC
Exon 10	GTAAAACGACGGCCAGTTTTAGGGGGTGAGTCACAGG	TAATACGACTCACTATAGGGGAACAGGAAATATGTCGAA
Exon 11	GTAAAACGACGGCCAGTGCAAATCCAATTTTCCCACTT	TAATACGACTCACTATAGGGCTTTGGCTGTGGTCAACTT
Exon 12	GTAAAACGACGGCCAGTCCACATGATTTTTCTTCTCCA	TAATACGACTCACTATAGGGGACCCATTAGAACCAACTCC
Exon 13	GTAAAACGACGGCCAGTTGTCTGTGTCACCCAAGGTC	TAATACGACTCACTATAGGCAACGCAAGGGGATTAAAGA
Exon 14	GTAAAACGACGGCCAGTGAGTCCCAACTCCTTGACCA	TAATACGACTCACTATAGGCACTGTTCGGCTCTCTGTGAA
Exon 15	GTAAAACGACGGCCAGTGAACATTTTTCTCCACCTTGG	TAATACGACTCACTATAGGATTTGTTGCCGGAAAACTTG
Exon 16	GTAAAACGACGGCCAGTACCACCAATCCAACATCCAG	TAATACGACTCACTATAGGTTCTTGACGAGGTCCATGTG
Exon 17	GTAAAACGACGGCCAGTACCCTTCCTTGTTCCTCCAC	TAATACGACTCACTATAGGAACTGCTAATGGCCCGTTC
Exon 18	GTAAAACGACGGCCAGTCAAGTGCCGTGTCCTGGCACCCAAGC	TAATACGACTCACTATAGGGCCAAACACTCAGTGAAACAAAGAG
Exon 19	GTAAAACGACGGCCAGTCCTTAGGTGCGGCTCCACAGC	TAATACGACTCACTATAGGGCATTTAGGATGTGGAGATGAGC
Exon 20	GTAAAACGACGGCCAGTGAAACTCAAGATCGGCATTCATGC	TAATACGACTCACTATAGGGGCAAACTCTTGCTATCCCAGGAG
Exon 21	GTAAAACGACGGCCAGTCAGCCATAAGTCCTCGACGTGG	TAATACGACTCACTATAGGGCATCCTCCCCTGCATGTGTTAAAC
Exon 22	GTAAAACGACGGCCAGTCACTGCCTCATCTCTCACCA	TAATACGACTCACTATAGGTTCCTCTGCCGTGTTTTCTC
Exon 23	GTAAAACGACGGCCAGTCCACTGCCTTCTTTTCTTGC	TAATACGACTCACTATAGGCAGCTAGGCAGTGTGGACAG
Exon 24	GTAAAACGACGGCCAGTGCATCACCAATGCCTTCTTT	TAATACGACTCACTATAGGAATGGAAGCACAGACTGCAA
Exon 25	GTAAAACGACGGCCAGTTAGCATCTCTACGGGCCATT	TAATACGACTCACTATAGGCACAGCTTGAGAGAGAGAGAGAGA
Exon 26	GTAAAACGACGGCCAGTCCATGGGCAACTTCTCTGTT	TAATACGACTCACTATAGGGAAAAACCCACACAGGAAG
Exon 27	GTAAAACGACGGCCAGTCCGGAGTAACCTTCCCTCAT	TAATACGACTCACTATAGGGAGCAGGACTGTTTCCAGA
Exon 28	GTAAAACGACGGCCAGTGGATGGTGCTTTGCTGATTA	TAATACGACTCACTATAGGTTGGTCCTGGGTATCGAAAG

