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Chapter

Experimental Results Help Shape the Development of Personalized Medicine in Colorectal Cancer

Rania B. Georges, Hassan Adwan and Martin R. Berger

Abstract

With estimated 700,000 deaths each year, colorectal carcinoma (CRC) continues to be the fourth leading cause of cancer-related deaths worldwide. Fortunately, the mortality of CRC is considered to be most avertable; hence, it is essential to develop new approaches for more accurate and early diagnosis of primary as well as metastatic CRC, including genetic and biomarker tests. In this regard, the intercellular junctions and the insulin-like growth factor (IGF) axis attract increasing attention, since they are involved in several stages of cancer and for their vital role in regulating cell survival and growth; furthermore, constituents of intercellular junctions and of the IGF axis could be used as tumor and/or metastasis markers, which are becoming the focus of increasing research activities. Our experimental results highlight the importance of gene expression changes in the tight junction proteins claudins, and in the IGF-binding proteins IGFBP3 and IGFBP7. They show additionally that claudins and IGFBPs cannot be simply defined in terms of favoring or antagonizing cancer progression but have additional properties and activities, which become apparent only in the context of liver colonization. Furthermore, their intensive modulation during the initial phase of liver colonization may suggest them as early metastasis-related markers.

Keywords: colorectal cancer, liver metastasis, personalized medicine, influence on treatment, claudins, IGFBPs, tumor cell reisolation, metastasis marker

1. Introduction

With estimated 700,000 deaths each year, colorectal carcinoma (CRC) continues to be the fourth leading cause of cancer-related deaths in both sexes worldwide [1]. The 5-year relative survival rate for stage IV metastatic CRC is about 11%, while in stage I this number rises to nearly 90%. These figures reflect the fact that despite the high incidence and mortality rate of CRC, its mortality is among the most avertable ones. In addition, the fact that liver metastasis is the cause of most deaths from CRC [2], underlines the significance of (early) metastasis prevention. In other words, it is of great importance to develop new approaches for more accurate and early diagnosis of primary CRC on one hand and of its metastasis on the other; including screening programs as well as genetic, molecular and biomarker tests.

Colorectal cancer progression is driven by increasing or recurring growth of the primary carcinoma as well as hematogenic and lymphatic spread. For hematogenic spread, the liver is most important as it constitutes the first vascular bed in which disseminating CRC cells can be trapped after their dissemination. Hence, this organ is affected in up to 10–20% of CRC patients at the time of presentation. Another 20–25% will develop overt liver metastasis during the course of their illness [3, 4].

The main purpose of our experimental studies was first to develop a suitable model for investigating the efficacy of novel drugs [5–7]. One of the few well-characterized animal models for hepatic CRC benefits from the rat CC531 cell line. After injecting the cells, liver metastases develop and their growth has been frequently used for studying effects of various anti-cancer treatments [8–10].

The second aim was to identify genes, which are instrumental in the survival and metastasis formation of disseminated CRC cells. In addition, we reasoned that there are genes, which are necessary for the primary tumor as well as those, which are essential for metastasis initiation and formation. We furthermore hypothesized that the latter genes would be modulated in expression during the cells' colonization of the liver. Consequently, temporal changes in gene expression of CRC cells homing into the liver were investigated using an *in vivo* rat model, which is characterized by a definite metastatic proliferation-onset in rat liver after intra-portal inoculation of CC531 rat colorectal cancer cells. This model relies on the successful reisolation of CC531 cells at various time intervals after their injection into the mesenteric vein of syngeneic rats and allows exploring the chronological modulation of gene expression, from the very beginning of cancer cell homing into the liver to their final colonization of the whole organ. Based on this procedure, a cDNA microarray was performed to analyze gene expression profiles of several thousand genes in the reisolated CC531 cells. Upon analysis of microarray's data, candidates from gene families being significantly up- or down-regulated were chosen for further study by using different *in vitro* models. These candidate genes included claudins and insulin like-growth factor binding proteins. It was hoped that the emerging genes or their products would be useful as target of a specific therapy or as a biomarker.

The National Institutes of Health defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention [11]. From a therapeutic point of view, genome variations are recognized as the main cause of variable response to and side effects of drugs and a "one size fits all" approach is not the best solution any more. The individual's genetic and molecular makeup will be devoted to improve and develop more specific and "personal" diagnostic and therapeutic approaches.

