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ΜΑΓΔΑΛΗΝΗ Σ. ΝΑΚΟΥ

Ο ΡΟΛΟΣ ΤΟΥ TGFβ ΚΑΙ ΤΟΥ ΣΗΜΑΤΟΔΟΤΙΚΟΥ ΜΟΝΟΠΑΤΙΟΥ ΣΤΗΝ ΗΠΑΤΟΚΑΡΚΙΝΟΓΕΝΕΣΗ

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ΕΥΧΑΡΙΣΤΙΕΣ

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ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide with a poor prognosis and an annual incidence of one million cases. The available treatments so far have not proved very efficient therefore there is a growing need for a better understanding of the biology of the disease and careful analysis of the molecular and cellular pathways involved in hepatocarcinogenesis. Angiogenesis has a critical role in the development of cancer since early neoplasias must develop angiogenic ability to facilitate their expansion to a larger size. One of the factors implicated in the process of angiogenesis in TGFβ, which also controls a wide range of cellular functions. Several studies have established the dual role of TGFβ in cancer because it can either act as a tumor suppressor via its autocrine action or as tumor enhancer, since it can induce angiogenesis and promote tumor growth.

In our study we wanted to investigate the role of all three TGFβs (TGF1,2,3) and their signaling receptors (TGFβ1R,-2R,-3R) in human hepatocellular carcinoma using semi-quantitative RT-PCR analysis. Our goals were: a) to evaluate the mRNA expression profile of TGFβ1,2,3 and the receptors TGFβ type I, II and III (TGFβ1R, TGFβ2R TGFβ3R) in a total of 45 hepatocellular carcinoma samples and compare the transcript levels of the TGFβ family in the tumor samples with those in the normal samples and b)to compare mRNA expression of TGFβ1,2,3 and receptors in the hepatocellular carcinoma group, in order to find co-expression patterns in this group.

Our results indicate that a part of the HCC samples do not express any of the TGFβ ligands and receptors but this was not the case in normal samples where we found significant levels of expression of all the ligands and receptors. We observed that about 31% of hepatocellular tumors express TGFβ1 and TGFβ2R but lack any detectable expression of the other two receptors, type I and type III. This co-expression of TGFβ1 and TGFβ2R was further established since Spearman's analysis revealed a positive correlation between these two members of the TGFβ family ($P < 0.0001$).

Our findings show that the signal transduction pathway is not functional since TGFβ1 is unable to form a complex in the absence of a second receptor. Therefore we suggest that TGFβ has lost its growth inhibitory role and probably promotes tumor

proliferation, by a variety of mechanisms, in the process of hepatocellular carcinogenesis.

ΠΕΡΙΛΗΨΗ

Το ηπατοκυτταρικό καρκίνωμα είναι από τους πιο κοινούς καρκίνους ανά τον κόσμο με περιστατικά που φτάνουν το ένα εκατομύριο ετησίως. Ο καρκίνος του ήπατος έχει κακή πρόγνωση και οι υπάρχουσες έως τώρα θεραπείες δεν είναι επαρκείς, για αυτό το λόγο η προσεκτική ανάλυση των μοριακών και βιολογικών μονοπατιών που εμπλέκονται στην ηπατοκαρκινογένεση θα βοηθήσει στην καλύτερη κατανόηση της βιολογίας της ασθένειας. Η αγγειογένεση έχει κυρίαρχο ρόλο στην ανάπτυξη του καρκίνου αφού η υπέρμετρη ανάπτυξη νεοπλασιών σε αρχικά στάδια στηρίζεται στην αγγειογενετική ικανότητα των όγκων αυτών. Ένας από τους παράγοντες που εμπλέκονται στην διαδικασία της αγγειογένεσης είναι ο μετασχηματίζων αυξητικός παράγοντας TGFβ, που επίσης ελέγχει ένα μεγάλο εύρος κυτταρικών δραστηριοτήτων. Σε πολλές μελέτες έχει επίσης αναφερθεί ο διπλός ρόλος του TGFβ στον καρκίνο, γιατί άλλοτε δρα ως ογκοκαταστολέας μέσω του αυτοκρινούς μηχανισμού, και άλλοτε ογκογονικά, προωθώντας την αγγειογένεση και την ανάπτυξη του όγκου.

Σκοπός της παρούσας μελέτης ήταν η διερεύνηση του ρόλου των TGFβ συνδετών και των υποδοχέων τους στο ηπατοκυτταρικό καρκίνωμα, με τη μέθοδο της ημιποσοτικής RT-PCR ανάλυσης. Στόχοι της μελέτης ήταν: α) να εκτιμήθουν τα επίπεδα έκφρασης των συνδετών TGFβ1,2,3 και των υποδοχέων τύπου I, II και III σε σύνολο 45 δειγμάτων καρκίνου του ήπατος και να συγκρίθουν αυτά τα επίπεδα mRNA μεταξύ των φυσιολογικών δειγμάτων και των καρκινικών και β) να διερευνηθεί η συσχέτιση της έκφρασης μεταξύ των παραγόντων στην καρκινική ομάδα, δηλαδή κατά πόσο συνεκφράζονται οι παράγοντες αυτοί.

Τα αποτελέσματα αυτής της μελέτης έδειξαν ότι ένα μέρος των καρκινικών δειγμάτων δεν εκφράζει κανέναν TGFβ συνδέτη ή υποδοχέα, το οποίο όμως δεν ίσχυε στην περίπτωση των φυσιολογικών δειγμάτων, τα οποία έδειξαν σημαντικά επίπεδα έκφρασης όλων των συνδετών και υποδοχέων. Επίσης παρατηρήθηκε ότι το 31% των ηπατοκυτταρικών όγκων εκφράζουν τους TGFβ1 και TGFβ2R αλλά κανέναν από τους άλλους δυο υποδοχείς. Αυτή η συνέκφραση του β1 συνδέτη με τον υποδοχέα τύπου II επιβεβαιώθηκε και στατιστικά με την μέθοδο Spearman ($P< 0.0001$).

Βάσει των αποτελεσμάτων αυτών, είναι εμφανές ότι το σηματοδοτικό μονοπάτι δεν είναι λειτουργικό, αφού είναι αδύνατη η δημιουργία του συμπλέγματος εν τη

απουσία δεύτερου υποδοχέα. Η παρατήρηση αυτή επιβεβαιώνει και τον διπλό ρόλο του TGFβ, συνεπώς προτείνουμε ότι ο TGFβ έχει χάσει τον ογκοκατασταλτικό του ρόλο και πιθανόν προωθεί την ογκογένεση του ηπατοκυτταρικού καρκινώματος μέσω ποικίλων μηχανισμών.

1. INTRODUCTION

1.1 *Hepatocellular carcinoma*

Hepatocellular carcinoma (HCC) is cancer that arises from hepatocytes, the major cell type of the liver. This type of carcinoma is one of the most common malignant tumors worldwide, with an annual incidence of one million cases (Ince N, 1999). It is especially prevalent in parts of Africa and Asia and occurs more often in men than women, mostly in people 50 to 60 years old. In most cases (around 80-90%) hepatocellular carcinoma is associated with cirrhosis. Among the many risk factors for HCC (Stuver, 1998) are chronic infections with the hepatitis B virus (HBV) and hepatitis C virus (HCV). Another major risk factor for the development of hepatocellular carcinoma is the exposure to aflatoxin (Aflatoxin B1), which is produced by a mold that is a contaminant of nuts, beans and grains. Other causes of liver cancer are various metabolic abnormalities, such as hemochromatosis, certain autoimmune diseases of the liver, alcohol abuse, which is the most common cause for cirrhosis in the U.S. and a whole host of other diseases that result in chronic inflammation of the liver leading to scarring.

