AUS DEM LEHRSTUHL
FÜR INNERE MEDIZIN I
PROF. DR. MED. JÜRGEN SCHÖLMERICH
DER MEDIZINISCHEN FAKULTÄT
DER UNIVERSITÄT REGENSBURG

DOWNREGULATION OF METHYLTHIOADENOSINE PHOSPHORYLASE PROMOTES PROGRESSION OF HEPATOCELLULAR CARCINOMA VIA ACCUMULATION OF 5´-DEOXY-5´-METHYLTHIOADENOSINE

Inaugural – Dissertation
zur Erlangung des Doktorgrades
der Medizin

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vorgelegt von
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Für meine Familie

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1 Introduction

1.1 Hepatocellular carcinoma

1.1.1 Liver cells and the role of hepatic stellate cells for hepatic fibrosis and HCC progression

The principal cell type found in the liver is the hepatocyte. Overall, hepatocytes represent approximately 94 vol. percent of the liver. The rest comprises various non-parenchymatic cells - Kupffer cells (resident liver macrophages), sinusoidal endothelial cells, liver lymphocytes and hepatic stellate cells (HSC). HSC in the normal liver are perisinusoidal cells of still uncertain embryological origin, responsible for the synthesis of basal membrane like-extracellular matrix (ECM), components of the subendothelial space of Disse and for storage and metabolism of vitamin A and retinoids (1). As HSC are less known outside the field of hepatology but are extensively used in the present work, their crucial role in liver disease is to be shortly explained.

Activation of HSC, following liver injury, progresses in sequential stages of initiation and perpetuation (2). Initiation is an early response stimulated by a number of paracrine signals, leading to a transient and potentially reversible contractile and profibrogenic phenotype, characterized by rapid induction of certain growth factor receptors and consequently increased responsiveness to growth factors and mediators, which are responsible for eliciting phenotypic responses operated by fully activated myofibroblast (MF)-like phenotype (perpetuation). These include most notably enhanced proliferation, migration/chemotaxis, contractility, excess deposition and altered remodeling of ECM (3).

The accumulation of ECM proteins in progressive liver fibrosis alters the hepatic architecture by forming a fibrous scar, and the subsequent development of nodules of regenerating hepatocytes defines cirrhosis. Cirrhosis causes hepatocellular dysfunction and increased intrahepatic resistance to blood flow. The consequences are hepatic insufficiency and portal hypertension (4).

Ultimately, cirrhosis leads to a clinically overt loss of liver function (decompensated cirrhosis), and most importantly, liver cirrhosis is the main risk factor for the development of hepatocellular carcinoma (HCC) (5).

Thus, activated HSC are a driving force of liver fibrosis progression and consequently of origination of HCC. However, once HCC develops, HSC also play a direct role in tumor progression. They are able to infiltrate the HCC stroma and as shown by a recent study, HSC promote HCC growth and invasiveness and diminish central necrosis *in vitro* and *in vivo* probably via activation of nuclear factor kappa B (NFκB) and extracellular-regulated kinase (ERK) in HCC cells, two signaling cascades that play a crucial role in HCC progression (6).

1.1.2 Epidemiology of HCC

1.1.2.1 Incidence and regional differences in etiology of HCC

Hepatocellular carcinoma (HCC) is the most common form of primary liver cancer with 85-90% (7). There are considerable differences in HCC incidence around the world.

The highest incidence rates are reported from China (46 per 100,000 population per year, total annual incidence of 137,000 cases. The rest of Eastern Asia and most of Africa are also high-endemic regions with incidence rates of 35.5 and 24.2, respectively. Regions of intermediate incidence areas include several countries in Eastern and Western Europe, Thailand, Indonesia, Jamaica, Haiti, New Zealand and Alaska. North and South America, most of Europe, Australia and parts of the Middle East are low incidence areas with less than 3/100,000 cases per year (7).

These marked differences are explained by studying the various etiological factors for HCC development and their regional distribution. The regional differences in HCC incidence mostly reflect the different incidence of chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) (8). In most of the Asia-Pacific regions as well as in Africa endemic HBV is the most important etiological factor, with the notable exception of Japan, where HCV is by far the most common risk factor. Within Europe there are also differences in regard to

etiology. In Southern Europe viral etiology accounts for as much as 76% of HCC cases (9) while in Central and Northern Europe HBV and/or HCV infections are present in only about half of patients (10;11). The fact that within a single country there may be regional and ethnic differences in HCC incidence patterns further adds to the considerable intra- and international variability of HCC etiology (12).

1.1.2.2 Risk factors

In the vast majority (70-90% according to region) of cases HCC develops in a cirrhotic liver. Thus, all causes for liver cirrhosis can be considered as separate risk factors for HCC. This differentiation is important because the cause of liver cirrhosis significantly affects the associated risk to develop HCC, clinicopathological tumor features, the choice of treatment, and consequently the prognosis (13).

It should be noted that all factors that can lead to HCC development in the setting of liver cirrhosis can do so in the absence of cirrhosis if the level of hepatocellular toxicity necessary to lead to liver cirrhosis is not yet reached, but the carcinogenic effect is already strong enough to induce HCC (14).

- **HBV**

The single most common HCC risk factor is hepatitis B virus (HBV) infection responsible for > 60 % of all cases worldwide. The hepatocarcinogenic potential of HBV is evidenced by a multitude of clinical studies with an overall odds ratio (OR) of up to 48 in a study from India (15). Major factors in HBV-induced hepatocarcinogenesis are chronic inflammation and the effects of cytokines in the development of fibrosis and liver cell proliferation, further, the role of integration of HBV DNA into host cellular DNA, which, in some situations, acts to disrupt or promote expression of cellular genes that are important in cell growth and differentiation. In addition, expression of HBV proteins may have a direct effect on cellular functions, and some of these gene products can favor malignant transformation (16).

The risk of developing HCC upon HBV infection varies greatly and is influenced by host as well as viral-related factors in addition to external or environmental factors. Some of the most important include an advanced stage of the disease,

longer duration of cirrhosis, the activity of the underlying hepatitis and high HBV load (17;18). Further, HBV genotype, specific alleles and mutations in the viral basal core promoter are associated with an increased risk to develop HCC (19-21).

- **HCV**

Worldwide, HCV infection is the second most common etiological factor for HCC. The overall odds ratio is estimated to be 17 (22). The oncogenic process of HCV infection itself, which probably requires multiple steps of genetic alterations as result of virus-specific and virus-non-specific immune responses, and probably a direct cytopathic effect of HCV itself is still poorly understood (23;24).

As acute HCV infection often remains undetected it is difficult to determine whether longer duration of HCV infection plays a role as observed in HBV. In contrast to HBV infection, the HCV genotype or the viral load are no major factors for the course of the disease (5). A higher degree of fibrosis, male gender and the presence of co-risk factors such as alcohol and concomitant HBV infection increase the risk of developing HCC due to HCV infection (25;26).

- **Alcohol**

In Western Europe, high consumption of alcohol is one of the major risk factors for developing liver cirrhosis. The risk of developing alcohol-related HCC depends largely on the quantity of alcohol consumption. Overall, alcohol consumption is associated with a 2-fold increase of the individual risk for HCC development, but in cases of > 80 g day this increases to a 6-fold risk (27;28).

Proposed mechanisms for the hepatocarcinogenic properties of alcohol include the chronic inflammation that leads to stimulation of apoptosis and through enhanced cell injury to an increased cellular proliferation and hyperregeneration. Further, inflammation increases oxidative stress that may cause DNA damage. Another important mechanism involves the presence of toxic metabolites like acetaldehyde capable of binding to the DNA, consequently leading to constant genomic alterations and interference with DNA-repair enzymes (29). The high levels of acetaldehyde also lead to a decreased antioxidant response, e.g. by binding of glutathione and reduction of the mitochondrial glutathione content causing mitochondrial dysfunction, lipid peroxidation and impairment of the

cellular tumor necrosis factor (TNF) tolerance (30). All these mechanisms are supported by general alimentary deficiency for vitamins and supplementary factors like folic acid and retinol in case of chronic alcohol abuse (31;32).

- **Obesity and NAFLD**

Epidemiological studies have shown that obesity is a risk factor for hepatocellular carcinoma (33). The relationship between obesity and non-alcoholic fatty liver disease (NAFLD) is well established, and case reports have shown progression of NAFLD to cirrhosis and hepatocellular carcinoma (34-36). Although no study has clearly tied all of these variables together, it is likely that the association of hepatocellular carcinoma with obesity represents the progression of underlying NAFLD to cirrhosis (33). Putative oncogenic mechanisms are the initiation by lipid peroxidation and free radical oxidative stress, compensatory proliferation of small oval progenitor cells , fat-induced up-regulation of anti-apoptotic factors, alterations in growth factors and cytokines e.g. transforming growth factor (TGF) and tumor necrosis factor (TNF), stimulating oval cell proliferation (37-39). Moreover, lipid peroxidation encourages the development of cancer-promoting mutations e.g. mutations of the p53 tumor suppressor gene seem to be potentiated by the 4-hydroxynonenal aldehyde, a product of omega-6 fatty acid peroxidation (40).

With the growing worldwide obesity epidemic this risk factor is likely to gain more significance in the future.

- **Hemochromatosis and iron metabolism**

In hereditary hemochromatosis the hepatotoxic effects of iron overload can lead to progressive fibrosis and eventually cirrhosis and HCC (41). As some cases of HCC have occurred in non-cirrhotic patients with hematochromatosis, a direct role for iron in carcinogenesis has been suggested (42;43). The latter has been supported by animal studies, where pre-neoplastic nodules and even HCC in the absence of fibrosis developed in rats fed a high-iron diet (44;45). The molecular mechanisms involve epigenetic alterations of genes characteristically hypermethylated in HCC, suggesting that epigenetic changes due to iron loading are an early event in hereditary hemochromatosis, and may lead to the increased risk of progression to HCC (46).

- **Rare risk factors for HCC**

Other rare causes of HCC include autoimmune hepatitis, chronic dietary intake of aflatoxine, metabolic disorders (e.g. alpha antitrypsine deficiency, glycogenosis Type I), biliary diseases (e.g. primary biliary sclerosis), certain hepatotoxic medications (e.g. anabolic steroids) and others.

1.1.2.3 Frequency of HCC risk factors - an example from a German university hospital

The human HCC samples used in this work originate from the largest consecutive HCC patient series in Germany at the University Hospital Regensburg. **Figure 1** gives the relative contribution of major etiologic factors for the development of HCC for this cohort.

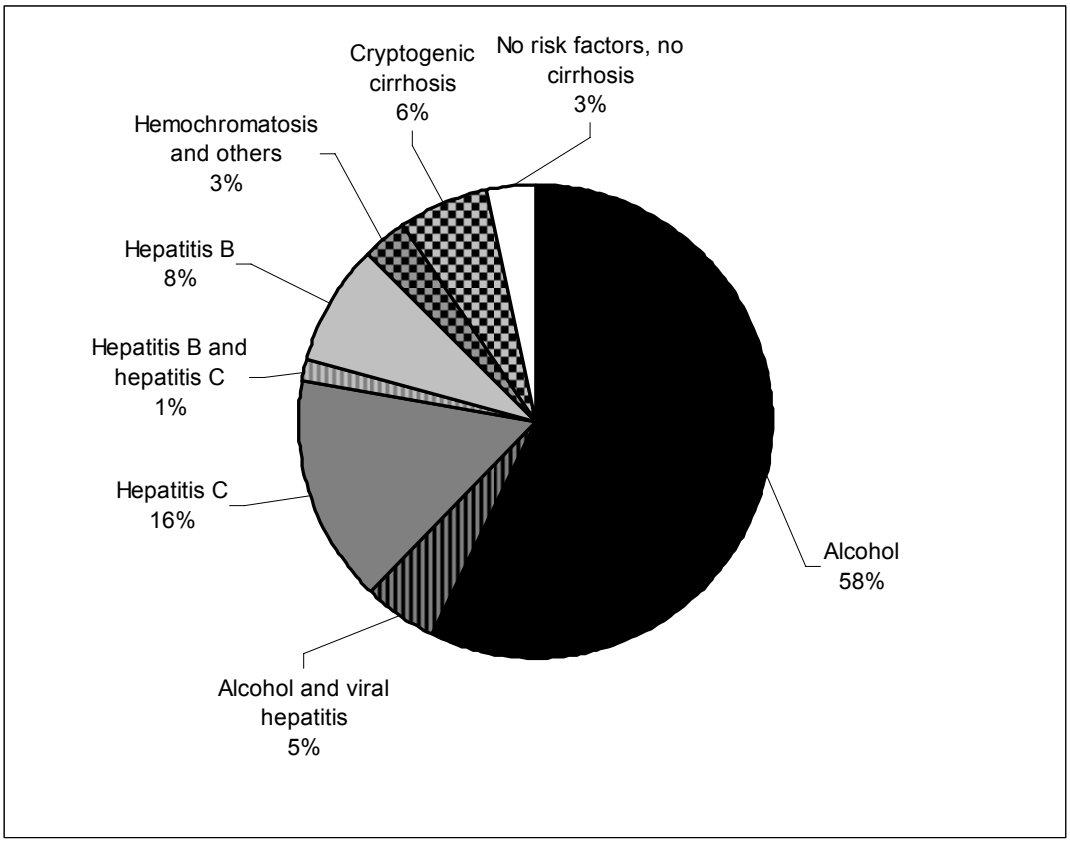

Figure 1: Etiology of underlying liver disease of HCC patients. Data are depicted of 374 patients.

