

Diploma Thesis

PTEN and the PI3K/Akt Pathway in Biliary Tract Cancer

submitted by

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3 May 2014

Affidavit

I hereby declare that I authored the present thesis independently and that I did not use any sources other than the sources declared. I explicitly identified all the passages that were taken, either literally or in spirit, from other works.

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Signature

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Abstract

Introduction: Biliary tract cancer and its most frequent type, gallbladder cancer, are relatively seldom diseases with a very poor outcome. There are high risk areas, and the global incidence will increase notably in the future. The tumor suppressor PTEN and the proto-oncogenic PI3K/Akt pathway play an important role in many human cancer entities. This thesis investigates the influence of PTEN and the PI3K/Akt pathway on biliary tract cancer to understand the molecular mechanisms that are responsible for cancer formation.

Material & Methods: Eight biliary tract cancer cell lines, eight frozen gallbladder cancer samples, and nine frozen tumor-free gallbladder specimens were investigated by qRT-PCR and Western blot. Furthermore, an immunohistochemical staining of 396 gallbladder cancer samples, 101 tumor-free gallbladder samples, and 3 mild chronic cholecystitis specimens was conducted.

Results: PTEN was downregulated or upregulated and, in the latter case, simultaneously inactivated in all biliary tract cancer cell lines. The inactivation of PTEN led to mTOR activation in an Akt-independent manner. The transition from normal gallbladder epithelium to gallbladder cancer as well as loss of differentiation, invasion, and poor prognosis were characterized by the overexpression of PTEN. Decreasing levels of nuclear phospho-p70S6K and phospho-mTOR correlated with tumor progression and reduced survival.

Discussion: The inactivation of PTEN due to phosphorylation apparently correlates with its overexpression and could be a promising objective for targeted therapies in the future. The alteration of the PI3K/PTEN ratio may lead to the activation of Akt, mTOR as well as p70S6K and may promote gallbladder carcinogenesis in chronic cholecystitis. Nuclear phospho-p70S6K and phospho-mTOR could be used to estimate the likelihood of metastasis by comparing the expression levels in cancer and tumor-free tissue of patients with gallbladder cancer. Additional studies are required that pursue these findings and thereby provide further insights into the PI3K/Akt pathway in gallbladder carcinogenesis.

Zusammenfassung

Einleitung: Karzinome des Gallentrakts inklusive ihrer häufigsten Entität, dem Gallenblasenkarzinom, sind relativ seltene und prognostisch ungünstige Erkrankungen. Neben vorhandenen Hochrisikogebieten wird die weltweite Inzidenz in Zukunft deutlich ansteigen. Der Tumorsuppressor PTEN und der malignitätsfördernde PI3K/Akt-Signalweg spielen in vielen Tumorentitäten des Menschen eine entscheidende Rolle. Diese Arbeit untersucht PTEN und den PI3K/Akt-Signalweg hinsichtlich ihres Einflusses auf Karzinome des Gallentrakts, um die zugrundeliegenden molekularen Mechanismen der Tumorentstehung zu verstehen.

Material & Methoden: Acht Zelllinien von Gallentraktkarzinomen, acht gefrorene Gallenblasenkarzinomproben sowie neun gefrorene Proben nichtmalignen Gallenblasengewebes wurden mittels qRT-PCR und Western Blot untersucht. Zudem wurden 396 Gallenblasenkarzinomproben, 101 tumorfreie Gallenblasengewebestücke und 3 geringgradige chronische Cholezystitisfälle immunhistochemisch analysiert.

Ergebnisse: PTEN war in Gallentraktkarzinomzelllinien herunterreguliert oder hochreguliert und in letzterem Fall gleichzeitig inaktiviert. Die Inaktivierung von PTEN führte zu einer Akt-unabhängigen Aktivierung von mTOR. Der Übergang von normalem Gallenblasengewebe in ein Gallenblasenkarzinom sowie eine Entdifferenzierung, Invasion und ungünstige Prognose waren charakterisiert durch die Überexpression von PTEN. Abnehmende Expressionen von nukleärem phosphorylierten p70S6K und phosphoryliertem mTOR korrelierten mit Tumorprogression und geringerer Überlebensrate.

Diskussion: Die durch eine Phosphorylierung bedingte Inaktivierung von PTEN geht anscheinend mit einer Überexpression einher und könnte ein vielversprechender Ansatz für eine zukünftige zielgerichtete Tumorthherapie darstellen. Eine Änderung des PI3K/PTEN-Verhältnisses könnte zur Aktivierung von Akt, mTOR und p70S6K führen und die Gallenblasenkarzinomentstehung auf dem Boden einer chronischen Cholezystitis fördern. Nukleäres phosphoryliertes p70S6K und phosphoryliertes mTOR könnten zur Bestimmung einer möglichen Metastasierung in Patienten mit

Gallenblasenkarzinomen herangezogen werden, indem Expressionsraten zwischen Tumor und tumorfreiem Gewebe dieser Patienten verglichen werden. Weitere Studien werden benötigt, die diese Ansätze verfolgen und damit weitere Einblicke in den PI3K/Akt-Signalweg in der Gallenblasenkarzinomentstehung ermöglichen.

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List of Abbreviations

A	Adenine
AEBSF	4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride
APS	Ammonium persulfate
Ars2	Arsenic resistance protein 2
BAD	Bcl-2-associated death promoter
BAK	Bcl-2 antagonist/killer
BAX	Bcl-2-associated X protein
BRAF	V-raf murine sarcoma viral oncogene homolog B
BSA	Bovine serum albumin
BTC	Biliary tract cancer
C	Cytosine
CC	Chronic cholecystitis
CCI-779	Cell cycle inhibitor 779
Co	Control
C _T	Threshold cycle
DAB	Diaminobenzidine
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle medium
dNTP	Deoxynucleotide triphosphate
DPBS	Dulbecco's phosphate-buffered saline
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
eNOS	Endothelial nitric oxide synthase
fw	Forward
G	Guanine
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

GC	G uanine- c ytosine content
GSK3	G lycogen s ynthase k inase 3
HCl	H ydro c hloride
I	I ntensity
IGF-1	I nsulin-like g rowth f actor 1
IKK	I κ B k inase
Jak	J anus k inase
JNK	C - J un N -terminal k inase
KRAS	K irsten r at s arcoma viral oncogene homolog
L	L ymphatic vessel invasion
M	M olar
mA	M illiampere
MAPK	M itogen- a ctivated p rotein k inase
MDM2	M ouse d ouble m inute 2
min	M inute
mL	M illiliter
mM	M illimolar
MMAC1	M utated in m ultiple a dvanced c ancers 1
mPGES-1	M icrosomal p rostaglandin E s ynthase 1
mTOR	M ammalian t arget o f rapamycin
mTORC1	M ammalian t arget o f rapamycin c omplex 1
mTORC2	M ammalian t arget o f rapamycin c omplex 2
MW	M olecular w eight
n	S ample size
NaCl	S odium c hloride
NF-κB	N uclear f actor κ appa B
NGF	N erve g rowth f actor

p27Kip1	Cyclin-dependent kinase inhibitor 1B
p53	Protein 53
p70S6K	70 kDa ribosomal protein S6 kinase
PBM	Pancreaticobiliary malunion
PDGF	Platelet-derived growth factor
PDK1	PIP₃-dependent protein kinase 1
PI3K	Phosphatidylinositide 3-kinase
PIP ₂	Phosphatidylinositol-4,5-bisphosphate
PIP ₃	Phosphatidylinositol-3,4,5-trisphosphate
PKB	Protein kinase B
pM	Picomolar
PRDX1	Peroxiredoxin 1
P-Rex2a	Phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 2a
PSC	Primary sclerosing cholangitis
PTEN	Phosphatase and tensin homolog deleted on chromosome ten
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
rev	Reverse
rpm	Revolutions per minute
RTK	Receptor tyrosine kinase
s	Second
SAPK	Stress-activated protein kinase
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	Serine
SIPL1	Shank-interacting protein-like 1
SMAD4	SMAD family member 4

STAT	S ignal transducer and a ctivator of t ranscription
SUMO1	S mall u biquitin-like m odifier 1
T	T hymine
TBS	T ris- b uffered s aline
TBST	T ris- b uffered s aline with T ween 20
TEMED	T etramethylethylenediamine
Thr	T hreonine
T _m	M elting t emperature
TMA _s	T issue m icroarrays
TNM	T umor extent, lymph n ode metastasis, distant m etastasis
Tris	T ris(hydroxymethyl)aminomethane
TSC1/2	T uberous s clerosis c omplex 1 and 2
UICC	U nion for I nternational C ancer C ontrol
V	V enous invasion
XIAP	X -linked inhibitor of a poptosis p rotein
μg	M icrogram
μL	M icroliter
μM	M icromolar

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1 Introduction

Biliary tract cancer and its most frequent type, gallbladder cancer (1–3), are uncommon diseases in comparison to breast or prostate cancer (4). Nonetheless, there are areas and ethnic groups that show high incidences (4–6). In contrast to more common cancer entities such as breast and prostate cancer, effective diagnostic and therapeutic strategies are still limited, because the molecular pathogenesis of biliary tract cancer is poorly understood in many aspects. Proto-oncogenic pathways, which play a well-established role in the formation of numerous cancer types, remain almost unexplored in biliary tract cancer. The PI3K/Akt signaling cascade is one such pathway, and its function in biliary tract cancer, especially in gallbladder cancer, is therefore the subject of this diploma thesis.

1.1 Biliary Tract Cancer

Biliary tract cancer is a rare disease with a very poor prognosis due to early lymph node and distant metastases (6,7). The frequency of the identification of biliary tract cancer increases, probably due to advances in diagnostic procedures (8), an increasing percentage of the elderly, and the growth of the world population (4). Despite all efforts, there has been no considerable improvement in the life expectancy of these patients over the last years (6).

1.1.1 Classification of Biliary Tract Cancer

Biliary tract cancer can be distinguished into gallbladder cancer, intrahepatic, and extrahepatic cholangiocarcinoma as well as the cancer of the ampulla of Vater (6). However, some authors regard the intrahepatic cholangiocarcinoma as liver cancer (5). Intrahepatic cholangiocarcinomas are also not considered in the epidemiological data given below (see 1.1.2 Epidemiology of Biliary Tract Cancer and Figures 1, 2, and 4). More than 95 % of all biliary tract malignancies are carcinomas, which originate from epithelial cells (9). By contrast, sarcomas (mesenchymal origin), neuroendocrine tumors (neuroendocrine origin), and lymphomas (derived from lymphocytes) are extremely rare (9,10). There are a large number of different histological subtypes, and the most frequent histological type is the adenocarcinoma,

which appears in more than 95–98 % of cases (6,10). The histological types differ in survival time; nevertheless, the prognosis is generally very bad (9).

1.1.2 Epidemiology of Biliary Tract Cancer

Biliary tract cancer rarely occurs in most parts of the world, whereas there are higher incidences in some countries, especially in Chile, Bolivia, Nepal, and South Korea (see Figure 1) (4).

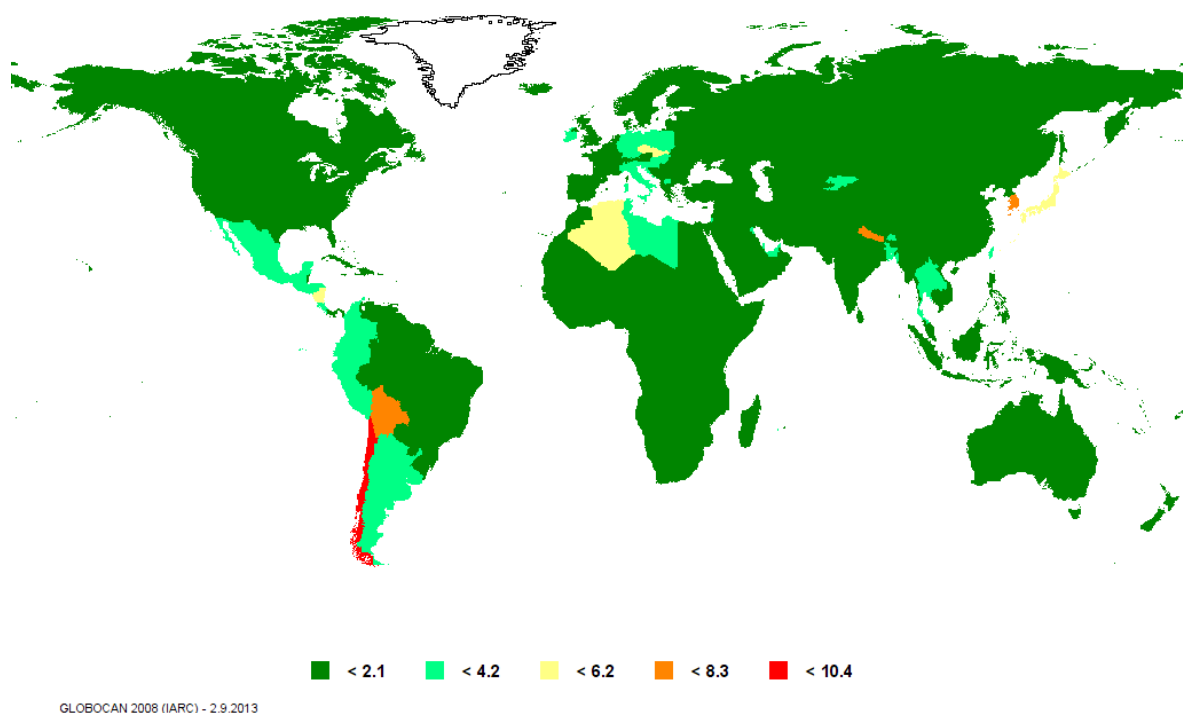


Figure 1: Global Incidence of Biliary Tract Cancer in 2008

The estimated age-standardized incidence rate of biliary tract cancer (excluding intrahepatic cholangiocarcinomas) per 100 000 persons was calculated for both sexes and all ages by GLOBOCAN 2008 (4). An age standardization using the world standard population was performed to allow a comparison of worldwide incidences because different age structures of the populations have an enormous effect on cancer incidences (4).

The high risk country Chile had an age-standardized mortality rate (2000–2004) of 16.6 per 100 000 females and 7.8 per 100 000 males (11). In 2012, the age-standardized mortality rate was even 10.3 per 100 000 women and 5.0 per 100 000 men (12). By contrast, in Austria, the mortality rate (2000–2004) was 2.2 per

100 000 females and 1.9 per 100 000 males (11). In 2012, it was 1.1 per 100 000 women and 0.9 per 100 000 men (12). These data explain that biliary tract cancer plays a major role in high risk areas and was even the sixth most common cancer in Chile in 2008 (see Figure 2) (4).