7.3 Genetic alterations in *EGFR*

	skin toxicity	Patient	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
teaching-set	0	1	-	-	-	c.474C>T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0	3	-	-	-	c.474C>T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0	4	-	-	-	c.474C>T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0	5	-	-	-	c.474C>T	-	-	-	-	-	-	x	-	-	c.1662G>G/A	-	c.1887T>T/A	-	-	-
	0	6	-	-	-	c.474C>T	-	-	-	-	-	-	x	-	-	-	-	x	x	-	x
	0	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0	8	-	-	-	c.474C>T	-	-	-	-	-	-	-	-	c.1562G>A	-	-	-	-	-	-
	0	9	-	-	c.289G>A	c.474C>T	-	-	x	-	-	-	-	c.1350A>G	-	-	-	-	-	-	x
	0	10	-	-	-	c.474C>T	-	-	-	-	-	c.1171G>A	-	-	-	-	-	c.1887T>T/A	-	-	-
	3	11	-	-	-	c.474C>T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	12	-	-	-	c.474C>T	-	-	-	-	-	-	-	-	c.1562G>G/A	-	-	c.1887T>T/A	-	-	-
	3	13	-	-	-	x	-	x	x	-	-	x	x	x	-	x	x	x	x	x	x
	3	14	-	-	-	c.474C>T	-	-	-	-	-	-	-	-	-	-	c.1839C>C/T	-	-	-	-
	3	15	-	-	-	c.474C>T	-	-	-	-	-	-	x	x	c.1562G>A	-	-	-	c.2039G>G/A	-	-
	3	16	-	-	-	c.474C>T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	c.2277C>T
	3	17	-	-	-	c.474C>T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	18	-	-	-	-	-	-	-	-	-	x	-	x	-	-	c.1839C>T	-	-	-	x
	3	19	-	-	-	c.474C>C/T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
validation-set	0	21			-							-			-				-	-	
	0	22			-							-			-				-	-	
	0	23			-							-			c.1562G>A				-	-	
	0	24			-							-			-				-	-	
	0	25			-							-			-				-	-	
	0	26			-							-			-				c.2016C>T	-	
	0	27			-							-			-				-	-	
	0	28			c.304G>G/A							-			-				c.1930C>C/T	-	
	0	29			-							-			c.1562G>G/A				-	-	
	3	30			-							-			-				-	-	
	3	31			-							-			c.1562G>A				-	-	
	3	32			-							-			-				c.1975G>G/A	-	
	3	33			-							-			c.1562G>G/A				-	-	
	3	34			-							-			-				-	-	
	3	35			-							-			-				-	-	
	3	36			-							-			-				c.2047C>C/T	-	
	3	37			-							-			-				-	-	
	3	38			-							c.1172A>G			c.1541G>A				-	-	
	3	39			-							-			c.1562G>G/A				-	-	
	3	40			-							-			c.1519C>C/T				-	-	
	3	41			-							-			-				-	-	
	3	42			-							-			-				c.2035C>C/T	-	
	3	43			-							-			c.1562G>A				c.1989G>A	-	
	3	44			-							-			-				-	-	
	3	45			-							-			-				-	-	

20	21	22	23	24	25	26	27	28
c.2361G>A	-	-	c.2709C>C/T	-	-	-	-	-
c.2361G>A	-	-	-	-	-	-	-	-
c.2361G>A	-	-	-	-	-	-	-	-
c.2317C>C/T	-	-	c.2709C>C/T	-	-	-	-	c.3468C>T,c.3520C>T
c.2361G>A,c.2362C>T	-	-	-	-	-	-	-	c.3553G>A
c.2361G>A	-	x	c.2709C>T	-	x	x	-	-
c.2361G>A,c.2317C>T	-	-	-	-	-	-	-	-
c.2361G>A	-	-	-	-	-	-	-	-
-	x	x	c.2709C>C/T	-	-	-	-	x
c.2361G>G/A	-	-	c.2709C>C/T	-	-	-	-	-
-	-	-	c.2709C>C/T	-	-	-	-	-
c.2361G>A,c.2400C>C/T,c.2448C>C/T	-	-	-	-	-	-	-	-
x	-	x	-	-	-	-	x	x
c.2361G>A	-	c.2697C>C/T	-	-	c.2982C>C/T	-	-	c.3434C>C/T
c.2361G>A	-	c.2681A>A/G	c.2709C>C/T	-	-	-	-	-
c.2361G>A,c.2439C>T	-	-	-	-	c.2982C>T	-	-	-
c.2361G>A	-	-	-	-	c.2982C>C/T	-	-	-
x	x	-	-	-	-	-	-	x
c.2361G>A	-	-	-	-	-	-	-	c.3475C>T
c.2361G>A	-	-	-	-	-	-	-	-

