# Acquired genetic and functional alterations associated with transforming growth factor β type I resistance in Hep3B human hepatocellular carcinoma cell line

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## Abstract

During the neoplastic process tumour cells frequently acquire resistance to the antiproliferative signals of transforming growth factor β (TGF-β). Here we examined a human hepatocellular carcinoma cell line (Hep3B-TS) sensitive to TGF-β signalling, and a derivative line (Hep3B-TR) rendered resistant to TGF-β by stepwise exposure to TGF-$\beta_1$. Comprehensive molecular cytogenetic analysis revealed that the acquisition of TGF-β- resistance by Hep3B-TR cells was due to loss of TGF-β receptor 2 (TGFβRII) gene. As demonstrated by spectral karyotyping and array-based comparative genomic hybridization, and in difference to Hep3B-TS cells, which have three rearranged and two normal copies of chromosome 3 that harbour the TGFβRII gene, Hep3B-TR cells have four rearranged and one apparently normal chromosome 3, which nonetheless underwent a critical microdeletion at the site of TGFβRII gene. Gene expression analysis using an oligonucleotide microarray of 21,397 genes showed that Hep3B-TR differentially expressed 307 genes, out of which 197 and 110 were up- and down-regulated, respectively, compared to Hep3B-TS. Six of differentially expressed genes were identified as downstream targets of the tumour necrosis factor (TNF) gene, suggesting that loss of TGFβRII triggered activation of the TNF pathway known to be regulated by TGF-$\beta_1$ network. On the functional level, the TGF-β-resistant Hep3B-TR cells displayed significantly enhanced capacity for anchorage independent growth and cell migration *in vitro*, and also increased tumorigenicity *in vivo* and *in vitro* and *in vivo* tumorigenicity compared with parental sensitive cells.

**Keywords:** HCC • TGF-β • TGFβRII • Hep3B • resistance • expression profiling • FISH • SKY karyotype • aCGH profile • microdeletion • chromosomal rearrangements • migration • invasion • tumorigenicity

## Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, accounting for 90% of all liver carcinomas. HCC has a high mortality rate, as many tumours are asymptomatic until the later stages [1] Like many other forms of cancer, HCC overexpress transforming growth factor-$\beta_1$ (TGF-$\beta_1$) [2]. TGF-$\beta_1$ is a member of a family of cytokines that controls several aspects of cell behaviour such as proliferation, migration, adhesion, differentiation and immune function. In early stages of tumour development TGF-β has tumour suppressive activity, while during advanced stages it promotes growth, invasiveness and dissemination of tumour cells [3–6]. Based on the dual effects of TGF-β on oncogenesis, a recent study from our laboratory demonstrated that gene expression signature discriminates early and late effects of TGF-β in HCC, thus establishing a reliable molecular classification of tumours as a new basis for targeted therapy [7]. TGF-β elicits its effect through two transmembrane serine/threonine kinase receptors, TGFβRI and TGFβRII. After binding TGF-β, the TGFβRII phosphorylates the TGFβRI receptor, which then phosphorylates the intracellular Smad proteins that serve as effectors

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for signal transduction [6, 8–10]. During the neoplastic process many tumour cells acquire resistance to the antiproliferative signals of TGF-β [4]. The absence of TGF-β inhibitory effect on cell growth correlates with loss of TGFβRI and/or TGFβRII [11].

Hep3B-TS, a well-characterized HCC cell line, is sensitive to the inhibitory effect of TGF-β on cell proliferation. In an elegant experiment [11] stepwise treatments of this cell line with increasing concentrations of TGF-β1 resulted in loss of the TGFβRII gene and acquired resistance to the inhibitory action of TGF-β on cell growth. The lost of sensitivity to TGF-β antiproliferative signalling in this derivative cell line (Hep3B-TR) occurred in the absence of any treatments with clastogenic/mutagenic agents commonly used to generate TGF-β-resistant cells, or to induce point mutation in the *TGFβ* protein [11]. Since the mechanism responsible for TGFβRII loss and the biological consequences of the acquisition of TGF-β resistance by Hep3B-TR remained unknown, we decided to undertake a genetic and functional characterization of Hep3B-TS and Hep3B-TR isogenic cell lines. Molecular cytogenetic analysis of these lines revealed significant karyotypic changes associated with the acquisition of TGF-β resistance, and provided evidence for a new mechanism responsible for the biallelic deletions of the TGFβRII through a translocation and interstitial microdeletion of chromosome 3p. Our study also shows that the loss of the TGFβRII gene in Hep3B-TR cells triggered the activation of tumour necrosis factor (TNF) pathway and resulted in promotion of cell migration *in vitro*, and increase in tumorigenicity *in vivo*.

# Materials and methods

## Cell culture

Hep3B-TS and Hep3B-TR cell lines were grown in DMEM/F12 medium supplemented with glutamine (2 mM) and 10% foetal bovine serum. Both cell lines were negative for mycoplasma.