1.1.3 Therapy of HCC

The therapeutic options for HCC fall into five main categories: (1) surgical interventions, including tumor resection and liver transplantation, (2) percutaneous interventions, including ethanol injection and radiofrequency thermal ablation, (3) transarterial interventions, including embolization and chemoembolization, (4) radiation therapy, and (5) drugs as well as gene and immune therapies (47). Curative treatment modalities are still the sole domain of surgery and comprise partial hepatectomy and liver transplantation (48). Liver transplantation is the ideal treatment for early HCC because both the malignant tumor and diseased liver can be eradicated by this treatment (49). However, because of organ shortage or continued alcohol abuse it cannot be performed often (50). Hepatectomy can be performed in timely fashion with less technical difficulty, but the risk of HCC recurrence is higher, even though 5-year survival in patients who meet certain criteria is the same as that with liver transplantation (51).

Therapy	N (%)
resection	124 (27.1%)
best supportive care (no specific therapy)	93 (20.3%)
local ablative therapy (TACE, RFA)	93 (20.3%)
chemotherapy alone	50 (10.9%)
liver transplantation	36 (7.9%)
chemotherapy and at least one ablative therapy	20 (4.4%)
multiple resections	11 (2.4%)
therapy with thymophysin	9 (2.0%)
combination of various ablative therapies	8 (1.7%)
percutaneous ethanol injection alone	7 (1.5%)
resection followed by liver transplantation	7 (1.5%)

Table 1: Frequency of HCC therapeutic modalities at the University Hospital Regensburg

Notable palliative options that have been shown to prolong survival include radiofrequency thermal ablation (RFA) and transarterial chemoembolization (TACE) (47).

Finally, patients who are at a very advanced stage of disease when no benefit is to be expected by any specific treatment typically receive so called “best supportive care” which generally comprises symptom-oriented treatment with e.g. analgesic drugs, antibiotics, transfusions and others as appropriate in each individual case. **Table 1** summarizes the treatment options for HCC at the University Hospital Regensburg.

1.1.4 Prognosis of HCC

Despite new therapies and attempts at early detection, HCC remains a disease with a very high mortality, a yearly fatality ratio close to 1 and in relation to other types of cancer an especially poor prognosis (52). This is illustrated by the fact that worldwide HCC has the 5th highest cancer incidence but the 3rd highest cancer mortality (52). In Europe, which is considered a low-endemic area, HCC was estimated to be the 14th most common cancer in 2006 but had the 7th highest mortality (53).

Overall survival of the largest consecutive German series of HCC patients, a source of HCC tumor samples used in this work, is shown in **Figure 2**. Corresponding survival rates are given in **Table 2.**

In general, there is considerable variation in survival rates reported in different studies. Thus, consecutive clinical series in low-endemic regions as Austria (10) or the United States (54) lie in the 2-10 months range, while other consecutive studies from Turkey (55) and Portugal (56) report between 17 and 24 month survival rates. The highest overall median HCC survival rates are found in studies of “selected“ HCC populations e.g. in an Italian study (25.7 months) of “early-intermediate” tumors identified in a HCC prevention program (57), and in Taiwan (26.8 months) in a cohort of patients undergoing hepatic resection (58).

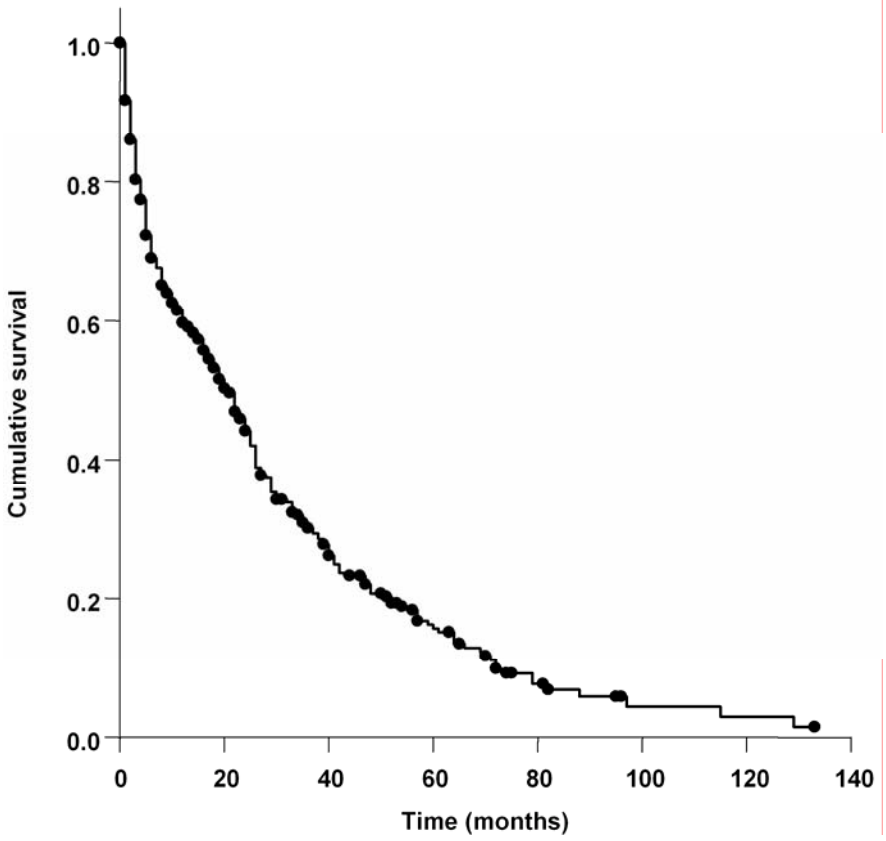

Figure 2: Kaplan-Meier survival curve of HCC patients. Data reflect 458 consecutive patients with HCC, both treated and untreated. Median survival was 19 months (95% CI: 15.3-22.7).

Survival rate at	Percentage of patients living
1-year after diagnosis	59 %
2-years after diagnosis	45 %
3-years after diagnosis	30 %
4-years after diagnosis	21 %
5-years after diagnosis	16 %

Table 2: 1-, 2-, 3-, 4- and 5- year survival rates of HCC patients at the University Hospital Regensburg

1.2 Methylthioadenosine phosphorylase (MTAP) and methylthioadenosine (MTA)

The molecular pathogenesis of HCC is not well understood. Studies have described many genomic aberrations and epigenetic modifications in gene expression in HCC but the relative importance and the precise role in the sequence of disease progression of many of these abnormalities are still to be elucidated (59;60). Methylthioadenosione phosphorylase (MTAP or 5´-deoxy- 5´-methylthioadenosine orthophosphate methylthio-ribosyltraexpressionnsferase) is one of the genes that has recently been implicated in HCC progression (61;62).

1.2.1 The human MTAP gene

The human MTAP gene (EC 2.4.2.28) resides on the short arm of chromosome 9, in the chromosomal region 9p21, from 21792543 to 22111094 (5´→ 3´). It consists of eight exons and seven introns (**Figure 3**).

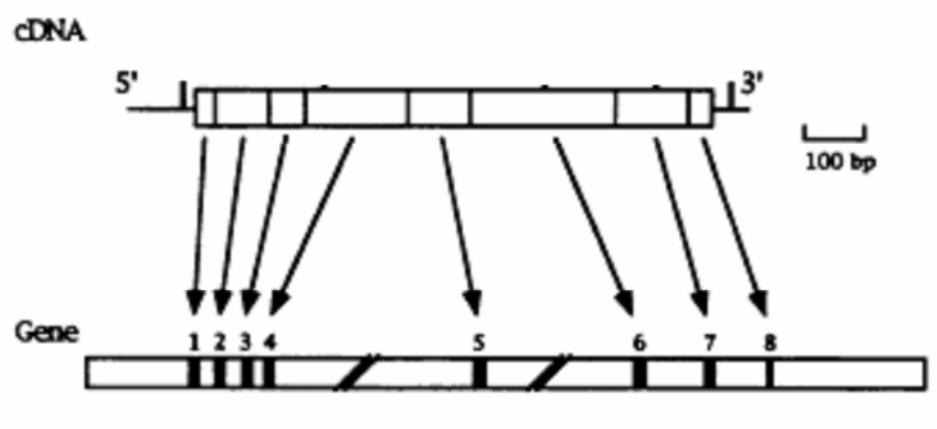

Figure 3: Intron-Exon structure of the human MTAP gene. Modified from (63)

Exon 1 encodes 11 amino acids and the 5' noncoding region, the sizes of exons 2-7 range from 79 to 240 bp. The last (8th) exon encodes the C-terminal 12 amino acids and the 3' noncoding region (63).

Biochemical and structural evidence indicate that human (and mammalian) MTAP protein is a trimer consisting of three identical subunits of 32 kDa that in humans contain 283 amino acid residues (64).

1.2.2 Functions of MTAP

MTAP catalyzes the phosphorylation of 5´-deoxy-5´-(methylthio)adenosine (MTA) which is formed as a by-product of polyamine synthesis to yield adenine and methylthioribose-1-phosphate (MTR-1P), (**Figure 4**).

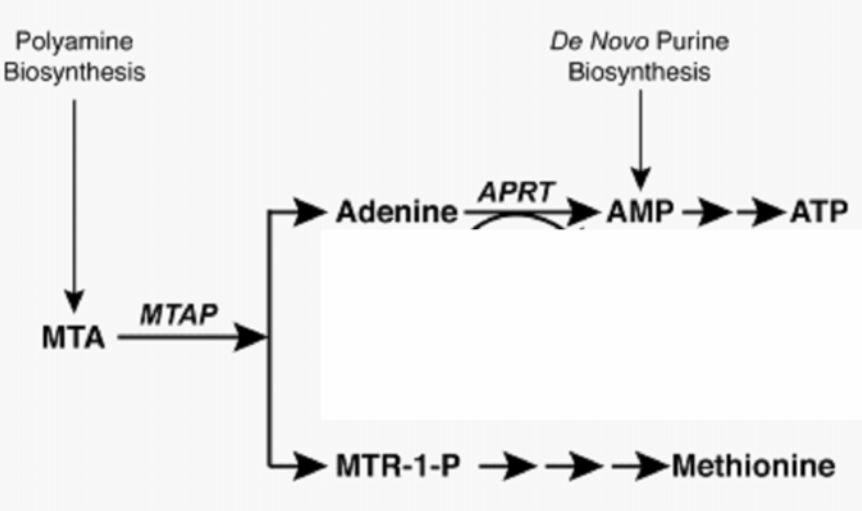

Figure 4: Schematic presentation of the MTAP metabolic pathway. Modified from (65)

Adenine is used to replenish the AMP and ATP pools while MTR-1P is converted in a series of reaction to regain methionine (66;67). Notably, MTAP is the rate-limiting step in these salvage pathways.

Besides its role in regaining adenine and methionine, MTAP is important for the proper functioning of polyamine metabolism. Loss of MTAP has been shown to cause a significant decrease in intracellular polyamine levels and alters the ratio of putrescine to total polyamines in vitro (68). The mechanism involves the accumulation of MTAP´s substrate, methylthioadenosine (MTA) which in higher concentrations acts as a potent inhibitor of spermine and spermidine synthase, two key enzymes of the polyamine synthetic pathway (69).

1.2.3 MTAP expression in normal and cancerous cells and tissues

Given the fundamental role of MTAP in the cellular processes outlined above, it is not surprising that MTAP is abundantly expressed in a wide range of healthy cells and tissues (70). Baseline MTAP expression in the liver is relatively high in comparison to other organs, which can be explained by the key role of the liver in methionine metabolism (71).

However, in cancer, MTAP expression abnormalities have been described by many studies. These are caused by both genomic and epigenetic changes. Partial or complete deletions of MTAP are quite frequent in a wide range of tumors including leukaemia (72), lung cancer (73), pancreatic adenocarcinoma (74;75), osteosarcoma (76), endometrial cancer (77). In contrast, epigenetic mechanisms are responsible for MTAP downregulation in melanoma and HCC (62;78) as well as lymphoma (79). However, MTAP may also be up-regulated in cancer as shown by a recent study in human colon carcinoma (80).