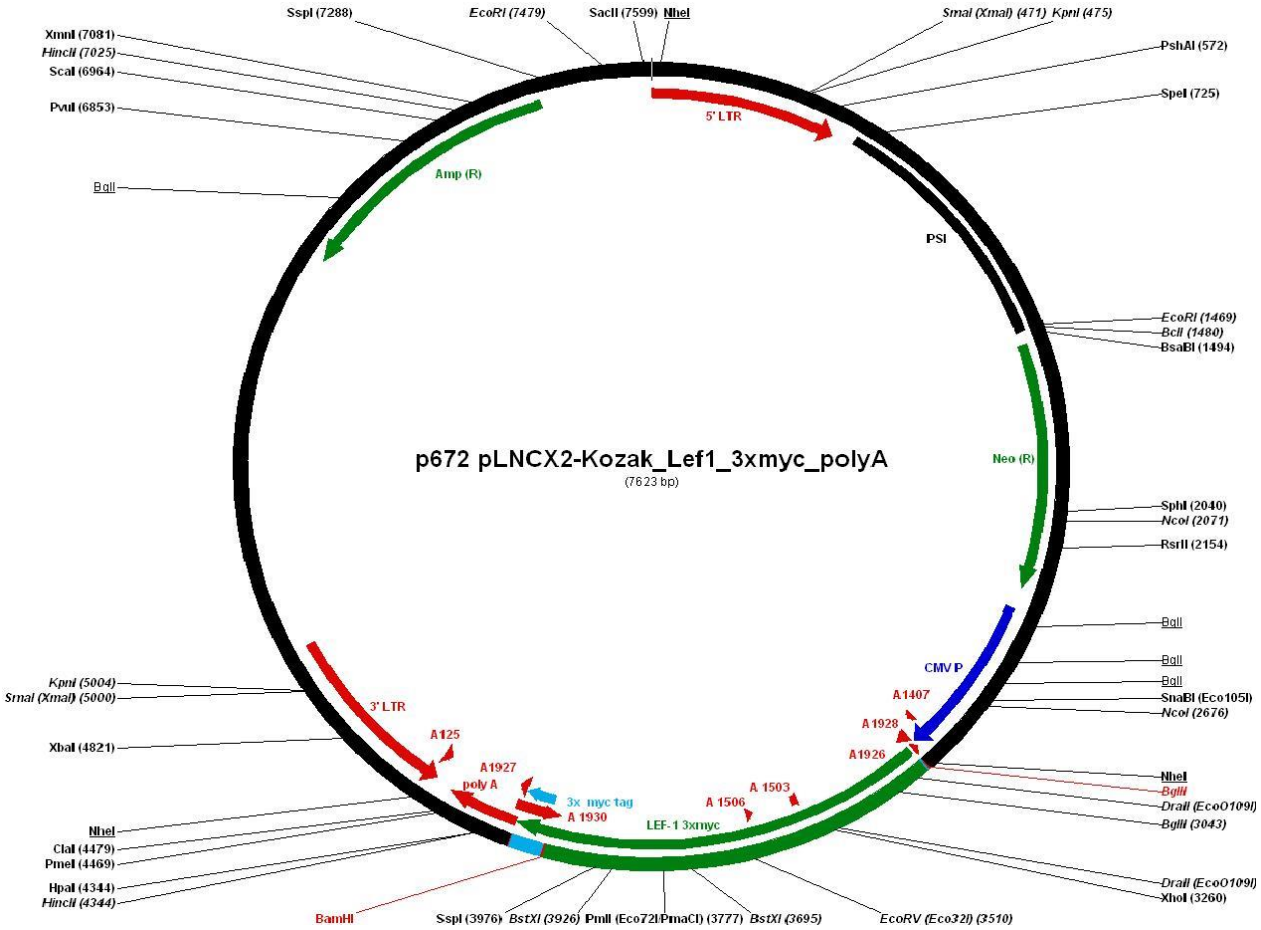
x	x							x
-	x							c.3532C>T
c.2361G>A	-							x
x	-							x
c.2361G>A	-							c.3439G>G/A
x	-							-
c.2361G>A	-							-
c.2324G>A	c.2664C>C/T							c.3465C>T,c.3604C>T
-	-							-
c.2391C>T	-							-
c.2361G>A	-							c.3471G>G/A
-	-							-
x	-							x
c.2361G>A	-							-
c.2361G>A	-							-
c.2361G>A	-							-
c.2443C>C/T	-							-
x	-							x
-	-							c.3292C>T
x	c.2657T>T/C							x
c.2361G>A	-							-
x	-							x
-	c.2640G>G/A							x
c.2361G>A	-							-
c.2361G>G/A	-							-