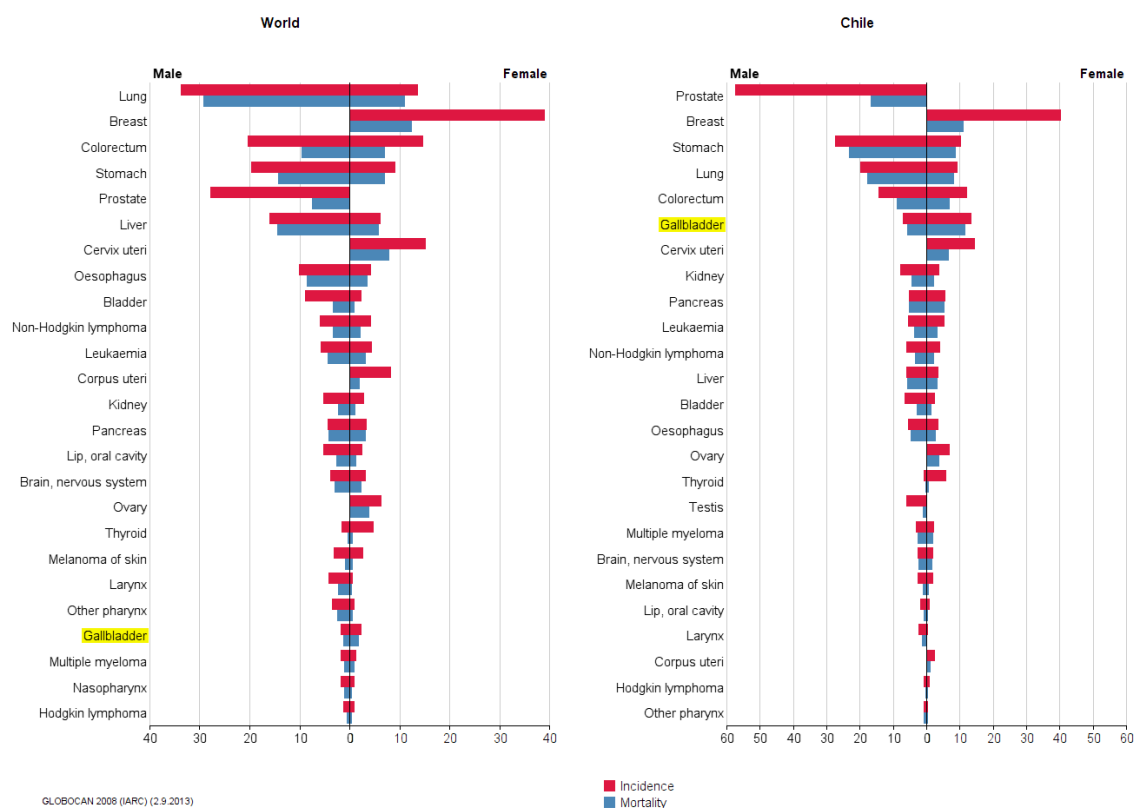


Figure 2: Incidence and Mortality of Cancers Worldwide and in Chile

The estimated age-standardized incidence and mortality rates of the 25 most common cancer entities per 100 000 persons were calculated by GLOBOCAN 2008 (4). Gallbladder cancer includes extrahepatic cholangiocarcinomas and ampullary cancers in this statistical evaluation.

There are also ethnic differences. For example, Native and Hispanic Americans suffer more often from biliary tract malignancies than the rest of the population of the United States (5,6). There is a male predominance in biliary tract cancer with the exception of gallbladder cancer (13). Women are approximately three times more likely to suffer from gallbladder cancer than men (14,15).

1.1.3 Etiology and Pathogenesis of Biliary Tract Cancer

Carcinomas of the biliary tract develop from deregulated epithelial cells that line the inner surface of the gallbladder and the bile ducts (7). The common final path of risk factors (see Table 1) is chronic inflammation by chemical and/or mechanical irritation (16).

Autoimmune Disorders	Calculosis	Carcinogens
Primary biliary cirrhosis	Bile duct stones	Betel nut
PSC	Cholelithiasis	Nitrosamines (Tobacco)
Ulcerative colitis		Thorotrast
Congenital Abnormalities	Infections	Miscellaneous
Bile duct cysts	Liver flukes	Aging
Caroli disease	Salmonella carrier state	Family history of BTC
PBM		Obesity

Table 1: Most Important Risk Factors for Biliary Tract Cancer

Caroli disease is characterized by a cystic dilatation of intrahepatic bile ducts. BTC (biliary tract cancer), PBM (pancreaticobiliary malunion), PSC (primary sclerosing cholangitis); Table modified according to Holzinger et al. (16) with the help of Michaud (5)

Some components of the bile and pancreatic juice seem to have carcinogenic potential, especially when they are not able to drain away normally (16). Chronic inflammation can finally lead to a metaplastic transformation of the epithelium, which can be the beginning of a metaplasia-dysplasia-carcinoma sequence owing to a gradual accumulation of molecular changes (8,17). In comparison to that, the malignant potential of adenomas appears to be very low (17). The changes of cellular processes in carcinogenesis are caused by the loss of diverse tumor suppressor genes, for example, *SMAD4* (**SMAD** family member **4**) and *p53* (**protein 53**) and/or by the enhanced activation of oncogenes, for example, *BRAF* (**v-raf** murine sarcoma viral oncogene homolog **B**) and *KRAS* (**Kirsten rat sarcoma viral oncogene homolog**) (16,17) and possibly also by epigenetic gene silencing (18). These changes include, for instance, the harmful effect of oxygen radicals associated with a disturbed cellular detoxification (16). The gene products interfere with each other and are

important components of different pathways (e.g., KRAS/MAPK pathway and PI3K/Akt pathway).

Although many gene and protein modifications have been identified, the molecular events in the oncogenesis of biliary tract malignancies are poorly understood so far. Furthermore, differences in epidemiology, pathogenesis, and sensitivity to therapy have become clear despite the mutual features of the distinct types of biliary tract cancer (5,17). Hence, this thesis also pays attention to possible differences between the biliary tract cancer types to avoid misplaced generalizations.

1.1.4 Clinical Presentation of Biliary Tract Cancer

The reasons for a late diagnosis and therefore, a poor outcome may be the unspecific signs and symptoms of biliary tract cancer, which appear only at later stages (6). These signs and symptoms are, for example, abdominal discomfort, maldigestion, jaundice, pruritus, reduction of weight, and fever (6,19) and do not differ from other diseases in this area (6). Not surprisingly, Dixon et al. reported in a retrospective review at a North American Center that only 38 % of patients had been correctly diagnosed with gallbladder cancer before surgery (20). Biliary tract cancer leads in the plurality of patients to biliary tract obstruction, which may be the cause of the above-mentioned signs and symptoms (6). Cholangitis, sepsis, liver failure, and death are the complications of biliary tract obstruction (6).

1.1.5 Diagnosis of Biliary Tract Cancer

Notably, radiological imaging technologies (i.e., sonography, computerized tomography, magnetic resonance imaging, and positron emission tomography) and optionally endoscopy strengthen the presumptive diagnosis of a biliary tract malignancy (6). However, a definite proof is only provided by the pathological evaluation of a biopsy, a fine needle aspiration, a brush cytology of the biliary tract, or an assessment of the resected tissue (21).

1.1.6 Therapy and Prognosis of Biliary Tract Cancer

Complete surgical resection is the sole possibility of a curable treatment, but is only practicable in 10–30 % of patients diagnosed as having advanced tumor stages (6,7). The close anatomical relationship of gallbladder, liver, portal vein, and intestine creates the barrier to successful surgical procedures (22). In special cases, liver transplantation can be the approach to a full recovery (21,23,24). Unfortunately, the patients who undergo surgery, apparently do not profit from either a neoadjuvant or an adjuvant chemotherapy with regard to the survival time (25). By contrast, cisplatin and gemcitabine combination chemotherapy is the established standard for unresectable locally advanced and metastatic biliary tract cancers (26,27). The median survival in these cases is, however, not even one year (7).

Apart from chemotherapy regimens, interventional radiology is also used from a palliative point of view (6). Interventional radiology is a low-risk method compared to surgery and is able to destroy tumor tissue via transarterial chemoembolization, chemicals (i.e., acetic acid, ethanol, and hot saline solution), and physical modalities (i.e., cryoablation, laser, microwaves, and radio frequency) (6). Despite additional therapies, such as radiotherapy, photodynamic therapy, and endoscopic brachytherapy, none made the breakthrough (6). That is why palliative and supportive care are the leading therapies in most patients. Palliative and supportive care primarily aim to improve life quality instead of extending life. Pain management, nutrition, and psychosocial support are the major tasks of these special services (6). They even include radiation and chemotherapy for pain relief and endoscopic procedures in case of a biliary obstruction.

Targeted therapy has already found its way into clinical practice for some cancer entities [e.g., trastuzumab in breast cancer (28) and gefitinib in non-small cell lung cancer (29)]. In parallel with the improvement of the above-mentioned therapeutic options, targeted therapy may revolutionize the treatment of biliary tract neoplasms in the near future. Several different agents that block oncogenic pathways are under way and already showed first successes (17). These include, among others, EGFR (epidermal growth factor receptor) inhibitors (e.g., cetuximab), EGFR tyrosine kinase

inhibitors (e.g., erlotinib), and antiangiogenic factors (e.g., bevacizumab) (7,21,22). Consequently, there is an urgent requirement to explore the carcinogenesis in biliary tract malignancies to find promising targets for specific therapies. In particular, it would be interesting whether the different types of biliary tract cancer participate in the same oncogenic pathways or not.

1.2 Gallbladder Cancer

In the following, a closer look at the details of gallbladder cancer is given. Facts that do not differ from general aspects of biliary tract cancer (e.g., clinical presentation) are not listed separately.

1.2.1 Classification of Gallbladder Cancer

Gallbladder cancers are in 99 % carcinomas, and almost 90 % of these carcinomas are adenocarcinomas (9). Gallbladder cancer invades the gallbladder wall, the gallbladder bed, and regional hilar lymph nodes and forms metastases, especially in the peritoneum and the liver (2,6).

1.2.2 Epidemiology of Gallbladder Cancer

Gallbladder cancer is the most common type of biliary tract cancer (1–3) and is the one with the worst prognosis (3). Gallbladder cancer mainly occurs in people older than 40 years (2), and the peak of gallbladder cancer is in the 8th decade of life (9). There is a similar geographical and ethnic distribution to biliary tract cancer. The incidence of gallbladder cancer was 1.43 per 100 000 people per year (analysis period from 1973 to 2005) in the United States of America with a female-male ratio of 1.74 (30). To the contrary, up to 7.5 per 100 000 male and up to 23 per 100 000 female Native Americans, North Indians, Poles, and South Americans will be affected by gallbladder cancer (31). The female-male ratio is generally three with a variation from one (Far East Asia) to five (Colombia, Spain) (14,15) probably because women are more frequently affected by the high risk factor cholelithiasis.

1.2.3 Etiology and Pathogenesis of Gallbladder Cancer

Although the above-mentioned risk factors for biliary tract cancer are also relevant for gallbladder cancer, cholelithiasis seems to be the principal risk factor (5) [pooled relative risk 4.9 (15)] with a presence of 69–80 % in gallbladder cancer (22,32). In line with this, regions with a high incidence of gallbladder cancer have also higher rates of cholelithiasis (1,14). Obesity, strongly correlated with cholelithiasis, is naturally also associated with gallbladder cancer (33,34). However, only 1–3 % of the patients who suffer from gallstones develop gallbladder cancer (31). Furthermore, it has not been proven with any certainty that gallstones are more than a cofactor in the pathogenesis of gallbladder cancer notwithstanding numerous studies (35). The chronic carrier status of *Salmonella* or *Helicobacter* in the gallbladder is positively correlated with the presence of gallstones (22). These bacteria seem to be also involved in the gallbladder carcinogenesis (36,37), maybe through the bacterial degradation of bile (38,39). Nevertheless, the etiology and pathogenesis of gallbladder cancer is very complex and still an unsolved puzzle.

1.2.4 Therapy and Prognosis of Gallbladder Cancer

The curative treatment of gallbladder cancer always includes the complete surgical resection of the tumor (3,6). In 1995, Carriaga and Henson reported a 5 year survival of 41.9 % for patients with localized disease, 3.8 % for patients with regional metastases, and only 0.7 % for stages with distant metastases (9). Regardless of some progress, the 5 year survival rate still does not exceed 35–38 % after aggressive surgery with curative intent (20,40). Aggressive surgery includes extended partial liver resection, biliary tree excision, and lymphadenectomy (20). By contrast, the incidental diagnosis of a T1a lesion after cholecystectomy (e.g., for cholelithiasis) has a cure rate of 85–100 % (22).

However, the prognosis is extremely poor at large because gallbladder cancer is often diagnosed at an advanced stage (9). The reasons for that are, among others, the pernicious nature of this type of cancer, the anatomic embedding of the gallbladder, and consequently the complicated surgical access (20). Cholecystectomy for cholelithiasis reduces the formation and with that the incidence of gallbladder

cancer (41). However, prophylactic cholecystectomy for asymptomatic cholelithiasis is not recommended on account of the very low likelihood of cancer development in these patients (42). On the other hand, in high incidence areas, such as Chile and India, prophylactic cholecystectomy may be beneficial (43).

1.3 PTEN

In normal development, cell survival and cell death are in a sensitive equilibrium. Both excessive cell death and unhindered cell growth are classic features of cancerous conditions. These conditions also include disturbed cell differentiation. The transition from normal cells to tumor cells is not characterized by only one mutation, but multiple changes (44). Owing to these mutations, there can be increased expressions of oncogenes and/or decreased expressions of tumor suppressor genes (44). This leads to excessive cell growth mediated by oncogenes, while downregulated tumor suppressor genes are no longer able to stop this event. (44)

In 1997, the identification of homozygous deletions on the human chromosome 10q23 led to the discovery of the tumor suppressor gene *PTEN* (*phosphatase and tensin homolog deleted on chromosome ten*), also known as *MMAC1* (*mutated in multiple advanced cancers 1*) (45,46). Mutations of *PTEN* were first found in brain, breast, and prostate cancers (45,46). A short time later, different research groups reported that *PTEN* was also mutated in malignant melanomas (47), endometrial carcinomas (48), and in less frequency in further tumor entities (49). In contrast to first assumptions (mutated in multiple **advanced** cancers 1), the inactivation of *PTEN* can also take place during early tumorigenesis (50–52). In mouse models it was demonstrated that homozygous *PTEN* inactivation caused early embryonic deaths, whereas heterozygous mutations were accompanied by tumorigenic processes in multiple organs (53,54). These investigations point out the fundamental importance of *PTEN* in embryonic development and oncogenesis (44).

Nowadays, it is a well-established fact that *PTEN* is deleted, mutated, or downregulated in a wide range of human cancers. Cantley and Neel even compared the significance of *PTEN* in carcinogenesis with *p53* (44). In accordance with this,

PTEN is characterized as the second most mutated tumor suppressor gene after *p53* (55). This is not surprising because *PTEN* as well as *p53* are both caretaker and gatekeeper genes (56). While caretaker genes account for the genome stability, gatekeeper genes intervene in the cell proliferation (56).

1.3.1 Functions of PTEN

PTEN is responsible for controlling cell proliferation, differentiation, migration, and apoptosis (57). Regarding these processes, the lipid phosphatase function of PTEN is the striking part in the downregulation of the proto-oncogenic PI3K/Akt pathway at the plasma membrane (58). The protein phosphatase of PTEN may also contribute to its tumor suppressor capacity (59–61). In addition to its tasks in the cytoplasm, PTEN has a nuclear activity, which is essential for the genome stability (62). In summary, the tumor suppressor activity of PTEN depends quite possibly on its cytoplasmic as well as on its nuclear functions (62).

Increasing numbers of cellular events are reported that are in direct connection with PTEN. For instance, PTEN initiates cell cycle arrest by activating the APC-CDH1 complex (63), and neutrophils prioritize chemoattractants in a PTEN-dependent manner (64). Future research will have to clarify whether and how all these activities contribute to the tumor suppressor function of PTEN.

1.3.2 Regulation of PTEN

Except for genetic loss or mutation, PTEN expression can also be reduced by the action of transcription factors, post-transcriptionally by microRNAs and the *PTEN* pseudogene, and post-translationally by various modifiers (65–67). The latter includes the polyubiquitylation of PTEN and its subsequent degradation through the ubiquitin-proteasome pathway in the cytoplasm; by contrast, the monoubiquitylation of PTEN is necessary for its nuclear function (68,69). Moreover, phosphorylation converts PTEN into a closed conformation with a decreased tumor suppressor activity (70–72). The disruption of PTEN causes centromeric breaks, DNA double-strand breaks, insufficient DNA repair mechanisms, cell survival, cell invasion,

and angiogenesis (58,62). The current level of knowledge regarding PTEN regulation is summarized in Table 2.