## Conventional fluorescence *in situ* hybridization

Cells were treated with colcemid for 4 hrs and chromosome preparations were made according to standard protocols. For fluorescent *in situ* hybridization (FISH), chromosomes spreads were hybridized with a biotin labelled-TGFβRII genomic probe as previously described [12]. Detection of the hybridization signal, digital image acquisition, and analysis were carried out as previously described [13].

## Spectral karyotyping

Chromosome hybridization and analysis for spectral karyotyping (SKY) were conducted according to a standard protocol with minor modifications [14]. Acquisition of interferograms and subsequent SKY analysis were performed with Spectral Imaging2.6 and SKY View™2.1.1 software (Applied Spectral Imaging, Inc., Vista, CA, USA), respectively, using SpectraCube™SD200 (Applied Spectral Imaging, Inc.), a Zeiss Axioscope II microscope (Zeiss Inc., Oberkochen, Germany) on a Windows XP Professional Workstation (Dell Computer, Inc., Round Rock, TX, USA).

## Microarray-based comparative genomic hybridization

For microarray-based comparative genomic hybridization (aCGH), human genomic DNA was isolated with a QIAamp DNA Mini Kit according to manufacturer protocol (Qiagen, Valencia, CA, USA). Test and reference (Promega, Madison, WI, USA) DNAs were labelled with Cy3 or Cy5 fluorescent dyes (Pharmacia, Piscataway, NJ, USA) according to BioPrime Array CGH Genomic Labeling protocol (Invitrogen, Carlsbad, CA, USA) and cleaned using Microcon YM-30 filters (Millipore, Billerica, MA, USA). Hybridization was carried out human genome CGH microarray 44K slides from Agilent Technologies (Santa Clara, CA, USA) according to CGH procedures for Genomic DNA Analysis (Agilent Technologies). Slides were hybridized for 20 hrs, washed, scanned with an Agilent microarray scanner and data were analysed using Feature Extraction® and CGH Analytics® software packages (Agilent Technologies). To ensure the test's reliability, dye-reversal experiments with reciprocal labelling of the test and reference DNA, were performed for each experiment.

## Northern blot analysis

The expression TGFβRII mRNA was examined by Northern blot hybridization as previously described [15].

## Microarray-based expression analysis

The Human Array-Ready Oligo Set™ (Version 2.0) containing 70-mer probes of 21,329 genes was obtained from Qiagen, Inc. (Valencia, CA, USA) and oligonucleotide microarrays were produced at the Advanced Technology Center of the National Cancer Institute. To minimize the contribution of cell density to differential gene expression, total RNAs were isolated with Trisol (Gibco-BRL, Rockville, MD, USA) from about 80% confluent Hep3B-TS and Hep3B-TR cells. Total RNAs from 19 normal livers were pooled and used as the reference. As previously described [16, 17] total RNAs were used to derive fluorescently (Cy5 or Cy3) labelled complementary DNAs (cDNA). Two hybridizations were carried out for each cell line using dye-swap strategy to eliminate labelling bias of the fluorescent intensity measurement. Hybridized arrays were scanned at 10 μm resolution on a GenePix 4000A scanner (Axon Instrument, Union City, CA, USA) at variable PMT voltage to obtain maximal signal intensities with less than 1% probe saturation. Resulting images were analysed in GenePix Pro v3.0 (Axon Instrument) as described in the manufacturer's manual. Signal intensities between the two fluorescent images were normalized by applying median Cy3/Cy5 ratio of all well-measured spots. Gene expression ratios were transformed to log2 and gene features that had missing values in any of four experiments were removed from further analysis. Only genes with an expression ratio that had at least more than twofold difference between Hep3B-TS and Hep3B-TR were selected for analysis. PathwayAssist (v2.5, Ariadne Genomics, Rockville, MD, USA) was used to find common regulators of gene expression in cell lines.

### Cell proliferation assay

Hep3B-TR and Hep3B-TS cells were cultured in 6-well plates and at different time intervals, from 1 to 10 days after seeding, cell were trypsinized, stained with trypan blue and counted.

### Wound-healing migration assay

Hep3B-TS and Hep3B-TR cells were seeded in 6 well plates and allowed to grow to 90% confluence. Subsequently, a plastic pipette tip was used to scratch the cell monolayer to create a cleared area, and the wounded cell layer was washed with fresh medium to remove loose cells. The 'wounded' areas were marked for orientation and photographed by phase contrast microscopy before and after 24 and 48 hrs of incubation.

