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STAT-3 activity in chemically-induced hepatocellular carcinoma

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Abstract

The signal transducer and activator of transcription (STAT)-3 regulates basic biological processes and it has been reported to be constitutively active in different types of malignant tumours. STAT-3 is active during the regenerative growth of the liver, but there are hardly any data about its presence in liver tumours. We investigated and found a high activity of STAT-3 using an electrophoretic mobility shift assay (EMSA) in chemically-induced rat hepatocellular carcinomas (HCCs). Dexamethasone treatment downregulated both STAT-3 activity and cell proliferation in the tumours. Therefore, the activity of the STAT-3 signal transduction pathway seems to be required for the growth of HCCs and could be a potential new target for therapeutic trials of this tumour type.

Keywords: STAT-3; Liver tumour; Dexamethasone; c-myc

1. Introduction

Hepatocellular carcinoma (HCC) is among the most common tumours worldwide and there is no effective treatment for this disease [1]. New potential targets are needed for future therapeutical approaches.

The investigation of interferon-induced transcription led to the discovery of the signal transducer and activator of transcription (STAT) family of transcriptional factors [2]. The STATs constitute a family of signal transduction proteins that are activated in the cytoplasm by the binding of extracellular polypeptides to transmembrane receptors. Activation of STATs results in the expression of genes that control critical cellular functions such as cell proliferation, survival, differentiation and development [3]. In addition to physiological functions, increasing evidence is supporting an important role for STATs in tumorigenesis, especially for STAT-3 and -5 [5–9]. Active STAT-3 has been reported in different forms of human and experimental tumours [6–8] but, as far as we know, it has not been studied in HCCs.

This is surprising, since rapid activation of the STAT-3 transcriptional complex has been reported in the regenerating liver following partial hepatectomy [10]. No STAT-3 activity could be detected in interleukin-6 (IL-6) [11] and tumour necrosis factor receptor type I (TNFR-I)-deficient mice [12] following a partial hepatectomy. The livers of these animals also failed to regenerate. Yamada and colleagues [12] proposed—based on this information—that the TNF–nuclear factor κB (NFκB)–IL-6–STAT-3 pathway is an important regulator of hepatocyte proliferation. We have also observed that this mechanism was equally important in driving the proliferation of the so-called oval cells in the rat liver (data not shown).

As a next step, we decided to study the activity of STAT-3 in HCCs.

2. Materials and methods

HCCs were induced by the Solt Farber protocol [13]. In brief, tumours were initiated in 160–180-g male F-344 rats by a single dose (200 mg/kg) diethylnitrosamine

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(DEN). Following a 2-week recovery period, 10 mg/kg of 2-acetylaminofluorene (AAF) was administered to the rats daily by gavage for 2 weeks. Partial hepatectomy (Ph) was performed in the middle of this time period. The rats were sacrificed 10 months after the initiation. Half of the rats were injected with 2 mg/kg dexamethasone intraperitoneally (i.p.) 24 h before sacrifice. Several well-formed tumours were found in each of the livers. The tumours were separated from the surrounding liver. Tissue samples were taken for histological analysis, the rest was immediately processed for nuclear protein isolation or frozen for RNA preparation. Each animal was numbered: 1–5 untreated rats, 6–10 dexamethasone-pretreated ones. The samples (lanes), both from the tumours and the surrounding tissues, on Figs. 1–4 were marked by these numbers. Those samples are shown only on the figures from which enough nuclear extract/RNA could be isolated. All the experiments were done at least in duplicate. Histologically, the tumours proved to be full-blown HCCs with a trabecular or pseudoglandular pattern.

Animal study protocols were conducted according to National Institute of Heath (NIH) guidelines for animal care.