Genetic Changes of <i>PTEN</i>	<i>PTEN</i> Expression
- Deletions	+/- Transcription factors
- Point mutations	- Methylation-mediated gene silencing
	- MicroRNAs
Modifications of <i>PTEN</i>	<i>PTEN</i> -Protein Interactions
+/- Ubiquitylation	+ PRDX1
- Acetylation	- P-REX2a
- Oxidation	- SIPL1
- Phosphorylation	

Table 2: Regulation of *PTEN*

Transcription factors can intensify (e.g., p53) or reduce (e.g., NF-κB) the transcription of PTEN. + (PTEN activity stimulated by), - (PTEN activity suppressed by), PRDX1 (peroxiredoxin 1), P-Rex2a (phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 2a), SIPL1 (shank-interacting protein-like 1); Table generated according to McCubrey et al. (73), Maehama (74), Salmena et al. (65), and Georgescu (55)

1.3.3 Mutations of *PTEN*

There are germline and somatic mutations of *PTEN*. Heterozygous germline mutations of *PTEN* cause autosomal dominant inherited hamartoma syndromes [e.g., Cowden disease (75–77) and Bannayan-Zonana syndrome (77)]. Cowden disease is well known for the occurrence of multiple hamartomas and is a high risk factor for breast and thyroid cancer (78). Somatic mutations of *PTEN* occur in multiple sporadic tumor entities (see 1.3 PTEN).

1.4 The PI3K/Akt Pathway

PTEN influences cell growth, cell survival, DNA damage repair, and apoptosis by intervening in the PI3K/Akt pathway (69). PTEN dephosphorylates PIP₃ (phosphatidylinositol-3,4,5-trisphosphate) at position three on the inositol ring to generate PIP₂ (phosphatidylinositol-4,5-bisphosphate) (79). Therefore, PTEN is the antagonist of PI3K (phosphatidylinositide 3-kinase), which phosphorylates PIP₂ to

PIP₃ and thereby initiates the PI3K/Akt signaling pathway (80). In 2011, *PIK3CA*, the catalytic subunit alpha of PI3K, was described as the most commonly mutated kinase in human cancer (81). Growth factors, for example, IGF-1 (insulin-like **g**rowth **f**actor **1**), PDGF (**p**latelet-**d**erived **g**rowth **f**actor), and NGF (**n**erve **g**rowth **f**actor) result in the production of PIP₂ (44). PIP₃ allows the phosphorylation and consequently the activation of Akt by PDK1 (**PIP**₃-**d**ependent protein **k**inase **1**) at threonine 308 (82) and by mTORC2 (**m**ammalian **t**arget **o**f **r**apamycin **c**omplex **2**) at serine 473 (83,84).

The serine-threonine-specific protein kinase encoded by the *Akt* proto-oncogene (85), also known as PKB (**p**rotein **k**inase **B**) (86), is frequently upregulated in human cancer in view of disturbed upstream signaling components (81). Besides, an increased amplification or mutations of *Akt* were also reported (81). Akt activates the oncoproteins IKK (**I**kB **k**inase) and MDM2 (**m**ouse **d**ouble **m**inute **2**) (69). IKK prevents apoptosis by activating NF-κB (**n**uclear **f**actor **κ**appa **B**) (69), whereas the activation of MDM2 results in the proteasome-dependent degradation of p53 (87). As a consequence, PTEN is capable of keeping the normal function of p53 by blocking the PI3K/Akt pathway (88). On the other hand, the transcription of *PTEN* can be intensified by p53 (89). This means that the two most important tumor suppressors are closely linked to each other (88).

PTEN stops cell cycle progression in the G1 phase by the upregulation of p27Kip1 (cyclin-dependent **k**inase **i**nhibitor **1**B) and the downregulation of cyclin D1 (90). By contrast, Akt prevents apoptosis, among others, by blocking the Bcl-2 family member BAD (**B**cl-2-**a**ssociated **d**eath promoter) (91). In addition, Akt stimulates p70S6K (**70** kDa ribosomal **p**rotein **S6** **k**inase) (92) by the activation of mTORC1 (**m**ammalian **t**arget **o**f **r**apamycin **c**omplex **1**) (73). p70S6K eventually slows down the PI3K/Akt pathway due to a negative feedback loop (93). mTOR (**m**ammalian **t**arget **o**f **r**apamycin) can, however, also lead to a further enhancement of Akt by mTORC2 (73). An overview of the PI3K/Akt pathway with further downstream targets is illustrated in Figure 3.

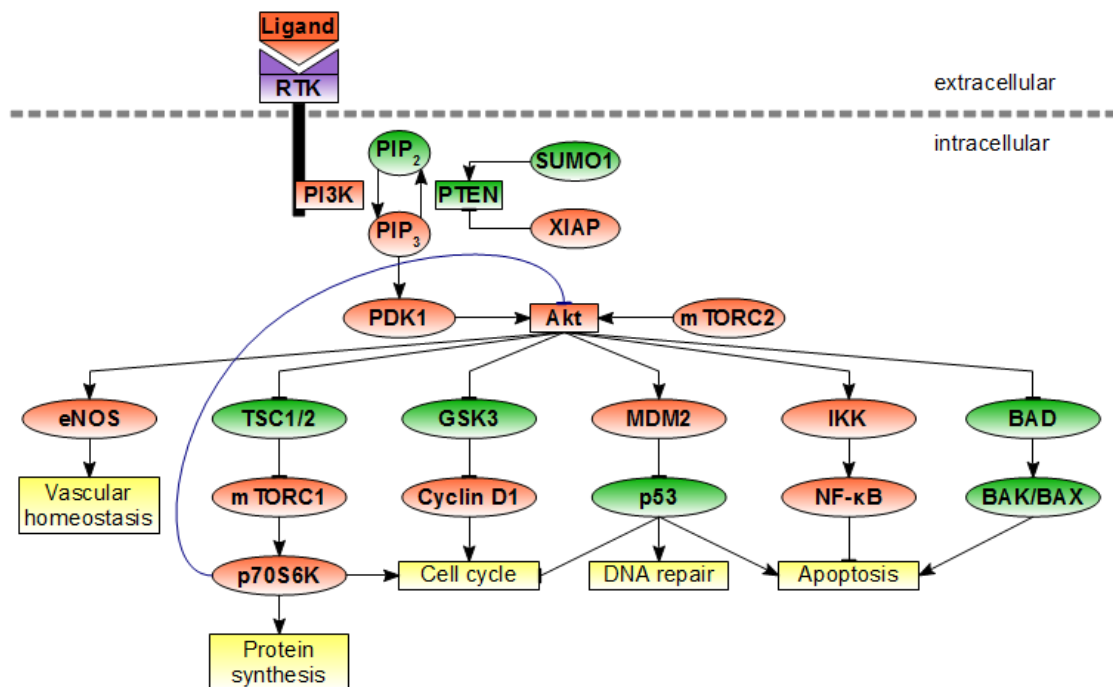


Figure 3: The PI3K/Akt Signaling Pathway

Various growth factors and insulin are ligands of receptor tyrosine kinases. → (activate), ⊥ (inhibit), BAK (**B**cl-2 antagonist/killer), BAX (**B**cl-2-associated **X** protein), eNOS (endothelial **n**itric **o**xide **s**ynthase), GSK3 (**g**lycogen **s**ynthase **k**inase **3**), RTK (**r**eceptor **t**yrosine **k**inase), SUMO1 (**s**mall **u**biquitin-like **m**odifier **1**), TSC1/2 (**t**uberous **s**clerotic **c**omplex **1** and **2**), XIAP (**X**-linked **i**nhibitor of **a**poptosis **p**rotein); Figure generated according to Govender and Chetty (69), Chalhoub and Baker (94), McCubrey et al. (73), Jiang and Liu (95), and Huang et al. (96)

Taken together, the tumor suppressor PTEN stops the PI3K-dependent activation of Akt (44) or in other words, higher levels of PIP₃ can result from the loss of PTEN function and on account of that, Akt is more activated (79). This connection was proven by studies that showed strong Akt activation in *PTEN*-deficient tumor cell lines (97) and Akt inhibition in *PTEN*-deficient tumor cell lines with reintroduced wild type *PTEN* gene (98). Furthermore, high Akt activation was found in tumors of *PTEN*-deficient mice (99). Not surprisingly, the upregulation of the PI3K/Akt pathway plays a leading role in human carcinogenesis.

Apart from the PI3K/Akt pathway, PTEN is involved in another cellular signaling cascade. The MAPK (mitogen-activated protein kinase) pathway, also known as Ras/Raf/MEK/ERK pathway, is also connected with cell proliferation and cell differentiation (100,101). Beyond that, the PI3K/Akt pathway communicates with further pathways, among others, with the Jak/STAT (Janus kinase/signal transducer and activator of transcription) pathway.

1.4.1 Targeting the PI3K/Akt Pathway

As deletion or mutation of *PTEN* is frequently found in a variety of cancer entities, the development of targeted therapies may be worthwhile. Loss of PTEN notably causes a hyperactivation of the PI3K/Akt pathway. Fortunately, the PI3K/Akt pathway offers numerous possible targets. In 2001, Neshat et al. showed the sensitivity of *PTEN*-deficient cancer cell lines to the rapamycin derivative inhibitor CCI-779 (cell cycle inhibitor **779**) (102). Since 2007, CCI-779, otherwise known as temsirolimus, has been licensed for the therapy of renal cell carcinoma (103). Aside from the first benefits concerning targeted chemotherapies in general, therapeutic limitations have also emerged. For instance, drug resistance correlated with a loss of PTEN in the breast (104) and colon cancer (105). However, the combination of trastuzumab with inhibitors of the PI3K/Akt pathway can avoid PTEN-dependent resistance in breast cancer (106).

It is also known that Akt mediates cisplatin resistance, and the PI3K inhibitor LY294002 can abolish this fact (107,108). Therefore, many inhibitors of the PI3K/Akt pathway are currently under development, and the combination of these inhibitors and conventional anticancer therapies (e.g., chemotherapy or radiotherapy) could improve treatment-refractory conditions in the future (109–111). These advances demonstrate the importance of further investigations of PTEN and the PI3K/Akt signaling pathway in oncogenesis. Particularly the establishment of targeted therapies may be the secret to success in tumor entities, such as biliary tract cancer, that show a dismal prognosis because there is a lack of therapy options.

1.4.2 The PI3K/Akt Pathway in Biliary Tract Cancer

The investigation of the PI3K/Akt pathway in biliary tract cancer is quite a new approach for a better understanding of the biliary carcinogenesis. The few studies that exist showed highly promising results. Therefore, further investigations are urgently needed in this area of research.

The liver-specific disruption of *PTEN* and *SMAD4* caused intrahepatic cholangiocarcinomas in a mouse model (112). Downstream targets of PTEN such as phospho-Akt and phospho-mTOR had increased levels in this study (112). Activated Akt and mTOR (i.e., phospho-Akt and phospho-mTOR) were also detected in further bile duct cancer studies (113–115), and phospho-mTOR correlated with a shorter overall survival (115). Interestingly, phospho-mTOR (116), phospho-Akt (116), and Akt (117) expressions were associated with a better prognosis in other studies. Moreover, the reduction of PDK1 and Akt activation by celecoxib led to apoptosis in cholangiocarcinoma cells (118). PTEN had also decreased phosphorylation levels in this study, but this is known for an enhanced tumor suppressor activity of PTEN (118).

In another murine model, the simultaneous deletion of *PTEN* and the activation of *KRAS* resulted in various biliary neoplasms (119). mPGES-1 (microsomal prostaglandin E synthase 1), Ars2 (arsenic resistance protein 2) and its target microRNA-21 were upregulated in cholangiocarcinomas and negatively influenced PTEN expression (120–122). Consequently, the regulation of PTEN is extremely complex. Whereas *KRAS* mutations have been widely established in biliary tract cancer by several research groups, the function of PTEN remains poorly understood.

1.4.3 The PI3K/Akt Pathway in Gallbladder Cancer

p53, the guardian of the genome, is mutated in more than 50 % of human tumors, including gallbladder cancer (1). Mutations of *p53* are highly associated with *p53* overexpression in tumors (123). The immunohistochemical expression of *p53* increased from 33 % in dysplasia to 59 % in invasive carcinoma and was neither detectable in normal nor metaplastic epithelium (124,125). Interestingly, the loss of heterozygosity of *p53* was already found in normal (25 %) and metaplastic epithelium

(17 %) and was detected in 91 % of invasive carcinomas (124). In summary, it was already considered almost 20 years ago that p53 was an important factor in the pathogenesis of gallbladder cancer. Since then, no great progress could be made notwithstanding many p53-related studies in gallbladder cancer. Consequently, more attention has been paid to other genes and proteins in the last few years.

In 2011, Liu and Yang detected a stronger immunostaining of PTEN in cases of cholecystitis (100 %), gallbladder polyps (93.3 %), and peritumoral tissue (87 %) compared to gallbladder adenocarcinomas (48.2 %) (126). Tumor growth, the loss of differentiation, and dissemination independently correlated with a lower PTEN expression (126). Moreover, PTEN expression was especially cytoplasmic and associated with a better outcome (126).

Besides the loss of PTEN function, mutations of *PI3K* (12.5 %) could also be responsible for the activation of the protumorigenic PI3K/Akt pathway in gallbladder cancer (127). The immunohistochemical staining intensity of PI3K in adenocarcinoma (50.9 %) was significantly higher than in benign tissue (20 %) or cases of cholecystitis (8.6 %) (128). Advanced stages and poor prognosis were associated with stronger PI3K expression (128). The downstream target phospho-mTOR was expressed in 64.1 % of gallbladder cancer samples, in 32.4 % of cases with cholecystitis including metaplasia, and only in 16 % of specimens with cholecystitis lacking metaplasia (129). Therefore, mTOR seems to play an important role in the early phase of the carcinogenesis of gallbladder cancer and is furthermore associated with a decreased overall survival (129).

1.5 Objective of this Thesis

The exploration of proto-oncogenic pathways generates the understanding of the molecular mechanisms that are responsible for cancer formation. The understanding of these mechanisms is the basis of cancer diagnostics, therapy, and prevention. The pathogenesis of biliary tract malignancies is poorly understood so far. As a result, favorable diagnostic procedures and therapeutic strategies are still lacking. Furthermore, the incidence of biliary tract cancer, as well as the cancer incidence in

general, will increase considerably in the future (see Figure 4) owing to the growth and aging of the world population (4). Therefore, cancer research is more important than ever before.

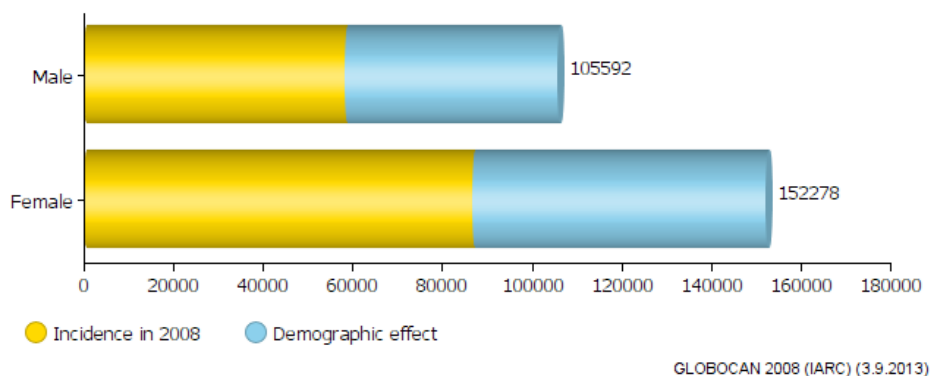


Figure 4: Predicted Incidence of Biliary Tract Cancer in 2030

This prediction compares the number of new biliary tract cancer cases (excluding intrahepatic cholangiocarcinomas) in 2008 with which in 2030 for all ages and was provided by GLOBOCAN 2008 (4).

1.5.1 Experimental Approach

The present work addresses this gap of research by investigating the influence of PTEN and the PI3K/Akt pathway on the carcinogenesis in the biliary tract. Various research methods were used to gain a comprehensive insight into the underlying molecular processes. Frozen gallbladder cancer samples and frozen non-tumorous gallbladder tissue, as well as various biliary tract cancer cell lines, were evaluated at both the gene and protein level. In order to achieve reliable results, qRT-PCRs (**q**uantitative **r**everse **t**ranscription **p**olymerase **c**hain **r**eaction) and Western blots were performed. Moreover, an immunohistochemical staining of a high quantity of gallbladder cancer samples was conducted. The study design was approved by the Ethics Committee of the Medical University of Graz (24-177 ex 11/12). In addition, the gallbladder cancer tissue from the University Hospital of Innsbruck was already utilized in other research studies (130,131), and the use of gallbladder tissue from the Yonsei University College of Medicine was in accordance with South Korean research guidelines.