Claudins (CLDNs) are tight junction (TJ) proteins that serve an intercellular adhesion function. The aberrant expression of individual claudins is well documented in different stages of various human cancers [12]. In addition, some claudins were proven to be useful as biomarkers [13, 14].

The insulin-like growth factor (IGF) axis attracts increasing attention since it is involved in several stages of cancer [15–17], and for its vital role in regulating cell survival and growth [18, 19] as well as the possible use of constituents of this axis as tumor and/or metastasis markers, which is becoming the focus of increasing research activities.

The insulin-like growth factors IGF-I and –II orchestrate their roles through the interaction with other members in this system, namely their receptors IGF-IR and -IIR, their binding proteins (IGFBPs) and the IGFBP proteases including matrix metalloproteinases (MMPs), cathepsins, and kallikreins [20]. Type I receptor mediates the growth promoting effects of IGFs [21], which are further modulated by 6 binding

proteins (IGFBPs 1–6) with high affinity for IGFs [22] as well as at least 4 IGFBPs with low affinity [23, 24], also known as IGFBP-related proteins (IGFBP-rp-1-4).

Based on the observation that the increased expression of IGFBPs attenuates the proliferative and anti-apoptotic effects of IGFs, they have been long considered as tumor suppressors, mostly due to their IGF-dependent roles. Interestingly, however, in addition to these IGF-dependent actions, IGFBPs were found to exert IGF-independent effects, as was reported for IGFBP1 [25], IGFBP3 [26–28], IGFBP5 [29] and IGFBP7 (or IGFBP-rp1) [30–32].

In this report, we have used the technique of cancer cell reisolation from rat liver, which permitted to monitor for the first time the expression profile of numerous candidate genes in a time-dependent manner. Based on these results we summarize our knowledge on claudins and IGFBP members and delineate their potential as tumor and/or metastasis markers.

2. Results

2.1 Modulation of selected genes in reisolated CC531 tumor cells

For identifying genes, which enable tumor cells to metastasize and colonize the liver, the CC531 cells were reisolated from rats, which had been implanted intraportally with these tumor cells. After various periods following tumor cell implantation, the CC531 cells were reisolated with a specific technique [33]. In subsequent experiments, these cells were used for mRNA and protein isolation and the mRNA screened by cDNA microarray and RT-PCR, and the proteins by Western blot.

As shown in **Table 1**, the microarray analysis revealed a significantly increased expression of insulin like growth factor binding proteins (*Igfbp3* and *Igfbp7)* and significantly decreased expression levels of claudins (*Cldn1* and *Cldn4*) in the beginning of liver colonization (days 3 and 6 after tumor implantation). These results were further confirmed by RT-PCR (for all four genes) and Western blot (for the two claudins and igfbp7) (**Figure 1**).

2.2 Effects of genes' knockdown in colorectal cancer cells

To investigate the knockdown effect(s) of each gene on various functions of colorectal cancer cell lines, siRNA experiments for transient knockdown were performed.

Gene	Time point of cell reisolation (days)[a]						
	3	6	9	14	21	14 (*in vitro*)	22 (*in vitro*)
Igfbp3	6.88[b]	13.62	6	18.03	17.29	1.56	*0.9*
Igfbp7	90.02	101.57	38.62	49.13	42.03	19.46	1.47
Cldn1	0.16	0.12	0.31	0.63	0.55	1.29	1.53
Cldn4	0.15	0.09	0.87	1.11	1.37	1.47	1.11

[a]*The day of tumor cell implantation was counted day 0.*
[b]*The number denotes the fold change in expression versus an in vitro control.*

Table 1.
Gene expression profiles from members of two gene families, chosen from the microarray analysis of reisolated CC531 cells.