Diagnostic tests include multiple-phase abdominal ultrasounds (US), computerized tomography (CT) and measurement of alpha-fetoprotein (AFP). Serum α – fetoprotein is a sensitive and useful marker for the diagnosis of liver cancer. Almost 70% of the patients with HCC have elevated concentration of serum α – fetoprotein but this is not always specific for this condition. It is usually measured as a part of screening in patients with chronic hepatitis B or chronic hepatitis C and cirrhosis. A rising blood α-fetoprotein concentration in someone with chronic liver disease suggests the development of hepatocellular carcinoma. The definitive diagnosis of HCC is liver biopsy. New antitumor treatments such as lipiodol chemo-embolisation and cryotherapy have not proved very efficient. So far only aggressive surgery or liver transplantation can be curative treatments but there are limitations. These procedures can be applied if the tumor is small or slow –growing and if it is diagnosed in an early stage and it has not spread beyond the liver. Otherwise chemotherapy and radiation may relieve symptoms and prolong life or give the surgery a greater chance of success.

1.2 *Angiogenesis*

One of the key processes in the development of cancer is the angiogenesis. Neovascularization or angiogenesis is a complex procedure that is characterized by growth of new blood vessels by sprouting from existing ones, and which under physiological conditions is essential for cell function and survival. Almost all tissues develop a vascular network that provides cells with oxygen and nutrients and enables them to eliminate metabolic wastes. Nevertheless, almost all cells in a tissue must reside within 100-200 μm of a capillary blood vessel in order to take enough oxygen for their survival. In multicellular organisms where organogenesis takes part, this close proximity of cells to capillary blood vessels is ensured by coordinated growth of vessels and parenchyma (Carmeliet P, Jain RK, 2000). Once the vascular network is formed, it is a stable system, carefully regulated and slowly regenerated.

Under physiological conditions, angiogenesis occurs primarily in embryo development as well as wound healing where it induces a burst of capillary growth in the neighborhood of the damage, to satisfy the high metabolic requirements of the repair process. Angiogenesis has also a critical role in inflammation since local irritants and infections cause proliferation of new capillaries, most of which regress and disappear when inflammation subsides (Bergers G and Benjamin L, 2002).

Pathological angiogenesis or the abnormal proliferation of blood vessels is implicated in over 20 diseases, including cancer, psoriasis and age-related macular degeneration. Goldman made the first observation of angiogenic activity around a tumor at the beginning of the 20th century (Goldman E, 1907). Later on, experimental evidence proved the importance of angiogenesis in inducement and maintenance of a tumor (Bouck et al., 1996; Hanahan and Folkman 1996; Folkman 1997). Folkman and colleagues used in vivo bioassays to demonstrate the requirement of angiogenesis for explosive growth of tumor explants (Folkman, 1997). Furthermore he supported the important role of angiogenesis due to the ability of many antiangiogenic substances that impaired the growth in tumor cells inoculated subcutaneously in mice (Folkman J, 1992 and 1997).

Cells with abnormal proliferative lesions initially lack angiogenic ability, limiting their capability for expansion. To facilitate their expansion to a larger size, early neoplasias must develop angiogenic ability (Bouck et al., 1996; Hanahan and Folkman 1996; Folkman 1997). In more detail, solid tumors smaller than 1-2 mm are

not vascularised and in order to spread they have to be supplied by blood vessels that provide oxygen and nutrients. Beyond this critical stage of 2mm, the oxygen and nutrient supply in the center of the tumor is difficult, leading to a state of cellular hypoxia that marks the onset of tumoral angiogenesis (figure 1.1).

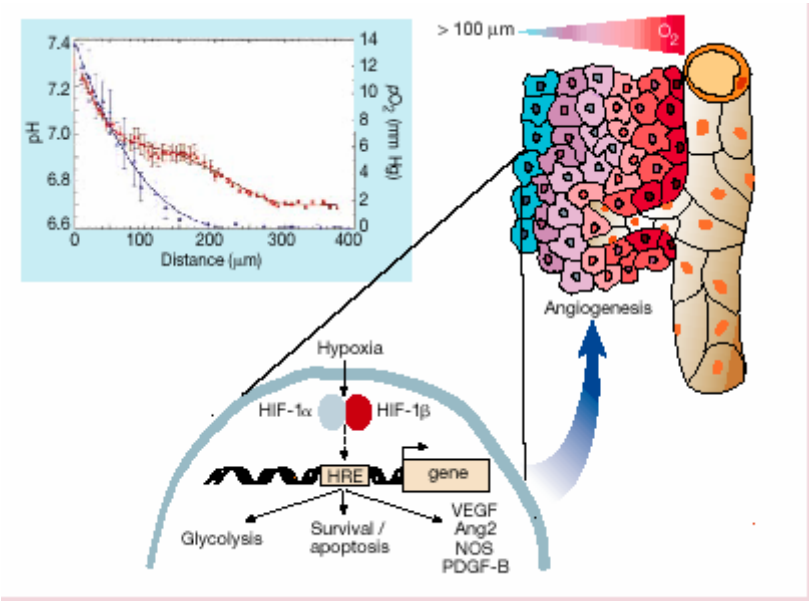


Figure 1.1
The role of hypoxia in angiogenesis

Tumor cells that are not close enough to blood vessels, become hypoxic (gradually turning from red to blue in the figure). The lack of oxygen results in the activation of HIF (hypoxia induced transcription factors) that induce angiogenesis through the transcription of growth factors.

The development of new blood vessels is an important process in tumor progression because it favors the passage from hyperplasia to neoplasia, meaning the transition from cellular multiplication to uncontrolled proliferation, which is a characteristic of tumor cells. Neovascularization also influences the distribution of cancer cells throughout the entire body eventually leading to metastasis formation. The vascularisation level of a solid tumor is thought to be an excellent indicator of its metastatic potential.

The mechanism of stimulation of blood vessel growth involves a series of events and a number of molecular factors (Liekens S et al., 2001; Cristofanilli M et al. 2002; Sharma R, 2001) (figure 1.2). A cell is activated due to the lack of nutrients and oxygen; therefore it releases angiogenic molecules that attract inflammatory and endothelial cells that promote their proliferation. When these inflammatory cells migrate, they also secrete molecules that intensify the angiogenic stimulus (Asahara T et al., 2000; Rafii S, 2000). The endothelial cells, which form the blood vessels, respond to the angiogenic stimulus by differentiating and by secreting matrix metalloproteases (MMP) that destroy the blood vessel walls. This event allows endothelial cells to escape and migrate toward the site of the stimulus. The digestion of the blood vessel walls results in the production of numerous protein fragments that increase the proliferative and migratory activity of endothelial cells, which then form a capillary tube by changing the arrangement of their adherence-membrane proteins. At the end, the continuous blood flow is ensured when the capillaries, originating from the arterioles, and the venules join.