1.5.2 Hypotheses

Before experiments began, the following hypotheses were advanced:

Firstly, PTEN is downregulated and its proto-oncogenic targets Akt, mTOR, and p70S6K are activated in biliary tract cancer cell lines.

Secondly, the transition from normal gallbladder epithelium to gallbladder cancer is characterized by the loss or downregulation of PTEN.

Thirdly, an increasing invasive capacity and a poor differentiation of gallbladder cancer correlated with the downregulation of PTEN.

Fourthly, the loss and the downregulation of PTEN lead to an enhanced expression of its proto-oncogenic targets Akt, mTOR, and p70S6K.

2 Material and Methods

2.1 Samples

2.1.1 Cancer Cell Lines

Eight different biliary tract cancer cell lines and one reference cell line were included in this thesis (see Table 3 and Figure 5). The biliary tract cancer cell lines were kindly provided by Dr. Tobias Kiesslich, who is a research fellow at the Institute of Physiology and Pathophysiology at the Paracelsus Medical University of Salzburg. HeLa cells were supplied by the Center for Medical Research at the Medical University of Graz.

Cell Line	Obtained From
GBC	Gallbladder mucosal carcinoma
Mz-ChA-1	Gallbladder adenocarcinoma abdominal wall metastasis
Mz-ChA-2	Gallbladder adenocarcinoma liver metastasis
BDC	Bile duct cancer
CC-SW-1	Intrahepatic biliary adenocarcinoma
EGI-1	Extrahepatic biliary adenocarcinoma
TFK-1	Extrahepatic biliary adenocarcinoma
SK-ChA-1	Malignant ascites in extrahepatic biliary adenocarcinoma
HeLa	Epidermoid carcinoma of the cervix

Table 3: Background of Cancer Cell Lines

All cell lines derived from human beings. Further information about the cell lines is provided by the following sources: GBC (132,133), Mz-ChA-1 (134), Mz-ChA-2 (134), BDC (133), CC-SW-1 (135), EGI-1 (136), TFK-1 (137), SK-ChA-1 (134), HeLa (138).

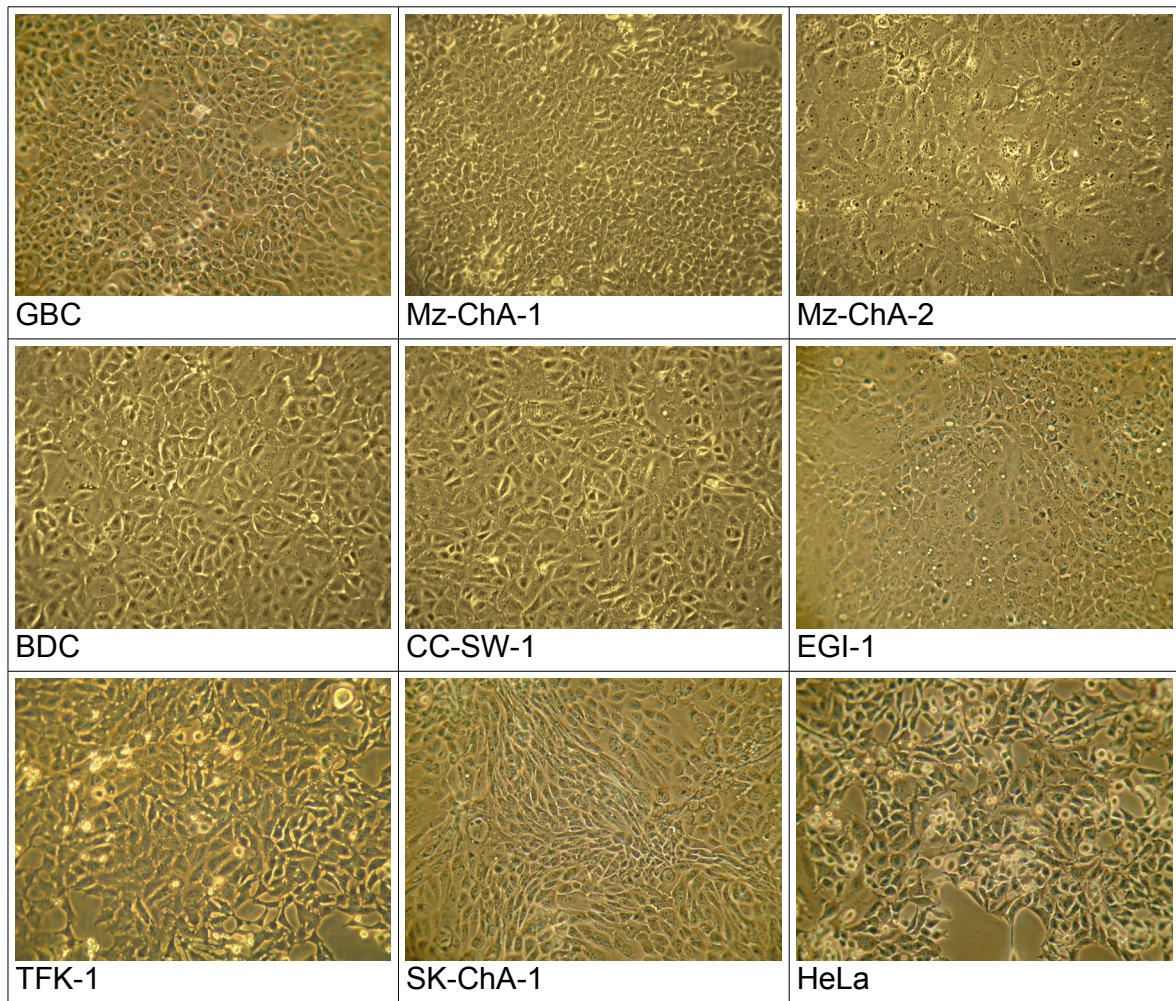


Figure 5: Morphological Characteristics of Cell Lines

All cell lines were photographed at 10 × magnification.

2.1.2 Frozen Gallbladder Tissue

Eight frozen gallbladder cancer samples and nine tumor-free gallbladder samples were provided by the Biobank Graz. The tumor-free tissue originated from gallbladders that were affected by cancer. These specimens included one acute cholecystitis and two samples with residues after cholecystitis.

2.1.3 Gallbladder TMAs

A total of 500 paraffin-embedded gallbladder tissue samples were investigated by immunohistochemical staining. These samples included 396 gallbladder cancer cases, 3 mild chronic cholecystitis cases (**CC**), and 101 samples of tumor-free tissue

from gallbladders that were affected by cancer. The latter was referred to as Control (**Co**). The paraffin-embedded tissue was provided by the University Hospital of Graz (Prof. Johannes Haybaeck), the Hospital Graz West (Prof. Sigurd Lax), the University Hospital of Innsbruck (Dr. Peter Obrist), and the Yonsei University College of Medicine (Prof. Young Nyun Park) (see Table 4). The tissue from the University Hospital of Graz was provided by the Biobank Graz. In order to facilitate immunohistochemistry, representative cores of the paraffin-embedded tissue were punched out to create tissue microarrays (**TMA**s). The TMAs of the University Hospital of Graz, the Hospital Graz West, and the University Hospital of Innsbruck were generated by Iris Halbwedl (Institute of Pathology, Medical University of Graz).

	Co	CC	Tumor
LKH	n=0	n=3	n=200
LKH West	n=0	n=0	n=29
Innsbruck	n=0	n=0	n=54
Seoul	n=101	n=0	n=113

Table 4: Cohort Composition

All tumor cases are hereinafter referred to as tumor. n (sample size)

2.2 Experiments

2.2.1 Cell Culture

In order to guarantee aseptic conditions, cell culture work was done using a laminar flow hood. Furthermore, the equipment and all materials were properly disinfected before use. Cell culture flasks were stored in an incubator with humidified atmosphere of 5 % CO₂ at 37 °C. DMEM (**D**ulbecco's **m**odified **E**agle **m**edium; Gibco, Life Technologies) with high glucose, L-glutamine, HEPES, and phenol red, and without sodium pyruvate was used as cell culture medium. Before use, 50 mL Fetal Bovine Serum Gold (GE Healthcare) and 5 mL penicillin-streptomycin (each 10 000 units/mL, Lonza) were added to 500 mL DMEM. DPBS (**D**ulbecco's **p**hosphate-**b**uffered **s**aline; Gibco, Life Technologies) without calcium and magnesium was used to wash cells.

DMEM, DPBS, and trypsin EDTA (ethylenediaminetetraacetic acid, PAA Laboratories) were stored at 4 °C and warmed in a 37 °C water bath before use.

2.2.1.1 Thawing Frozen Cells

Frozen cells were transported in liquid nitrogen. The cells were slightly defrosted and carefully mixed with 2 mL DMEM. Afterwards, cells were transferred into a 75 cm² or 162 cm² cell culture flask, which was prefilled with 10 mL or 20 mL DMEM, respectively. After 24 hours, the medium was changed to remove dead cells and metabolic products. Apart from that, DMEM was changed whenever the nutrients were exhausted.

2.2.1.2 Passaging Cells

Cells have to be subcultured when they cover the entire substrate of a cell culture flask. Otherwise, cell death would exceed cell growth. In order to subculture cells, the medium was aspirated, and the flask was gently rinsed with 10 mL DPBS to remove medium residues. The latter is necessary because medium residues inactivate the proteolytic enzyme trypsin. Cells were treated with 1000 µL trypsin EDTA to detach them. This process was facilitated by putting the flask into the incubator for up to 10 min. The detachment of the cells was eventually confirmed by microscopic examination. The loosened cells were resuspended in 10 mL DMEM and centrifuged at 800 rpm (revolutions per minute) for 7 min. After the centrifugation, the medium was aspirated, and the cell pellet was carefully dissolved in 10 mL fresh DMEM. This suspension was split up into new cell culture flasks.

2.2.1.3 Cryopreservation of Cells

Instead of splitting cells after centrifugation, cells can also be frozen for long-time storage. In case of a 162 cm² flask, a cell pellet was dissolved in 4 mL cold freezing medium, which consisted of fetal bovine serum and 10 % dimethyl sulfoxide (Sigma-Aldrich). Cryogenic tubes were filled with 1 mL of this suspension per tube and placed in an isopropanol chamber to ensure a gradual drop of temperature in a -80 °C freezer.

2.2.1.4 Harvesting Cells

The procedure was initially the same as performed to subculture cells (see 2.2.1.2 Passaging Cells). However, the cell pellet was not dissolved in DMEM, but in 4 mL ice-cold DPBS. After centrifuging at 800 rpm for 7 min, DPBS was discarded, and the cell pellet was resuspended in 4 mL fresh ice-cold DPBS and once more centrifuged. Thereafter, DPBS was aspirated, and the cell pellet was dissolved in fresh ice-cold DPBS and split up into several 2 mL tubes according to the cell pellet size. The tubes were centrifuged at 12 000 rpm for 10 min at 4 °C. The supernatants were thoroughly removed, and the tubes with the remaining cell pellets were snap frozen in liquid nitrogen and stored at -80 °C. Multiple washing steps with DPBS are required to guarantee the total removal of medium residues. Otherwise, the residues would disturb further procedures (e.g., purification of RNA).

2.2.2 qRT-PCR

qRT-PCR combines the reverse transcription of RNA into cDNA with the quantification of the latter. Therefore, the gene expression can be determined.

2.2.2.1 RNA Isolation from Cultured Cells

RNA isolation from cultured cells (see 2.1.1 Cancer Cell Lines) was performed by means of the RNeasy Plus Mini Kit (Qiagen) according to the corresponding RNeasy Plus Mini Handbook as of September 2010 (139). The whole procedure was performed at room temperature (i.e., 20–25 °C) as recommended. First, Buffer RLT Plus (Qiagen) was completed with 2-mercaptoethanol (Sigma-Aldrich), and Buffer RPE (Qiagen) was completed with pure ethanol (Merck Millipore) as indicated. A cell pellet originated from a 37.5 cm² growth area (i.e., approximately 5×10^6 cells) was transported in liquid nitrogen and slightly defrosted after removal. The detached cell pellet was carefully dissolved in 600 µL Buffer RLT Plus. The lysate was properly homogenized by vortexing and then transferred into a gDNA Eliminator spin column (Qiagen) to eliminate genomic DNA.

After centrifuging at 12 000 rpm for 30 s, the flow-through was thoroughly mixed with 70 % ethanol. 600 µL of this solution was transferred into an RNeasy spin column

(Qiagen) and centrifuged at 12 000 rpm for 15 s. The flow-through was discarded, and 700 μ L Buffer RW1 (Qiagen) was added to the RNeasy spin column. After centrifuging at 12 000 rpm for 15 s, the flow-through was removed, and 500 μ L Buffer RPE was added to the RNeasy spin column to wash the spin column membrane. After centrifuging at 12 000 rpm for 15 s, the flow-through was discarded, and 500 μ L fresh Buffer RPE was added to the RNeasy spin column. The following centrifugation at 12 000 rpm for 2 min guaranteed the total elimination of ethanol, which would have interfered with further reactions. The RNeasy spin column was placed into a fresh tube, and another centrifugation at 12 000 rpm for 1 min was performed to remove all residuals of Buffer RPE. Thereafter, the RNeasy spin column was once again placed into a new tube. 50 μ L RNase-free water (Qiagen) was directly pipetted on the column membrane to elute the RNA. After centrifuging at 12 000 rpm for 1 min, the concentration of the eluted RNA was measured by the NanoDrop 1000 Spectrophotometer V3 (Thermo Scientific). The eluted RNA was subsequently stored at -20°C .

2.2.2.2 RNA Isolation from Frozen Tissue

Every step of the RNA isolation from frozen tissue (see 2.1.2 Frozen Gallbladder Tissue) was performed on ice. The centrifuge was cooled down to 4°C . The frozen samples were transported in liquid nitrogen and cut into suitable pieces as quick as possible to prevent degradation. Up to 100 mg of each frozen sample was mixed with 1 mL TRIzol Reagent (Life Technologies). The mixtures were placed in tubes that were prefilled with ceramic beads (MagNA Lyser Green Beads, Roche). The tubes were centrifuged with the MagNA Lyser Instrument (Roche) for 30 s at 6500 rpm to homogenize the lysates. After an incubation period of 15 min at room temperature, 200 μ L chloroform (Merck Millipore) was added to each tube, and the samples were vigorously mixed by a vortex mixer for 15 s. After incubating for 3 min at room temperature, the samples were centrifuged at 12 000 rpm for 15 min. The supernatants were carefully aspirated and transferred into fresh tubes prefilled with 500 μ L isopropanol (Merck Millipore) and then incubated for 10 min at room temperature. After centrifuging at 12 000 rpm for 30 min, the resulting supernatants were thoroughly removed.

The RNA pellets were washed by mixing them with 70 % ethanol. Following the centrifugation at 12 000 rpm for 5 min, the supernatants were discarded, and the washing procedure was repeated two times. The pellets were dried at 37 °C for 5 min and thereafter dissolved in approximately 50 µL DEPC-treated water according to the respective pellet size. DEPC (**diethyl pyrocarbonate**) is used to inactivate RNase enzymes and thereby prevent RNA degradation. After an incubation period of 10 min at 58 °C with 500 rpm in a thermal mixer, the concentration of RNA was determined by the NanoDrop 1000 Spectrophotometer V3. Afterwards, the RNA was stored at -20 °C.

2.2.2.3 cDNA Synthesis

cDNA synthesis from cultured cells and frozen tissue was performed by means of the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Life Technologies) according to the corresponding High-Capacity cDNA Reverse Transcription Kits Protocol as of June 2010 (140). The following procedure was performed on ice. First, samples with a concentration of 2 µg RNA per 10 µL were prepared. According to the RNA concentration, the appropriate amount of RNA was diluted with Aqua bidest (Fresenius Kabi). Thereafter, the 2 × Reverse Transcription Master Mix was prepared (see Table 5).