### *In vivo* tumorigenicity assay

Hep3B-TS and Hep3B-TR cells were harvested and stained with trypan blue to assess the cell viability. Viable cells ($2 \times 10^6$) were injected subcutaneously at the proximal dorsal midline of 6-week-old male athymic nu/nu mice (Harlan). The size of the resulting tumours was measured in two dimensions twice a week for up to 6 weeks and volume of tumours was calculated with the formula ($L \times W^2$) $\times$ 0.5, where L is length and W is width. Histological preparations from tumours that developed in nude mice were examined for their morphology.

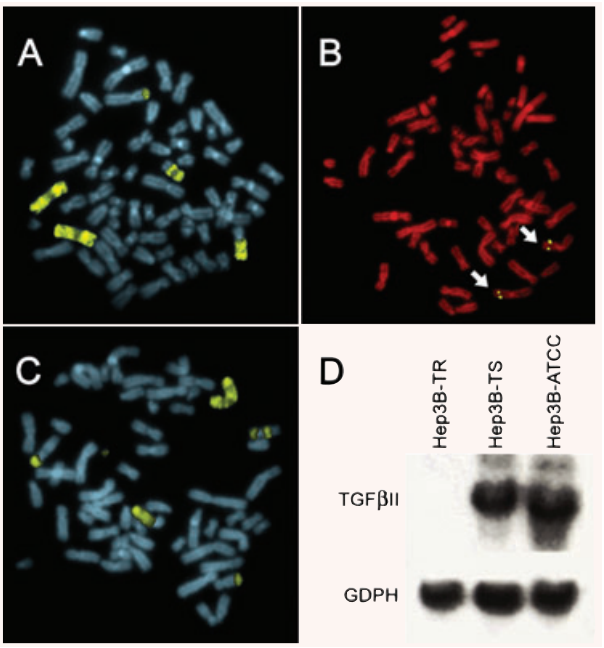


**Fig. 1** FISH and Northern blot analysis of Hep3B-TS and Hep3B-TR cells. Status of chromosome 3 in Hep3B-TS (**A**) and Hep3B-TR (**C**) was examined by FISH with whole chromosome 3 painting probe. Whereas Hep3B-TS cells exhibit two sets of TGFβRII signals (**B**) Hep3B-TR cells show no hybridization signals and no TGFβRII RNA (**D**).

## Results

### Molecular cytogenetic analysis uncovers a novel mechanism for loss of TGFβRII gene

Using conventional FISH, SKY and aCGH techniques we analysed and compared the genetic make up of the a well established Hep3B-TS cell line that has been extensively used in a variety of genetic and functional studies of HCC, and its TGF-β-resistant derivative, Hep3B-TR. Hep3B-TS FISH analysis with gene-specific probe targeting TGFβRII locus at the short arm of chromosome 3, and with chromosome 3 painting probe revealed a significant difference between these two cells lines.

While Hep3B-TS exhibited two normal chromosomes 3 (Fig. 1A) which carried two sets of fluorescent signals indicative of two TGFβRII alleles (Fig. 1B), Hep3B-TR cells showed only one apparently normal chromosome 3 (Fig. 1C), but neither fluorescent signals in FISH with TGFβRII probe, nor the presence of TGFβRII RNA in Northern-blot analysis were detected (Fig. 1D). Like in other HCC cell lines, the SKY analysis of Hep3B-TS cell line (Fig. 2A) showed abnormal karyotype nearly identical to that reported by others [18], with a near triploid chromosome number and multiple complex structural and numerical alterations that left just a few chromosomes with normal appearance. Yet, a comparison of our chromosomal CGH analysis of Hep3B-TS cells from 1999 (12) with microarray-based CGH data obtained in this study – after several extended culturing episodes in the meantime – points to a marked genomic stability of this cell line.

Although TGF-β-resistant Hep3B-TR cells (Fig. 2C) retained most of the structural and numerical abnormalities seen in Hep3B-TS cells, several new and complex alterations, such as translocations t(2; X) and t(2; 18), occurred as a result of exposure to TGF-β during selection of TGF-β-resistant cells. We found a remarkable concordance between SKY karyotypes of Hep3B-TS and Hep3B-TR cells (Fig. 2A, B) and their respective aCGH profiles of genomic imbalances (Fig. 2B, D), thus underscoring the power of combined approaches for a more accurate detection of genomic reorganizations in cancer cells. This combined analysis confirmed new rearrangements involving chromosome 3 (Fig. 3A) suggested by earlier chromosome 3 painting, and revealed that the only one apparently normal chromosome 3 in Hep3B-TR cells, has, in fact, undergone a unique microdeletion, not present in the Hep3B-TS cells (Fig. 3B), which affected a region spanning from 26.6MB to 31.2MB and effectively erased the only remaining copy of the TGFβRII gene (Fig. 3C).