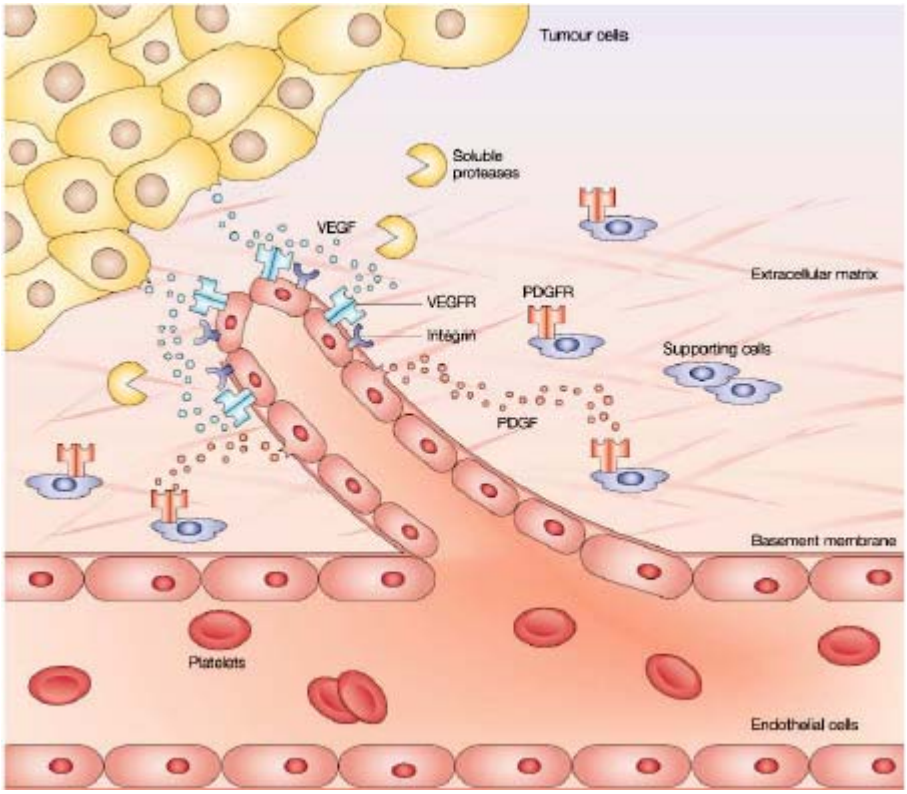


Figure 1.2

Mechanism of stimulation of blood vessel growth in a tumor

A fine balance between positive and negative signals that either encourage the formation of blood vessels or block the process governs the normal regulation of angiogenesis and changing of this balance activates the "angiogenic switch" (Hanahan and Folkman, 1996). Positive regulators of angiogenesis include growth factors, matrix metalloproteinases, cytokines and integrins. Growth factors, such as the vascular endothelial growth factor (VEGF), which has a predominant role in angiogenesis, fibroblast growth factor (FGF) and epidermal growth factor (EGF) induce the division of cultured endothelial cells thus signifying a direct angiogenic action (Carmeliet P, 2000; Gasparini G, 2001; Bergers G, 2003). However other factors, such as TGF-beta and TNF-alpha, have an indirect angiogenic action since they have been recognized as growth inhibitors.

1.3 *TGF-beta*

Transforming growth factor β (TGF-β) is a growth modulator with many cell and tissue activities including angiogenesis, cell cycle control, regulation of early development, chemotaxis, hematopoiesis, immune functions, extracellular matrix formation and induction of apoptosis (Blobe GC, 2000; Massague J, 1992, 1994, 1998; Hata A, 1998; Hartwell LH, 1994). The involvement of TGF-beta in these functions has been investigated in many cell types. More specifically it has been demonstrated in hepatocytes that TGF-β1 can inhibit cell proliferation and induce apoptosis *in vitro*; furthermore *in vivo*, it can control the maintenance of liver size and its excessive enlargement (Kanzler and Galle 2000). Evidence have shown that exogenous administration of TGF-β in rodents caused an important increase in hepatic cell death (Russel WE, 1988). In normal epithelial cells and human keratinocytes, TGF-beta can inhibit cell growth *in vivo* and *in vitro* via an autocrine action and promote its own expression (Shipley GD, 1986; Bascom CC, 1989). Furthermore TGFβ1 possesses both pro-inflammatory and anti-inflammatory activities and plays a significant role in the immune system since it can inhibit T and B cell proliferation as well as macrophage maturation and activation.

The transforming growth factor beta (TGF-β) superfamily consists of a large number of structurally related, secreted, dimeric proteins. They act either as hormones or usually as local mediators to control a wide range of biological functions. There have been identified five isoforms of TGF-β that share a homology of 64-82% but only three are expressed in mammalian cells (TGFβ1, TGFβ2 and TGFβ3), (Benson JR, 2004). The chromosomal locations of these 3 isoforms, TGFβ1, TGFβ2 and TGFβ3 are on chromosomes 19, 1 and on chromosome 14 respectively. The TGFβ1 gene consists 7 exons and 6 introns and its reading frame is 100kb. The 3 isoforms also differ in the degree of binding to the TGFβ receptors while their expression at the mRNA level depend on the type of tissue. In more detail TGFβ1 is expressed in endothelial cells, hemopoietic and connective tissue cells while TGFβ2 is expressed in endothelial and nerve cells and TGFβ3 mainly in mesenchymal cells.

The most abundant isoform is TGFβ1, a homodimer of 25kDa that is composed of two peptide subunits 112 amino acids long, joined by disulfide bonds (figure 1.3). The members of the TGFβ superfamily originate from inactive secreted precursor

proteins through proteolytic processing. These precursors have an N-terminal signal peptide; a central prodomain consisted of 50-375 amino acids and a C-terminal mature domain that forms the active growth factor. The monomeric form of these growth factors contain 110-140 amino acids and has a compact structure with four antiparallel β strands and three intramolecular disulfide linkages creating a structure called a cysteine knot. This cysteine knot domain that is quite resistant to denaturation can be a well suited structure for extracellular molecules. The regions where sequence variation is observed between different TGFβ proteins are the N-terminal regions, the α helices and the loops joining the β strands. The linking of TGFβ monomers into functional homodimers and heterodimers is achieved by the addition of an N-terminal cysteine.

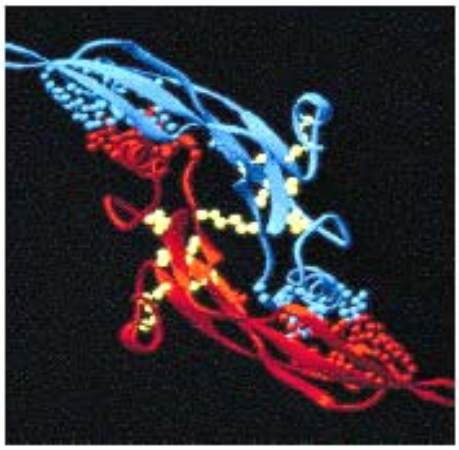


Figure 1.3
Molecular model of the TGFβ1 dimer

Three different polypeptides were identified and characterized as TGFβ receptors type I, II and III, after a cross-linking experiment with radio-iodinated TGFβ molecules bound to the surface of the surface of the cells. TGFβ receptors type I and II are transmembrane serine/threonine kinases with molecular weights of 55 and 85 kDa. Binding of TGFβ induces the formation of multimeric receptors, usually

heterotetramers, consisted of the type I and type II receptors leading to signal transduction (Massague et al. 1992 and 1994) (figure 1.4). The type II receptor subunit phosphorylates serine and threonine residues in a highly conserved sequence motif in the juxtamembrane region of the type I receptor subunit, thus activating its kinase activity (Wrana et al. 1994). Then the activated type I receptor phosphorylates a subgroup of Smads, the R-Smads (Receptor regulated Smads) and after the joining of the related protein Smad 4 they move into the nucleus resulting in the regulation of gene expression (Lagna et al, 1996; Hata et al, 1998; Massague and Wotton, 2000; Yingling et al., 2004). The type III TGFβ receptor is a cell surface proteoglycan, called β-glycan, which has no kinase activity but seems to regulate and facilitate the accessibility of TGF-beta to the signal transducing heterotetramer of the type I and type II receptor. Therefore, in the case of a mutation in the TGF-beta type II receptor gene or in the case of defective binding ability of the type II receptor, TGFβ3R forms a complex with TGFβ1R and TGFβ to regulate signal transduction (Kim SJ et al. 2000).