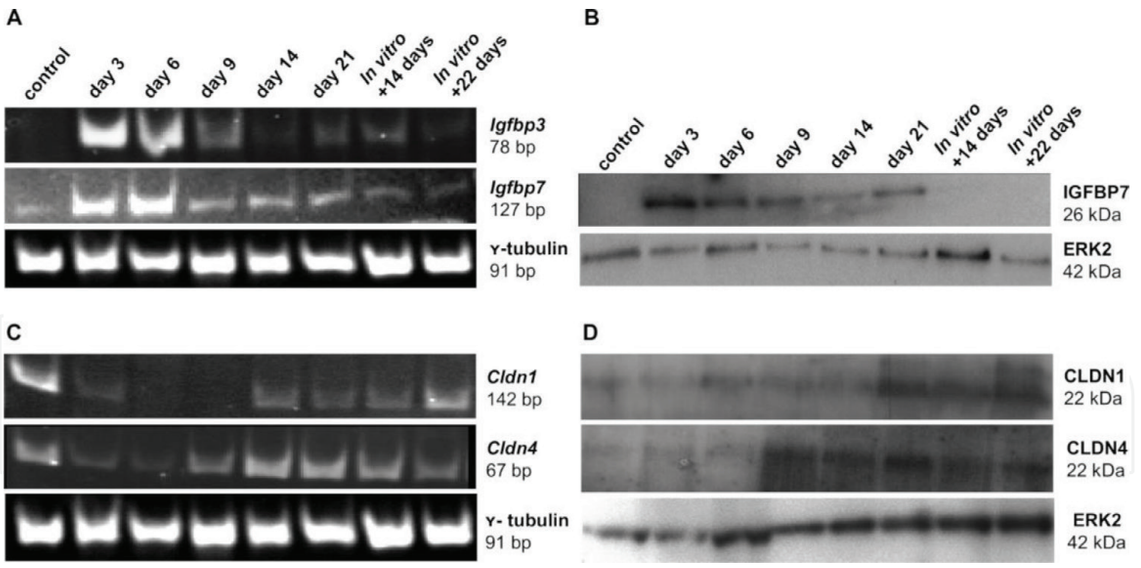

Figure 1.
Expression of Igfbp3, Igfbp7, cldn1, and cldn4 in reisolated CC531 cells. (A and C): Expression of Igfbp3 and Igfbp7 (A) and of Cldn1 and Cldn4 (C) in reisolated CC531 cells as shown by RT-PCR compared to the expression of the housekeeping gene γ-tubulin. (B and D): Expression of the proteins IGFBP7 (B), CLDN1 and CLDN4 (D) in the reisolated CC531 cells as shown by western blot compared to the expression of ERK2 loading control. 1st lane: CC531 cells (control); 2nd, 3rd, 4th, 5th and 6th lanes: CC531 cells (reisolated from rat livers after 3, 6, 9, 14 and 21 days, respectively); 7th and 8th lanes: CC531 cells (reisolated after 21 days and cultured in vitro for further 14 and 22 days, respectively).

Colorectal cancer cells (CC531, Caco2 or SW480) cultured in 6-well-plates were transfected with specific siRNA (200 nM) or negative control using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. The cells were harvested at 24, 48 and 72 h after treatment.

As shown for overview in **Table 2** (for details see **Figures 2–4**), knockdown of *igfbp3* or *igfbp7* was induced in cell lines with relevant expression only and caused significantly reduced proliferation rates (**Figure 2A–C**). Similarly, colony formation (**Figure 3A–C**) of CRC cells was diminished. Finally, cell migration was reduced in SW480 cells (**Figure 4B**), but not in CC531 (**Figure 4A**) and Caco2 (**Figure 4C**) cells.

Interestingly, different effects were noticed after *cldn1* or *cldn4* knockdown in CC531 cells. No significant effect on cell proliferation was observed, while a significant inhibition of colony formation and significant stimulation of cell migration resulted from the siRNA knockdown of each claudin (**Table 2**).

Target gene	Cell proliferation	Cell migration	Colony formation
Igfbp3 (in SW480)	↓*	↓*	↓*
Igfbp7 (in CC531 or Caco2)	↓*	ns	↓*
Cldn1 (in CC531)	ns	↑*	↓*
Cldn4 (in CC531)	ns	↑*	↓*

↓ and ↑* denote significant ($p < 0.05$) inhibition or stimulation of the investigated cell function (proliferation, migration or colony formation), respectively; ns denotes a nonsignificant effect.*

Table 2.
Overview of the siRNA knockdown effects of insulin like growth factor binding proteins 3 and 7 (Igfbp3 and 7) and claudins 1 and 4 (cldn1 and 4) on cellular functions (cell proliferation, migration and colony formation) of colorectal cancer cell lines (SW480, Caco2 and CC531).