Generally, by far the most common MTAP abnormality in cancer remains a deletion of MTAP gene. This can be explained by the location of the MTAP gene in the 9p21 chromosomal region which exhibits frequent deletions in a variety of human malignancies. This region contains, starting from the centromeric end, the genes p15—p16—MTAP—IFN-a—IFN-b (70). The former two genes encode for two cyclin-dependent kinase inhibitors which are recognized tumor suppressor genes (81). Co-deletions of MTAP and one or more of these genes are common (82;83), and the co-deletion frequencies vary between 35 and 70 % depending on the kind of tumor (65). This raises the question whether the observed MTAP deficiency in cancer has any relevant cancer-promoting effect on its own or is an associated feature of p15 and p16 tumor suppressor deletions.

One argument in favour of a unique tumor-promoting role of MTAP deficiency was the finding that loss of MTAP activity results in the activation of ornithine decarboxylase (ODC). This effect is probably mediated by 2-keto-4-methylthiobutyrate (MTOB) an intermediate in the methionine salvage pathway

(69). Elevated ODC activity is long recognized for its cancer-promoting effect in-vitro and in vivo (84;85). Further, in HCC ODC activity was shown to be correlated with the degree of malignancy (86;87).

Another relevant contribution suggesting a unique tumor-promoting role of MTAP deficiency was the discovery that MTAP deficiency results in higher MTA concentrations in vivo in melanoma and that MTA induces a number of pro-tumorigenic effects e.g. increased migration of cancer cells (88).

1.2.4 Clinical implications of MTAP deficiency for cancer therapy

After it was clear that MTAP is absent in a high percentage of tumors much of the research has focused on the possibilities to exploit this therapeutically. Initial experiments showed that inhibitors of *de novo* purine synthesis, methotrexate or azaserine, which are very commonly used chemotherapeutic agents, in combination with MTA, selectively killed MTAP-negative cells, while sparing MTAP-positive cells as the latter could derive their purine nucleotide requirements from MTA (89). A number of studies that followed up on this proposal examined other inhibitors of *de novo* purine synthesis, such as 5,10-dideazotetrahydrofolate and L-alanosine, an inhibitor of AMP synthesis from IMP (90).

Based on this promising in-vitro data several Phase I and II clinical trials with L-alanosin were started, however the treatment was considered ineffective (91;92).

In a different approach, it was proposed that if serum were depleted of methionine by a methioninase, normal cells, but not MTAP-negative tumor cells, could be rescued by providing MTA, which is cleaved to MTR-1-P and leads to methionine synthesis (93). To the best of our knowledge, there have been no reported clinical trials of this proposed therapy.

Finally, a very recent study suggested a combination therapy of MTA and a toxic adenine analog, e.g. 2-fluoroadenine (65). In MTAP-positive cells, abundant adenine, generated from supplied MTA, competitively blocks the conversion of an analog by adenine phosphoribosyltransferase (APRT), to its active nucleotide form. In MTAP-negative tumor cells, the supplied MTA cannot generate adenine;

hence conversion of the analog is not blocked. The study found that this combination therapy is effective in MTAP-deficient human breast and lung cancer cell lines, while fibroblasts expressing MTAP were protected (65).

1.2.5 Regulation of MTAP

Data on the regulation of MTAP are rather scarce and inconclusive.

As explained above, in most cases the loss of MTAP activity or mRNA is the result of homozygous deletions of the *MTAP* gene. The presence of a CpG island proximal to the transcription start site of the MTAP promoter suggests a transcriptional regulation of the gene through a methylation/demethylation mechanism (78).

This notion was confirmed in HCC (hepatocellular carcinoma) cell lines showing down-regulation of *MTAP* gene expression that could not be attributed to genomic losses or mutations but to promoter hypermethylation (62). As already mentioned, this mechanism is also present in a share of melanomas and lymphomas (78;79).

Additionally, it has been found that the transcriptional activation of the human MTAP gene is mediated by the binding of the CCAAT binding factor to a distal CCAAT motif in the promoter (94).

Besides the regulation at the transcriptional level, there is also evidence suggesting modulation of MTAP activity. Phosphorylation of Ser183 and Thr188 residues have been recently reported, although the biological significance of these modifications must be demonstrated (95).

Oxidation also arises as a mechanism to modulate MTAP activity as suggested by the requirement of the enzyme for thiol-reducing agents and its specific and rapid inactivation by thiol-blocking groups (96).

1.2.6 5´-Methylthioadenosine – a key player at a biochemical crossroads

It is plausible that alterations in MTAP activity as described in cancer may be associated with altered levels of MTAP´s substrate, 5´-methylthioadenosine (MTA). This was confirmed for melanoma cell lines and tissues where higher

MTA levels induced a number of pro-tumorigenic changes in melanoma cells (88).

The natural existence of MTA has been known for almost a century now (66) and its molecular structure was reported in 1924 (71).

MTA is a hydrophobic sulfur-containing adenine nucleoside in which the hydroxyl group in the 5´ position of the ribose is substituted by a methylthio moiety (**Figure 5**). This methylthio moiety is derived from the amino acid methionine, while the rest of the molecule comes from ATP.

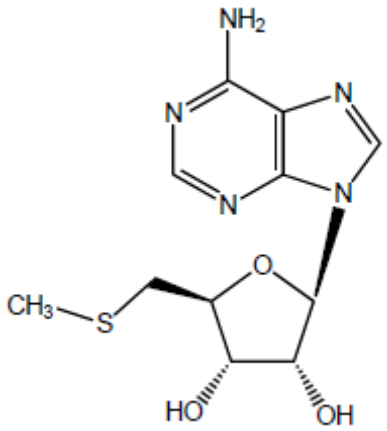

Figure 5: Structure of 5´-methylthioadenosine (MTA).

The major source of MTA is, as mentioned, polyamine biosynthesis which occurs in all mammalian cells, and starts with the decarboxylation of S-adenosylmethionine (SAM) by the enzyme SAM decarboxylase (SAMDC) **(Figure 6)**. Decarboxylated SAM (dSAM) is the immediate precursor of MTA. It is a substrate for the aminopropyltransferases. These enzymes transfer the aminopropyl group of dSAM to putrescine forming spermidine (spermidine synthase), and subsequently to spermidine forming spermine (spermine synthase), with MTA forming as a “by-product“ in both reactions.

MTA is metabolized by MTAP to adenine and 5-methylthioribose-1-phosphate (MTR-1P). Adenine is needed to ultimately replenish the AMP and ATP pools while MTR-1P undergoes a complex set of oxidations to give 2-keto-4-

methylthiobutyrate (MTOB), which is finally transaminated to methionine. Consequently, the two metabolites from which SAM and MTA are formed, namely methionine and ATP, are thus recovered (66;71)

As polyamine synthesis is a vital ubiquitous cellular process and since MTA is mainly produced during polyamine synthesis it is not surprising that MTA is found in small amounts in all mammalian tissues (and also in prokaryotes, yeast, plants and higher eukaryotes) (97).

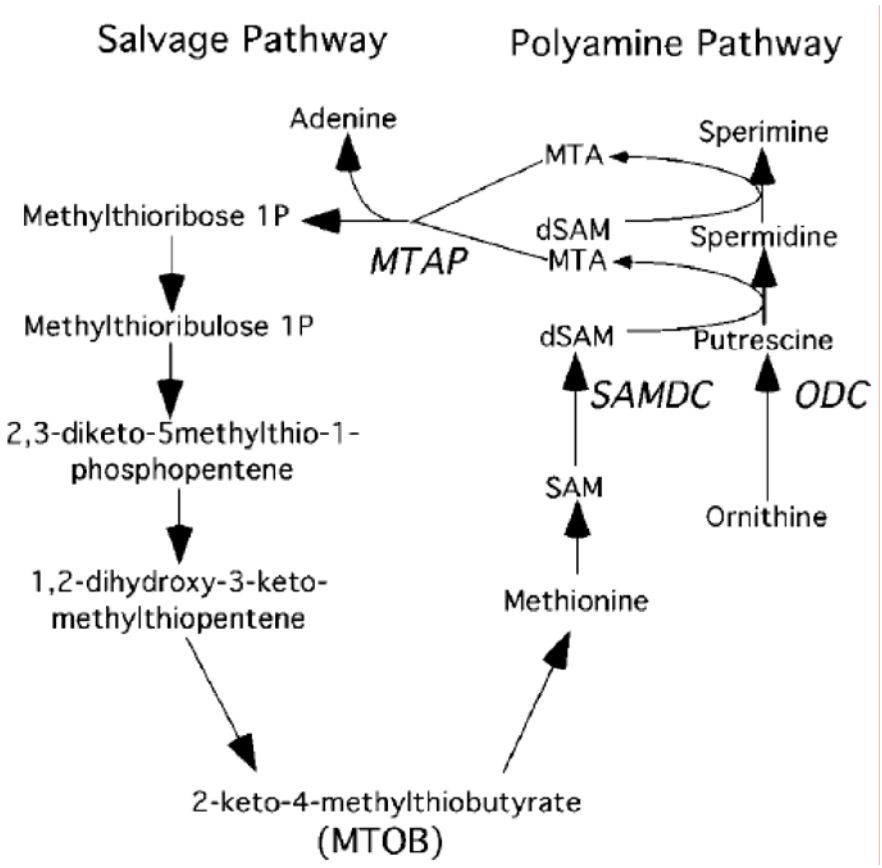

Figure 6: Methionine salvage and polyamine synthesis pathways**.** MTA is located at their crossroads forming as a by-product of the latter and being the initial substrate of the former. Enzymes are shown in italics; SAM, S-adenosylmethionine; SAMDC, S-adenosylmethionine decarboxylase, ODC, ornithine decarboxylase. Modified from (69).

1.2.7 Biological effects of MTA

MTA has notable influence on fundamental cellular functions. Earlier studies have focused on MTA effects on lymphocytes showing that MTA inhibits several

lymphocyte functions. Mitogen-stimulated activation of murine and human lymphocytes (98;99), lymphocyte-mediated cytolysis (100) and natural killer cell mediated cytotoxicity (66) are strongly inhibited by high (>1000 µM) concentrations of MTA. A later study showed that in a murine pre-B cell line MTA at a concentration of 2000 µM impairs LPS induced NFkB signaling (101).

There are convincing studies that MTA inhibits spermine synthase and to a lesser extent spermidine synthase and ODC (97;102).

More arguable are effects regarding some tumor-related cellular functions. Depending on the type of cell analyzed, and the MTA concentration used, studies describe various, partly opposite effects

In cultured melanoma cell lines for example, exogenous addition of 50-100 µM MTA caused up-regulation of tumor-promoting genes and enhanced invasiveness and vasculogenic mimicry while no similar genes up-regulation was observed in normal melanocytes (88).

However, decreased rather than increased invasiveness induced by MTA were suggested another study examining two rat ascites hepatoma cell lines, possibly due to alterations in the phospholipid composition and fluidity of the tumor cell membranes (103).

MTA effects on apoptosis are another controversial issue. A recent study observed that SAM as well as MTA at a concentration of 4000 µM protected rat hepatocytes from okadaic-acid induced apoptosis (104). However, at a lower concentration, MTA induced apoptosis in the human HCC cell lines Huh7 and HepG2. It is unclear whether this represents a differential effect on normal vs. malignant cells as cells originated from different species (murine vs. human) and different concentrations were used.

1.3 Aim of thesis

Based on previous *in vitro* and *in vivo* studies in our group (62) suggesting functional role of MTAP in HCC progression this work aimed to

(1) expand this analysis to human HCC tissue and establish associations of MTAP down-regulation with clinicopathological features of HCC tumors

(2) examine whether MTAP expression correlates with MTA concentrations in human liver cell lines and tissues

(3) elucidate the molecular mechanism of the tumor-promoting role of MTAP deficiency by analyzing the effects of MTA on tumor cells and their microenvironment.

2 Materials and Methods

2.1 Chemicals and reagents

Agarose SeaKem® LE	Biozym, Hess/Oldendorf, Germany
β-Mercaptoethanol	Sigma, Deisenhofen, Germany
DMEM medium	PAA Laboratories, Cölbe, Germany
DMSO	Sigma, Deisenhofen, Germany
FCS (fetal calf serum)	PAN-Biotech, Aidenbach, Germany
Milk powder	Carl Roth, Karlsruhe, Germany
Penicillin	Invitrogen, Karlsruhe, Germany
Streptomycin	Invitrogen, Karlsruhe, Germany
Trypsin/EDTA	PAA Laboratories, Cölbe, Germany

All chemicals not listed were purchased at VWR (Darmstadt, Germany).