7.4 Allelic frequencies *EGFR*

skin toxicity	Polymorphism	allelic frequency teaching set [%]					allelic frequenccy validation set [%]					
		G/G	G/A	A/A	C/C	C/T	T/T	G/G	G/A	A/A	C/C	C/T	T/T
0	c.289G>A	90	0	10	-	-	-	100	0	0	-	-	-
	c.304G>G/A	100	0	0	-	-	-	89	11	0	-	-	-
	c.1171G>A	90	0	10	-	-	-	100	0	0	-	-	-
	c.1562G>A	89	0	11	-	-	-	78	11	11	-	-	-
	c.1930C>C/T	-	-	-	100	0	0	-	-	-	89	11	0
	c.2317C>T	-	-	-	80	10	10	-	-	-	100	0	0
	c.2324G>A	100	0	0	-	-	-	86	0	14	-	-	-
	c.2362C>T	-	-	-	90	0	10	-	-	-	100	0	0
	c.3439G>G/A	100	0	0	-	-	-	83	17	0	-	-	-
	c.3520C>T	-	-	-	89	0	11	-	-	-	100	0	0
	c.3532C>T	-	-	-	100	0	0	-	-	-	83	0	17
	c.3553G>A	89	0	11	-	-	-	100	0	0	-	-	-
	c.3604C>T	-	-	-	100	0	0	-	-	-	83	0	17
3	c.1172A>G	0	0	100	-	-	-	6	0	94	-	-	-
	c.1519C>C/T	-	-	-	100	0	0	-	-	-	94	6	0
	c.1541G>A	100	0	0	-	-	-	94	0	6	-	-	-
	c.1562G>A	80	10	10	-	-	-	75	13	13	-	-	-
	c.1975G>G/A	100	0	0	-	-	-	94	6	0	-	-	-
	c.2039G>G/A	89	11	0	-	-	-	100	0	0	-	-	-
	c.2640G>G/A	100	0	0	-	-	-	94	6	0	-	-	-
	c.2657T>T/C	-	-	-	0	0	100	-	-	-	0	6	94
	c.2681A>A/G	0	11	89	-	-	-	0	0	100	-	-	-
	c.3292C>T	-	-	-	100	0	0	-	-	-	91	0	9
	c.3434C>C/T	-	-	-	88	13	0	-	-	-	100	0	0
	c.3471G>G/A	100	0	0	-	-	-	91	9	0	-	-	-
	c.3475C>T	-	-	-	88	0	13	-	-	-	100	0	0