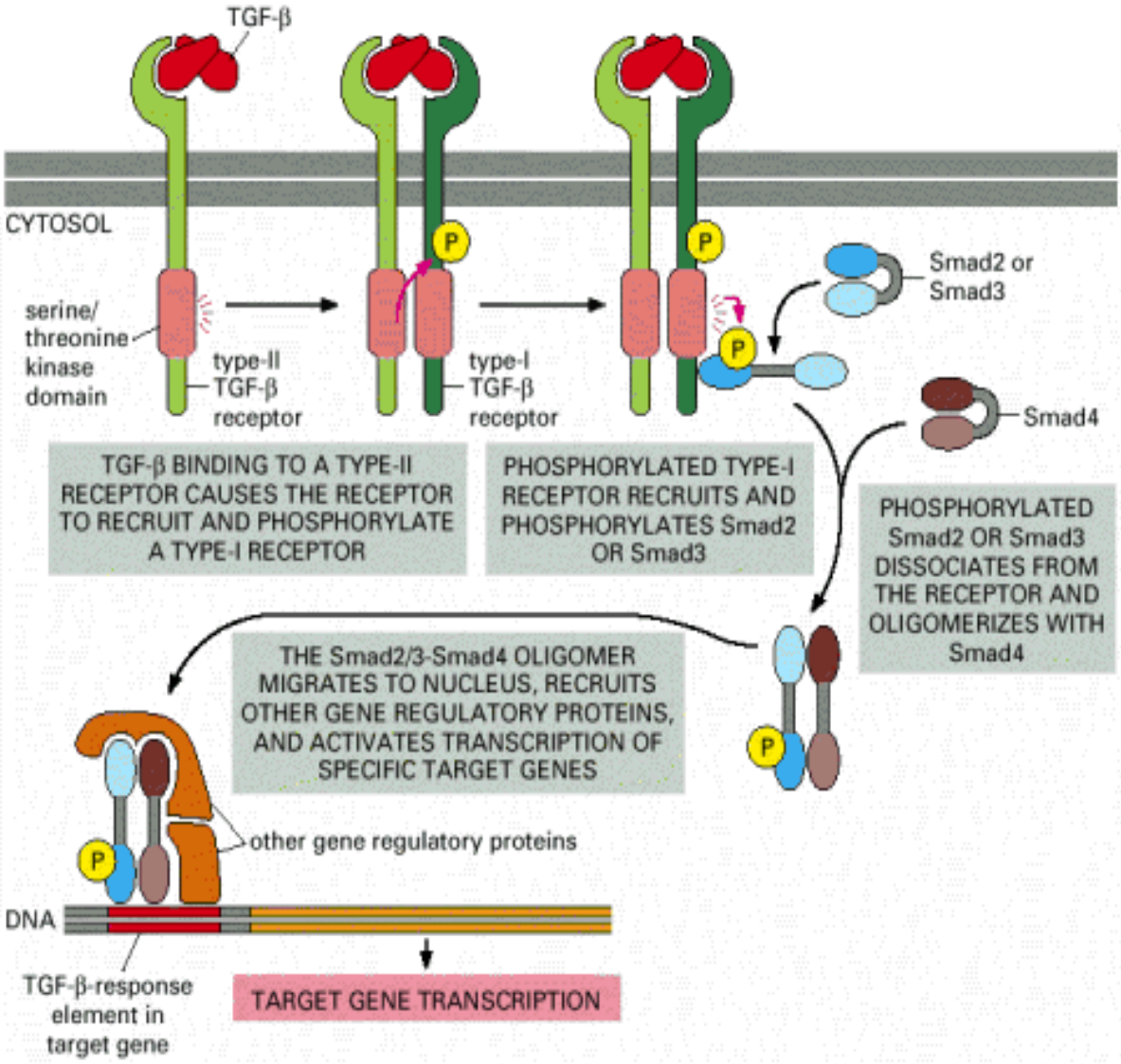


Figure 1.4
Mechanism of TGFβ signal transduction

In cancer, TGFβ has a dual role since it can either inhibit or promote tumor growth (Figure 1.5).

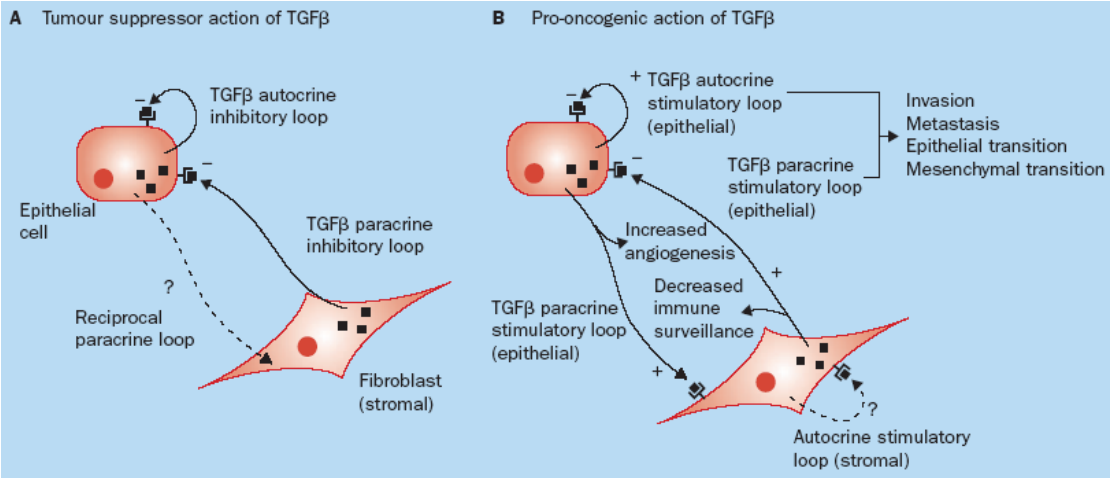


Figure 1.5
Schematic representation of the dual role of TGF-beta in cancer

(A). At the early stages of cancer, TGF-beta induces apoptosis and inhibits proliferation of endothelial cells, while in **(B)** late stages of cancer it induces angiogenesis and promotes tumor growth.

In healthy adult tissues, TGFβ act a tumor suppressor via its autocrine action that leads tumor cells to produce its own growth factors that suppress their development. The tumor suppressive role of TGF-beta is maintained at the early stages of cancer but at some point epithelial cells to do not respond to its tumor suppressive action. In the late stages of a malignancy, TGFβ indirectly promotes tumor growth by the induction of angiogenesis (figure 1.6) and immunosuppression (Roberts AB, 1998; Tang B et al., 2003).

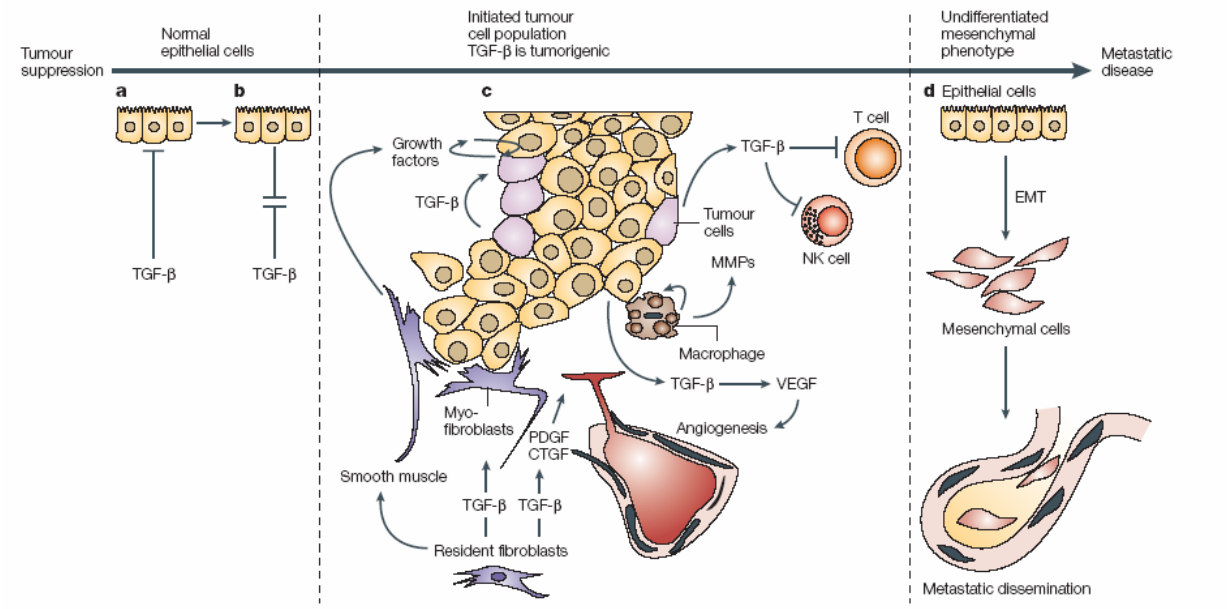


Figure 1.6
TGF-beta induces angiogenesis in the microenvironment of a tumor

Increased expression of TGF-β in the microenvironment of tumor favors tumor growth. This oncogenic function of TGF-β is also accompanied by immunosuppression and activation of growth factors as well as production of MMPs (matrix metalloproteases) that destroy the blood vessel walls.