2.1. Nuclear extracts and electrophoretic mobility shift assays (EMSA)

Nuclear proteins were extracted based on the sucrose gradient method [14], with slight modifications. Every procedure described below was performed at 4 °C. Five hundred milligrams of fresh liver tissue was homogenised in 5 ml homogenate buffer (10 mmol/l HEPES pH 7.5, 25 mmol/l (KCl), 1 mmol/l ethylene diamine tetraacetic acid (EDTA), 2 mmol/l sucrose, 10% glycerol, 0.5 mmol/l spermidine, 0.15 mmol/l spermine, 0.1% Nonidet (N)P-40, 1 mmol/l phenylmethyl sulphonyl fluoride (PMSF), 2 μg/ml aprotinin, 2 μg/ml leupeptin). The homogenised sample was filtered through gauze sponges and layered over 1 ml of NP-40-deficient homogenate buffer. After ultracentrifugation at 110 000*g* for 1 h in a SW40 Ti rotor, the pellet

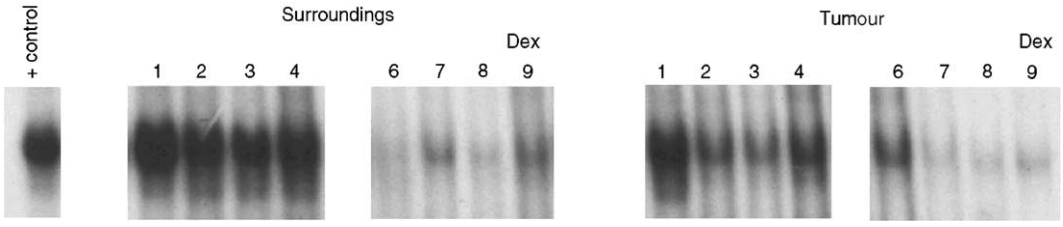

Fig. 1. Electrophoretic mobility shift assay (EMSA) analysis of signal transducer and activator of transcription (STAT-3) activity in liver tumours and in the surrounding liver tissue. Dex marked panel shows samples from the livers of dexamethasone-pretreated rats. Each lane represents individual animals, the numbers above the lanes mark the identity number of the experimental rats.

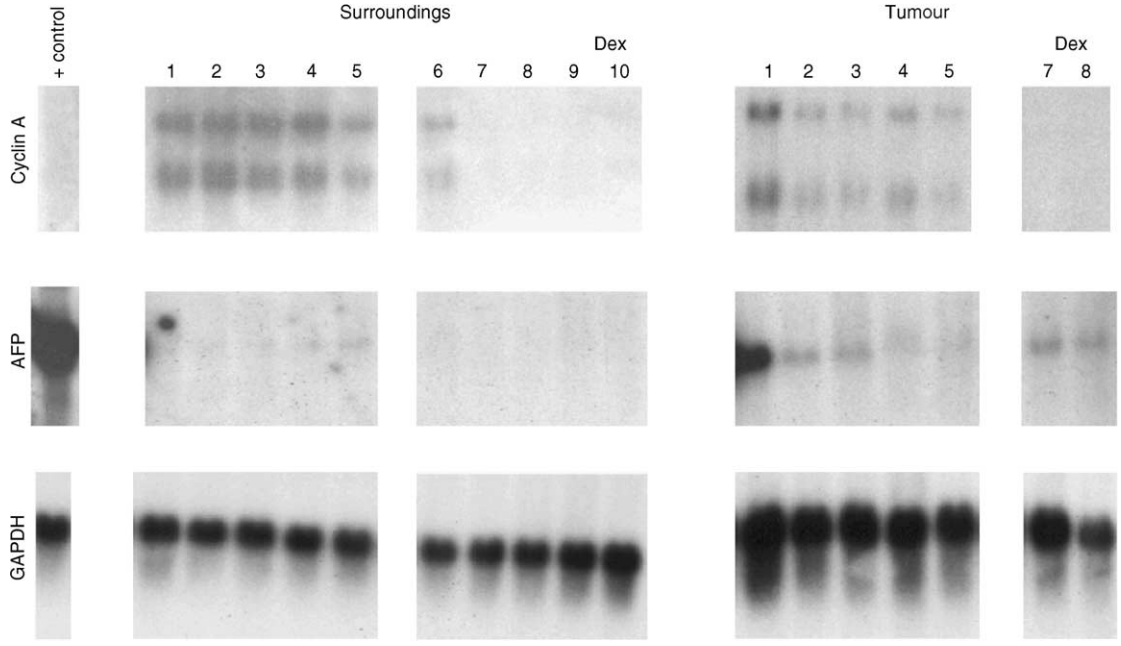

Fig. 2. Northern analysis of mRNA expression of *Cyclin A*, alpha-fetoprotein (*AFP*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in the tumour and surrounding liver samples.