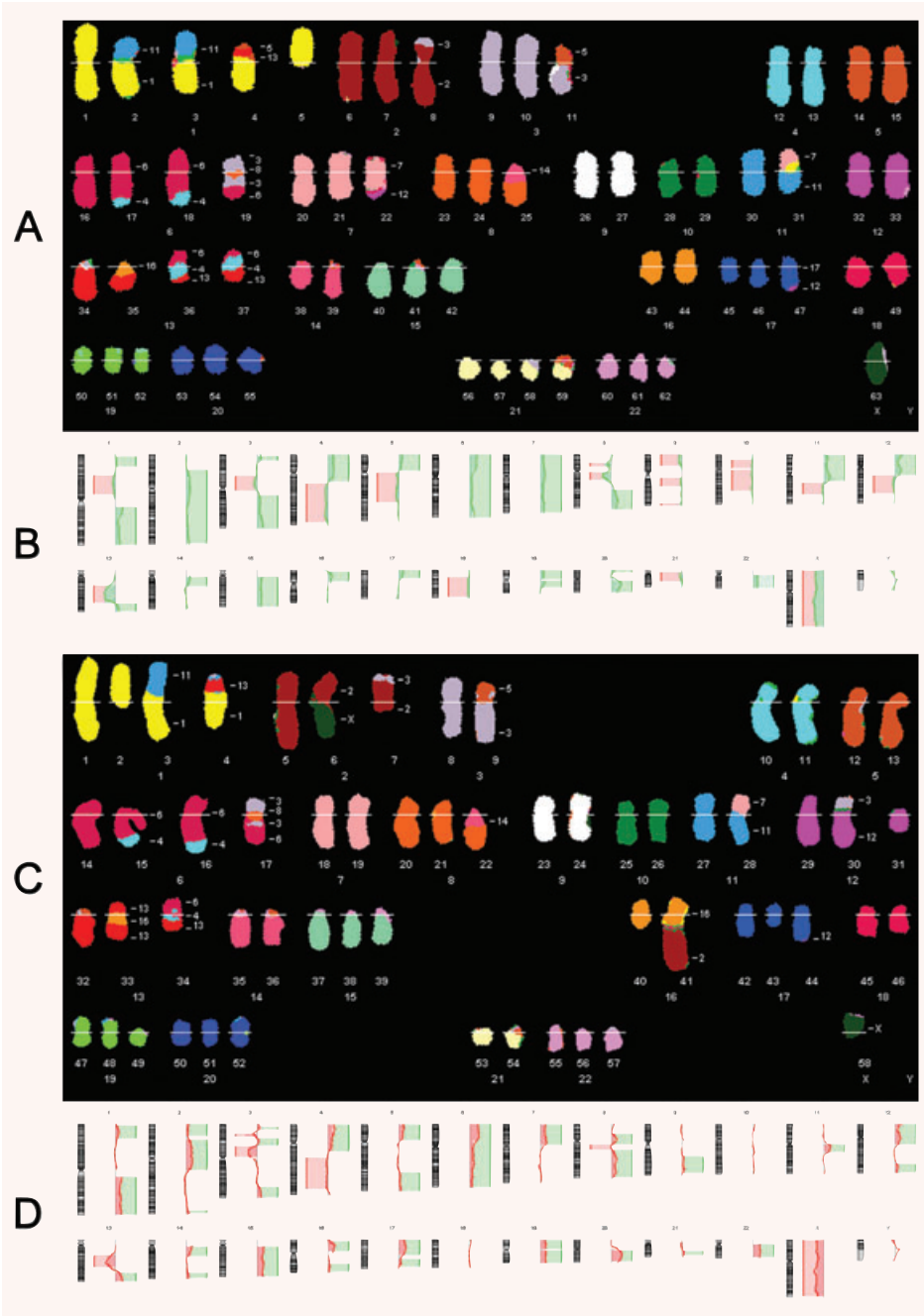


**Fig. 2** SKY and aCGH analyses of Hep3B-TS (**A**, **B**) and Hep3B-TR (**C**, **D**) reveal heavily rearranged near triploid karyotypes that, in spite of their apparent similarities, present significant differences in both recurrent specific chromosomal rearrangements and in DNA copygains and losses (highlighted in green and red, respectively).

## Expression profiling of Hep3B-TS and Hep3B-TR; TGF-β resistance activates TNF pathway

Using an oligonucleotide microarray of 21,397 gene targets we compared the gene expression profiles of Hep3B-TS and Hep3B-TR to identify differentially expressed genes. This analysis showed that Hep3B-TR, compared to Hep3B-TS, differentially expressed 307 genes, out of which 197 were up-regulated whereas the expression of other 110 was down-regulated. Using PathwayAssist™ we identified six of differentially expressed genes (Fig. 4) as downstream targets of the TNF gene.