The precise function of TGF-beta depends on the disease stage, the microenvironment and the presence of certain receptors, therefore its role in the process of angiogenesis in hepatocellular carcinoma is not well understood. However in several other cancer types, angiogenesis is linked with downregulation of TGF-beta, which acts as a growth inhibitor.

2. MATERIALS AND METHODS

2.1 *Patients and tissue samples*

Tissues from the tumour and from surrounding non-tumourous liver were obtained from 45 patients. Twenty of the samples were embedded in paraffin and the other twenty-five samples were frozen material stored at –80^{o} C. All tumors were from patients who were operated in the Department of Visceral Surgery and Transplantation at the University Hospital in Bern Switzerland. Tumors were categorized according to histological stage and grade. Most of the tumors (35/45) had stage of T3 and the rest T4. Concerning the grade, with the exception of two tumors, which had a grade of G3-4 the rest, had a grade of G1-2.

2.2 *RNA extraction*

Total RNA from the paraffin-embedded tissues was extracted with PURESCRIPT® RNA Isolation kit (Gentra) according to manufacturer's instructions. Paraffin-embedded tissue samples were deparaffinised using Xylene (0.3ml of xylene for 5-10mg of tissue) and centrifuged at 13,000 x g for 1-3 minutes. After 3 xylene washes, the tissue was washed twice with 100% ethanol. After the addition of 0.3ml Cell Lysis Solution (Gentra kit) and homogenization, the lysate was incubated at 65^{o}C for 15-60 minutes to complete lysis. Following protein-DNA precipitation, by adding 0.1ml of protein-DNA precipitation solution (Gentra kit), RNA was precipitated using isopropanol and centrifuged at 13,000 x g for 3 minutes. RNA was then washed with an equal volume of 70% ethanol and resuspended in RNA hydration solution (Gentra kit). Purified RNA samples were stored at –80^{o}C.

Total RNA from frozen tissues was extracted with TRI REAGENT ^{TM} (Sigma), according to manufacturer's protocol. Briefly, 1ml of reagent was added to each tissue sample (50-100mg of tissue), which was then homogenized using a microfuge tube pestle and transferred to a 1.5 ml eppendorf tube. A volume of 0.2ml of chloroform was added and the tube was vortexed and centrifuged at 12,000 x g for 15

minutes at 4°C. Following centrifugation the RNA was precipitated with the appropriate volume of isopropanol (0.5 ml of isopropanol per ml of TRI REAGENT™ used), and centrifuged at 12,000 x g for 10 minutes at 4°C. The RNA pellet was washed with 75% ethanol and resuspended in DEPC (diethyl pyrocarbonate) treated water to remove any RNase. Samples were stored at –80°C.

2.3 *Spectrophotometry of RNA*

RNA concentration and purity was determined on a UV spectrophotometer (Hitachi Instruments Inc., USA) by absorbance measurements at 260nm and 280nm. RNA quantity is determined based on the absorption at 260nm and by taking as a rule of thumb that an RNA solution with a concentration of 40μg/ml has an absorbance of 1 a.u. (absorbance unit). RNA purity in a sample, is determined by observing the difference and ratio of the absorbance at 260nm and 280nm under the corrected background (eg. contaminated with protein or phenol). A pure preparation of RNA provides a ratio of 2.0 but if the sample is impure the ratio becomes less.

2.4 *RNA electrophoresis on agarose gel*

RNA integrity was examined by agarose gel electrophoresis. A quantity of 5μg of RNA was resuspended in 25μl deionised formamide 50% (v/v), 1x MOPS solution (0,02 M MOPS, 50mM sodium acetate and 10μM EDTA, pH 8,0) and 16% formaldehyde. Samples were incubated at 65°C for 25 minutes and put on ice. A volume of 2.5 μl loading (50% glycerol, 1mM EDTA and 0.25% bromophenol blue) was added and the samples were resolved by electrophoresis on a 1% agarose gel (in 1x MOPS solution and 2.2M formaldehyde). After electrophoresis, the gel is put on a shaker in distilled water for 1 hour, to remove the formaldehyde and then stained with 0.5ng/ml ethidium bromide.

2.5 *Semi-quantitative RT-PCR analysis*

Reverse transcription reactions were conducted for the preparation of the first strand of cDNA. Two micrograms of total RNA from each tumor and nontumor tissue sample was subjected to RT by M-MuLV Reverse Transcriptase (New England Biolabs Inc.) with oligo(dN) primers (New England Biolabs Inc.) and RNaseOUT® (Invitrogen) in a 20μl reaction volume.

Transcribed products were subjected to PCR for the target of interest in a PTC-200 programmable thermal cycler (MJ Research Inc., USA). PCRs were performed with 1μl of RT product (cDNA) using Taq DNA polymerase (New England Biolabs Inc.) and in a final volume of 10 μl containing 10x PCR reaction buffer, 50mM $MgCl_2$, 10mM dNTPs and the appropriate quantities of each primer set (kindly provided by Gianna Soufla), which are summarized in Table 2.1. All primer pairs span at least one intron in order to avoid amplification of genomic DNA along with cDNA.

B2- microglobulin was used as an internal control in all PCR reactions.

The PCR program involved preheating at 94^{o}C for 3 minutes and the annealing temperature varied between 54^{o}-62^{o}C depending on the gene (Table 2.2) for 35-37 cycles and it was followed by a final 5-minute extension at 72^{o}C.

Genes	Oligonucleotide sequence (5'-3')	Primer quantities (pmol gene /pmol β2M)
TGFb1	(F): ACCAACTATTGCTTCAGCTC (R): TTATGCTGGTTGTACAGG	12 : 1,5
TGFb2	(F):CTGTCCCTGCTGCACTTTTGT (R):TCTTCCGCCGGTTGGTCTGTT	12 : 1,5
TGFb3	(F): CCTTTCAGCCCAATGGAGAT (R): ACACAGCAGTTCTCCTCCAA	30 : 1,5
TGFb1R	(F): TCGTCTGCATCTCACTCAT (R): GATAATCTCTGCCTCACG	20 : 1,5
TGFb2R	(F): GCGGGAGCACCCCTGTGTC (R):CCCGAGAGCCTGTCCAGATGC	12 : 1
TGFb3R	(F): AATCTGGGCCATGATGCAG (R): ACTGCTGTTTTCCGAGGCT	10 : 10
β2M **(β2microglobulin)**	(F):AGCGTACTCCAAAGATTCAGGTT (R):TACATGTCTCGATCCCACTTAACTAT	

Table 2.1. Oligonucleotide primer sequences and primer quantities according to the internal control (β2M).

GENES	Annealing temperature (^{o}C)	Amplification cycles	Product size (bp)
TGFb1	55	35	198
TGFb2	58	35	227
TGFb3	57	35	241
TGFb1R	54	35	344
TGFb2R	62	35	216
TGFb3R	57	35	287
B2M			297

Table 2.2. PCR cycling conditions of each primer pair and size of the PCR product

2.6 *PCR product electrophoresis and silver staining*

The resulting PCR products were resolved by electrophoresis in Tris-boric-EDTA (TBE) buffer (0.09M TrisHCl, 0.09M boric acid, 2.5mM EDTA, pH 8.3) on 8-9% acrylamide gel (acrylamide/bis-acrylamide 29:1). Prior electrophoresis samples were resuspended in loading buffer that contains 0.25% bromophenol blue, 0.25% xylene cyanide and 40% glycerol. Acrylamide gels were stained with silver nitrate ($AgNO_3$); a method called silver staining that is characterized for its great sensitivity. The gel is transferred in a solution containing 10% ethanol and 0.5 acetic acid for 10 minutes for fixation. Following fixation, silver nitrate solution 0.1% (w/v) is added for 15 minutes in the dark and finally the bands can be visualized after the addition of the developer consisted of 0.4 N NaOH and 0.15 (v/v) formaldehyde.