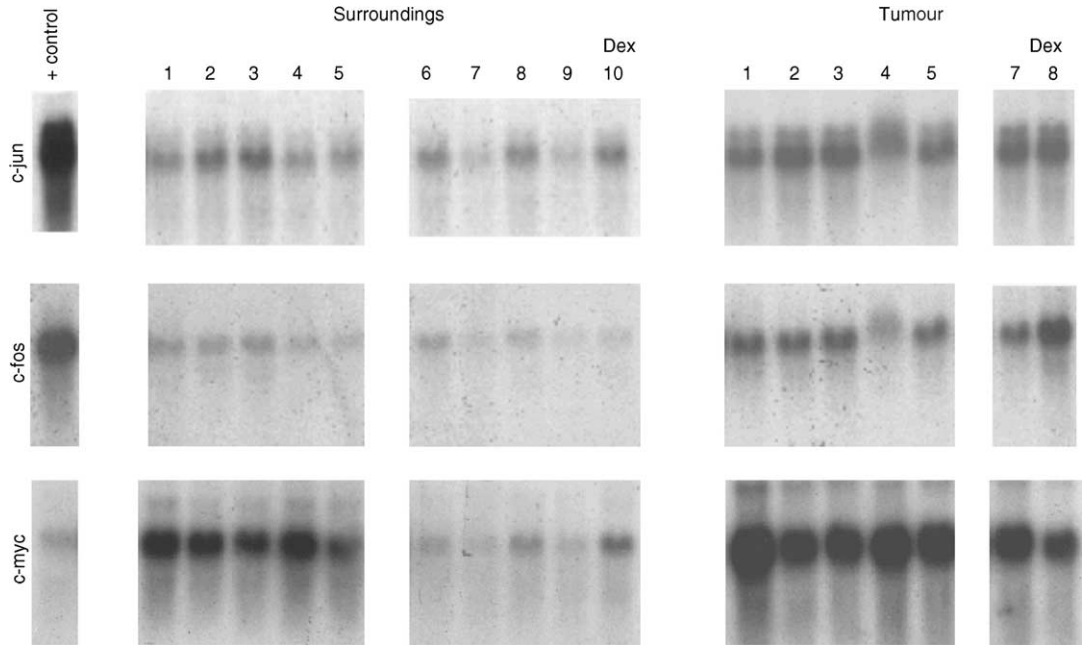

Fig. 3. Northern analysis of mRNA expression of *c-jun*, *c-fos* and *c-myc* in the tumour and surrounding liver samples.

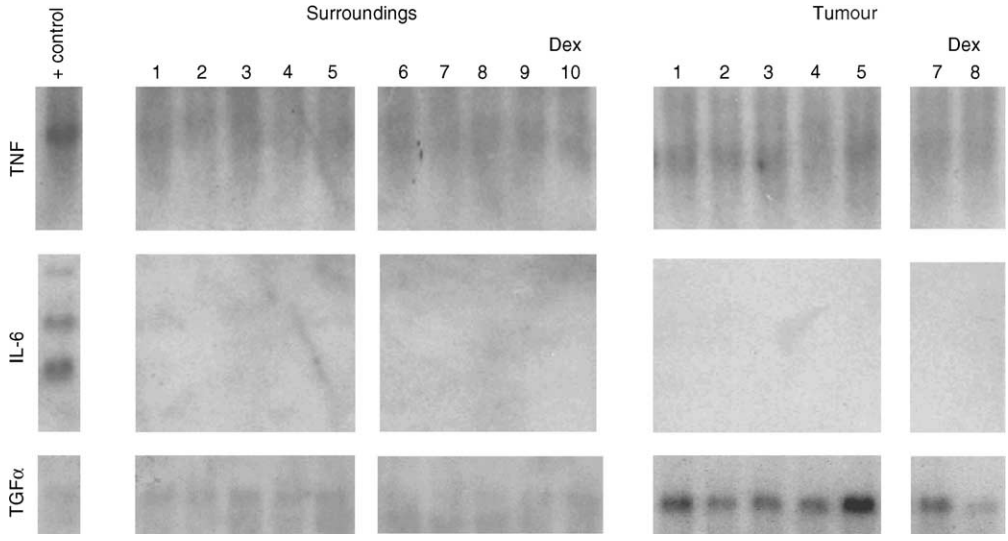

Fig. 4. Northern analysis of the mRNA expression of tumour necrosis factor (TNF), interleukin-6 (IL-6) and transforming growth factor α (TGFα) in the tumour and surrounding liver samples.