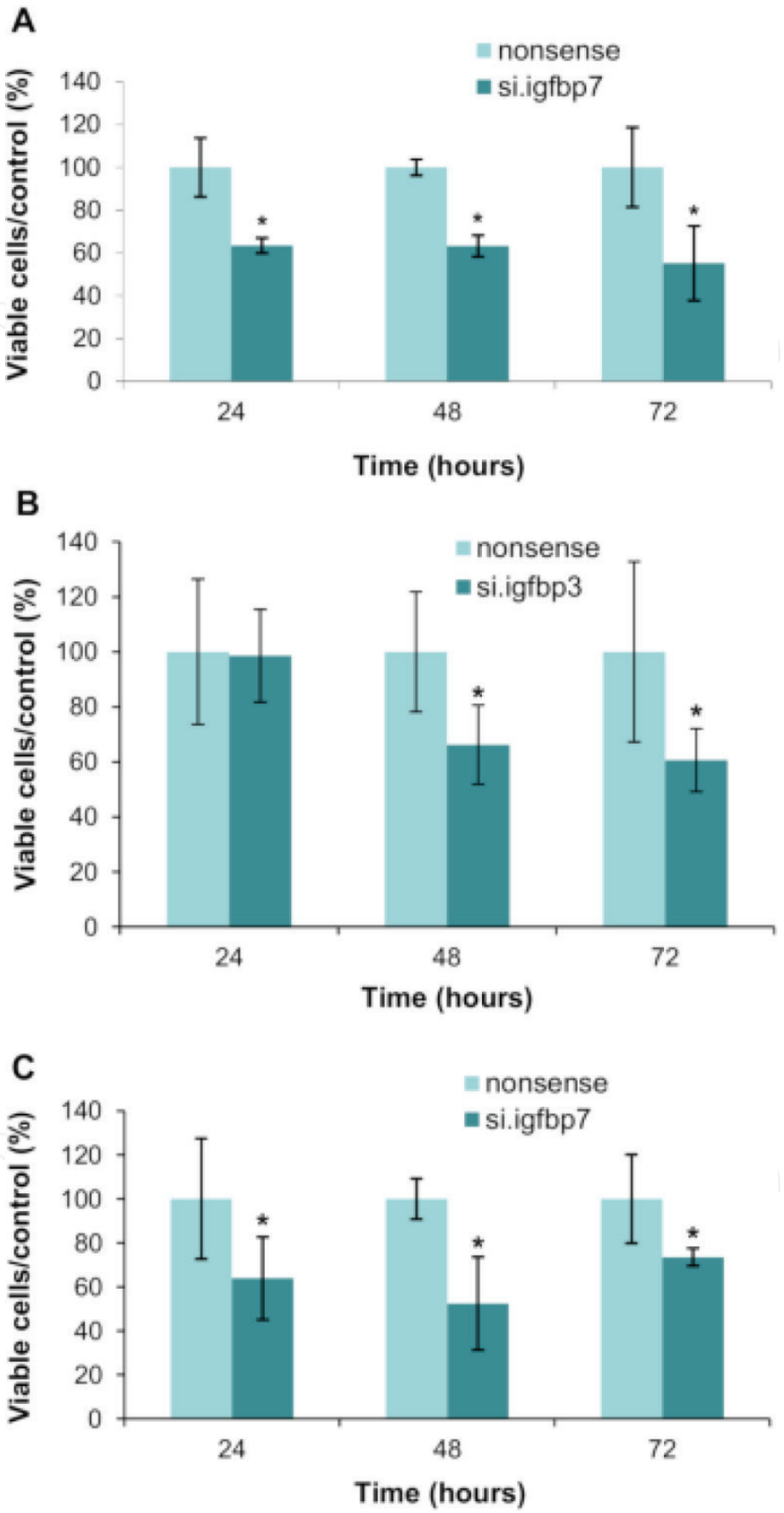

Figure 2.
Effects of Igfbp7 or Igfbp3 knockdown on proliferation of colorectal cancer cells. (A) Reduced proliferation of rat CC531 colorectal cancer cells after si.Igfbp7 treatment. (B) Reduced proliferation of human SW480 colorectal cancer cells after si.Igfbp3 treatment. (C) Reduced proliferation of human Caco2 colorectal cancer cells after si.Igfbp7 treatment. Data (n = 3) are shown as means ± S.D. in percentage of nonsense-treated cells. Asterisk (*) denotes a significant difference to controls ($p < 0.05$). Abbreviations: CF; colony formation.