Component	Volume (µL)
10 × RT Buffer	2.0
25 × dNTP Mix (100 mM)	0.8
10 × RT Random Primers	2.0
MultiScribe Reverse Transcriptase	1.0
RNase Inhibitor	1.0
Nuclease-free water	3.2
Total per Reaction	10.0

Table 5: Components of 2 × Reverse Transcription Master Mix

All components are included in the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor. dNTP (deoxynucleotide triphosphate)

10 µL of the 2 × Reverse Transcription Master Mix was transferred into the wells of a 96-well plate according to the number of samples. 10 µL of each RNA sample was added to the respective well. To exclude the possibility of genomic DNA contamination, 10 µL of the 2 × Reverse Transcription Master Mix without reverse transcriptase was transferred into a well, and all samples were added in equal parts to get a final RNA concentration of 2 µg per 20 µL. After thoroughly mixing and removing of air bubbles, the plate was sealed and put in a thermal cycler (see Table 6) to synthesize single-stranded cDNA.

	Step 1	Step 2	Step 3	Step 4
Temperature	25 °C	37 °C	85 °C	4 °C
Time	10 min	120 min	5 min	unlimited

Table 6: Thermal Cycler Protocol

cDNA was transferred into fresh tubes and mixed in a 1:32 ratio with Aqua bidest. Storage took place at –20 °C.

2.2.2.4 Primers

Primer design was performed by means of www.ncbi.nlm.nih.gov/gene, <http://pga.mgh.harvard.edu/primerbank>, and www.ncbi.nlm.nih.gov/tools/primer-blast. The designed primers were ordered from Eurofins MWG Operon (see Tables 7 and 8). According to the supplied protocol, the respective amount of Aqua bidest was added to the primers to get a final concentration of 100 pM. Thereafter, 10 µL of each primer was thoroughly mixed with 90 µL Aqua bidest. These dilutions were stored at –20 °C and used in the following procedures.

Primer		Sequence (5' → 3')	T _m (°C)	GC (%)
PIK3CA	fw	CCA CGA CCA TCA TCA GGT GAA	59.8	52.4
	rev	CCT CAC GGA GGC ATT CTA AAG T	60.3	50
PIK3CB	fw	TAT TTG GAC TTT GCG ACA AGA CT	57.1	39.1
	rev	TCG AAC GTA CTG GTC TGG ATA G	60.3	50
PTEN	fw	TGG ATT CGA CTT AGA CTT GAC CT	58.9	43.5
	rev	GGT GGG TTA TGG TCT TCA AAA GG	60.6	47.8
SUMO1	fw	TGA CCA GGA GGC AAA ACC TTC	59.8	52.4
	rev	AAT TCA TTG GAA CAC CCT GTC TT	57.1	39.1
XIAP	fw	ACC GTG CGG TGC TTT AGT T	56.7	52.6
	rev	TGC GTG GCA CTA TTT TCA AGA TA	57.1	39.1
PDK1	fw	CTG TGA TAC GGA TCA GAA ACC G	60.3	50
	rev	TCC ACC AAA CAA TAA AGA GTG CT	57.1	39.1
Akt	fw	AGC GAC GTG GCT ATT GTG AAG	59.8	52.4
	rev	GCC ATC ATT CTT GAG GAG GAA GT	60.6	47.8
eNOS	fw	TGA TGG CGA AGC GAG TGA AG	59.4	55
	rev	ACT CAT CCA TAC ACA GGA CCC	59.8	52.4
mTOR	fw	ATG CTT GGA ACC GGA CCT G	58.8	57.9
	rev	TCT TGA CTC ATC TCT CGG AGT T	58.4	45.5
p70S6K	fw	CGG GAC GGC TTT TAC CCA G	61	63.2
	rev	TTT CTC ACA ATG TTC CAT GCC A	56.5	40.9
Cyclin D1	fw	GCT GCG AAG TGG AAA CCA TC	59.4	55
	rev	CCT CCT TCT GCA CAC ATT TGA A	58.4	45.5
p53	fw	CAG CAC ATG ACG GAG GTT GT	59.4	55
	rev	TCA TCC AAA TAC TCC ACA CGC	57.9	47.6
BAK	fw	GTT TTC CGC AGC TAC GTT TTT	55.9	42.9
	rev	GCA GAG GTA AGG TGA CCA TCT C	62.1	54.5
BAX	fw	CCC GAG AGG TCT TTT TCC GAG	61.8	57.1
	rev	CCA GCC CAT GAT GGT TCT GAT	59.8	52.4

Table 7: Primers for Target Genes

PIK3CA and PIK3CB are the catalytic subunits alpha and beta of PI3K, respectively. A (adenine), C (cytosine), fw (forward), G (guanine), GC (guanine-cytosine content), rev (reverse), T (thymine), T_m (melting temperature)

Primer		Sequence (5' → 3')	T _m (°C)	GC (%)
Beta-Actin	fw	TGA CCC AGA TCA TGT TTG AG	55.3	45
	rev	CGT ACA GGG ATA GCA CAG	56.0	55.6
Cyclophilin A	fw	AAA TGC TGG ACC CAA CAC	53.7	50
	rev	CCA TCC AAC CAC TCA GTC	56.0	55.6
GAPDH	fw	ATG GGG AAG GTG AAG GTC G	58,8	57,9
	rev	GGG GTC ATT GAT GGC AAC AAT A	58,4	45,5
Lamin A/C	fw	TTA TTC TGC CAA GCT GGA C	54.5	47.4
	rev	CAG GTT GCT GTT CCT CTC	56.0	55.6

Table 8: Primers for Housekeeping Genes

Housekeeping genes are genes that are constantly expressed in all cells. For that reason, expression levels of target genes were normalized to housekeeping genes to ensure an accurate gene quantification. The primers for Beta-Actin, Cyclophilin A, and Lamin A/C were designed by Dr. Karl Kashofer (Institute of Pathology, Medical University of Graz). GAPDH (glyceraldehyde-3-phosphate dehydrogenase)

2.2.2.5 Quantitative PCR

The diluted cDNA samples were transferred into the wells of a 96-well plate, which was placed on ice. The master mixes were also prepared on ice, and one master mix contained 5 µL Power SYBR Green (Applied Biosystems, Life Technologies), 0.05 µL forward primer, 0.05 µL reverse primer, and 0.9 µL Aqua bidest per replicate. Three replicates of each sample were analyzed to evaluate PCR efficiency. SYBR Green is a fluorescent dye that binds to DNA. The quantification of DNA is consequently monitored by the fluorescence intensity. The Microlab STARlet Liquid Handling Workstation (Hamilton), provided by the Center for Medical Research at the Medical University of Graz, transferred the samples and the master mixes to a 384-well plate to guarantee a maximum degree of accuracy and repeatability. The 384-well plate was centrifuged for 1 min and then placed into the 7900HT Fast Real-Time PCR System (Applied Biosystems, Life Technologies) to start a quantitative polymerase chain reaction.

The evaluation of the resulting C_T (threshold cycle) values was conducted by means of the SDS v2.3 software (Applied Biosystems, Life Technologies). The C_T value is

defined as the number of PCR cycles at which the fluorescence emission crosses the threshold. Furthermore, the C_T value increases with a decreasing amount of cDNA in the sample. The threshold, which was placed in the exponential phase of the amplification, and the baseline values, were automatically generated for each gene by the software and manually verified according to the corresponding Relative Quantitation Using Comparative C_T Getting Started Guide as of June 2010 (141). In order to achieve the results for relative quantification, the comparative C_T method (see Table 9) was applied as described by Schmittgen and Livak (142). Relative quantification implies to quantify the differences between the expression levels of specific target genes in different samples. Three replicates of each sample were included in the calculations.

$\Delta C_{T \text{ test sample}}$	$= C_{T \text{ target gene of test sample}} - C_{T \text{ housekeeping gene of test sample}}$
$\Delta C_{T \text{ control sample}}$	$= C_{T \text{ target gene of control sample}} - C_{T \text{ housekeeping gene of control sample}}$
Fold Change	$= (2^{-\Delta C_{T \text{ test sample}}}) / (2^{-\Delta C_{T \text{ control sample}}})$
If Fold Change < 1	$\rightarrow \text{Fold Change}_{\text{revised}} = -1 / \text{Fold Change}$

Table 9: Calculations with the Comparative C_T Method

Test samples corresponded to biliary tract cancer cell lines or frozen gallbladder cancer tissue, whereas HeLa cells or non-tumorous gallbladder tissue were used as control samples, respectively. $C_{T \text{ housekeeping gene}}$ corresponded to the mean value of various housekeeping genes.

2.2.3 Western Blot

Western blot is a well-established procedure to detect and quantify proteins in cells or tissue. Firstly, the proteins will be separated by their size and secondly detected by specific antibodies. Interestingly, post-translational modifications of proteins such as phosphorylations can also be verified by Western blot.

2.2.3.1 Preparation of Samples

Every step of the sample preparation was performed on ice to prevent protein denaturation. Whenever the samples had to be centrifugalized, the centrifuge was cooled down to 4 °C. In order to extract proteins of cells or tissue, a lysis buffer was prepared (see Table 10).

Component		Volume
Aqua bidest		1 mL
PhosSTOP phosphatase inhibitor	(Roche)	1 tablet
Complete Mini protease inhibitor	(Roche)	1 tablet
Nonidet-P40 buffer		9 mL
1 M 1.4-Dithiothreitol	(Carl Roth)	10 µL
0.1 M Pefabloc SC (AEBSF)	(Roche)	100 µL

Table 10: Components of Lysis Buffer

The tablets were primarily dissolved in 1 mL Aqua bidest and thereafter mixed with the other components. Nonidet-P40 buffer consisted of 25 mL 1 M Tris HCl (pH 7.5, *tris*(hydroxymethyl)aminomethane hydrochloride, Merck Millipore), 15 mL 5 M NaCl (sodium chloride, Merck Millipore), and 2.5 mL IGEPAL CA-630 (Sigma-Aldrich), and the reagents were topped up to 500 mL with purified water. Purified water was provided by the TKA MicroPure Water System (TKA, Thermo Scientific). 1.4-Dithiothreitol and Pefabloc SC (AEBSF) [4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride] were diluted with purified water to get the above-mentioned concentrations.

Cell pellets of the cell lines (see 2.1.1 Cancer Cell Lines) were transported in liquid nitrogen. Every cell pellet was carefully dissolved in approximately 50 µL lysis buffer according to the respective cell pellet size. Afterwards, the cells were centrifuged at 12 000 rpm for 10 min. The resulting supernatants, which contained the extracted proteins, were transferred into fresh tubes. By contrast, the pellets, which contained cell debris, were discarded.

Frozen samples (see 2.1.2 Frozen Gallbladder Tissue) were transported in liquid nitrogen and cut into suitable pieces as quick as possible to prevent degradation. The pieces were dissolved in 250 µL lysis buffer in tubes that were prefilled with MagNA Lyser Green Beads. The tubes were centrifuged with the MagNA Lyser Instrument for 30 s at 6500 rpm. The centrifugation with the MagNA Lyser Instrument resulted in a homogenization of the tissue and thereby, facilitated the following extraction and analysis of the proteins. The supernatants were taken and centrifuged at 12 000 rpm for 10 min. The pellets were discarded, while the supernatants were transferred into fresh tubes.

2.2.3.2 Determination of Protein Concentration

In order to load the exact amount of proteins on the gel for electrophoresis, the protein concentrations were measured by means of the Bradford protein assay. One part of Bio-Rad Protein Assay (Bio-Rad) was diluted with four parts of Aqua bidest, and 998 μL of this reagent was filled in every cuvette (Brand). In addition, 2 μL of each sample (i.e., the resulting supernatants from the sample preparation, see 2.2.3.1 Preparation of Samples) was given into the respective cuvette. The cuvettes were covered with Parafilm M (American National Can) and gently swayed. After an incubation time of 15 min, the absorbance and with that, the protein concentrations, were measured by the BioSpectrometer (Eppendorf). The lysis buffer was used as the blank control.

The proteins were mixed in a 1:2 ratio with the sample buffer. The sample buffer was composed of 50 μL 2-mercaptoethanol and 950 μL Laemmli Sample Buffer (Bio-Rad). This step facilitated the later separation of proteins through gel electrophoresis. The samples were snap frozen in liquid nitrogen and stored at $-20\text{ }^{\circ}\text{C}$.

The procedures (i.e., cell culture, preparation of samples, and determination of protein concentration) to obtain proteins from the cell lines were partly performed by Eva Lederer, M. Sc.

2.2.3.3 Gel Electrophoresis

Gel electrophoresis was used to separate proteins according to their size by electric current. Gels, which consisted of a stacking and a separating gel, were prepared as described in Table 11 and stored overnight at $4\text{ }^{\circ}\text{C}$ before use.

	Stacking Gel	8 % Separating Gel	10 % Separating Gel
Purified Water	3.1 mL	4.6 mL	4 mL
1.5 M Tris pH 6.8	1.25 mL	–	–
1.5 M Tris pH 8.8	–	2.5 mL	2.5 mL
30 % Acrylamide/ Bis Solution, 37.5:1 (Bio-Rad)	500 µL	2.7 mL	3.3 mL
10 % SDS	50 µL	100 µL	100 µL
10 % APS	25 µL	100 µL	100 µL
TEMED (Bio-Rad)	7.5 µL	6 µL	7.5 µL

Table 11: Composition of Different Gels

Tris buffers were prepared from Tris and purified water. The pH-values were adjusted with HCl fuming 37 % (Merck Millipore). A lower concentration of acrylamide results in larger pores within the gel. Consequently, the separation of large proteins will be supported. By contrast, higher concentrations of acrylamide are beneficial to smaller proteins. 10 % SDS (sodium dodecyl sulfate) and 10 % APS (ammonium persulfate) were made from SDS (Sigma-Aldrich) and APS (AppliChem), respectively and purified water. TEMED (tetramethylethylenediamine)

Proteins were denatured by heating at 95 °C for 5 min in a thermal mixer. The protein denaturation is necessary to enable the antibodies recognizing the epitopes of the proteins. The gels were placed in an electrophoresis unit and filled with running buffer. The Running buffer was made from 50 mL Rotiphorese 10 × SDS-PAGE (**SDS polyacrylamide gel electrophoresis**, Carl Roth) and 450 mL purified water. Thereafter, 30 µg of each sample and 4 µL of the Novex Sharp Pre-Stained Protein Standard (Invitrogen, Life Technologies) were loaded on the gel. The latter marks molecular weights and thereby allows the protein classification. The electrophoresis ran with 80 V about two hours.

2.2.3.4 Transfer of Proteins

After the protein separation through gel electrophoresis, the proteins had to be transferred to a PVDF (**polyvinylidene difluoride**) membrane (Immobilon-P Membrane, Merck Millipore) for later analysis. The membrane was covered with methanol (Sigma-Aldrich) for 15 s, washed with Aqua bidest for 2 min, and finally placed in a tray with Towbin transfer buffer (see Table 12) for 5 min. Two pieces of Whatman filter

paper (Whatman, GE Healthcare) were also placed in the transfer buffer filled tray for 5 min. The tray was put on a rocking platform shaker to ensure a homogenous covering of the membrane and the pieces of Whatman filter paper during the incubation time. After the electrophoresis run, the gel was taken out, and the stacking gel was discarded, whereas the separating gel was placed on the top of the membrane. The two were sandwiched between the two pieces of Whatman filter paper. The sandwich was drenched by Towbin transfer buffer to avoid dehydration and to guarantee a smooth transfer. Air bubbles were removed by means of a roller, and the blotter unit was gently closed to ensure a proper connection between the gel and the membrane. The transfer of the proteins from the gel to the membrane, also known as blotting, took place by electric current at 80 mA and approximately 4 V.

The successful transfer was proven by staining the membrane with Ponceau S solution (Sigma-Aldrich) and methanol. In order to remove the staining, the membrane was washed three times with TBST (see Table 12) on a rocking platform shaker for 5 min. The following washing steps were likewise performed with TBST on a rocking platform shaker.