was washed with 200 μl nuclei storage buffer (50 mmol/l Tris–HCl pH 8.3, 5 mmol/l $MgCl_2$, 0.1 mmol/l EDTA, 40% glycerol, 1 mmol/l dithiothreitol (DTT), 0.2 mmol/l PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin) and collected by centrifugation at 1300*g* for 5 min. Nuclear proteins were extracted from the pellet by dissolving in 100 μl of extraction buffer (10 mmol/l HEPES pH:8.0, 0.42 mmol/l NaCl, 0.1 mmol/l EDTA, 0.1 mmol/l ethylene glycol-bis tetraacetic acid (EGTA), 1.5 mmol/l $MgCl_2$, 25% glycerol, 1 mmol/l DTT, 1 mmol/l PMSF, 2 μg/ml aprotinin and 2 μg/ml leupeptin) with a vigorous shake for 30 min. After centrifugation at 160 000*g* for 30 min, the supernatant was collected. The protein concentration was measured using a BCA protein assay (Pierce, Rockford, IL, USA).

Nuclear protein extract (5 μg) was incubated with P-32 labelled consensus STAT-3 binding oligonucleotide sequence (SantaCruz, Santa Cruz, CA, USA) in the presence of poly (dI-dC). DNA binding complexes were resolved by gel electrophoresis on 5% polyacrylamide/1x TBE. The gels were dried and exposed to Kodak X-AR film at −80 °C for approximately 12 h. In cases of competition, the nuclear extract was pre-incubated with cold consensus or mutated oligonucleotides.

2.2. RNA isolation and northern blot hybridization

RNA was extracted from the tissue with guanidine thiocyanate, followed by centrifugation in caesium chloride solution. Poly (A) RNA was selected by oligo(dT)-cellulose chromatography. Five micrograms of poly(A)RNA per lane was electrophoresed on 1% agarose gels containing 2.2 mmol/l formaldehyde and later transferred to nylon membranes (Nytran plus, Schleicher & Schuell, Keene, NH, USA). After ultraviolet crosslinking, the filters were hybridised with P-32 labelled cDNA probes. The following cDNA fragments were used for labelling: *c-myc*:mouse 1.4 kb *XhoI*; *c-fos*:rat 2.1 kb *Eco RI*, transforming growth factor α (*TGFα*): rat 0.31 kb *Eco RI*; *cyclin A*: mouse 0.8 kB *Eco RI*; *TNF*: mouse 2.1 kb *Eco R I.*, *IL-6*: rat 0.9 kb *BamHI-PstI*. The 651 bp cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was generated by reverse transcription and subsequent amplification by the polymerase chain reaction (PCR) using the following primers: 5′-ACCACAG-TCCATGCCATCAC-3′ and 5′-TCCACCACCCTGT-TGCTGTA-3′.

3. Results

3.1. STAT-3 activity is present in HCCs and downregulated by dexamethasone

Nuclear protein was isolated from the liver tumours and from the surrounding liver tissue. STAT-3 activity could be detected in each of the samples by EMSA. The bands ran in parallel with our positive control samples. They could be competed out with a cold consensus sequence, but not with mutated oligonucleotides (data not shown). Although there was variation among the individual animals, the tendency was clear that dexamethasone pretreatment decreased the intensity of the bands both in the tumour and surrounding liver samples (Fig. 1). Interestingly, the STAT-3 activity was higher in the surrounding tissue, than in the tumours. However, one should not forget that this surrounding tissue was not 'normal'. It was fibrotic and there was permanent liver damage in this tissue with constant regeneration due to the growing tumours. The cell proliferative activity of the surrounding liver was proven by bromodeoxyuridine (BrdU) incorporation (data not shown) and by Northern hybridisation (Fig. 2). The cyclin A level was more constant and slightly higher in the surrounding liver tissue, than in the tumour samples in the untreated animals (numbers 1–5). It is known that the normal proliferating liver has active STAT-3 [10]. The correlation between cyclin A expression and STAT-3 activity was most striking in the tumour samples (numbers 1–4).