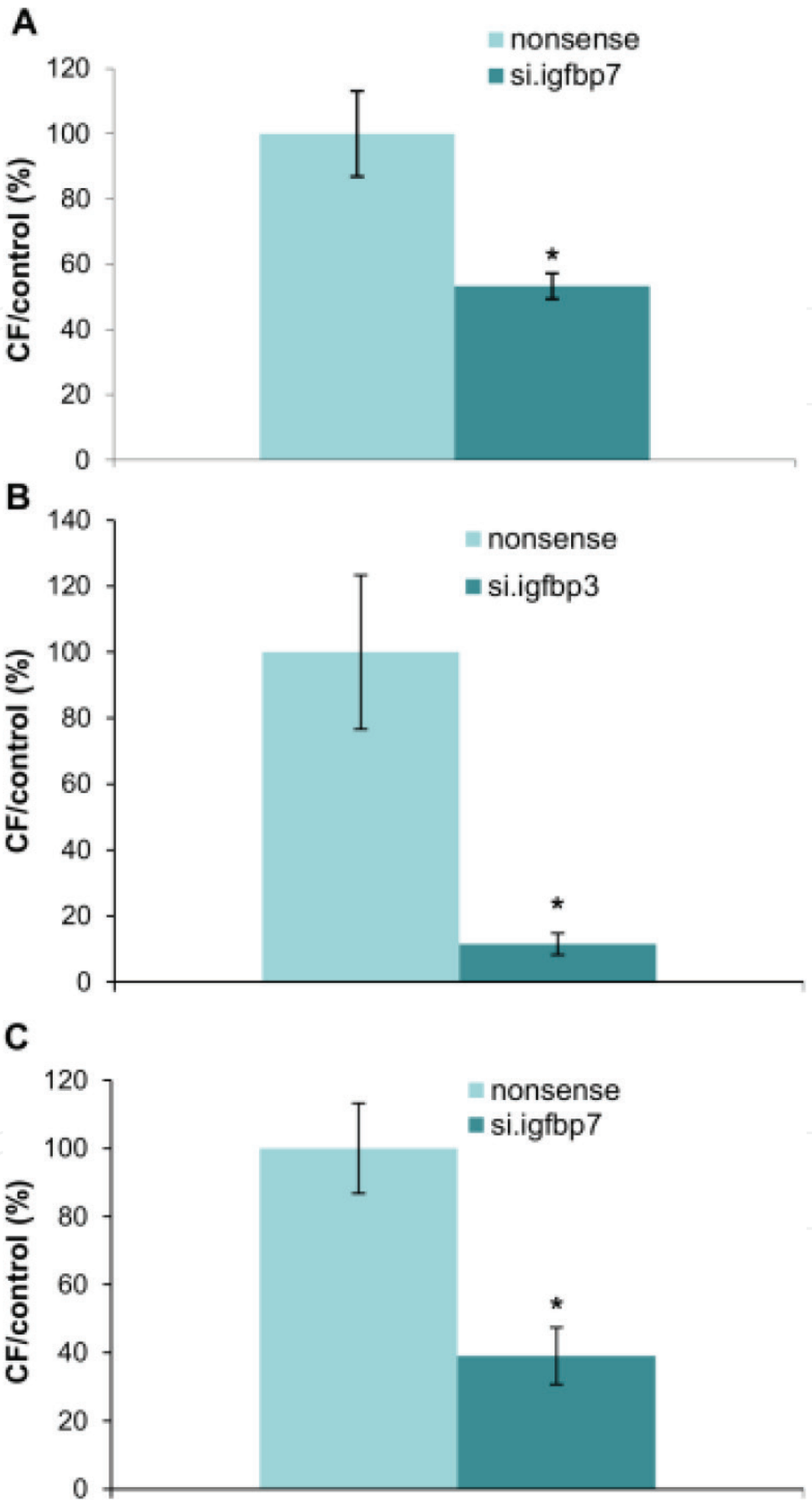

Figure 3.
Effects of Igfbp7 or Igfbp3 knockdown on colony formation of colorectal cancer cells. (A) Inhibited colony formation of rat CC531 colorectal cancer cells after si.Igfbp7 treatment. (B) Inhibited colony formation of human SW480 colorectal cancer cells after si.Igfbp3 treatment. (C) Inhibited colony formation of human Caco2 colorectal cancer cells after si.Igfbp7 treatment. Data (n = 3) are shown as means ± S.D. in percentage of nonsense-treated cells. Asterisk (*) denotes a significant difference to controls ($p < 0.05$). Abbreviations: CF; colony formation.