2.2 Laboratory expendables

CryoTube vials	Nunc, Roskilde, Denmark
Pipet tips (10, 20, 100 und 1000 µl)	Eppendorf, Hamburg, Germany
Falcon tubes (50 ml)	Corning, New York, USA
glassware (various)	Schott, Mainz, Germany
Multiwell plates	Corning, New York, USA
Pipettes (stripettes®) (5, 10, 25, 50 ml)	Corning, New York, USA
Reaction vessels (1.5 and 2 ml)	Eppendorf, Hamburg, Germany
Stripe tubes (0.2 ml)	Peqlab, Erlangen , Germany
Cell culture flasks T25, T75, T175	Corning, New York, USA

2.3 Laboratory instruments

Heating block:	
Thermomixer comfort	Eppendorf, Hamburg, Germany
PCR-cycler:	
GeneAmp® PCR System 9700	Applied Biosystems, Foster City, USA
Pipettes:	
Gilson (P2, P20, P200, P1000)	Gilson, Bad Camberg, Germany
Pipette controllers:	
Accu-jet®	Brand, Wertheim, Germany
Shaking devices:	
KS 260 Basic Orbital Shaker	IKA® Werke, Staufen, Germany
Power Supplies:	
Consort E145	Peqlab, Erlangen, Germany
Power Supply-EPS 301	Amersham Biosiences, Munich, Germany
Spectrophotometer:	
EMax® Microplate Reader	MWG Biotech, Ebersberg, Germany
SPECTRAFluor Plus Microplate Reader	Tecan, Männedorf, Switzerland
Scale:	
MC1 Laboratory LC 620 D	Sartorius, Göttingen, Germany
Water bath:	
Haake W13/C10	Thermo Fisher Scientific, Karlsruhe, Germany
Centrifuge:	
Biofuge fresco	Heraeus, Hanau , Germany
Megafuge 1.0 R	Heraeus, Hanau, Germany
Microscope:	
Olympus CKX41 with ALTRA20 soft imaging system	Olympus Hamburg, Germany

2.4 Buffers

PBS-Puffer	140 mM	NaCl	
	10 mM	KCl	
	6.4 mM	Na_2HPO_4	
	2 mM	KH_2PO_4	pH 7.4
TE-Puffer	10 mM	Tris/HCl	
	1 mM	EDTA	pH 8.0

2.5 Cell Culture

2.5.1 Cell culture medium

DMEM (high glucose/10%FCS)	4.5 g/l	Glucose
	300 µg/ml	L-Glutamine
	Supplemented with:	
	10% (v/v)	FCS
	400 U/ml	Penicillin
	50 µg/ml	Streptomycin
HSC medium	DMEM (high glucose/10%FCS)	
	Supplemented with:	
	10 µg/ml	Diflucan
	4 µg/ml	Ciprobay

2.5.2 Cultivation of cell lines

All cell culture work was conducted within a laminar flow biosafety cabinet (Hera Safe, Heraeus, Osterode, Germany). The cells were cultivated in a Binder series CB incubator (Binder, Tuttlingen, Germany) in 10% CO_2 atmosphere at 37 °C. DMEM containing 4.5 g/l glucose and 300 µg/ml L-glutamine supplemented with

10% (v/v) FCS, 400 U/l penicillin and 50 µg/ml streptomycin was used as cell culture medium. For cell passaging adherent cells were washed with PBS and detached with trypsin (0.05%)/EDTA (0.02%) (PAA Laboratories, Cölbe, Germany) at 37 °C. Trypsination was stopped by addition of DMEM containing 10% FCS. Subsequently, cells were centrifuged at 500 g for 5 min, and the obtained cell pellet was resuspended in fresh culture medium and distributed to new cell culture flasks achieving a cell density thinning factor of 5 to 10. Medium change took place every second day. Cell growth and morphology were controlled and documented with a microscope. Cell culture waste was autoclaved before disposal with a Sanoclav autoclave (Wolf, Geislingen, Germany).

2.5.3 Human hepatocellular carcinoma cell lines

The hepatocellular carcinoma cell lines PLC (ATCC CRL-8024) HepG2 (ATCC HB-8065) and Huh7 (JCR B0403) were obtained from the American Type Culture Collection (ATCC) and the Japanese Collection of Research Biosources (JCRB), respectively.

2.5.4 Isolation of primary human hepatocytes

Primary human hepatocytes (PHH) were isolated in co-operation with the Center for Liver Cell Research (Department of Surgery, University of Regensburg, Germany) from human liver resections using a modified two-step EGTA/collagenase perfusion procedure (105-109). The experimental procedures were performed according to the guidelines of the charitable state controlled foundation HTCR, with the informed patient's consent. For cell isolation only tissue which has been classified as not pathological after macroscopical and microscopical investigation was used. All used liver resections have been negatively tested for HBV, HCV and HIV infection. Viability of the isolated hepatocytes was determined by trypan blue exclusion, and cells with viability greater than 85% were used for further tests.

2.5.5 Isolation of human hepatic stellate cells

Human hepatic stellate cells (HSC) were isolated in co-operation with the Center for Liver Cell Research (Department of Surgery, University of Regensburg, Germany). After perfusion and separation of hepatocytes by an initial centrifugation step at 50 g (5 min, 4 °C) the supernatant containing the non-parenchymal cells was centrifuged at 700 g for 7 min (4 °C). The obtained cell pellet was resuspended in HSC medium, and cells were seeded into T75 flasks. Within the first week, the medium was replaced daily. From the second week on medium change took place every 2-3 days. By cultivation on uncoated plastic HSC activate within the first 2 weeks and transdifferentiate to myofibroblast-like cells. Liver disease mediated HSC activation can be simulated *in vitro* that way. After 2 weeks the cell culture was split 1:3 by incubating the cells with Trypsin (0.05%)/EDTA (0.02%) solution. Thereby, only HSC detach, whereas Kupffer cells remain adherent to the plastic surface. Therefore, after the first passage only activated HSC remain in the cell culture which was confirmed by previously done analysis (110).

2.5.6 Determination of cell number and viability

Cell number and viability was determined by trypan blue exclusion test. The cell suspension was diluted 1:2 with trypan blue solution (Sigma, Deisenhofen, Germany) and applied on a Neubauer hemocytometer (Marienfeld GmbH, Lauda-Königshofen, Germany). Cells with impaired cell membrane integrity are stained blue, and therefore, can be clearly distinguished from intact cells which appear white under microscopic inspection. The cell number could be calculated after counting cells in all four quadrants of the hemocytometer, each containing sixteen smaller squares, with the following equation:

$$\textit{Cell number/ml} = Z \times DF \times 10^4 \div 4$$

Z = counted cell number in all four quadrants

DF = dilution factor (in the described procedure the factor is 2)

The ratio of viable cells could be determined by setting the number of unstained cells in relation to the total cell number (blue und unstained cells).

2.5.7 Transfection of PLC cells with MTAP siRNA

MTAP siRNAa (numbered 1, 2, 3 and 4), negative control siRNA and HiPerFect Transfection Reagent® were purchased from Qiagen (Hilden, Germany).

Transfection of PLC cells was performed after the Qiagen fast-forward siRNA transfection protocol.

Shortly before transfection, 200,000 cells per well were seeded on a 6-well plate in 2,300 µl of DMEM culture medium containing 10 % fetal calf serum. 150 ng siRNA per well was diluted in 100 µl DMEM without serum and 12 µl of HiPerFect Transfection Reagent was added to the diluted siRNA and mixed by vortexing. The samples were incubated for 10 min at room temperature to allow the formation of transfection complexes which were then added drop-wise onto the cells. The plates were gently swirled to ensure uniform distribution of the transfection complexes. After 24 hours cell medium was changed and the cells were grown for another 24-72 hours (according to individual experimental set-up) and successful gene silencing was documented on the mRNA and protein levels by quantitative RT-PCR and Western blotting.

2.5.8 Collection of conditioned medium from MTAP silenced HCC cells

For the purpose of studying potential effects of MTAP-deficient HCC on HSC cells *in-vitro*, transfection of HCC cells with MTAP siRNA was performed as described in **2.5.7.** 24 hours after transfection HCC cells were washed twice with serum-free DMEM, and then incubated for 24 h with serum-free DMEM (1 ml per one well of a six-well plate) which was then collected and stored at -80° C until use. HCC cells transfected with negative control siRNA were used to generate control conditioned medium under otherwise identical conditions.

2.6 Human tissues and HCC tissue microarray (TMA)

Paired HCC and non-neoplastic liver tissues were obtained from HCC patients undergoing surgical resection in cooperation with the Center for Liver Cell Research, Department of Surgery, University Hospital Regensburg. Tissue

samples were immediately snap frozen and stored at -80°C until subsequent analysis. Human liver tissue was obtained and experimental procedures were performed according to the guidelines of the charitable state controlled foundation HTCR, with the informed patient's consent.

The immunohistochemical results presented in this work are based on a tissue microarray, which was described before (111). Generally, in the tissue microarray technique, a hollow needle is used to remove small tissue cores from both cancerous and adjacent non-cancerous regions in paraffin-embedded tissues such as clinical biopsies. These tissue cores are then inserted in a recipient paraffin block in a precisely spaced, array pattern. Sections from this block are cut using a microtome, mounted on a microscope slide and then analyzed by any method of standard histological analysis

For the presented HCC tissue microarray, immunohistochemical staining of 5-µm sections of the TMA blocks was performed using a monoclonal mouse anti-human MTAP antibody (ab55517, abcam, Cambridge, UK) at a dilution of 1:500 and an indirect immunoperoxidase protocol according to the LSAB2-kit (Dako, Hamburg, Germany). For negative control, the primary antibody was omitted and IgG isotype control antibodies did not reveal any detectable staining. For analysis of the tissue microarray, positivity for MTAP was defined as any detectable staining, while cases designated as MTAP negative did not reveal any immunohistochemical staining for MTAP.

2.7 MTA extraction and analysis

For analysis of MTA in cell culture medium, cells were cultured in serum free medium for 24 h. Subsequently, medium was collected, centrifuged, and the supernatant was snap-frozen and stored at -80°C. Further, cell number in corresponding cell culture plates was determined by counting the trypsinised cells.

For intracellular MTA measurements, cells were harvested by incubation in a solution containing 0.05% (w/v) trypsin and 0.02% (w/v) EDTA. Trypsination was stopped after 5 min with cell culture medium. Following centrifugation, the supernatant was removed, the cell pellet was washed with PBS buffer,

centrifuged again, snap-frozen and stored at -80°C.

Samples were further processed by the Institute of Functional Genomics, University of Regensburg, as described in greater detail in (112;113). Briefly, cell culture medium was spiked with stable isotope labeled MTA, dried by means of an infrared evaporator, and the residues reconstituted in 0.1 mol/L acetic acid. Frozen cell pellets were extracted by three repeated freeze/thaw cycles in 600 µL of MeOH / 0.1 mol/L acetic acid (80:20, v/v) after the addition of stable isotope labeled MTA. After centrifugation the supernatant was transferred in a glass vial and the protein pellet was washed twice with MeOH/acetic acid. The combined extracts were dried and reconstituted in 0.1 mol/L acetic acid.

Tissue samples were weighed and then homogenized in 600 µL of MeOH/ 0.1 mol/L aqueous acetic acid (80:20, v/v) using “Precelly-Keramik-Kit 1.4 mm” vials (Peqlab Biotechnologie GmbH, Erlangen, Germany). The samples were centrifuged at 9,000 g for 5min at 4°C. Subsequently, the supernatant was transferred to a 1.5 mL glass vial and the pellet was washed twice. After solvent evaporation, the residues were reconstituted in 0.1 mol/L acetic acid.

Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) was carried out as described (113). The analysis was performed using an Agilent 1200 SL HPLC system (Böblingen, Germany) and a PE Sciex API 4000 QTrap mass spectrometer (Applied Biosystems, Darmstadt, Germany). An Atlantis T3 3 µm (2.1mm i.d.×150mm) reversed-phase column (Waters, Eschborn, Germany) was used. LC separation was carried out using a water-acetonitrile gradient consisting of 0.1% acetic acid and 0.025% HFBA in both solvents at a flow-rate of 400 µL/min with an injection volume of 10 µL. The API 4000 QTrap mass spectrometer was operated in positive mode and quantitative determination was performed with multiple reaction monitoring (MRM).

2.8 Isolation and analysis of RNA

2.8.1 RNA isolation and determination of RNA concentration

RNA isolation was performed with the RNeasy® mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The principle of RNA

isolation is based on the adsorption of RNA to hydrophilic silicon-gel membranes in presence of suitable buffer systems. Biological samples were first lysed and homogenized in the presence of a highly denaturing guanidine isothiocyanate containing buffer, which immediately inactivates RNases to ensure isolation of intact RNA. To homogenize tissue samples a hand-held rotor-stator homogenizer (Xenox Motorhandstück 2.35 with a Roti®-Speed-Rührer Ø 7 mm, Carl Roth, Karlsruhe, Germany) has been used. After lysis, ethanol has been added to provide ideal conditions for the binding of RNA to the silica-gel membranes. Contaminants have been washed away with suitable buffers before RNA was eluted in water and stored at -80 °C. The concentration of RNA was measured with the NanoDrop® ND-1000 UV/VIS spectrophotometer (Peqlab, Erlangen, Germany).