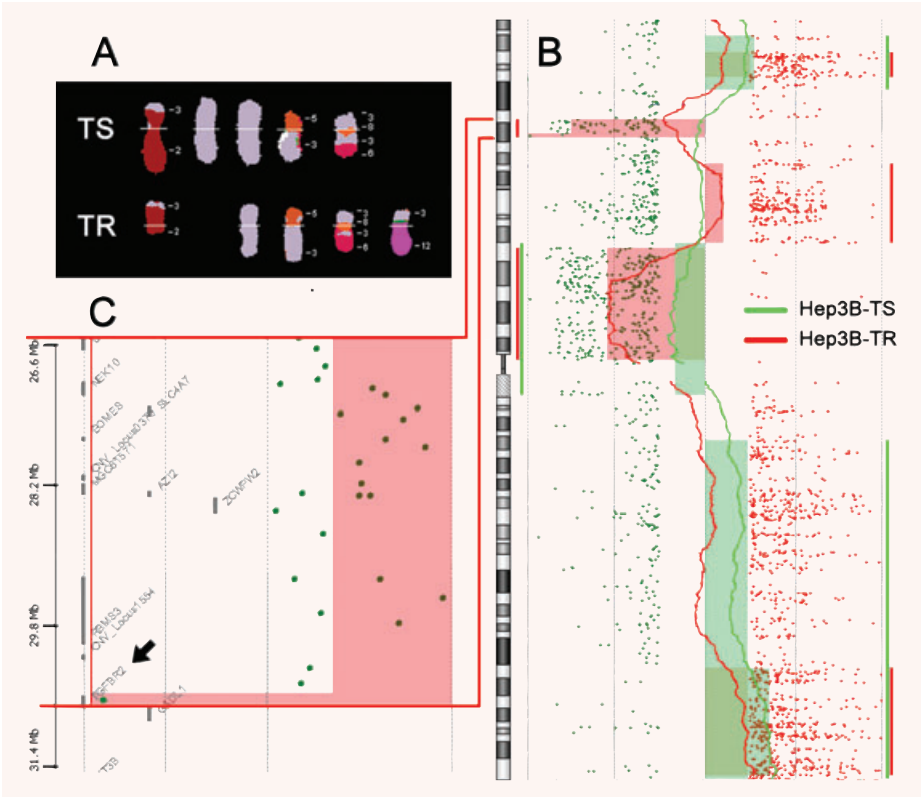


**Fig. 3** SKY analysis reveals differences in reorganizations involving chromosome 3 between Hep3B-TS and Hep3B-TR cell lines (**A**). A microdeletion of short arm of chromosome 3, detected by aCGH (**B**) occurs only in Hep3B-TR and involves DNA segment harbouring TGFβRII gene (**C**).

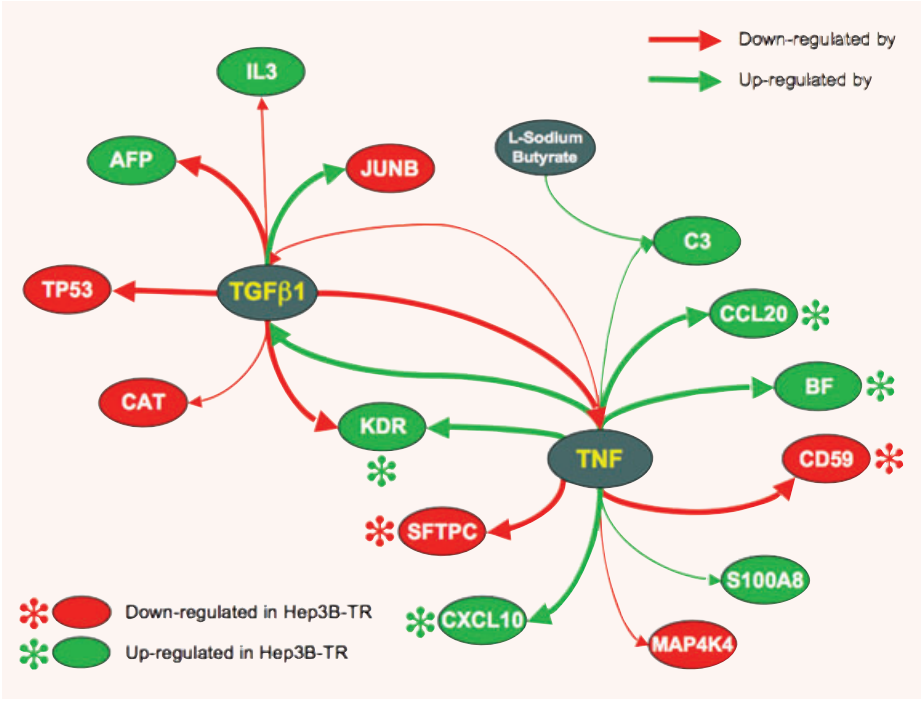


**Fig. 4** Array-based expression profiling reveals differences in expression patterns of Hep3B-TS and Hep3B-TR cell lines. Among all differentially expressed genes, six (labelled with asterisk) are positively or negatively regulated by tumour necrosis factor.