2.7 *Processing of the results and statistical analysis*

Acrylamide gels were scanned at a high resolution on an Agfa Snap-Scan 1212u. scanner. Gel electrophoresis images were saved using Corell Grafics 11 software and the original intensity of each specific band was quantified with Adobe Photoshop software. The integrated intensity of each band was the result of the intensity of the background minus the intensity of the band of the product. The intensity of β2-microglobulin was used as an internal standard. The ratio of the integrated density of the gene of interest to that of β2-microglobulin was used for the semi-quantification of the results.

Non-parametric procedures were applied to the set of data for the evaluation of significant statistical differences. The Spearman's rank correlation was used to evaluate the significance of mRNA of the TGF-beta co-expression pair wise. Probability values less than 0.01 were considered statistically significant. Statistical calculations were performed using the SPSS software, version 11.

3. RESULTS

In this study, we evaluated the mRNA expression profile of TGFβ1,2,3 and the receptors TGFβ type I, II and III (TGFβ1R, TGFβ2R TGFβ3R) in a total of 45 hepatocellular carcinoma samples. In the first part we assessed the transcript levels of TGFβ1,2,3 and the receptors in the tumor samples and compare it with the transcript levels of the TGFβ family in normal samples (Figure 3.1). In the second part of this study we compared mRNA expression of TGFβ1,2,3 and receptors in the hepatocellular carcinoma group, in order to find co-expression patterns in this group.

3.1 *mRNA levels of TGFβ1,2,3 and receptors*

The results from this study indicate that in many of the tumor samples, the growth factors TGFβ1,2,3 and the receptors type I,II and III, were not expressed at all, but this was not the case in the normal samples which expressed all the TGFβ ligands and receptors. In more detail, TGFβ1 ligand was expressed in the normal, non-tumorous samples but almost 44% of the hepatocellular carcinoma samples (20/45 HCC samples) did not express this particular ligand (Fig.3.2 upper left panel). Nevertheless, TGFβ1 was expressed in 29% of the tumors (13/45 HCC samples) and its transcript levels were about the same as the TGFβ1 transcript levels in the normal samples. In three out of 45 HCC samples, the TGFβ1 expression level was higher compared to normal tissue, while the remaining 20% of the tumor samples (9/45 HCC samples) had relatively lower expression levels of TGFβ1.

The TGFβ2 ligand was expressed in the normal tissues but we found no sign of expression in the tumor samples. All 45 hepatocellular carcinoma samples did not express TGFβ2.

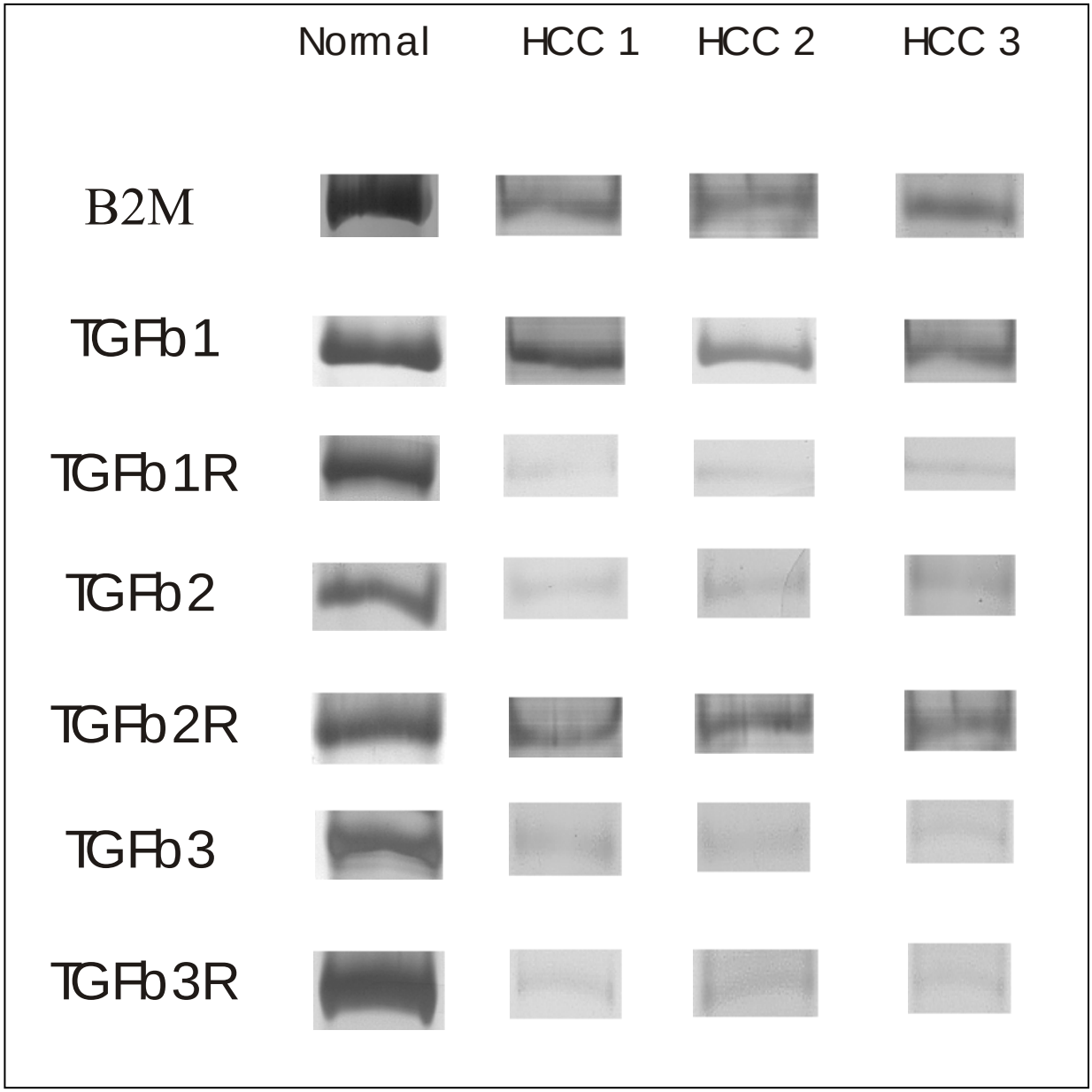
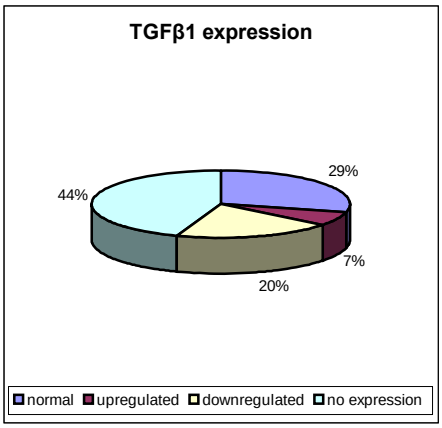
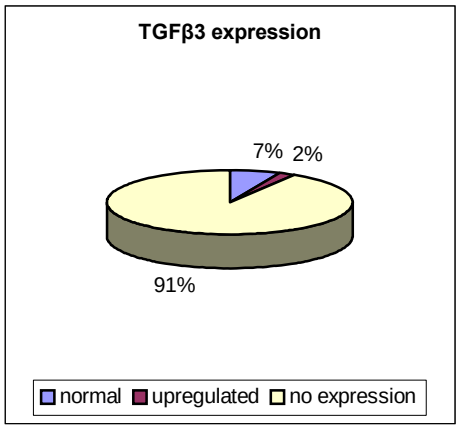
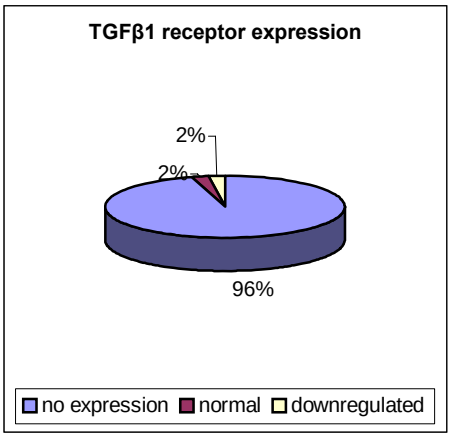
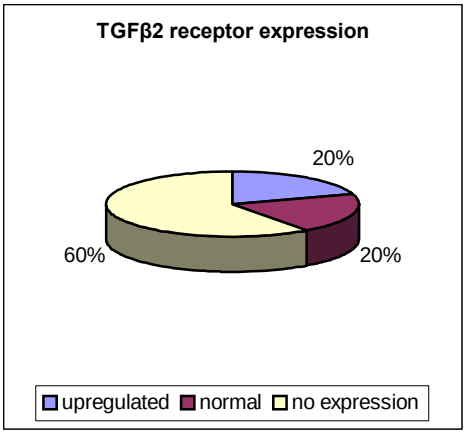