2.8.2 Reverse transcription of RNA to cDNA

Transcription of RNA to complementary DNA (cDNA) was conducted with the Reverse Transcription System Kit (Promega, Mannheim, Germany) which uses avian myeloblastosis virus reverse transcriptase (AMV RT). The working solution was pipetted with contamination-free aerosol filter pipet tips after the following pipetting scheme:

0.5 µg	RNA
5 µl	$MgCl_2$ (25 mM)
2.5 µl	10x reverse transcription buffer
2.5 µl	dNTP mix (10 mM)
1.25 µl	random primer
0.625 µl	RNasin ribonuclease inhibitor
ad 25 µl	$H_2O_{dest.}$

For reverse transcription the samples have been incubated in a GeneAmp® PCR cycler (Applied Biosystems, Foster City, USA) for 30 min at 42 °C. For denaturation of the AMV RT the temperature has been raised to 99 °C for 5 min.

After cooling down to 4 °C the obtained cDNA was diluted with 75 µl $H_2O_{dest.}$ and used immediately or stored at -20 °C.

2.8.3 Quantitative real time polymerase chain reaction

To quantify the expression of specific mRNA, quantitative real time polymerase chain reaction (qRT-PCR) has been performed with the LightCycler II system (Roche Diagnostics, Mannheim, Germany). The qRT-PCR is principally based on a conventional polymerase chain reaction (PCR), but offers the additional possibility of quantification, which is accomplished by fluorescence measurements at the end and/or during a PCR cycle. As fluorescent reagent SYBR® Green (QuantiTect SYBR® Green PCR Kit, Qiagen, Hilden, Germany) has been used. SYBR® Green intercalates with double-strand DNA whereby the fluorescence emission rises significantly. Therefore, the fluorescence signal increases proportionally with the amount of PCR products. To quantify the expression of a specific gene of interest, the results have been normalized to the housekeeper gene β-actin for human samples and to 18s rRNA for murine samples, respectively. The results were evaluated with the LightCycler software version 3.5 (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions. The qRT-PCR was performed according to the QuantiTect® SYBR® Green PCR Master Mix protocol (Qiagen, Hilden, Germany):

10 µl	QuantiTect® SYBR® Green PCR Master Mix
2 µl	cDNA
0.5 µl	forward primer (20 µM)
0.5 µl	reverse primer (20 µM)
7 µl	$H_2O_{dest.}$

The following standard scheme has been used and adapted to particular primer melting point temperature:

Initial denaturation:	20 °C/s to 95 °C, 900 s

Three step PCR:	20 °C/s to 95 °C, 15 s
	20 °C/s to 55-65 °C, 20 s, 40 cycles
	20 °C/s to 72 °C, 20 s
Analysis of melting curve:	20 °C/s to 95 °C, 0 s
	20 °C/s to 65 °C, 15 s
	0.1 °C/s to 95 °C, 0 s
	20 °C/s to 40 °C, 30 s

For validation, 5-10 µl of the PCR product has been mixed with loading buffer (Peqlab, Erlangen, Germany) and loaded on a agarose gel with ethidium bromide (50 µg/100 ml gel) to determine the PCR product length. Each experimental condition was performed in triplicates and experiments were repeated at least three times.

Primers were synthesized by SIGMA Genosys (Hamburg, Germany). The lyophilized primers were solved in $H_2O_{dest.}$ (SIGMA Genosys primers) and stored at -20 °C.

2.8.4 Sets of primers used for quantitative PCR analysis

name	forward primer	reverse primer
β-actin	5'-CTA CGT CGC CCT GGA CTT CGA GC	5'-GAT GGA GCC GCC GAT CCA CAC GG
MTAP	5´- GCG AAC ATC TGG GCT TTG	5´- GCA CCG GAG TCC TAG CTT C
IL8	5'-TCT GCA GCT CTG TGT GAA GGT GCA GTT	5'- AAC CCT CTG CAC CCA GTT TTC CT
MMP-1	5´- TCA CCA AGG TCT CTG AGG GTC AAG C	5´- GGA TGC CAT CAA TGT CAT CCT GAG C
MMP-9	5´- GAG GTG CCG GAT GCC ATT CAC GT	5´- CCG AGC TGA CTG GAC GGT GAT G
MMP-14	5´- GGA ACC CTG TAG CTT TGT GTC TGT C	5´- TCT CTA CCC TCA AC AAG ATT AGA TTC C
VEGFB	5´- TGG TGG TGC CCT TGA CTG TGG AGC	5´- CTG TCT GGC TTC ACA GCA CTG TCC
FGF-2	5´- AGC CTA GCA ACT CTG CTG CTG ATG G	5´- ACG TAG GAG ACA CAG CGG TTC GAG

Table 3: Primers for qRT-PCR. IL-8, interleukin 8; MMP, matrix metalloproteinase; VEGF, vascular-endothelial growth factor; FGF-2, fibroblast growth factor 2.

2.9 Protein analysis

2.9.1 Preparation of whole cell protein extracts

Used buffer:

RIPA buffer	50 mM	Tris/HCl; pH 7.5
	150 mM	NaCl
	1% (v/v)	Nonidet® P40
	0.5% (w/v)	Sodium desoxycholate
	0.1% (w/v)	SDS

To extract whole cell protein from cell lines the cell culture medium was discarded and cells were washed once with PBS, then scraped off with a cell scraper (Corning, NewYork, USA) and taken up into 1 ml of cooled PBS. After centrifugation (1,700 g, 5 min, 4 °C) the cell pellet was resuspended in 200 µl RIPA buffer and treated with an ultrasonoscope (Sonoplus hp 70, Bandelin electronics, Berlin, Germany) 10 x 3 s at an intensity of 40% for cell lysis. Subsequently, the solved proteins were separated from the non soluble cell components by centrifugation at 20,000 g (10 min, 4 °C). The protein solution was transferred into new reaction tubes and stored at -20 °C.

2.9.2 Determination of protein concentration

To determine the protein concentrations of protein solutions the BCA Protein Assay Kit (Pierce, Rockford, USA) was used. The assay combines the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation Cu^{1+} by bicinchoninic acid (BCA). The first step is the chelation of copper with protein in an alkaline environment to form a blue-colored complex. In this reaction, known as the biuret reaction, peptides containing three or more amino acid residues form a colored chelate complex with cupric ions in an alkaline environment. One cupric ion forms a colored coordination complex with four to six nearby peptides bonds. In the second step of the color development reaction, BCA, a highly sensitive and

selective colorimetric detection reagent reacts with the cuprous cation Cu^{1+} that was formed in step 1. The purple-colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion. The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 562 nm with increasing protein concentrations. 200 µl of alkaline BCA/copper(II) solution (50 parts of solution A mixed with 1 part of solution B) was added to 2 µl of protein solution using a 96-well plate and have been incubated for 1 h at RT. Thereafter the purple color was measured at 562 nm with a spectrophotometer (EMax® Microplate Reader, MWG Biotech, Ebersberg, Germany). The optical absorbance values could be translated into specific protein concentrations by parallel quantification of a BSA standard.

2.9.3 SDS polyacrylamid gel electrophoresis

Used buffers:

Laemmli buffer	62.5 mM	Tris/HCl, pH 6.8
	2% (w/v)	SDS
	10% (v/v)	Glycerine
	5% (v/v)	β-Mercaptoethanol
Running buffer	25 mM	Tris/HCl, pH 8.5
	200 mM	Glycine
	0.1% (w/v)	SDS
10% Resolving gel	7.9 ml	$H_2O_{dest.}$
	5.0 ml	1.5 M Tris/HCl pH 8.8
	0.2 ml	10% (w/v) SDS
	6.7 ml	Acrylamide/Bisacrylamide 30%/0.8% (w/v)
	0.2 ml	Ammonium persulfate 10% (w/v)
	0.008 ml	TEMED

5% Stacking gel	2.7 ml	$H_2O_{dest.}$
	0.5 ml	1.0 M Tris/HCl pH 6.8
	0.04 ml	10% (w/v) SDS
	0.67 ml	Acrylamide/Bisacrylamide (30%/0.8% w/v)
	0.04 ml	Ammonium persulfate 10% (w/v)
	0.004 ml	TEMED

The protein solutions were heated at 95 °C for 5 min in Laemmli buffer and applied on a SDS polyacrylamid gel for protein fractionation by size at 35 mA/150 V (XCell SureLock™ Mini-Cell, Invitrogen, Karlsruhe, Germany). As size marker the Full Range Rainbow Molecular Weight Marker (GE Healthcare, Freiburg, Germany) was used.

2.9.4 Western blotting

Used buffer:

Transfer buffer	10% (v/v)	Methanol
	25 mM	Tris
	190 mM	Glycine

To detect the proteins after SDS-PAGE by use of specific antibodies the proteins were transferred electrophoretically to a nitrocellulose membrane (Invitrogen, Karlsruhe, Germany) at 220 mA/300 V for 1.5 h (XCell II Blot Module, Invitrogen, Karlsruhe, Germany). To block unspecific binding sites, the membrane was bathed in PBS containing 3% BSA or 5% milk powder for 1 h at RT. Then, the membrane was incubated with a specific primary antibody (**Table 4**) over night at 4 °C. After washing, the membrane was incubated with a secondary horseradish peroxidase (HRP) conjugated antibody (**Table 4**) for 1 h at RT.

Primary Antibody	Manufacturing company	Product #	Dilution
Mouse anti-human Actin	Sigma, Deisenhofen	A1978	1:20,000
Mouse anti-human MTAP	Abcam, Cambridge/UK	abcam55517	1:3,000
Secondary Antibody	**Manufacturing company**	**Product #**	**Dilution**
Goat anti-mouse HRP	Santa Cruz, Heidelberg	sc-47047	1:3,000

Table 4: Used primary and secondary antibodies for Western blot analysis; used dilution; manufacturing company. Secondary antibodies are conjugated with horseradish peroxidase (HRP)

Thereafter, the membrane was washed with PBS and incubated with the ECL Plus Western Blotting Detection System (GE Healthcare, Freiburg, Germany) for 1 min. This system utilizes chemiluminescence technology for the detection of proteins. It consists of the acridan substrate Lumigen PS-3, which is converted to an acridinium ester intermediate when catalyzed by HRP. The ester intermediate reacts with peroxide in alkaline conditions and emits light, which was detected by autoradiography using a Biomax film (Kodak, Stuttgart, Germany) and a Curix 60 automatic film developer (Agfa, Cologne, Germany). All incubation steps were done on a KS 260 Basic Orbital Shaker (IKA®; Staufen, Germany). Western blot experiments were repeated at least three times.

2.10 Cell-based functional assays

2.10.1 XTT-proliferation assay

Cell proliferation was quantified with the XTT kit (Roche Diagnostics, Mannheim, Germany). The assay is based on the ability of metabolic active cells to reduce the tetrazolium salt XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[phenylamino)carbonyl]-2H-tetrazolium hydroxide) to orange colored compounds of formazan. The dye formed is water soluble and dye intensity can be read with a spectrophotometer at a wavelength of 450 nm and a reference absorbance

wavelength of 650 nm. The intensity of the dye is proportional to the number of metabolic active cells.

To quantify the effects of certain reagents on cell proliferation, cells were seeded in 96-well tissue culture plates (4,000 cells per well) and incubated for 4 h in medium containing 10% FCS. Thereafter, medium was taken off and cells were starved for 16 hours in FCS free medium. After a further medium change and addition of specific stimulatory reagents, cells were incubated for different time intervals (1-7 days). At the chosen time points XTT reagent was added and the intensity of the forming dye was measured two hours thereafter with an EMax Microplate Reader (MWG Biotech, Ebersberg, Germany). Values of optical density (OD) at individual time points were corrected for background by subtracting the OD value of a blank well without cells. Each experimental condition was performed in triplicate and experiments were repeated three times.

2.11 Statistical analysis

Values are presented as mean ± standard error (SEM). Comparisons between groups were made using the unpaired t-test (or one-way ANOVA with Bonferroni correction for more than 2 groups) or in the case of comparisons between HCC/non-HCC pairs using the paired t-test. Fisher's exact test was used to study the statistical association between clinicopathological and immunohistochemical variables. A p-value < 0.05 was considered statistically significant. Calculations were performed using the statistical computer packages SPSS 15.0 and GraphPad Prism 9.

3 Results

3.1 MTA concentration in human HCC cell lines and tissues

Previously our group had shown that *MTAP* expression is strongly reduced in human HCC cell lines and tissues as compared to primary human hepatocytes (PHH) and non-tumorous tissue (62).

One of the aims of this thesis was to analyze whether this downregulation of *MTAP* affected MTA levels in HCC. Liquid chromatography tandem mass spectrometry (LC-ESI-MS/MS) revealed significantly higher MTA levels in HCC as compared to non-tumorous liver tissue (**Figure 7**).