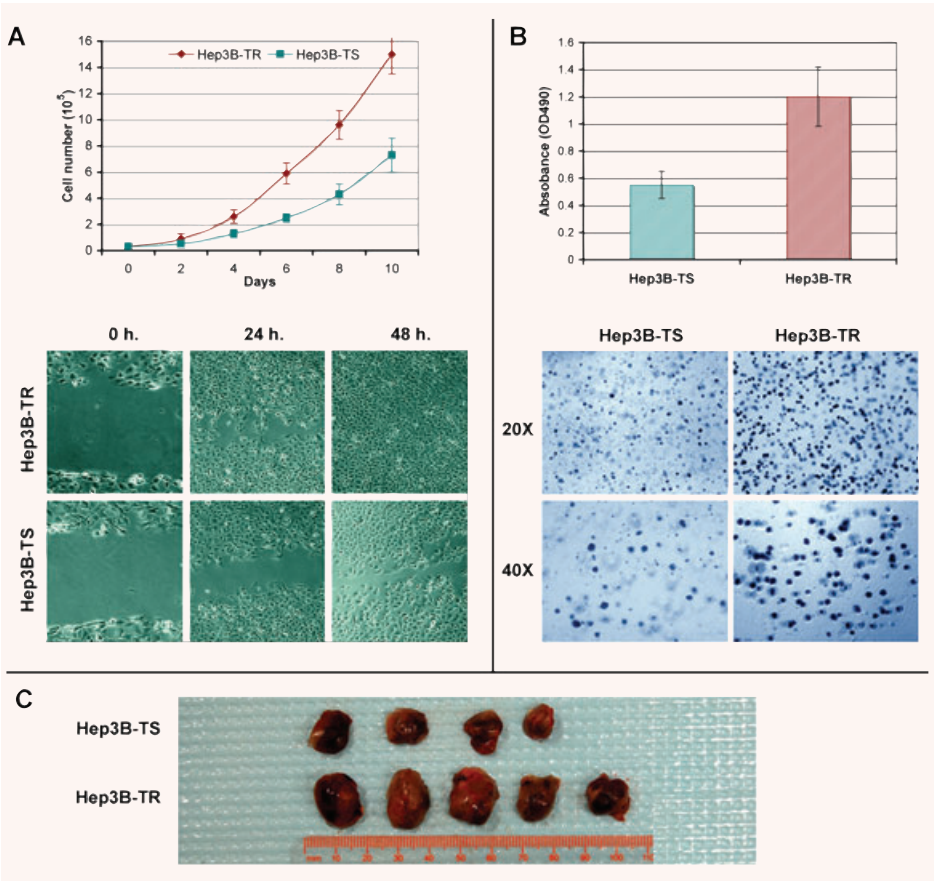


**Fig. 5** Hep3B-TS and Hep3B-TR cells proliferate with different dynamics and show different capacity to migrate (**A**). They also differ in propensity for colony formation (**B**) and tumorigenicity *in vivo* (**C**), since Hep3B-TR cells induce greater number and bigger size of tumours in nude mice.

## Cell proliferation, migration, anchorage independent growth *in vitro*, and tumorigenicity *in vivo* associated with loss of TGFβRII and TGF-β-resistance

To see whether loss of TGFβRII influenced tumour cell behaviour, we compared the *in vitro* cell proliferation, migration and ability to form colonies in soft agar and *in vivo* tumorigenicity of two cell lines. We found that Hep3B-TS grew considerably slower than Hep3B-TR cells over a period of 10 days in culture, and that the migratory behaviour of the cells was also different (Fig. 5A) since quantitative analysis revealed a significant difference in the extent and the speed of wound closure between the two cell lines. While Hep3B-TR cells closed 80% of the wound gap within 24 hrs and 100% after 48 hrs, Hep3B-TS cells failed to close the monolayer even after 48 hrs. We also examined growth of both cell lines in semisolid medium. Quantification of anchorage-independent cell growth on the basis of the number and size of colonies produced in soft agar showed that Hep3B-TR cells formed 50% more colonies of larger size than Hep3B-TS cells (Fig. 5B) Since anchorage-independent growth is often associated with the cell's ability to induce tumours *in vivo*, we tested the tumorigenicity of both cell lines in nude mice. Whereas all 10 mice injected with Hep3B-TR cells developed tumours in 21 days, inoculation with Hep3B-TS cells resulted in tumour formation in eight mice while the remaining two mice remained free of tumours throughout 5 weeks of observation (Table 1). The size of tumours that developed after inoculation with Hep3B-TS was more than a half the size of tumours formed in mice injected with Hep3B-TR (Fig. 5C).