Figure 3.1
Representative examples of TGFβ1,2,3 and TGFβ1R, -2R and –3R expression in hepatic tissue. Ratio: integrated density of the band of each gene divided by the integrated density of the internal standard band (β2-microglobulin).









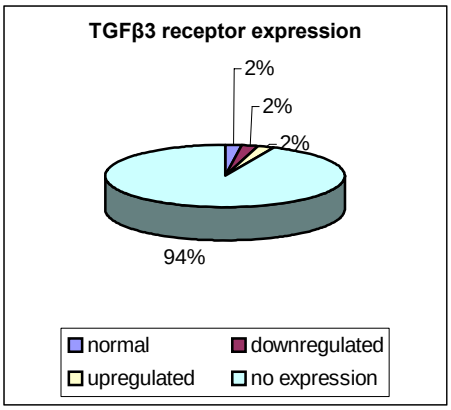


Figure 3.2
Levels of expression of TGFβ1,3 and receptors type I, II and III in the group of tumor samples. TGFβ2 is not represented because it was not expressed in these samples.

The vast majority of the tumor samples (about 91% of HCC samples) did not express TGFβ3 (Fig 3.2 upper right panel). Transcript levels of TGFβ3 were significantly higher in only one out of 45 tumor samples when compared with normal samples and three more tumor samples (7% of HCC samples) expressed TGFβ3 at the same level as normal tissues.

There are three types of TGFβ receptors: receptors type I, type II and type III (TGFβ1R, TGFβ2R and TGFβ3R). In our study we evaluated the expression levels of all three receptors and our findings were quite interesting. TGFβ1R, which is an essential receptor to form the complex and regulate signal transduction, is not expressed in 96% of the hepatocellular carcinoma samples (43/45 samples) (Fig 3.2 middle left panel). There was only one sample from cancer tissue that exhibited the same TGFβ1R transcript levels as in normal tissue, and one more sample that expressed considerably lower TGFβ1R mRNA levels compared to controls.

TGFβ2 receptor is another important receptor for signal transduction. Almost half of the tumor samples (27/45 HCC samples) did not express the TGFβ2 receptor (Fig 3.2 middle right panel). Nevertheless, a significant increase of TGFβ2 receptor mRNA expression was observed in 20% of the hepatocellular carcinoma samples (9/45 samples) compared to normal, while the remaining 20% of the tumor samples revealed same expression levels as in the normal tissues.

The TGFβ3 receptor has no kinase activity but seems to facilitate in the signal transduction pathway. Our findings suggest that the vast majority of the HCC samples (42/45 samples) do not express this particular receptor (Fig 3.2 bottom left panel). The highest transcript levels of TGFβ3R were observed in only one tumor sample and one more sample had significantly lower transcript levels compared to normal samples. Finally the remaining 2% of the HCC samples had the same TGFβ3R expression levels as normal samples.

3.2 *mRNA co-expression analysis pair wise*

In the present study we wanted to evaluate if there is any correlation between the mRNA expression of growth factors TGFβ1,2,3 and TGFβ1R,-2R and –3R in the cancer group, in order to establish a pattern of expression of these factors in the hepatocellular carcinoma. We used the Spearman's rank correlation to evaluate the

significance of mRNA of the TGF-beta co-expression pair wise. Probability values less than 0.01 were considered statistically significant.

Our results revealed that 13 hepatocellular carcinoma samples have similar TGFβ1 expression levels as normal samples, but after evaluating the expression levels of the other growth factors in the same HCC samples we observed that 7 of these samples simultaneously express TGFβ2R at normal levels while 3 of 13 did not express growth factors TGFβ2, TGFβ3 and the receptors TGFβ1R, -2R and 3R. The remaining 3 of 13 samples, which have normal levels of expression of TGFβ1, also have elevated transcript levels of TGFβ2R and one of them simultaneously expresses the TGFβ1 Receptor at normal levels. This leads us to the assumption that in this particular tumor sample, binding of the receptors and therefore complex formation can be achieved, which is essential for TGFβ1 signaling action.

There were only 3 HCC samples of 45 that had higher levels of TGFβ1 expression but one of them did not express any of the other ligands or receptors. The other 2 of 3 samples, both had elevated levels of TGFβ2R. When the TGFβ1 ligand and the TGFβ2R are expressed and bind together they establish the first step for their signaling role, which is inhibited in the absence of another receptor that will assist the complex formation. Our findings suggest that one of these two samples that had elevated levels of TGFβ1 and TGFβ2R, also had high TGFβ3R mRNA levels, meaning that ligand binding to the receptor was facilitated by receptor type III but there was still absence of TGFβ1R.

We also observed that TGFβ1 was downregulated in 20% (9 of 45) of the hepatocellular carcinoma samples but in 2 of 9 samples, TGFβ2R was upregulated. In 5 of 9 tumors, apart of the low levels of expression of TGFβ1, we did not observe any expression of the other ligands TGFβ2,3 or receptors TGFβ1R,-2R and TGFβ3R. The remaining 2 samples both expressed TGFβ3 but one of them had elevated TGFβ3 transcript levels while the other sample expressed TGFβ3 at normal levels accompanied by underexpression of the TGFβ3 receptor.

The majority of the samples in the cancer group (20 of 45) did not express TGFβ1 and interestingly enough 13 of 20 did not express any other ligand or receptor either. Therefore the 29% of all HCC samples does not express any of the members of the TGFβ family leading us to the assumption that the function of the signal transduction mechanism is totally lost in these samples. Despite the fact that there is loss of TGFβ1 expression, 7of 20 samples express other ligands and receptors. In more

detail, 2 of 7 express TGFβ3 ligand at normal levels in the absence of TGFβ1. Furthermore, one sample exhibits normal transcript levels for TGFβ3R only and 3 of 7 tumors express the TGFβ2R only, and in one of them TGFβ2R is upregulated. There was also the case in one sample, that in the absence of a ligand the receptors were present, although TGFβ1R was downregulated and TGFβ2R was upregulated.

The Spearman correlations for evaluation of TGFβ1,2,3 and TGFβ1R,-2R and –3R co-expression patterns in the hepatocellular carcinoma group are demonstrated in Table 3A. Our analysis revealed that TGFβ1 mRNA was strongly co-expressed with TGFβ2 Receptor in the tumor samples (P<0.0001 and Spearman's rho = 0.500) but there was no other correlation established between the members of the TGFβ family.