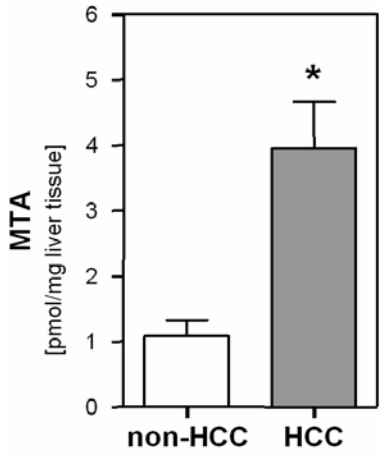

Figure 7: MTA concentration in human HCC tissue.
MTA levels in human HCC tissues (HCC) compared to adjacent non-tumorous liver tissues (non-HCC). 14 paired samples were analyzed.
(*: P≤0.05 compared to non-HCC)

Further, higher amounts of MTA were found in lysates of 4 different HCC cell lines as compared to PHH (**Figure 8A**).

Levels of MTA were also higher in the corresponding supernatant of HCC cell cultures (**Figure 8B**).

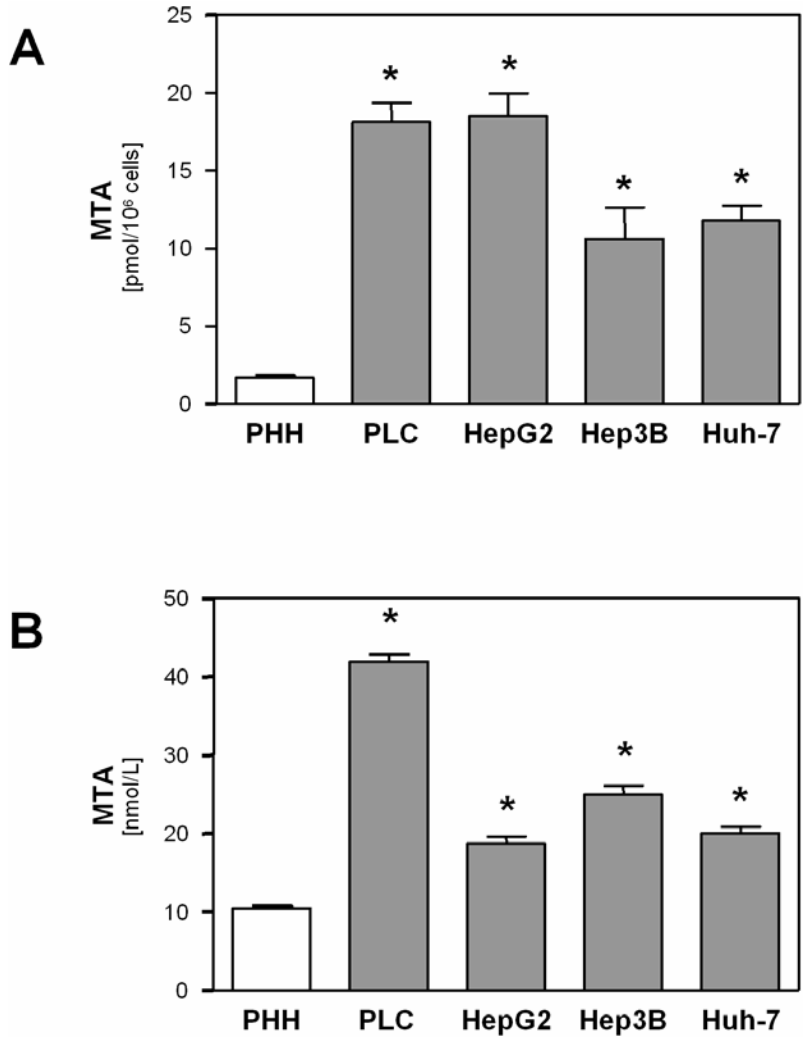

Figure 8: MTA concentration in human HCC cell lines.
MTA levels in cell lysates **(A)** and cell supernatants **(B)** of primary human hepatocytes (PHH) and the HCC cell lines PLC, HepG2, Hep3B and Huh-7. Cells were cultured for 24 h in FCS-free DMEM.
(*: P≤0.05 compared to PHH)

To confirm that MTA accumulation in HCC cells is caused by downregulation of *MTAP* we analyzed HCC cells that had been transfected transiently with *MTAP* siRNA. *MTAP* suppression led to higher MTA concentrations both in cell lysates (**Figure 9A**) and in supernatants (**Figure 9B**).

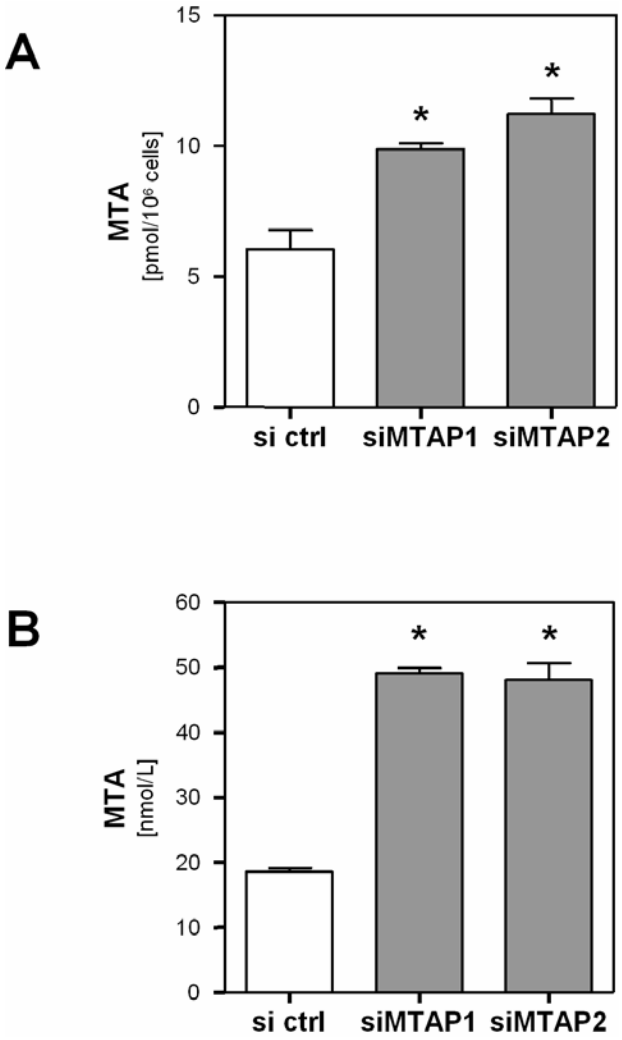

Figure 9: Effect of MTAP suppression on MTA concentration in human HCC cells.
The HCC cell line PLC was transiently transfected with negative control siRNA (si ctrl) or 2 different *MTAP* siRNAs (siMTAP1 and siMTAP2). MTA levels were measured in cell lysates **(A)** and supernatants **(B)** 48 h after transfection.
(*: P≤0.05 compared to si ctrl).

Corresponding successful *MTAP* suppression in the samples was documented on both MTAPsiRNA (**Figure 10A)** and protein **(Figure 10B**) levels.

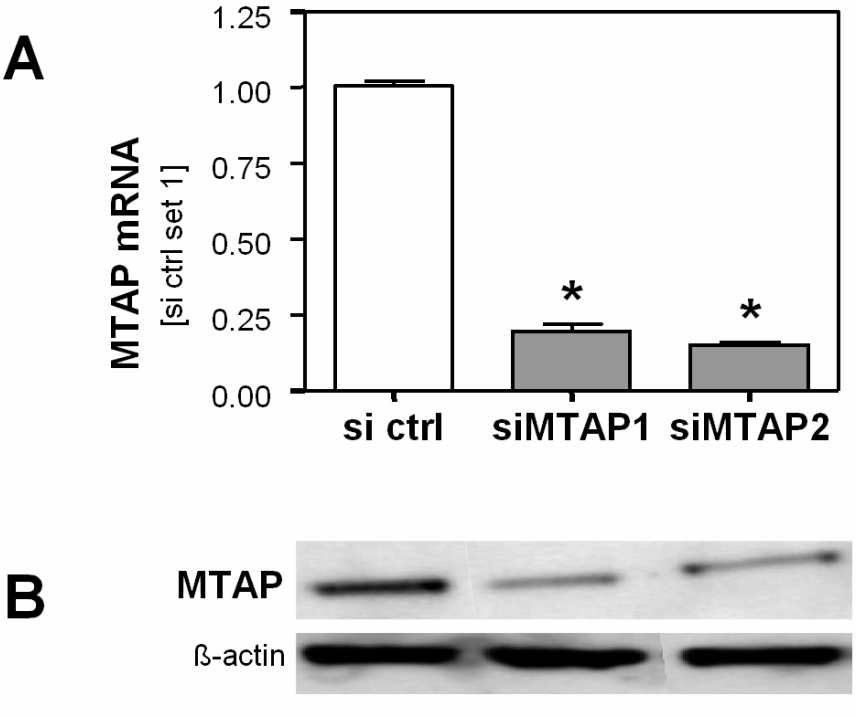

Figure 10: MTAP suppression after transfection with MTAPsiRNA.
MTAP mRNA **(A)** and protein (Western blot, **B**) expression in the cell samples from Figure 9.
(*: P≤0.05 compared to si ctrl).

3.2 MTA effects on HCC cells

The functional effect of MTA on HCC cells was investigated by treating the HCC cell lines HepG2 and PLC with MTA doses similar to those determined in HCC tissue.

Incubation with MTA led to a dose-dependent induction of the proliferation of HCC cells (**Figure 11**).

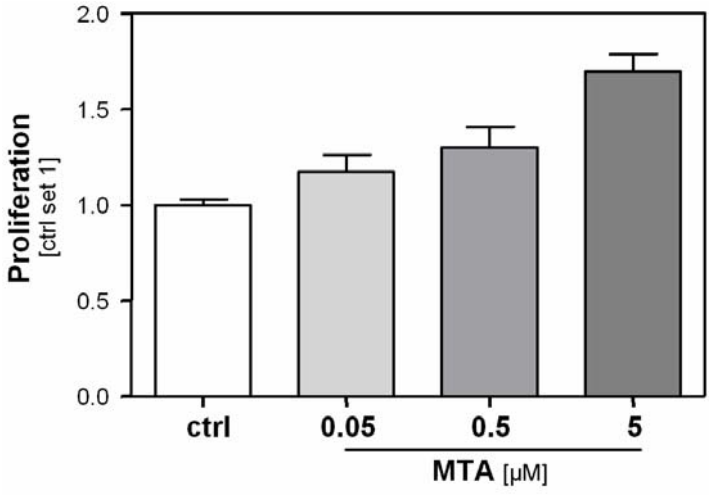

Figure 11: MTA effect on proliferation of HCC cells.
Proliferation of the HCC cell line HepG2 after stimulation with MTA at different concentrations (0.05, 0.5 and 5 µmol/L) compared to untreated control cells (ctrl).

Moreover, we analyzed the effect of MTA on the expression of several genes known to correlate with HCC progression and aggressiveness.

Thus MTA induced *IL8* (MIM 146930, **Figure 12**) expression.

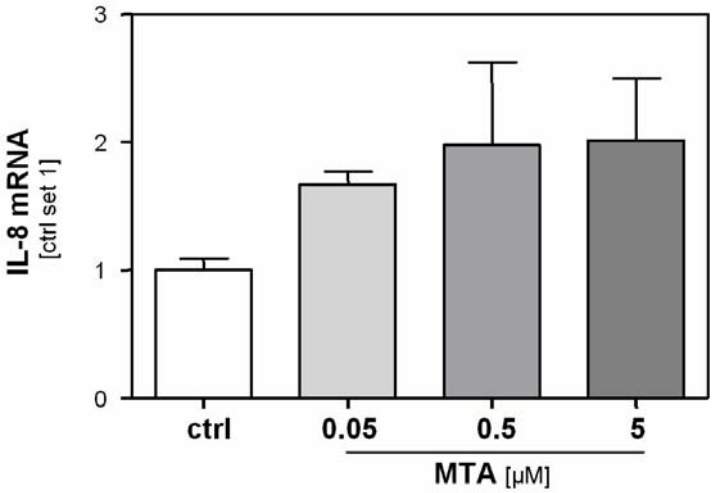

Figure 12: MTA effect on IL-8 expression of HCC cells.
IL-8 mRNA expression of PLC cells after treatment (24h) with MTA at different concentrations (0.05, 0.5 and 5 µmol/L) compared to untreated control cells (ctrl).

Likewise, the expression of the matrix-metalloproteinases *MMP1* (MIM120353), and *MMP9* (MIM 120361) was also induced (**Figure 13A** and **Figure 13B**, respectively).