Towbin Transfer Buffer	TBST	TBS 10 ×
100 mL Rotiphorese 10 × SDS-PAGE	100 mL TBS 10 ×	< 1 L Purified water
200 mL Methanol	900 mL Purified water	0.2 M Tris
700 mL Purified water	1 mL Tween 20 (Merck Millipore)	1.4 M NaCl

Table 12: Ingredients of Different Buffers

The pH-value of TBS 10 × (concentrated Tris-buffered saline) was adjusted with HCl to 7.6 and topped up to 1 L with purified water. TBST (TBS with Tween 20)

2.2.3.5 Detection of Proteins

Before incubating the membrane with primary and secondary antibodies to detect the targeted proteins, the membrane was blocked on a rocking platform shaker for one hour. Blocking is absolutely necessary to prevent non-specific background caused by antibodies. The blocking reagent was prepared by dissolving 0.5 g non-fat dry milk

(Blotting-Grade Blocker, Bio-Rad) in 10 mL TBST. After blocking, the membrane was washed three times for 5 min. The membrane was incubated with the diluted primary antibody solution overnight at 4 °C on a rocking platform shaker for immunological detection. Primary antibodies (see Table 13) were generally used at dilutions of 1:1000. 10 mL TBST was mixed with either 0.5 g BSA (**b**ovine **s**erum **a**lbumin, Roche) or 0.5 g non-fat dry milk according to the datasheet of the concerning antibody.

Antibody	Product Number	MW (kDa)	Purification
Phospho-PTEN (Ser380)	9551	54	Polyclonal
PTEN	9559	54	Monoclonal
Phospho-Akt (Ser473)	4058	60	Monoclonal
Akt	9272	60	Polyclonal
Phospho-mTOR (Ser2448)	5536	289	Monoclonal
mTOR (1)	2983	289	Monoclonal
mTOR (2)	4517	289	Monoclonal
Phospho-p70S6K (Thr421/Ser424)	9204	70, 85	Polyclonal
p70S6K	9202	70, 85	Polyclonal
Phospho-p53 (Ser15)	9286	53	Monoclonal
p53	2527	53	Monoclonal
GAPDH	2118	37	Monoclonal
Beta-Actin	A5441	42	Monoclonal

Table 13: Primary Antibodies

Phospho-specific antibodies detect proteins only when phosphorylated at bracketed sites. Phospho-p70S6K and p70S6K antibodies also detect phospho-p85S6K and p85S6K, respectively. All antibodies were ordered from Cell Signaling Technology apart from Beta-Actin, which was ordered from Sigma-Aldrich. MW (molecular weight), Ser (serine), Thr (threonine)

On the next morning, the primary antibody was removed, and the membrane was washed five times for at least 5 min to dispose redundant primary antibody. According to whether the primary antibody was produced from rabbit or mouse, the corresponding secondary antibody solution was made from 10 mL TBST, 0.5 g non-fat dry milk, and either 2 µL anti-rabbit or 3 µL anti-mouse secondary antibody (see Table 14). The membrane was incubated for one hour at room temperature and thoroughly washed five times for 5 min under agitation after antibody removal.

Antibody	Product Number	Target
Anti-rabbit	NA934 (GE Healthcare)	IgG
Anti-mouse	NXA931 (GE Healthcare)	IgG

Table 14: Secondary Antibodies

Secondary antibodies are conjugated to the enzyme horseradish peroxidase. Horseradish peroxidase allows the chemiluminescent detection of proteins by the production of visible light.

Depending on the expected signal strength, the membrane was covered with either 1000 µL SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) or Amersham ECL Prime Western Blotting Detection Reagent (RPN2236 or RPN2209, GE Healthcare) for 5 min. The membrane was subsequently placed in a sheet protector in an X-ray cassette, and chemiluminescence films (Amersham Hyperfilm ECL, GE Healthcare) with various exposure times were developed to achieve optimal results. Film processing was performed by means of a developer machine in a darkroom with the following reagents: X-ray developer ROENTOROLL 25 (Tetena), X-ray fixing solution SUPERFIX 25 (Tetena), and tap water.

After film processing, the membrane was washed three times and subsequently stripped of bound antibodies with the Restore PLUS Western Blot Stripping Buffer (Thermo Scientific) for 30 min. Thereafter, the membrane was washed three times for 5 min. After stripping and washing, the membrane could be blocked again, and the detection protocol was performed with further antibodies. However, stripping can damage post-translational modifications of proteins such as phosphorylations. Therefore, the membranes were incubated with phospho-specific antibodies before stripping.

2.2.3.6 Controls for Western Blot

Antibodies were verified by positive and negative controls (see Table 15). Furthermore, GAPDH was used as a loading control to allow a comparison of the results with the protein amounts that were loaded on the gels. Because GAPDH did not seem to be suitable as a loading control in frozen gallbladder samples, Beta-Actin was used to demonstrate an even protein loading in these cases. Moreover, all results were confirmed by at least two independent Western blots.

Control	Product Number	Positive Control	Negative Control	Antibodies
HeLa cells	–	HeLa cells without treatment	–	Phospho-PTEN, PTEN
Akt Control Cell Extracts	9273	Jurkat cells, serum starved overnight and then treated with calyculin A	Jurkat cells, serum starved overnight and then treated with 50 μ M LY294002	Phospho-Akt, Akt
p70S6K Control Cell Extracts	9203	MCF-7 cells treated with insulin	MCF-7 cells without treatment	Phospho-p70S6K, p70S6K, Phospho-mTOR, mTOR
SAPK/JNK Control Cell Extracts	9253	293 cells treated with ultraviolet light	293 cells without treatment	Phospho-p53, p53

Table 15: Controls for Antibodies

Positive controls should be detected by both phosphorylated and non-phosphorylated antibodies, whereas negative controls should only be detected by non-phosphorylated antibodies. However, negative controls can also be detected by phosphorylated antibodies with a weaker signal than non-phosphorylated antibodies. All control cell extracts were ordered from Cell Signaling Technology. JNK (c-Jun N-terminal kinase), SAPK (stress-activated protein kinase)

2.2.4 Immunohistochemistry

Apart from Western blot, proteins of the PI3K/Akt pathway were furthermore detected in paraffin-embedded gallbladder tissue (see 2.1.3 Gallbladder TMAs) by immunohistochemistry. Immunohistochemistry allows the investigation of a multitude of samples and is therefore a well-established procedure in both research and diagnostics.

2.2.4.1 Immunohistochemistry Procedure

Immunohistochemical staining was kindly performed by Margit Gogg-Kamerer (Institute of Pathology, Medical University of Graz). Following deparaffinization with xylol (Merck Millipore) and hydration with a series of decreasing concentrations of ethanol, the slides were washed with Dako Wash Buffer (Dako) and consequently blocked with Peroxidase-Blocking Solution (Dako) for 5 min. After washing with Tris buffer, immunohistochemical staining was performed by the Autostainer Plus

instrument (Dako) or the BenchMark XT automated staining system (Ventana). PTEN (dilution 1:10), phospho-Akt (dilution 1:100), and phospho-mTOR (dilution 1:40) antibodies were used on the Autostainer Plus instrument (see Table 16). Before staining, heat-induced epitope retrieval was performed using a microwave for 40 min with sodium citrate (10 mM, pH 6.0), Target Retrieval Solution (pH 9.0, Dako), and Tris HCl buffer containing 5 % urea (pH 9.5), respectively. After cooling down for 20 min at room temperature, the REAL EnVision Detection System Peroxidase/DAB (diaminobenzidine) Rabbit/Mouse (K5007, Dako) was used to achieve optimal results on the Autostainer Plus instrument. The BenchMark XT automated staining system was used with phospho-p70S6K (1:200 dilution, see Table 16), and the tissue was pretreated with Cell Conditioning 1 Solution (Ventana) for 32 minutes. Phospho-p70S6K was finally identified by the iVIEW DAB Detection Kit (Ventana).

Antibody	Product Number	MW (kDa)	Purification
PTEN	MS-1797	60	Monoclonal
Phospho-Akt (Ser473)	4051	60	Monoclonal
Phospho-mTOR (Ser2448)	2971	289	Polyclonal
Phospho-p70S6K (Ser411)	8416	70	Monoclonal

Table 16: Antibodies for Immunohistochemistry

Phospho-Akt and phospho-mTOR antibodies were ordered from Cell Signaling Technology, whereas PTEN was ordered from Thermo Scientific and phospho-p70S6K from Santa Cruz Biotechnology.

After the immunohistochemical staining of the target antigens, the slides were counterstained with Mayer's Hemalum Solution (Gatt-Koller) for 1 min and consequently blued in tap water to get the best contrast. Thereafter, the slides were dehydrated with increasing concentrations of ethanol and treated with xylol to allow the embedding with Entellan (Merck Millipore).

2.2.4.2 Evaluation of Immunohistochemical Staining

The intensity and density of the nuclear and cytoplasmic staining of tumor or secretory epithelial cells were assessed by both Prof. Johannes Haybaeck and me by means of a light microscope as described in Table 17.

Score	Intensity	Score	Density
0	No staining	0	No staining
1	Weak staining	1	< 10 %
2	Moderate staining	2	10 – 50 %
3	Strong staining	3	51 – 80 %
		4	> 80 %

Table 17: Assessment of Immunohistochemical Staining

The strength of the intensity was allocated to a score between zero and three, whereas the density (i.e., the percentage of positive cells) was allocated to a score between zero and four.

Three replicates of each sample were investigated. In the case of the samples from the Yonsei University College of Medicine, two cancer replicates and one tumor-free sample of each patient were analyzed. The intensity scores of one sample were summarized and divided by the number of intensity values of this sample. The result was classified as 0, low, or high as described in Table 18. The sum of all intensity and density scores of one sample was divided by the number of intensity and density values of this sample. This result was called tissue score, which was also classified as 0, low, or high as indicated in Table 18.

Classification	Intensity	Tissue Score
0	< 0.5	< 0.5
Low	≥ 0.5	≥ 0.5
High	≥ 2.5	≥ 3

Table 18: Evaluation of Immunohistochemical Staining

The following data were used for the analysis: gender, date of surgery, date of death or last follow-up, diagnosis, grading, the TNM (tumor extent, lymph node metastasis, distant metastasis) classification according to the UICC (Union for International Cancer Control), and further parameters [i.e., L (lymphatic vessel invasion) and V (venous invasion)]. The statistical calculations were performed by Robert Reihs as follows. The data were fed in a relational database scheme (PostgreSQL 9.1). R and RStudio with the RPostgreSQL package were used for all calculations and graphs. Kaplan-Meier survival estimates were calculated by means of the R survival package.

The likelihood ratio test in the Cox regression analysis was used to calculate p-values. In case of ties, the Breslow method was applied. The Mann-Whitney test was calculated with the Wilcoxon rank-sum and signed-rank tests from R to determine the significance levels of mean intensities and tissue scores. P-values < 0.05 were considered as statistically significant.

3 Results

3.1 Cancer Cell Lines

3.1.1 qRT-PCRs of Cancer Cell Lines

Four housekeeping genes (*GAPDH*, *Beta-Actin*, *Cyclophilin A*, *Lamin A/C*) were determined in eight biliary tract cancer cell lines and in HeLa cells (see 2.1.1 Cancer Cell Lines). *GAPDH*, *Beta-Actin*, and *Cyclophilin A* showed a similar expression pattern, whereas the expression of *Lamin A/C* clearly differed in Mz-ChA-2 in proportion to EGI-1 and Mz-ChA-1 compared to the other housekeeping genes (see Figures 6 and 7). Therefore, *Lamin A/C* was excluded in further calculations. By contrast, a mean value of the C_T mean values of *GAPDH*, *Beta-Actin*, and *Cyclophilin A* was calculated for each cell line and used for the evaluation with the C_T method (see 2.2.2.5 Quantitative PCR).

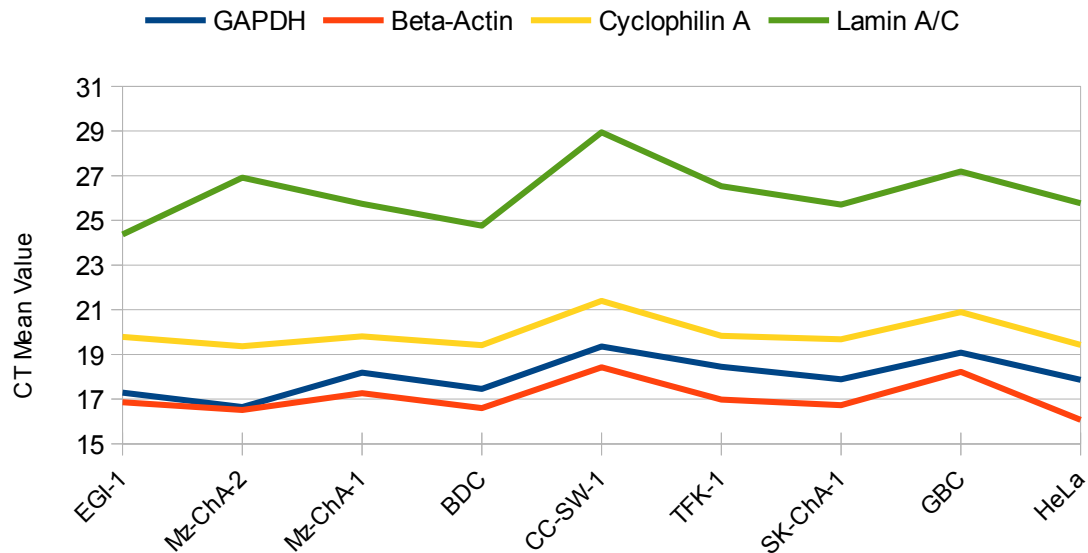


Figure 6: Housekeeping Gene Expressions in Cell Lines

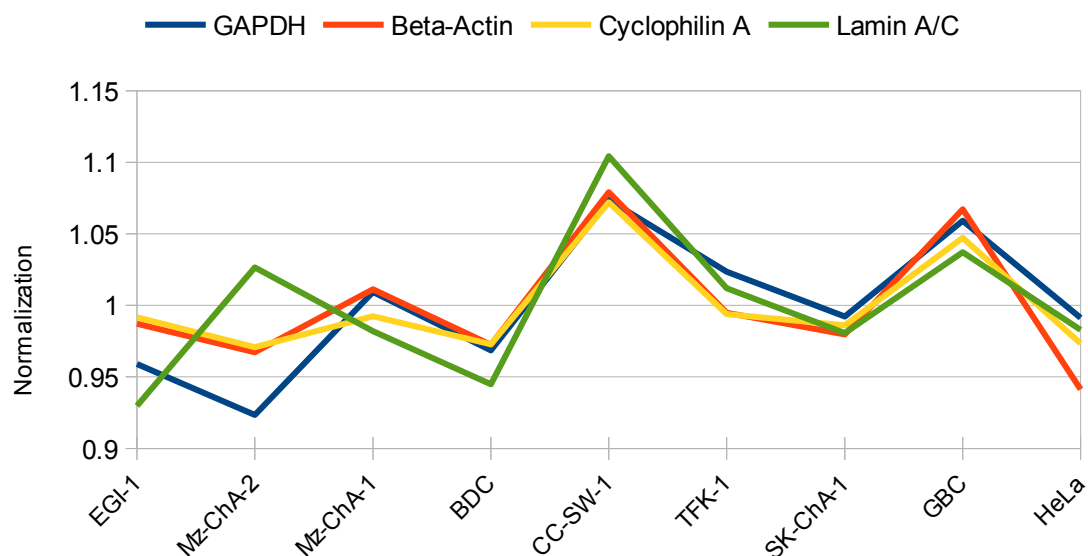


Figure 7: Normalization of Housekeeping Gene Expressions in Cell Lines

The CT mean value of each cell line was divided by the mean value of the CT mean values of all cell lines of the respective housekeeping gene.