**Table 1** *In vivo* tumorigenicity of Hep 3BTR and Hep 3BTS cells

| Cell line | Latency (days) | Tumour size (mm) | No. of tumours/no. of mice |
|---|---|---|---|
| Hep 3BTR | 21 | 15 × 18 | 10/10 |
| Hep 3BTS | 21 | 7 × 8 | 8/10 |

Nude mice were injected subcutaneously with $2 \times 10^6$ viable Hep 3BTR and Hep 3BTS cells. The size of the tumours (width × length) and the number of tumours observed *versus* the number of mice inoculated, 35 days after cell injection were determined.

## Discussion

Lack of TGFβRII mRNA expression in Hep3B-TR cells, as demonstrated by Northern blot analysis, and consistent with the previous results [11] is caused by interstitial microdeletion of chromosome 3p at the region spanning from 26.6MB to 31.2MB. Compelling evidence has been provided for the ablation of TGFβRII function by mutations in colon, gastric, biliary, pulmonary, oesophageal and head and neck carcinoma [reviewed in 6]. In HCC, however, the mechanisms responsible for down-regulation and/or inactivation of TGFβRII are not known [reviewed in 2]. With this respect, genomic reorganization involving the locus of TGFβRII at chromosome 3p identified in our study represents a novel mechanism that may be more common in HCC.

Of all differentially expressed genes six were identified as downstream targets of the TNF gene. TNF was demonstrated to induce the expression of KDR (kinase insert domain receptor) [19], CCL20 (chemokine ligand 200 [20], CXCL10 (chemokine ligand 10) [21] and BF (B-factor, properdin) [22]. On the other side, TNF was shown to suppress the expression of SFTPC (surfactant, pulmonary-associated protein C) [23] and CD59 (complement regulatory protein) [24]. Differential expression of these genes in Hep3B-TR cells, compared to Hep3B-TS cells, points to the possibility that loss of TGFβRII may have affected some components of the TNF pathway believed to be regulated by TGF-$\beta_1$ pathway [25, 26]. The nature and extent of this putative connection remains to be further investigated and clarified.

The TGFβRII gene has tumour suppressor activity and its loss in cancer cells could lead to the loss of TGF-β response resulting in enhanced tumour growth and invasiveness. Loss of functional TGFβRII in non-malignant intestinal polyps, mammary tumours or premalignant lesions initiated by oncogenes, leads to development of carcinoma [reviewed in 3, 6]. Current results with liver carcinoma cells add to the existing evidence on the role of loss of the tumour suppressive component within the TGF-β pathway. Clearly, loss of the TGFβRII gene in Hep3B-TR HCC cells was associated with significant increase in cell proliferation, migration and enhanced anchorage independent growth *in vitro*, and in increased *in vivo* tumorigenicity. Consistent with our observations are the results generated in two independent studies using *in vitro* assays for migration and invasion. In these studies it has been found that Hep3B-TS cells are either non-invasive or exhibits low invasiveness capacity [7, 27].

Despite the complexity of TGF-β signalling in oncogenesis, many components of the TGF-β pathway have been characterized, thus allowing the identification of markers for diagnosis and prognosis of certain cancers as well as for providing molecular targets for some subset of cancers [3, 6]. Given our limited knowledge of the factors that mediated the switch between the tumour promoter and suppressor functions of TGF-β, it currently appears that immune cells from the tumour host may hold the best prospects for a novel therapeutic approach for advanced cancer [28].