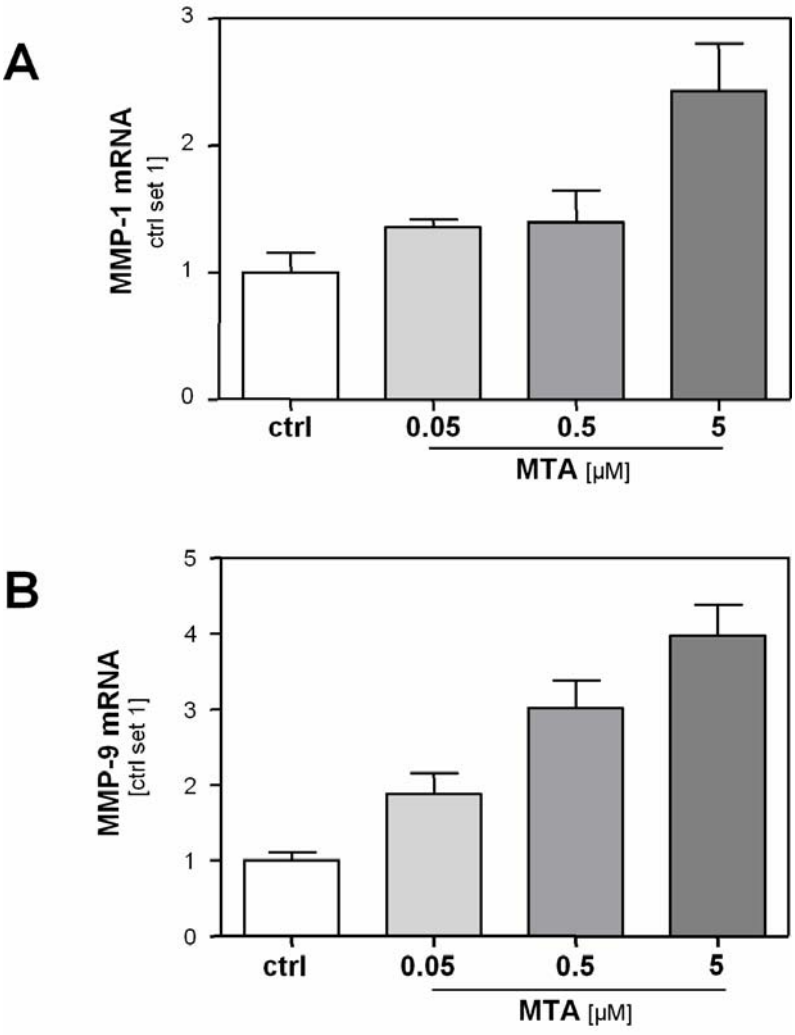

Figure 13: MTA effect on MMPs expression of HCC cells.
MMP-1 **(A)** and MMP-9 **(B)** mRNA expression of PLC cells after treatment (24h) with MTA at different concentrations (0.05, 0.5 and 5 µmol/L) compared to untreated control cells (ctrl).

3.3 MTA effects on activated HSC

Since down-regulation of *MTAP* in HCC also led to higher extracellular MTA levels, we investigated whether MTA also exerted an effect on stromal cells. Here, we focused on activated hepatic stellate cells (HSC). These myofibroblast-like cells are located around tumor sinusoids and fibrous septa, and infiltrate the HCC stroma (114;115). Quantitative RT-PCR analysis revealed that incubation of HSC with MTA induced the expression of the *MMP1* (**Figure 14A**) and *FGF2* (MIM 134920) genes (**Figure 14B**).

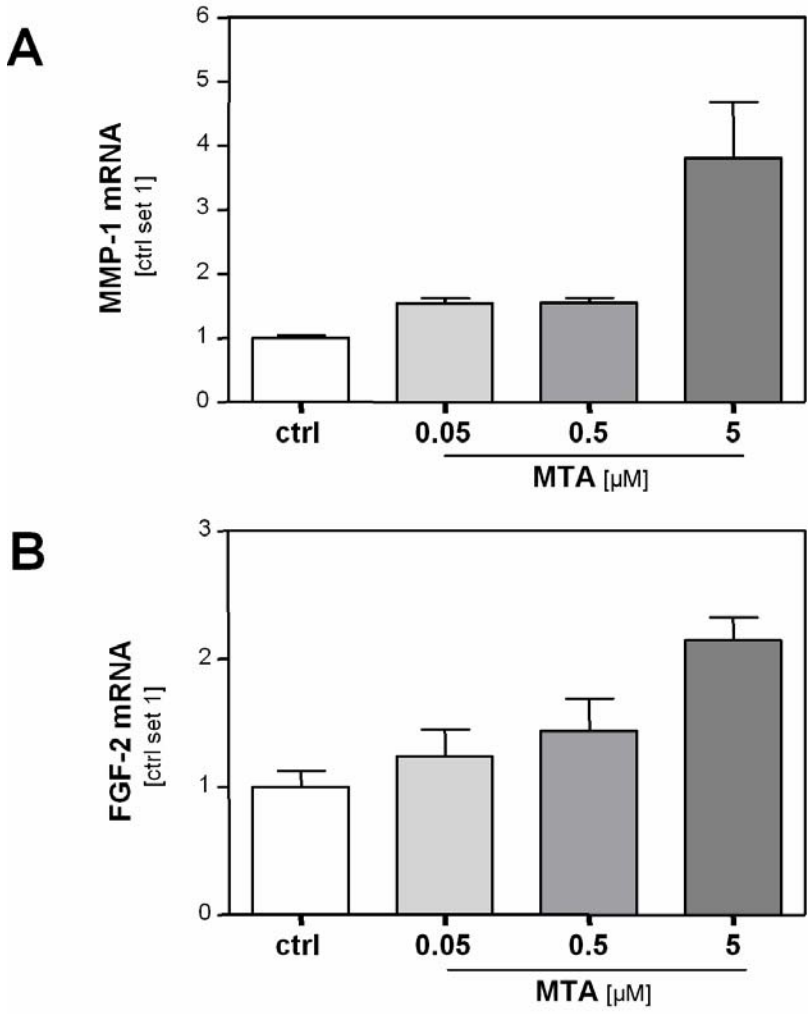

Figure 14: **MTA effect on activated HSC.**
MMP1 **(A)** and FGF2 **(B)** mRNA expression in activated hepatic stellate cells (HSC) after treatment (24 h) with MTA at different concentrations (0.05, 0.5 and 5 µmol/L) compared to untreated control cells (ctrl).

Notably, *MMP1* and *FGF-2* expression levels had been found to correlate with HCC progression (116;117).

Furthermore, HSC were incubated with conditioned media (CM) collected from HCC cells transiently transfected with MTAP-siRNA or control-siRNA, respectively (see **Figure 10**). CM from HCC cells with suppressed *MTAP* gene expression induced significantly higher *MMP1* and *FGF2* gene expression than CM derived from HCC cells transfected with control-siRNA (**Figure 15**).

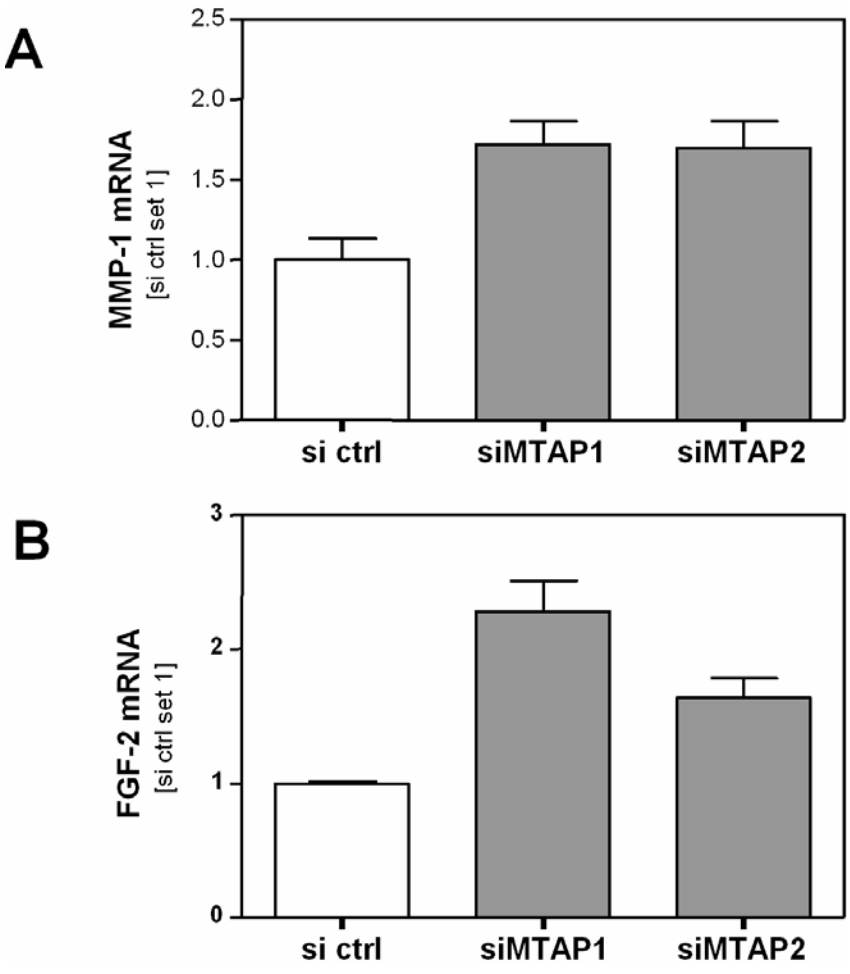

Figure 15: Effect of conditioned medium derived from HCC on activated HSC.
MMP1 **(A**) and FGF2 **(B)** mRNA expression in HSC after stimulation (24 h) with conditioned media derived from PLC cells in which MTAP expression was depleted by transfection with *MTAP* siRNA (siMTAP1 and siMTAP2) or from PLC cells transfected with control siRNA (si ctrl).

These findings indicate that downregulation of *MTAP* and consecutive elevation of intracellular and extracellular MTA levels promotes tumorigenesis of HCC cells directly as well as indirectly via induction of pro-cancerous genes in stromal cells.

3.4 MTAP expression in human HCC tissue

To further evaluate the functional effects of *MTAP* downregulation in HCC *in vivo* we analyzed MTAP protein expression in a series of 140 human HCC tissues applying tissue microarray (TMA) technology. In 57 HCCs (40.7%) immunohistochemistry revealed no MTAP immunosignal. In contrast, MTAP expression was visible in all non-tumorous tissue (data not shown), which confirms that in a considerable portion of human HCC MTAP protein expression is significantly downregulated or lost, respectively.

For descriptive data analysis, HCC were separated into tissues with positive and negative MTAP immunosignal (representative examples are depicted in **Figure 16A**). Matched data of mRNA expression and semiquantitative protein expression analyzed on the TMA were available from 25 HCC patients. MTAP mRNA expression was significantly higher in HCC cases with positive MTAP immunosignal (n=15) compared to cases, where no MTAP was detectable (n=10; **Figure 16B**). This finding indicates that MTAP expression is accurately detected by immunohistochemistry.

A correlation of immunohistochemical results with clinico-pathological tumor characteristics (**Table 5**) revealed that loss of MTAP expression was significantly associated with higher tumor stage (P=0.032) and tumor grading (P=0.019). No correlation was found between MTAP expression and age, gender or tumor size.