PTEN was less expressed in all biliary tract cancer cell lines compared to its expression in HeLa cells. The expression of *PTEN* was even reduced by almost 5-fold in the gallbladder cancer cell lines Mz-ChA-2 and GBC (see Figure 8). Consistent with these facts, the proto-oncogenic PI3K/Akt pathway members *Akt*, *mTOR*, *p70S6K*, *PI3K*, *XIAP*, *eNOS*, and *cyclin D1* were mainly more expressed in biliary tract cancer cell lines than in HeLa cells (see Figures 8, 9, and 11). Interestingly, the proapoptotic Bcl-2 family members *BAK* and *BAX* also showed predominantly higher expression levels (see Figure 10).

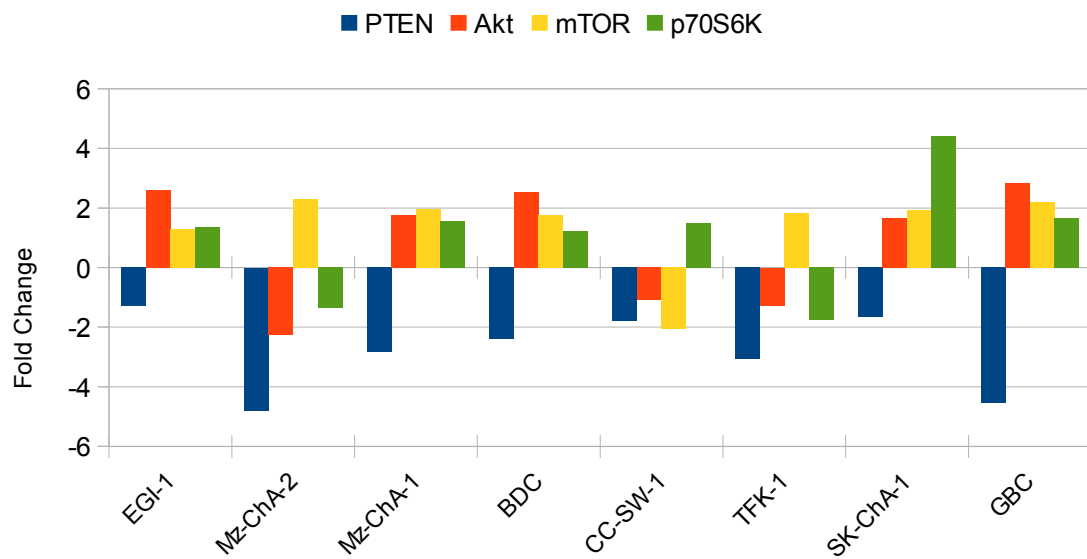


Figure 8: Gene Expressions of PTEN, Akt, mTOR, and p70S6K in Cell Lines

Illustration of gene expressions in biliary tract cancer cell lines when compared to the expression of these genes in HeLa cells.

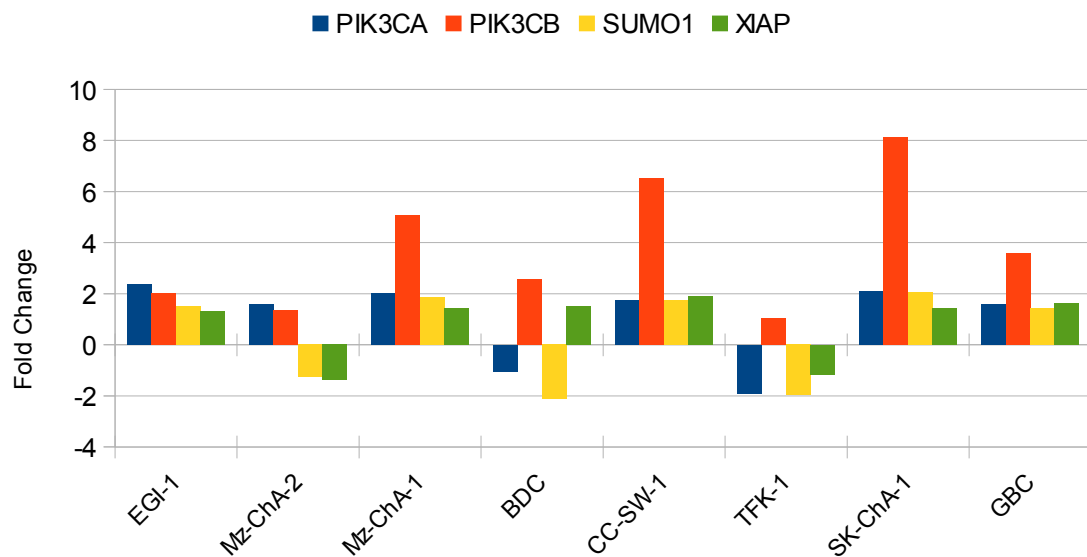


Figure 9: Gene Expressions of PIK3CA, PIK3CB, SUMO1, and XIAP in Cell Lines

Illustration of gene expressions in biliary tract cancer cell lines when compared to the expression of these genes in HeLa cells.

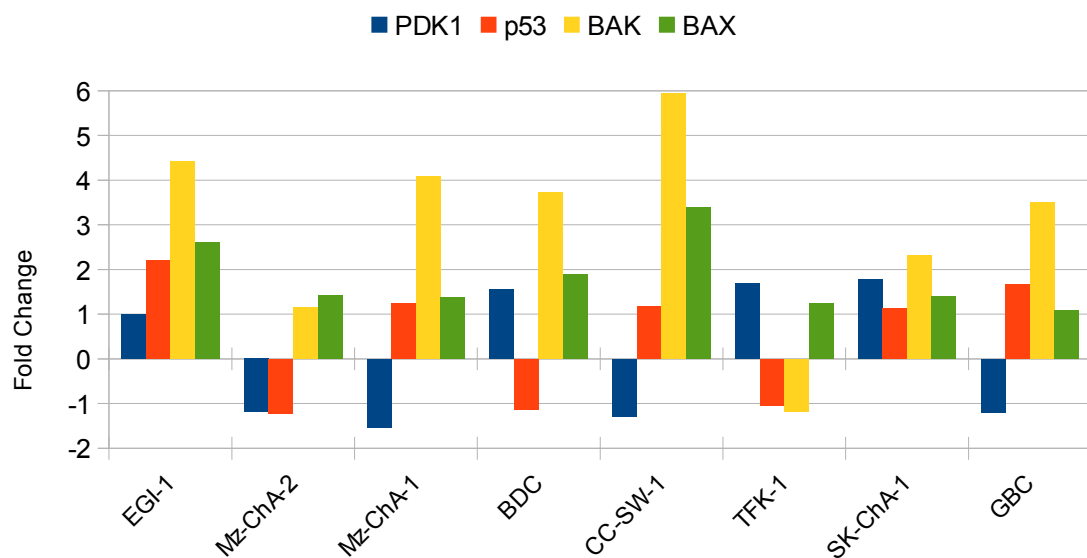


Figure 10: Gene Expressions of PDK1, p53, BAK, and BAX in Cell Lines

Illustration of gene expressions in biliary tract cancer cell lines when compared to the expression of these genes in HeLa cells.

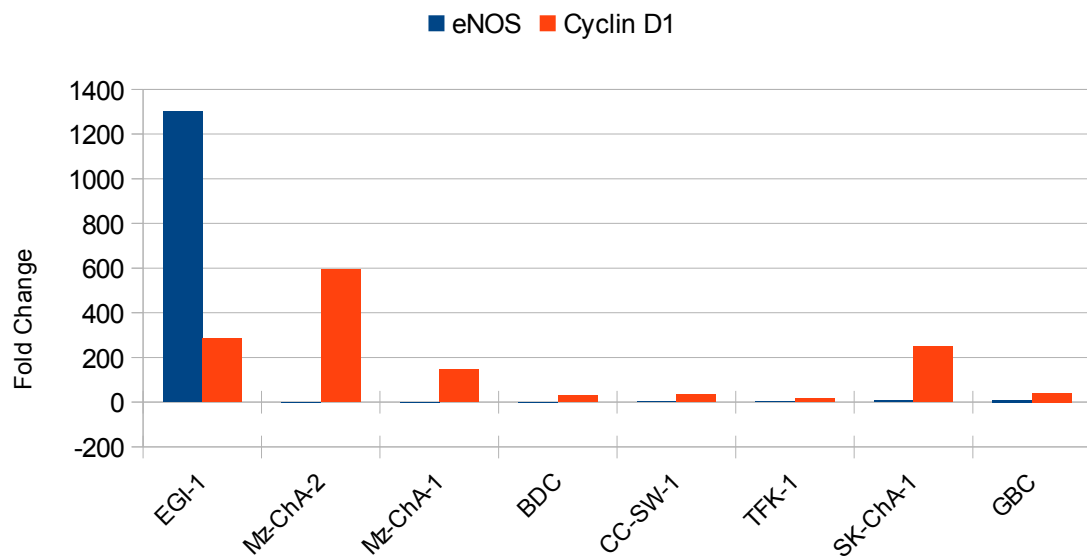


Figure 11: Gene Expressions of eNOS and Cyclin D1 in Cell Lines

Illustration of gene expressions in biliary tract cancer cell lines when compared to the expression of these genes in HeLa cells.

3.1.2 Western Blots of Cancer Cell Lines

The expression levels of the key proteins of the PI3K/Akt pathway in biliary tract cancer cell lines and HeLa cells are illustrated in Figure 12. The housekeeping genes GAPDH and Beta-Actin confirmed that equal protein amounts were loaded on the gels.

PTEN was not detectable in the gallbladder cancer cell lines Mz-ChA-1 and GBC, whereas all other cell lines showed both PTEN and phospho-PTEN (Ser380) expressions. Akt was present in all cell lines. However, activated Akt (i.e., phospho-Akt, Ser473) was highly expressed in the PTEN-deficient cell lines Mz-ChA-1 and GBC and less strongly expressed in EGI-1 and Mz-ChA-2. Activated-Akt was not present in all the other cell lines. One phospho-mTOR (Ser2448) and two mTOR antibodies showed quite unspecific signals. Therefore, the represented expression levels of mTOR and phospho-mTOR must be studied with reservations. However, the expression levels of mTOR and activated mTOR (i.e., phospho-mTOR) were approximately balanced in all cancer cell lines except for HeLa cells. p70S6K was present in all cell lines, whereas phospho-p70S6K (Thr421/Ser424) was not detectable in HeLa cells and was present with different expression levels in all the other cell lines.

Interestingly, both phospho-p70S6K and phospho-p53 (Ser15) were highly expressed in the PTEN-deficient cell lines Mz-ChA-1 and GBC. In addition, phospho-p53 was also highly expressed in EGI-1 and weakly present in SK-ChA-1, whereas p53 was even verifiable in Mz-ChA-2 and BDC.

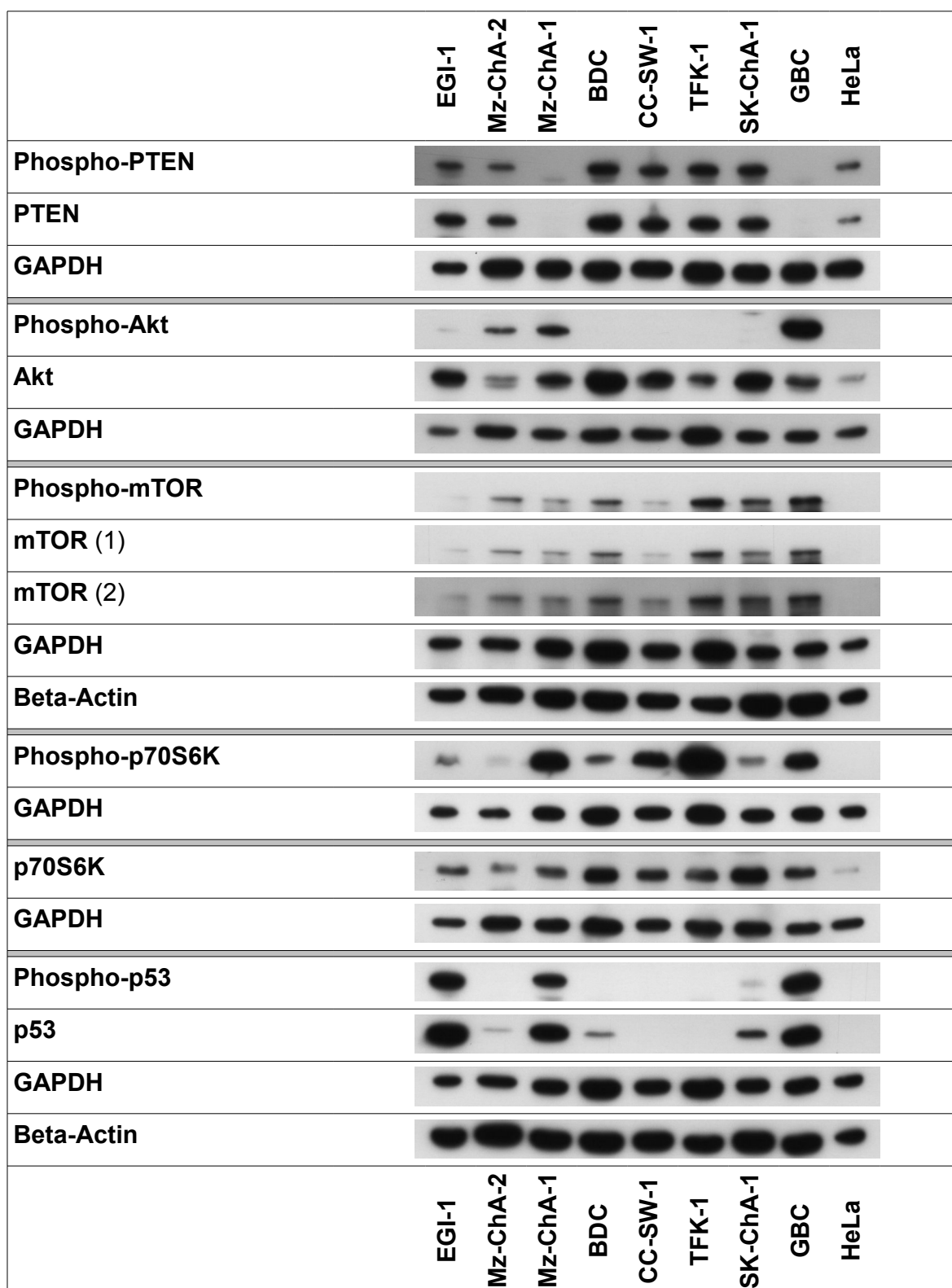


Figure 12: Western Blots of PI3K/Akt Pathway Members in Cell Lines

Different membranes are separated by gray bars. mTOR (1) and mTOR (2) are two different antibodies (see Table 13).

3.2 Frozen Gallbladder Tissue

3.2.1 qRT-PCRs of Frozen Gallbladder Tissue

The expression levels of four housekeeping genes (*GAPDH*, *Beta-Actin*, *Cyclophilin A*, *Lamin A/C*) were detected in five frozen samples of non-tumorous gallbladder tissue and in eight frozen gallbladder cancer samples. The housekeeping genes showed similar expression patterns in all samples (see Figures 13 and 14). Hence, a mean value of the C_T medians of all housekeeping genes was calculated for each sample and used for the evaluation with the C_T method.