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## References

1. **Thorgeirsson SS, Grisham JW.** Molecular pathogenesis of human hepatocellullar carcinoma. *Nat. Genet.* 2002; 31: 339–46.
2. **Grisham JW.** Molecular genetic alterations in primary hepatocellular neoplasm: hepatocellular adenoma, hepatocellular carcinoma and hepatoblastoma. In: Coleman WB, Tsogalis GJ, editors. The molecular basis of human cancer. Totowa, NJ: Humana Press; 2001. pp. 269–346.
3. **Brattain MG, Markowitz SD, Willson JK**. The type II transforming growth factor-beta receptor as a tumor-suppressor gene. *Curr Opin Oncol.* 1996; 8: 49–53.
4. **Derynck R, Akhurst RJ, Balmain A.** TGF-beta signaling in tumor suppression and cancer progression. *Nat Genet.* 2001; 29: 117–29.
5. **Roberts AB, Wakefield LM.** The two faces of transforming growth factor beta in carcinogenesis. *Proc. Natl. Acad. Sci. USA.* 2003; 100: 8621–23.
6. **Massague J**. TGFbeta in Cancer. *Cell.* 2008; 134: 215–30.
7. **Coulouarn C, Factor VM, Thorgeirsson SS.** Transforming growth factor-beta gene expression signature in mouse hepatocytes predicts clinical outcome in human cancer. *Hepatology.* 2008; 47: 2059–67.
8. **Zimonjic DB, Durkin ME, Keck-Waggoner CL, *et al.*** SMAD5 gene expression, rearrangements, copy-number and amplification at fragile site FRA5C in human hepatocellular carcinoma. *Neoplasia.* 2003; 5: 390– 96.
9. **Massague J, Wotton D.** Transcriptional control by the TGF-beta/Smad signaling system. *EMBO J.* 2000; 19: 1745–54.
10. **Itoh S, Itoh F, Goumans MJ, *et al.*** Signaling of transforming growth factor-beta family members through Smad proteins. *Eur J Biochem.* 2000; 267: 6954–67.
11. **Inagaki M, Moustakas A, Lin HY, *et al.*** Growth inhibition by transforming growth factor beta (TGF-beta) type I is restored in TGF-beta-resistant hepatoma cells after expression of TGF-beta receptor type II cDNA. *Proc Natl Acad Sci USA.* 1993; 90: 5359–63.
12. **Zimonjic DB, Keck CL, Thorgeirsson SS, *et al.*** Novel recurrent genetic imbalances in human hepatocellular carcinoma cell lines identified by comparative genomic hybridization. *Hepatology*, 1999; 29: 1208–14.
13. **Zimonjic DB, Rezanka L, Popescu NC.** Refined localization of the erbB−3 proto−oncogene by direct visualization of FISH Signals on LUT−inverted and contrast- enhanced digital images of DAPI-banded chromosomes. *Cancer Genet Cytogenet.* 1995; 80: 100–2.
14. **Zimonjic DB, Keck-Waggoner CL, Popescu NC.** Novel genomic imbalances and chromosome translocations involving c-myc gene in Burkitt's lymphoma. *Leukemia*, 2001; 15: 1582–8.

15. **Durkin ME, Ullmannova V, Guan M, *et al.*** Deleted in Liver Cancer 3 (DLC-3), a novel Rho GTPase activating protein, is downregulated in cancer and inhibits tumor cell growth. *Oncogene.* 2007; 26: 4580–9.
16. **Lee JS, Thorgeirsson SS.** Functional and genomic implications of global gene expression profiles in cell lines from human hepatocellular cancer. *Hepatology.* 2002; 35: 1134–43.
17. **Lee JS, Chu IS, Heo J, *et al.*** Classification and prediction of survival in hepatocellular carcinoma by gene expression profiling. *Hepatology*. 2004; 40: 667–76.
18. **Wong N, Lai P, Pang E, *et al.*** A comprehensive karyotypic study on human hepatocellular carcinoma by spectral karyotyping. *Hepatology*. 2000; 32: 1060–8.
19. **Chang B, Zheng SL, Isaacs SD, *et al.*** Linkage and association of CYP17 gene in hereditary and sporadic prostate cancer. *Int J Cancer*. 2001; 95: 354–9.
20. **Fujiie S, Hieshima K, Izawa D, *et al.*** Proinflammatory cytokines induce liver and activation-regulated chemokine/ macrophage inflammatory protein-3alpha/CCL20 in mucosal epithelial cells through NF-kappaB [correction of NK-kappaB]. *Int Immunol.* 2001; 13: 1255–63.
21. **Kapás L, Hong L, Cady AB, *et al.*** Somnogenic, pyrogenic, and anorectic activities of tumor necrosis factor-alpha and TNF-alpha fragments. *Am J Physiol.* 1992; 263: R708–15.
22. **Zhao YX, Andoh A, Shimada M, *et al.*** Secretion of complement components of the alternative pathway (C3 and factor B) by the human alveolar type II epithelial cell line A549. *Int J Mol Med*. 2000; 5: 415–9.
23. **Bachurski CJ, Pryhuber GS, Glasser SW, *et al.*** Tumor necrosis factor-alpha inhibits surfactant protein C gene transcription. *J Biol Chem*. 1995; 270: 19402–7.
24. **Li WX, Tada T, Miwa T, *et al.*** mRNA expression of complement components and regulators in rat arterial smooth muscle cells. Microbiol. & Immunol. 1999; 43: 585–93.
25. **Campos SP, Wang Y, Koj A, *et al.*** Divergent transforming growth factor-beta effects on IL-6 regulation of acute phase plasma proteins in rat hepatoma cells. *J Immunol*. 1993; 151: 7128–37.
26. **Benveniste EN, Kwon J, Chung WJ, *et al.*** Differential modulation of astrocyte cytokine gene expression by TGF-beta. *J Immunol*. 1994; 153: 5210–21.
27. **Giannelli G, Bergamini C, Fransvea E, *et al.*** Human hepatocellular carcinoma (HCC) cells require both alpha3beta1 integrin and matrix metalloproteinases activity for migration and invasion. *Lab Invest.* 2001; 81: 613–7.
28. **Yang L, Moses HL.**Transforming growth factor B: tumor suppressoror promoter? Are host immune ceel the answer? *Cancer Res.* 2008; 68: 9107–11.