3.2. Northern analysis

Fig. 2 shows the result of the hybridisation with the *GAPDH*, alpha-fetoprotein (*AFP*) and cyclin A probes. GAPDH demonstrates the approximately equal loading of the lanes. The 2.3 kB *AFP* mRNA is not expressed in the normal liver tissue, but is expressed in the oval cells and tumours. The presence of this band in the tumour and the lack of expression in the surrounding samples proves the correct separation of the tumour tissue. Cyclin A is an established marker of cell proliferation. Our results demonstrate that dexamethasone pretreatment sharply reduced the expression of this proliferation marker, both in the tumour and in the surrounding liver tissue. BrdU incorporation detected by immunohistochemistry showed the same results (data not shown).

Potential target genes of STAT-3 were probed and the results are shown in Fig. 3. All of them were expressed at slightly higher levels in the tumour samples. Dexamethasone pretreatment did not influence the steady-state level of *c-fos* and *c-jun* mRNAs. Perhaps even more surprisingly, *c-myc* expression was reduced in the surrounding tissue following dexamethasone treatment, while it was practically unaffected in the tumour samples.

Fig. 4 shows the steady-state messenger RNA level of genes that might be responsible for STAT-3 activation. *TNF* and *IL-6* could not be detected in any of our samples, although the bands were clearly seen in the positive control lanes. However, *TGFα* MRNA was barely detectable in the surrounding tissue but its level was higher in the tumour samples. Dexamethasone pretreatment did not influence *TGFα* expression.

4. Discussion

We could demonstrate an active STAT-3 complex in *in vivo* HCC samples. This activity and the cell proliferative activity were downregulated by dexamethasone. *c-jun*, *c-fos* and *c-myc*, potential target genes of STAT-3, did not follow this pattern. *TNF* and *IL-6* mRNA could not be detected in the tumours, while *TGFα* was expressed.

STATs are a relatively recently discovered family of transcriptional factors responsible for regulating basic biological processes, e.g. cell proliferation, survival and differentiation [3,15]. Therefore, it is not surprising that the deregulation of several members of this family has been reported in a wide variety of tumours [4]. Furthermore, inhibition of STAT-3 has also been reported to reduce the growth of some tumours [6] and STAT-3 has been suggested to be a target for new chemotherapeutic approaches [9]. Yoshikawa and colleagues [16] observed that SOCS-1, a negative regulator of the jak/

STAT pathway, is silenced by methylation in human HCCs. Moreover, the restoration of SOCS-1 suppressed both the growth rate and reversed constitutive STAT-3 activation in HCC cell lines. It has been recently discovered that the HBx protein of the Hepatitis B virus [17] and the core protein of the Hepatitis C virus [18] may activate STAT-3 and this has further increased the interest in this signal transduction pathway in HCC.

As far as we know, we are the first to report STAT-3 activity in *in vivo* HCC samples. The chemical carcinogens used in the recent experiments (DEN, AAF) are hepatocarcinogens in several different species [19]. DEN proved to be the most potent liver tumour-inducing chemical in non human-primates [20]; therefore, we can presume that our results have relevance for human tumours. Pretreatment of the tumour-bearing animals with dexamethasone reduced both the STAT-3 activity and cell proliferation. Although this observation does not prove the causal relationship between HCC growth and STAT-3 activity, data from the literature suggest this connection is highly probable. It would be worthwhile to challenge the tumours with more specific inhibitors of STAT-3 to see if they can influence the growth of HCCs.

The steady-state level of three potential target genes of STAT-3: *c-jun*, *c-fos* and *c-myc* was higher in the untreated tumour samples than in the corresponding surrounding tissues (Fig. 3). Dexamethasone also did not significantly reduce the *c-jun* and *c-fos* signal, either in the tumour or in the surrounding tissue. These trends contradict the STAT-3 data in Fig. 1. These observations indicate that although STAT-3 may contribute to the regulation of these genes, it is definitely not the only factor determining their expression level. The behaviour of c-myc in our study was the most interesting. The upregulation of *c-myc* in HCC has been known for decades [21,22], but its function is still uncertain. The *c-myc* level was downregulated in the non-tumorous tissue by dexamethasone, as was cell proliferation. Dexamethasone was equally efficient in decreasing cell proliferation and STAT-3 activity in the tumours, but failed to reduce the *c-myc* level. This observation serves as a further proof for the deregulation of *c-myc* in HCC and supports the notion that c-myc is not functionally equivalent with cell proliferation.