Table 3A.
Spearman correlation rho and *P* values in the HCC group of patients

			TGFB1	TGFB2	TGFB3	TGFBR1	TGFBR2	TGFBR3
Spearman's rho	TGFB1	Correlation Coefficient	1,000	.	-,139	,037	,500(**)	-,023
		Sig. (2-tailed)	1,000	.	,364	,811	,000	,881
		N	.	45	45	45	45	45
	TGFB2	Correlation Coefficient			.	.	.	.
		Sig. (2-tailed)			.	.	.	.
		N			45	45	45	45
	TGFB3	Correlation Coefficient			1,000	-,067	-,247	,222
		Sig. (2-tailed)			.	,661	,102	,143
		N			45	45	45	45
	TGFBR1	Correlation Coefficient				1,000	,170	-,058
		Sig. (2-tailed)				.	,263	,707
		N				45	45	45
	TGFBR2	Correlation Coefficient	(**)				1,000	-,070
		Sig. (2-tailed)					.	,646
		N					45	45
	TGFBR3	Correlation Coefficient						1,000
		Sig. (2-tailed)						.
		N						45

** Correlation is significant at the 0.01 level (2-tailed).

4. DISCUSSION

Homeostasis in human tissues is achieved through the balanced and cooperative interactions between cells and the extracellular matrix. A number of diseases are the outcome of the interruption of balance, in which many cytokines take part. One of these cytokines is the transforming growth factor β (TGFβ), which takes part in many processes as proliferation, differentiation and wound healing. TGFβ regulates these processes through signal transduction, by binding to high-affinity cell-surface receptors. Overproduction or decrease of TGFβ, as well as mutations in the TGFβ genes and its receptors, associate with pathogenesis of cancer and other diseases.

The available studies so far have been focused in the effect of TGFβ1 expression solely or combined with TGFβ2 Receptor's expression in cancer tissue function, and provide us with limited information on the combined mRNA expression levels of the TGFβ family.

In our study we evaluated the combined mRNA expression of TGFβ1,2,3 and TGFβ1R,-2R and –3R in a group of hepatocellular carcinoma patients. According to our data the only members of the TGFβ family that exhibited elevated transcript levels compared to normals were TGFβ1 and TGFβ2R. The presence of these factors is required for signal transduction but this is not enough. Signal transduction is activated when one of the TGFβ ligands (β1, β2, β3) bind type II receptor indirectly through type III receptor or directly on the cell membrane. Binding of the ligand to TGFβ2R induces formation of the complex since binding of TGFβ1R is achieved (Blobe et al. 2000). It is important to highlight that the mechanism does not work in the presence of only one receptor since complex formation is essential for signal transduction.

Overproduction of TGFβ1 in human hepatocellular carcinoma patients and high plasma and urine levels of TGFβ1 in HCC patients, were common observations in previous publications (Ito et al. 1991 and 1995, Tsai et al. 1997, Bedossa et al. 1995, Shirai et al. 1994, Matsuzaki et al. 2000). Increased production of TGFβ1 has also been documented in gastric, pancreatic, thyroid, and brain cancers as well as in breast cancer progression (Sue SR et al. 1995). In normal cells, TGFβ activates the signaling pathway and arrests the cell cycle at the G1 stage. This results to inhibition of proliferation, differentiation and apoptosis. During carcinogenesis, cells as well as

fibroblasts, become resistant to the effects of TGFβ and proliferate in an unregulated manner leading to an increased production of TGFβ that causes immunosuppression, angiogenesis and tumor invasion. As the cancer progresses, the increased TGFβ production has been linked with greater invasiveness of the tumor (Maehara Y et al. 1999, Picon A et al. 1998). This loss of cell's responsiveness to TGFβ1-mediated growth inhibition has also been implicated in hepatocarcinogenesis (Inagaki M et al. 1993). During this process, certain growth factors that are normally present at low or undetectable levels in normal liver tissue in humans become highly expressed in human hepatocellular carcinoma, hepatoma cell lines and in chemical models of hepatocarcinogenesis (Rizzino A, 1993).

Our data reveal that TGFβ1 is kept at normal levels of expression in 29% of tumor samples and 7% is upregulated, while TGFβ2 ligand is not expressed in any of the samples. Interestingly enough, we observed the presence of TGFβ3 ligand in some of the samples, when there was no expression of TGFβ1 and TGFβ2. This could be explained as an attempt of the system to form the complex, but we observed absence of one or both receptors needed to form the heterotetramer therefore TGFβ3 has no signaling action.

In the present study we found no significant expression and in many cases absence of TGFβ receptors type I and type III, but this was not the case with TGFβ2R, which was expressed in 40% of HCC samples. In the study of Abou-Shady et al., the authors reported that TGFβ1,2,3 mRNAs were prominently increased in HCC tissues after immunohistochemistry and Northern blot analysis. However they did not observe any increase of the signaling TGFβ receptors compared to normal controls and in addition to the highly proliferating cell rate of the tumor, they suggested that these cells have lost growth inhibition control of TGFβ. Examples of resistance to growth inhibitory effects of TGFβ1 have been demonstrated in many spontaneously transformed rat liver cells and this acquisition of resistance appears to be correlated with the degree of tumorigenicity of some transformed cell lines (Huggett AC et al. 1991).

In hepatocytes, the expression of TGFβ1 protein was decreased in advanced hepatocellular carcinoma compared with small or early HCC and downregulation of TGFβ receptors was correlated with disease progression (Paik et al. 2003). During hepatocarcinogenesis, the inhibitory effects of TGFβ1 protein in HCC cells was

outweighed by its effects on stromal cells, which overall had an indirect role to a tumor growth stimulatory environment (Paik et al. 2003).

Our results indicate that about 31% of HCC patients expressed TGFβ1 and the TGFβ2 type II receptor (TGFβ2R) in their tissues but lack any detectable expression of TGFβ1R and TGβ3R. The increase of TGFβ2R mRNA was accompanied by an increase of TGFβ1 expression in some samples while the others were kept at normal levels. This relationship was also supported by the resulting positive correlation of TGFβ1 and TGFβ2R in cancer samples (P<0.0001). According to signal transduction pathway, TGFβ2R is able to bind a TGFβ ligand in the absence of TGFβ1R but signaling is inhibited since there is absence of the second receptor to bind the complex. Therefore we believe that TGFβ1 has lost its signaling action. There was only one exception where TGFβ1R was also expressed at normal trancript levels, which could be characterized as an attempt for activation of the signal transduction pathway.

Due to their high affinity, the cell surface receptors bind to activated TGFβ ligands and exert their biological effects. Breast cancer cells as well as other tumor cell lines, such as neuroblastoma, retinoblastoma and pheochromocytoma do not express either TGFβ1R or TGFβ2R and show no growth inhibition by TGFβ (Bassing et al. 1994). Furthermore, TGFβ1 is closely associated with the expression level of TGFβ2R and the expressions of these members of the TGFβ family, changed significantly during the promotion of hepatocarcinogenesis in rats, and these alterations might contribute to the development and progression of preneoplastic lesions (Park et al. 2001).

The non-signaling action of TGFβ is further supported since 29% of HCC samples in our study did not express any ligand or receptor. This means that the pathway is not functional therefore TGFβ has lost its growth inhibitory role and this loss of TGFβ expression is the cause of the loss of normal phenotype.

Interestingly, we observed that although there were elevated or undetectable levels of expression of TGFβ ligands and receptors in the hepatocellular carcinoma group, in the normal control group we show expression of all 3 TGFβ ligands (TGFβ1,2,3) and their receptors (TGFβ1R,-2R and –3R). These results support the dual role of TGFβ, which acts as a tumor suppressor in normal cells and early stages of cancer but it can also act as a tumor enhancer during carcinogenesis.

We suggest that signal transduction of growth inhibition is not functional in the human hepatocellular carcinoma samples of this study; therefore TGFβ probably

promotes tumor cell proliferation, possibly by a variety of mechanisms. These mechanisms may include changes in ligand concentration, modulation of the number and affinity of the receptors, altered activation of latent forms of the ligand and changes of post-receptor pathways (Grupposo et al. 1990).

Summarizing, our findings give indirect evidence that the dysregulation of TGFβ mRNA expression is implicated in the development and progression of hepatocellular carcinogenesis and we speculate that TGFβ acts as tumor enhancer in HCC *in vivo.*

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