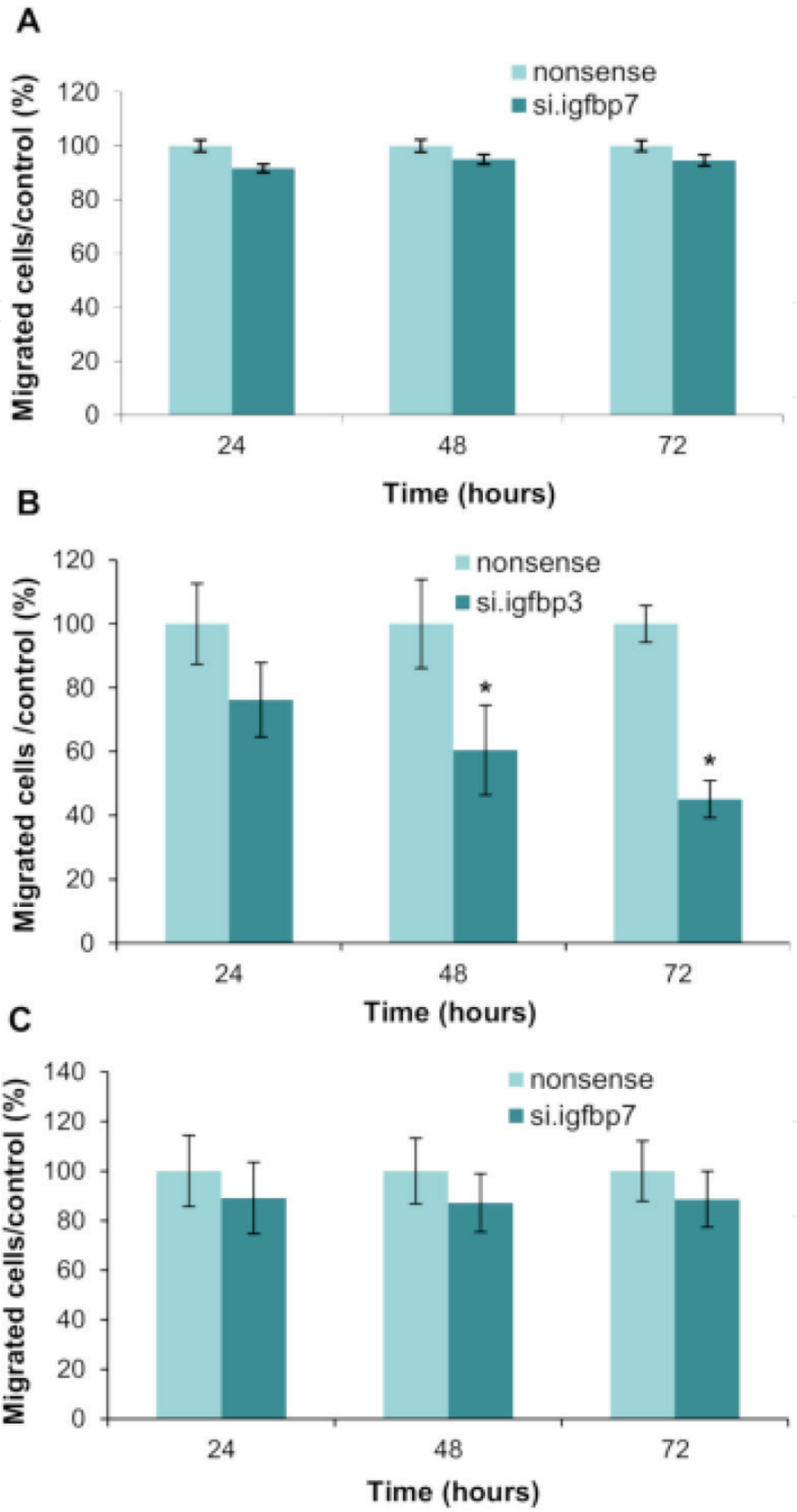

Figure 4.
Effects of Igfbp7 or Igfbp3 knockdown on migration of colorectal cancer cells. (A) Migration of rat CC531 colorectal cancer cells after si.Igfbp7 treatment. (B) Reduced migration of human SW480 colorectal cancer cells after si.Igfbp3 treatment. (C) Migration of human Caco2 colorectal cancer cells after si.Igfbp7 treatment. Data (n = 3) are shown as means ± S.D. in percentage of nonsense-treated cells. Asterisk (*) denotes a significant difference to controls ($p < 0.05$). Abbreviations: CF; colony formation.