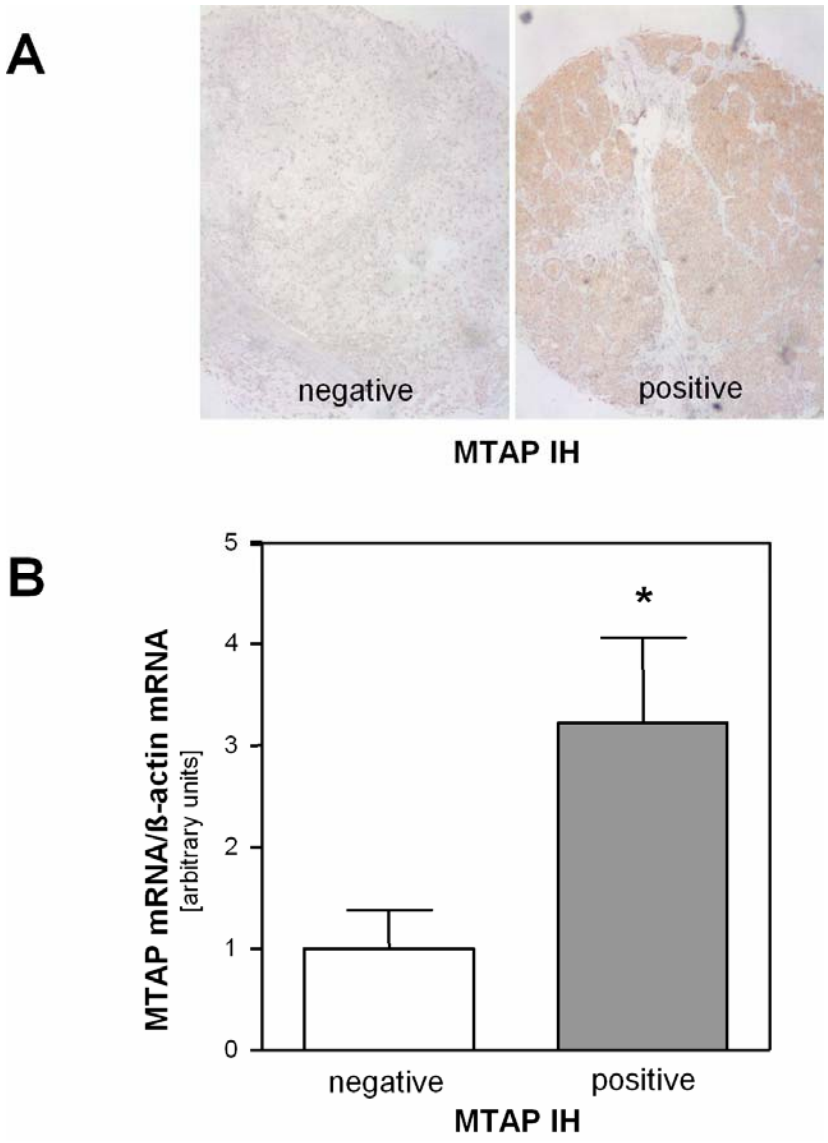

Figure 16: **MTAP expression in human HCC tissue.**
(A) Representative MTAP immunohistochemical (IH) staining results of HCC tissue samples arranged on a tissue microarray (left panel: MTAP negative; right panel: MTAP positive).
(B) *MTAP* mRNA expression in 10 HCC tissues with negative and 15 HCC tissues with positive MTAP immunohistochemical staining (IH).
(*: P≤0.05 compared to MTAP IH negative).

Variable	Categorization	n	%	MTAP IR negative	MTAP IR positive	P*
Clinicopathological characteristics						
Age at diagnosis						
	<60 years	52	*37.1*	17	35	0.157
	≥60 years	88	*62.9*	40	48	
Gender						
	female	21	*15.0*	10	11	0.483
	male	119	*85.0*	47	72	
Tumor stage						
	pT1	47	*33.6*	13	34	**0.032**
	pT2	42	*30.0*	15	27	
	pT3	46	*32.9*	25	21	
	pT4	3	*2.1*	2	1	
	nd	2	*1.4*			
Histological grade						
	G1	53	*37.9*	15	38	**0.019**
	G2	70	*50.0*	31	39	
	G3	17	*12.1*	11	6	
Tumor size						
	≤ 5 cm	70	*50.0*	23	47	0.339
	> 5 cm	40	*34.3*	20	28	
	nd	30	*15.7*			

Table 5: MTAP immunoreactivity (IR) in HCC-tissue of 140 patients in relation to clinicopathological characteristics
* Fisher's exact test (2-sided); bold face representing P-values ≤0.05.
(nd: no data available; IR: immunoreactivity)

From HCCs on the TMA no suitable tissue samples for MTA analysis were available. However, analysis of HCC specimens from another 19 patients revealed an inverse correlation between *MTAP* mRNA expression and MTA levels (r= -0.46; P= 0.047; **Figure 17**).

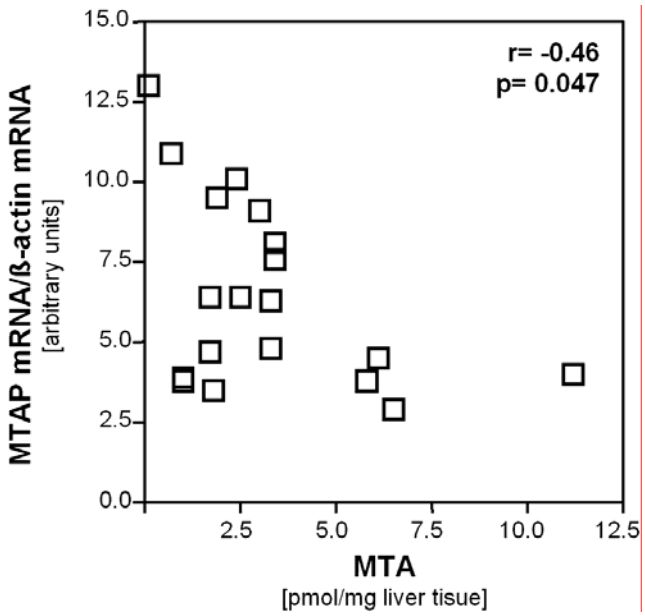

Figure 17: Correlation of MTA levels with *MTAP* mRNA expression in human HCC tissue.
Data are based on 19 human HCC samples showing a significant (p=0.047) inverse correlation of medium size (Pearson´s r=-0.46).

In summary, these data indicate that also *in vivo* MTAP downregulation leads to increased MTA levels, which functionally affect HCC progression.

4 Discussion

In a previous study by our group it was shown that downregulation of *MTAP* in HCC cell lines enhanced tumorigenicity *in vitro* (62). Due to the lack of a sensitive detection method, it was not possible to determine at that time the amounts of MTA in or secreted by HCC cells, and thus, the molecular mechanism for the tumor-promoting effect of *MTAP* deficiency in HCC remained unsolved. A later study examining MTAP expression in malignant melanoma using LC-ESI-MS/MS to measure MTA found an elevation of MTA levels in skin cancer sell lines and tumor samples (88). We speculated that similar changes might be found in HCC and indeed we could show that cell media as well as intracellular MTA levels were significantly higher in 4 different HCC cells than in primary hepatocytes. Experimental inhibition of *MTAP* expression in HCC lines with 2 siRNAs caused inverse changes of MTA levels, indicating that enhanced MTA levels in HCC were caused by downregulation of *MTAP*.

The difference in MTA concentrations between HCC cell lines and primary hepatocytes was more pronounced in the cell media than intracellularly which is line with the finding that tumor MTAP deficient cells are able to excrete MTA indicating that MTAP deficiency is likely to influence not only intracellular processes but also the hepatocytes´ environment (63;66;118).

Importantly, this study found an induction of the gene expression of *MMP1, MMP9*, and *IL8* upon treatment of HCC cells with MTA in doses similar to those found *in vivo* in HCC. The increased expression of these genes is a well documented event in hepatocarcinogenesis and correlates with various more aggressive clinicopathological HCC features, specifically with invasive potential (116;119). It was previously shown that MTAP over-expressing HCC cells in culture have reduced invasive potential (62) and it may now be speculated that in MTAP-deficient HCC cells, MTA-induced up-regulation of MMP1 and 9 is at least one of the underlying mechanisms.

Moreover, effects of MTA were not only observed in the tumor cells, but also in activated HSC that constitute an important cellular component of the tumor microenvironment. They are located around tumor sinusoids, fibrous septa and

capsule, if the latter is present (114;115) and recently, in our group it was shown that activated HSC promote tumorigenicity of HCC (6). Thus, the observed up-regulation of MMP1 and FGF2 by HSC caused by MTA stimulation is of importance, because in addition to MMP1, FGF2 is an important angiogenic factor shown to be associated with the capsular infiltration of HCC (119), with more invasive HCCs (117) and also with enhanced proliferation of HCC cells in vitro (120). Further, similar MTA induced gene expression changes are found in melanoma cells as well (88) which supports that these MTA induced effects are possibly not confined to the liver but may be common to many malignant cells.

Together these findings indicate that the tumor-promoting effect of *MTAP* deficiency in HCC is at least in part mediated by enhanced MTA levels.

Most importantly, this study could demonstrate the clinical relevance of *MTAP* downregulation in HCC. Although *MTAP* deficiency is documented in various human malignancies, only a few studies (and none on HCC) include a representative number of tumor samples to assess its frequency and possible clinical significance. Already previously, our group found lower MTAP mRNA and protein expression in a limited number of HCC tissue samples compared to adjacent non-cancerous tissue (62). Here, we confirmed downregulation of MTAP in a significant number of human HCCs, and most strikingly, MTAP deficiency correlated with more advanced tumor staging and poorer histopathological grading.

Remarkably, also in human HCC tissue MTAP expression correlated inversely with MTA levels, suggesting that also *in vivo* enhanced MTA levels account at least to a certain extent for the tumor promoting effect of MTAP deficiency. Studies by others revealed that MTA exhibited an anti-inflammatory activity on macrophages and lymphocytes, thus, further supporting a direct tumor-promoting effect of MTA (121-123).

In contrast to our findings, some groups have reported pro-apoptotic effects of MTA on hepatoma cells, and inhibitory effects on proliferation and invasion of various cell lines as well as differentiation and growth factor receptor activation (71;104;124-127). However, in most of those studies significantly higher, pharmacological doses had been administered, whereas the MTA levels

achieved here mirrored endogenous tumor levels. In addition, the therapeutic effect of MTA seems to be dependent on the cell type and the *MTAP* level in the cells (128). Further, in line with our results, previous studies suggested a correlation of *MTAP* deficiency with poor differentiation in non-Hodgkin's lymphoma (129). In contrast, in a large lung cancer study (73) this association was not observed showing that the role of *MTAP* deficiency in tumor progression is likely tumor-specific.

In conclusion, we demonstrated that the downregulation of *MTAP* in HCC leads to increased MTA levels, which functionally affect cancerous cells as well as tumor stromal cells in a tumor supportive way. This work advances *MTAP* as a tumor suppressor in HCC, and MTA as potential biomarker for the progression of this highly aggressive tumor.

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6 Appendix

6.1 Zusammenfassung (deutsch)

Titel: Die verminderte Expression von Methylthioadenosin-phosphorylase fördert das Fortschreiten des hepatozellulären Karzinoms über eine Anhäufung von 5´-deoxy-5´-methylthioadenosin

Einführung: Das hepatozelluläre Karzinom (HCC) nimmt weltweit den dritten Platz unter den krebsbedingten Todesursachen ein. Die häufigsten Risikofaktoren für die HCC Entstehung sind chronische Hepatitis B und C Infektion sowie chronischer Alkoholabusus, die zu einer Leberzirrhose führen. Weniger bekannt ist jedoch die molekulare Pathogenese des HCC. Viele genomische Veränderungen im HCC Verlauf sind bereits beschrieben worden aber ihre funktionelle Bedeutung und genaue Rolle für das Fortschreiten des HCC bleiben in vielen Fällen noch unklar.

Vor kurzem wurde eine verminderte Expression der Methylthioadenosin-phosphorylase (MTAP) in HCC Zelllinien beschrieben. MTAP spielt eine Schlüsselrolle im Polyaminmetabolismus und stellt den geschwindigkeits-bestimmende Schritt in der Wiedergewinnung von Methionin und Adenin dar.

Ziele der Arbeit waren die Untersuchung der klinisch-pathologischen Bedeutung der Herabregulation von MTAP im HCC und die Erforschung der Mechanismen, die der pro-tumorigenen Wirkung der MTAP-Defizienz im HCC zugrundeliegen.

Methoden and Ergebnisse: Sowohl intrazellulär als auch in Überständen von 4 HCC-Zellinien fanden sich im Vergleich zu primären humanen Hepatozyten signifikant erhöhte 5´-deoxy-5´-methylthioadenosin (MTA) Konzentrationen, welche durch stabile Isotopenverdünnungsanalyse bestimmt wurden. Weiterhin führte eine Suppression der MTAP-Expression *in vitro* in HCC Zellen mittels siRNA zu erhöhten MTA Spiegeln. Ferner induzierte MTA in HCC-Zellen die Expression von Matrixmetalloproteinasen (MMPs) und IL-8 sowie deren Proliferation. In aktivierten hepatischen Sternzellen, die aus humanem HCC Gewebe isoliert wurden, induzierte MTA die mRNA Expression von MMP1 und Fibroblast-Growth Factor 2 (FGF-2).

Die immunohistochemische Analyse von einem Gewebemicroarray von 140 humanen HCC Proben zeigte eine signifikante Korrelation zwischen verminderter MTAP Proteinexpression und fortgeschritteneren histopathologischen HCC-Stadien bzw. schlechterer Tumordifferenzierung. Weiterhin zeigte sich auch *in vivo* in 19 humanen HCC Proben ein signifikanter Zusammenhang zwischen verminderter MTAP-Expression und erhöhten MTA Spiegeln.

Diskussion: Eine verminderte MTAP Expression im HCC fördert die Tumorigenität über eine Anhäufung von MTA. MTA beeinflusste sowohl HCC Zellen als auch die Tumorumgebung. Die Arbeit zeigt, dass MTAP im HCC einen Tumorsuppressor darstellt.

6.2 Lebenslauf

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