Before STAT-3 can be selected as a potential target for therapeutic intervention, it is important to determine the mechanism of its regulation. TNF and IL-6 are responsible for its activation during liver regeneration and in oval cells. Dexamethasone inhibits the growth and STAT-3 activity in these models through its anti-inflammatory effect, reducing the synthesis of IL-6 and TNF [23] and directly inhibiting NFκB [24]. This does not seem to be the mechanism used here, since neither *TNF* nor *IL-6* mRNA could be detected in our samples. The expression of these cytokines is usually not very high, but they could be easily detected in our positive controls (oval cell-containing rat livers). Thus, even if they are expressed, their expression is significantly weaker than in the above-mentioned models. Increased TNF and/or IL-6 levels are relatively common in the sera of tumour patients, but the source of these cytokines is usually the tumour or the tumour-surrounding inflammatory reaction. However, these tissues did not express a detectable level of these cytokines in our experimental model. There are a wide variety of other cytokines which may participate in the regulation of STAT-3. Increased TGFα expression in HCC has been reported a long time ago [25]. A causal role for TGFα in liver tumorigenesis has been proven by the high rate of liver tumours in transgenic mice that overexpress TGFα [26]. TGFα is also among those cytokines that may be responsible for activating STAT-3 [27]. In fact, selective inhibition of TGFα activity in a head and neck squamous cell carcinoma model effectively reduced STAT-3 activity and cell proliferation [7]. TGFα was also expressed in our HCC samples, but its level did not decrease in the dexamethasone-pretreated samples. Thus, if TGFα is responsible (at least partly) for the STAT-3 activity in HCC, then dexamethasone influences this pathway somewhere downstream from the TGFα production. TGFα executes its function by binding to the epidermal growth factor receptor (EGFR). EGFR is a classic thyrosine kinase receptor activating the mitogen-activated protein kinase (MAPK) pathway [28]. It has been recently reported that EGFR upregulates NFκB activity via the action of the Akt kinase [29] and this mechanism seemed to be an important growth regulator of HCC. If our hypothesis is correct then TGFα would increase HCC proliferation by at least three different pathways (Fig. 5): MAPK, NFκB and STAT-3. In this scenario, EGFR plays a central role in

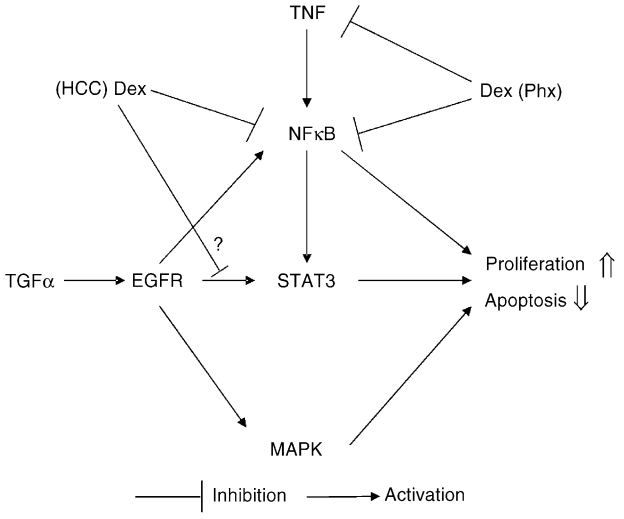

Fig. 5. Schematic representation of the proposed mechanism of dexamethasone's inhibition in case of the liver tumours (hepatocellular carcinomas, HCC) and regeneration (Phx).

the growth regulation of HCC and recently discovered specific inhibitors [30] of this receptor should be tried in the treatment protocols of HCC patients.

In conclusion, we found an increased *TGFα* expression and STAT-3 activity in chemically-induced HCCs. If further experiments support the role of the TGFα–STAT-3 pathway in the growth of liver tumours, then both EGFR and STAT-3 could be potential targets for future chemotherapeutic trials.

Acknowledgements

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