3. Discussion

"If it were not for the great variability among individuals, medicine might as well be a science and not an art" with these words the famous Canadian physician Sir William Osler anticipated and acknowledged the concept of personalized medicine since the nineteenth century.

Personalized medicine aims to optimize and tailor preventive and therapeutic approaches in favor of the best outcome for each patient, by using genetics, proteomics, and biological information, including biomarkers [34]. It attempts to sub-categorize patients into different groups according to their "molecular make up", i.e. using biomarkers.

Here we have used a rat model of liver metastasis to identify genes with importance for organ colonization, which could be used as biomarkers or therapeutic targets.

The selected model for this experiment is characterized by a defined metastatic proliferation-onset in rat liver after intra-portal inoculation of CC531 cancer cells. Consequently, this allows exploring the chronological modulation of gene expression, from the very beginning of cancer cell homing into the liver to their final colonization of the whole organ. The technique of cancer cell reisolation from rat liver permitted, for the first time, monitoring the expression profile of numerous candidate genes from the whole genome in a time-dependent manner.

The initial observation of these studies was based on cDNA microarray analysis of CC531 colon cancer cells, which allowed selecting candidates from gene families with significant up- or down-regulation. These candidates were further analyzed by different *in vitro* studies.

We have been focusing on the detection and evaluation of biomarkers for the last decades [33, 35–43]. From the several gene groups, which were highly modulated in expression during liver colonization we focused on two families. Both were characterized by dramatic initial changes in expression, with claudins being down modulated and IGFBPs being up-regulated.

The first group (claudins; CLDNs) form the structural backbone of tight junctions (TJs), one type of cell-cell adhesion, and comprise at least 27 members of integral transmembrane proteins ranging in size from 20 to 34 kDa [12]. In recent years, the up- or down-modulated expression of several claudins has been associated with the progression of various cancers in humans, even in a tissue specific manner [12].

Interestingly, individual claudins are being used as therapeutic targets [44, 45] as well as diagnostic biomarkers [13, 14], making them a very interesting molecule to be investigated and characterized.

The second group of insulin like growth factor binding proteins (IGFBPs) differs in its importance from claudins, as they belong to the insulin-like growth factor (IGF) axis, which has a vital role in regulating cell survival and growth and is involved in several stages of cancer.

The expression of the two IGFBP genes, igfbp3 and igfbp7, was intensely upregulated at the beginning of liver colonization (days 3 and 6 after tumor implantation). Subsequently, however, this increased expression returned gradually to normal, hence we assume that the up-regulation of IGFBPs is essential for dissemination and homing of tumor cells into the liver during early metastasis formation. This strongly suggests that the tumor/metastasis microenvironment has a crucial impact on the regulation of igfbp3 and igfbp7. Furthermore, these results along with previous studies [28, 46–48] show that the balanced expression of IGFBP3 and IGFBP7 is very essential for several cellular functions as both, up- and down-regulation of these two genes were related to malignant properties. This further suggests that the aberrant expression of these two genes can be an early indicator of CRC progression.

Further verification of their value arises from studies that demonstrate involvement of the IGF axis in several stages of cancer and for its vital role in regulating cell and tissue survival, growth and differentiation [18, 19]. In addition, the possible use of constituents of this axis as tumor and/or metastasis markers is becoming the focus of increasing research activities [49–51]. Most *in vitro* studies, reported a tumor suppressor function of IGFBP3 and IGFBP7 through IGF-dependent and/or independent mechanisms [27, 31, 52, 53]. At variance to these studies, our experiments on silencing *IGFBP3* and *IGFBP7* in three CRC cell lines uniformly show reduced proliferation, colony formation, and for *IGFBP3*, also reduced migration. Our observations are in agreement with few reports, which related IGFBP3 and IGFBP7 to growth promoting functions [28, 54]. Accordingly, these and our results support the idea that IGFBP3 and IGFBP7 are multi-functional.