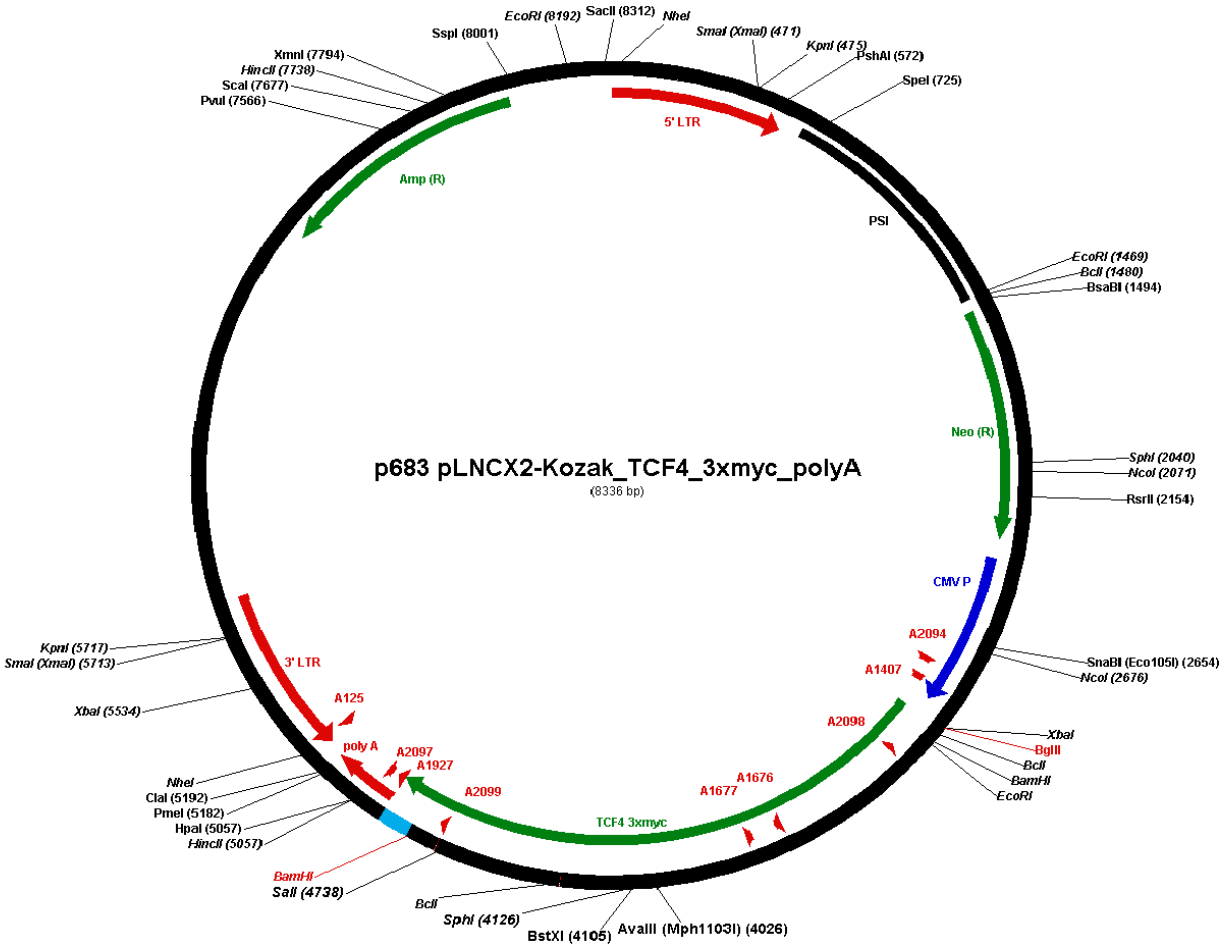
7.5 Plasmids

- pLNCX2-Lef1_3xmyc



- pLNCX2-EGFP_3xmyc

- pLNCX2-TCF4_3xmyc



- Renilla Luciferase plasmid: phRL-tk TBE mut (modified by insertion of a mutation into a TBE from pRL-TK Luciferase Reporter Vector; Promega)
- M50 Super 8x TOPFlash, M51 Super 8x FOPFlash (TOPFlash mutant) (addgene deposited by Randall Moon)
- pEGFP-C1 (Clontech, Mountain View, USA)
- pLNCX2-TCF4-HA (cloned by Silvio Scheel, original plasmid pLNCX2 from Clontech, Mountain View, USA)
- pcDNA3-CAT (Chloramphenicol acetyltransferase; Invitrogen, Life Technololgies, Carlsbad, USA)
- pcI-neo-ßcatenin-D45 (addgene deposited by Bert Vogelstein)

7.6 Curriculum Vitae

7.7 Publications

7.7.1 Manuscripts

Jaitner S, **Reiche JA**, Hiendlmeyer E, Herbst H, Schindler A, Brabletz T, Kirchner T, Jung A (2012) Human telomerase reverse transcriptase (hTERT) is a target gene of β-catenin in human colorectal tumors. *Cell Cycle, 11(17)3331-8)*

Kriegl L, Horst D, **Reiche JA**, Engel J, Kirchner T, Jung A (2010) LEF-1 and TCF4 expression correlate inversely with survival in colorectal cancer. *J Transl Med, 8(123)*

Kriegl L, Jung A, Engel J, Jackstadt R, Gerbes AL, Gallmeier E, **Reiche JA**, Hermeking H, Rizzani A, Bruns CJ, Kolligs FT, Kirchner T, Göke B, De Toni EN (2010) Expression, cellular distribution, and prognostic relevance of TRAIL receptors in hepatocellular carcinomas. *Clin Cancer Res 16(22) 5529-38*

Wiebe JC, Schüller C, **Reiche JA**, Kramer K, Skerra A, Hock B (2010) An expression system for the E. coli fermentation of recombinant antibody Fab fragments from mice and rabbits. *J AOAC Int 93(1) 80-8*

7.7.2 Poster

Stintzing S, Jung A, Kapaun C, **Reiche JA**, Modest D, Giessen C, Vehling U, Stauch M, Hass H, Fischer von Weikersthal L, Kirchner T, Heinemann V; Ligand expression of amphiregulin and epiregulin and EGFR-FISH expression predict for treatment efficacy in KRAS wildtype metastatic colorectal cancer (mCRC) patients treated with cetuximab plus CAPIRI or CAPOX analysis of the randomized trial of the german AIO CRC study group: KRK-0104. *ASCO annual meeting, Chicago 2012*

Reiche JA, Schütz EM, Stintzing S, Heinemann V, Kirchner T, Jung A Polymorphisms in the EGFR gene are not associated with anti-EGFR induced skin rash by cetuximab in metastatic colorectal cancer. *95. Jahrestagung der Deutschen Gesellschaft für Pathologie e.V., Leipzig 2011*

Kapaun C, Stintzing S, **Reiche JA**, Jung A, Heinemann V; Expression of egfr ligands amphiregulin and epiregulin predicts outcome in metastatic colorectal cancer treated with cetuximab. *Neuntes Wissenschaftliches Symposium, Herrsching 2011*

Reiche JA, Schütz EM, Stintzing S, Heinemann V, Kirchner T, Jung A; Polymorphisms in the EGFR gene are not predictive for the effective treatment of metastatic colorectal cancer with the monoclonal antibody cetuximab. *<interact> München 2011*

Reiche JA, Scheel SK, Hiendlmeyer E, Herbst H, Kirchner T, Jung A; hTERT (human telomerase RT-component) expression is regulated by β-CATENIN in human colorectal cancer. *94. Jahrestagung der Deutschen Gesellschaft für Pathologie e.V., Berlin 2010*

7.8 Danksagung

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Eidesstattliche Versicherung

Leuchs, Jana

Ich erkläre hiermit an Eides statt,

dass ich die vorliegende Dissertation mit dem Thema

„Die Signalwegskomponenten LEF-1 und EGFR als Biomarker in der Karzinogenese des kolorektalen Karzinoms“

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

Ort, Datum

Unterschrift Doktorandin