IGFBP3 is well known in the literature: It is the predominant IGFBP in plasma, hence plays a crucial role in regulating the bioavailability of plasma IGFs, and it is expressed locally in most tissues including the intestine [55]. Additionally, IGFBP3 induces apoptosis and inhibits proliferation in human colon [56], prostate [57], breast [58], and lung cancer cells [26] *in vitro* and in experimental CRC animal models [59]. Furthermore, wild type p53 can induce IGFBP3 expression [60], thus enhancing the p53-dependent apoptotic response of CRC cells to DNA damage [56]. Reduced levels of IGFBP3 and elevated circulating levels of IGF-I were associated with increased risk of prostate [61], breast [62], and colorectal [63, 64] cancers. Nevertheless, this association was not confirmed in all conducted studies [65–67]. It was observed that TGF-β can induce IGFBP3 and mediates its proliferative response in aggressive CRC cells, which exempts the studies reporting a tumor suppressor function of IGFBP3 [28].

In contrast to IGFBP3, the regulation and functions of IGFBP7 are less investigated. This gene was originally cloned as a gene, which is down-regulated in meningioma cell lines [68]. IGFBP7 is usually expressed by colonic mucosa [65], however both, up- and down-regulation patterns were recorded in the context of cancer [46, 47]. Lately, it was shown that IGFBP7 is a direct p53 target and the DNA methylation mediated-epigenetic silencing of IGFBP7 was associated with the absence of p53 mutations in CRC [30]. Until now, *in vitro* experiments demonstrated a negative effect of IGFBP7 on the growth of various cancer cells, including cervical carcinoma (HeLa) [69], osteosarcoma (Saos-2) [69], and breast [70]. Furthermore, in human CRC cell lines, expression of IGFBP7 was detectable in Caco2 and SW480 cells only, whereas its expression in invading tumor cells associated with poor prognosis in CRC patients [71]. In addition, immunohistochemistry and RT-PCR showed IGFBP7 over-expression in CRC tissues as compared to the respective normal tissues [36].

As also known from the literature, several members of the IGF axis were found to be prognostic markers for various tumor types, including IGFBP5 and IGFBP7 for nonsmall cell lung cancer (NSCLC) [72], IGFBP2 and IGFBP3 as compensatory biomarkers for CA19-9 in early-stage pancreatic cancer [51], IGF-1 for metastatic uveal melanoma [49] and IGF-IR for glioblastoma [50]. In addition, a recent study showed that certain single nucleotide polymorphisms (SNPs) in IGF1R and IGF2R were associated (positively or inversely, respectively) with adenomas in Caucasian, but not in African American CRC patients [73]. Similarly, specific SNPs in the IGF-l gene were suggested as risk assessment markers of gastrointestinal cancers [74]. All these studies emphasize the crucial role of the patient's genetic background in tailoring the therapeutic approach to fit the "size" of this particular patient.

With regard to using the above family members as prognostic marker, the past experience should be considered. Contrasting with our expectations and reports from the literature, no significant correlation was found between the increased expression levels of four known tumor progression-associated genes (Opn, Tgf-β,

Mmp-2 and Cox-2) and the prognostic value of these genes in CRC patients [43]. This raises an important to answer question, i.e. what would be the best procedure to apply personalized medicine effectively and reliably? Could it be a minimum number of (bio)-markers for each cancer type to be tested, or should it be a complex approach as high throughput genome sequencing, as it is increasingly performed?

Ideally, a few markers would be better suited regarding costs of analysis and time until a patient can benefit from the results. However, there are currently only few markers which succeeded to be applied accordingly. Therefore, even more methods are being approached to assess the specific changes inherent to the full genome.

4. Conclusion and perspectives

Here we show a new high throughput approach in exploring genes relevant to CRC progression in terms of liver metastasis. Our method has yielded initial results related to the importance of claudins and IGFBP in liver colonization. Nevertheless, we reason that other genes, which result from this model, might be even more valuable. For instance, one of the very important and interesting gene families that resulted from this model, which is extensively investigated, is the endothelin system with all its components (endothelins, their converting enzymes and their receptors). Several members of this system could prove useful as tumor/metastasis markers. Future experiments will show whether such a functional model can compete or complement, at least partially, other techniques, including whole genome sequencing.

Conflict of interest

The authors confirm that there are no conflicts of interest.

Author details

Rania B. Georges[1*], Hassan Adwan[2] and Martin R. Berger[1]

1 German Cancer Research Center (DKFZ), Heidelberg, Germany

2 German University of Cairo (GUC), Cairo, Egypt

*Address all correspondence to: r.georges@dkfz-heidelberg.de

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