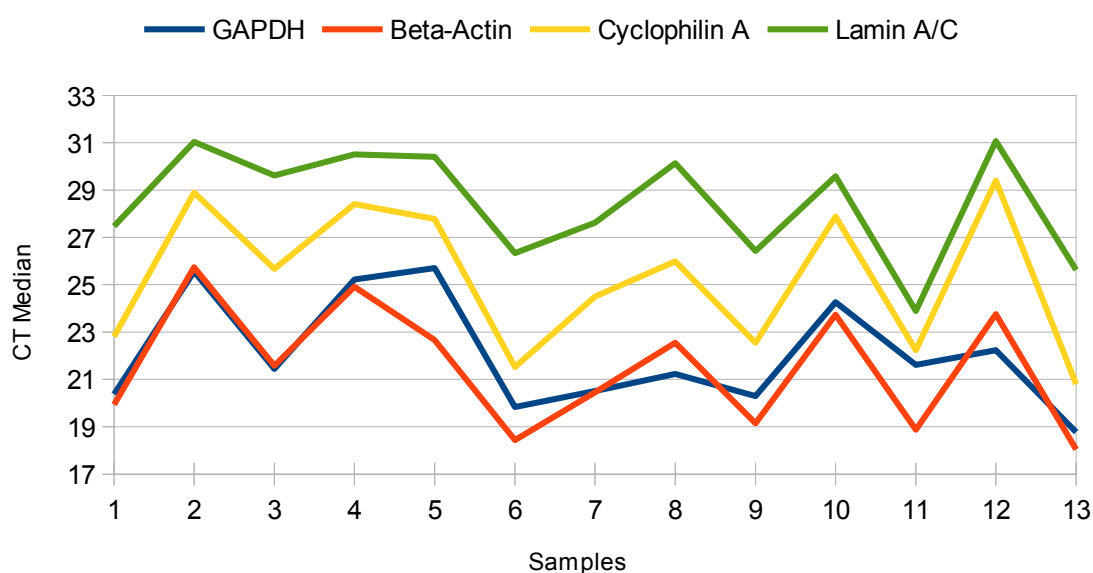


Figure 13: Housekeeping Gene Expressions in Gallbladder Tissue

1–5 (non-tumorous gallbladder tissue), 6–13 (gallbladder cancer samples)

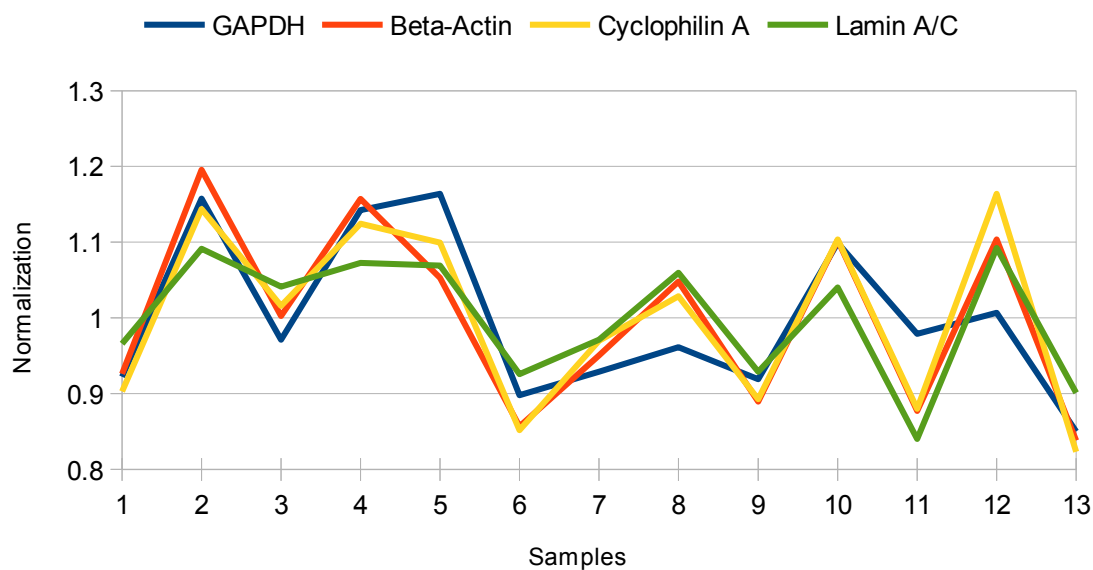


Figure 14: Normalization of Housekeeping Gene Expressions in Gallbladder Tissue

The CT median of each sample was divided by the mean value of the CT medians of all samples of the respective housekeeping gene. 1–5 (non-tumorous gallbladder tissue), 6–13 (gallbladder cancer samples)

Especially the expression of *PTEN* and the expression of the majority of the other PI3K/Akt pathway members were increased in gallbladder cancer samples compared to non-tumorous gallbladder tissue. By contrast, the expression of *eNOS* was reduced by nearly 4-fold in gallbladder cancer samples (see Figure 15).

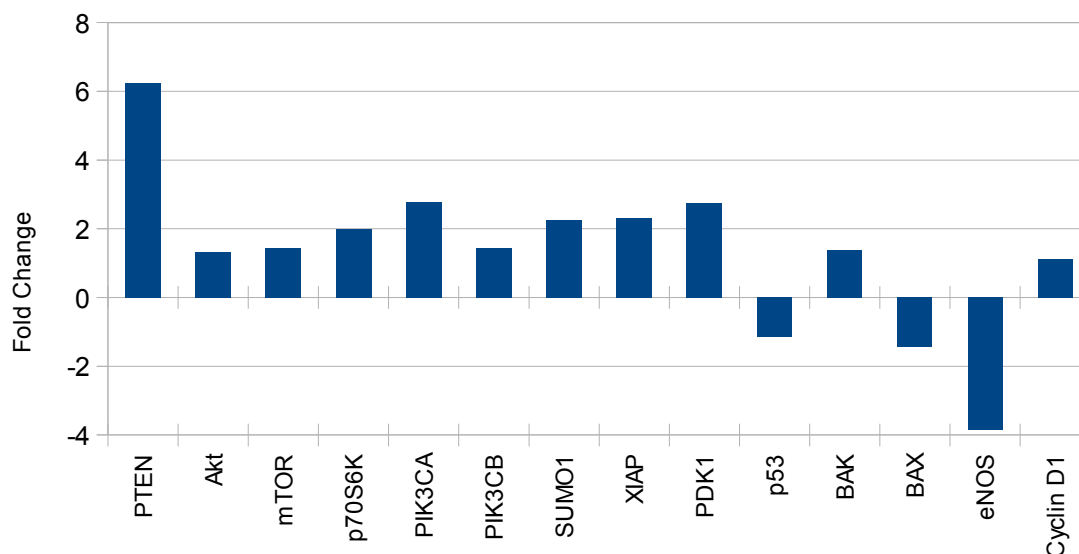


Figure 15: Gene Expressions of PI3K/Akt Pathway Members in Gallbladder Cancer

Illustration of gene expressions in gallbladder cancer samples when compared with the expression of these genes in non-tumorous gallbladder tissue.

3.2.2 Western Blots of Frozen Gallbladder Tissue

The expression levels of the key proteins of the PI3K/Akt pathway in non-tumorous gallbladder tissue and gallbladder cancer are charted in Figure 16. Two Controls were used for each antibody to ensure proper protein detection. However, phospho-mTOR (Ser2448) and mTOR antibodies showed a high background noise, and neither the control for non-phosphorylated nor the control for non-phosphorylated and phosphorylated antibodies showed a positive signal. Therefore, the results with phospho-mTOR and mTOR should be considered with caution. Apart from that, the controls for non-phosphorylated antibodies showed a weak signal of phospho-Akt (Ser473), phospho-p70S6K (Thr421/Ser424), and phospho-53 (Ser15). This was caused by the fact that the controls for non-phosphorylated as well as the controls for non-phosphorylated and phosphorylated antibodies were obtained from the same cell lines (see Table 15). None of the proteins were detected with noteworthy differences between the non-tumorous tissue group and the tumor group. Interestingly, GAPDH was unevenly expressed, whereas Beta-Actin showed a balanced protein loading.

	Non-Tumor				Tumor				C					
Phospho-PTEN														
PTEN														
GAPDH														
Beta-Actin														
Phospho-Akt														
Akt														
GAPDH														
Beta-Actin														
Phospho-p70S6K														
p70S6K														
GAPDH														
Beta-Actin														
Phospho-mTOR														
mTOR (1)														
mTOR (2)														
GAPDH														
Beta-Actin														
Phospho-p53														
p53														
GAPDH														
Beta-Actin														
	A	B	C	D	E	F	G	H	I	J	K	L	-	+

Figure 16: Western Blots of PI3K/Akt Pathway Members in Gallbladder Tissue

In case of PTEN, - corresponds to a complete negative control (loading buffer). + (Control for non-phosphorylated and phosphorylated antibodies), - (Control for non-phosphorylated antibodies), A-F (tumor-free gallbladder tissue), A+E (with residues after cholecystitis), C (Control), D (acute cholecystitis), G-L (gallbladder cancer samples)

3.3 Gallbladder TMAs

3.3.1 Representative Stainings of TMAs

Figures 17–21 display representative stainings of all used antibodies with different intensities as described in Table 17 (see 2.2.4.2 Evaluation of Immunohistochemical Staining). PTEN was exclusively detectable in the nucleus, whereas phospho-mTOR was only present in the cytoplasm. By contrast, phospho-Akt and phospho-p70S6K were localized in the nucleus as well as in the cytoplasm.

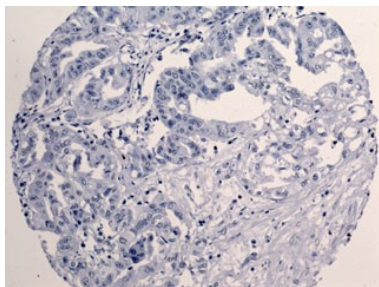
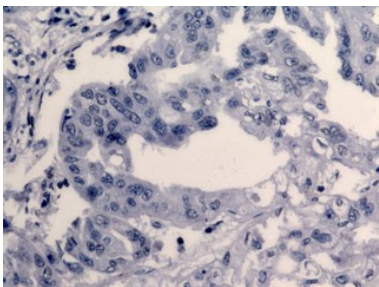
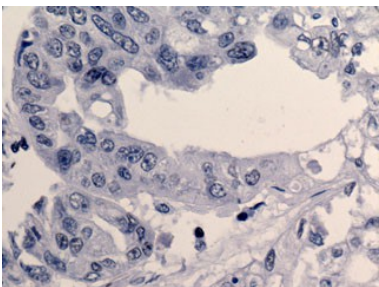
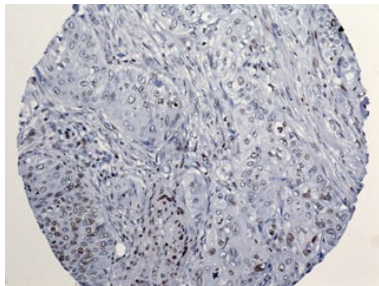
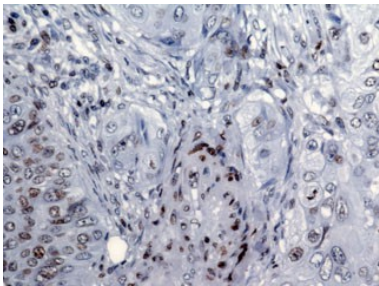
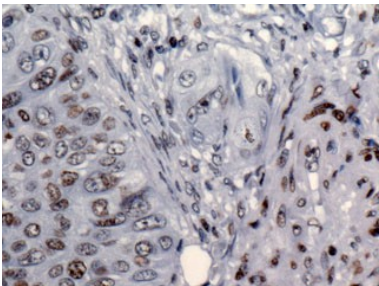
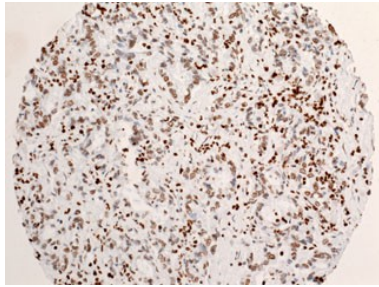
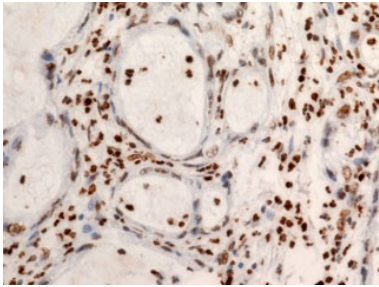
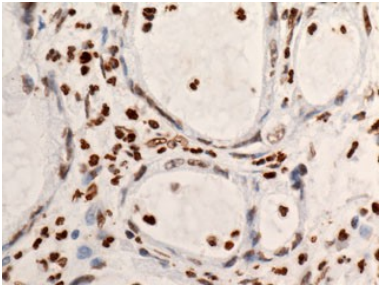
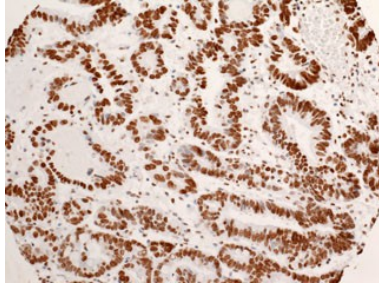
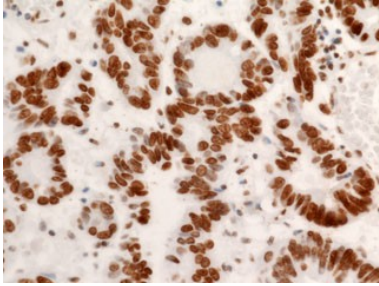
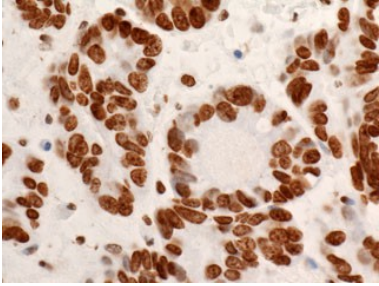
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Nuclear Intensity 0, Cytoplasmic Intensity 0		
		
Nuclear Intensity 1, Cytoplasmic Intensity 0		
		
Nuclear Intensity 2, Cytoplasmic Intensity 0		
		
Nuclear Intensity 3, Cytoplasmic Intensity 0		

Figure 17: PTEN in Gallbladder Cancer TMAs

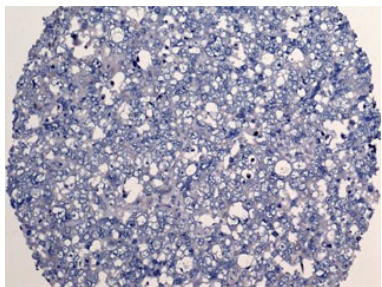
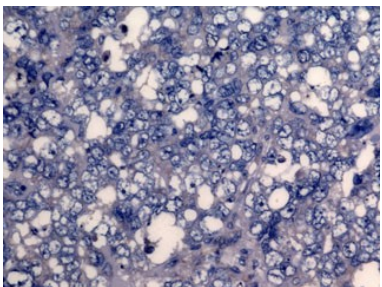
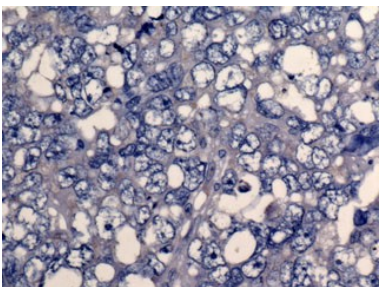
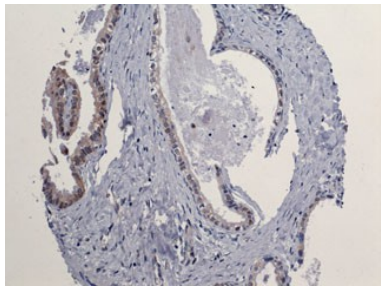
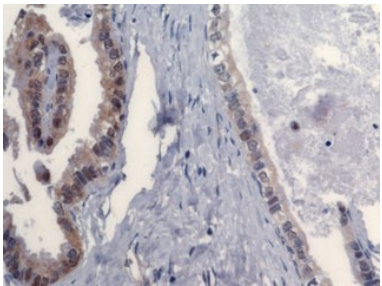
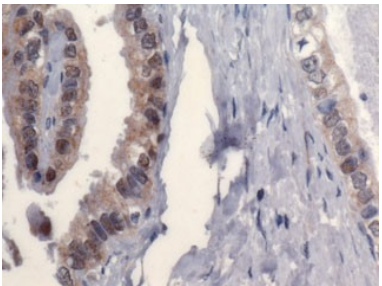
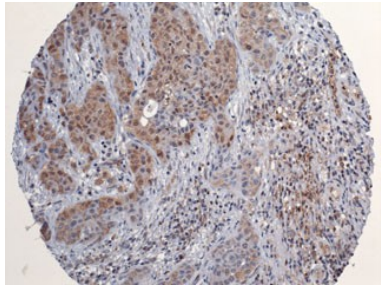
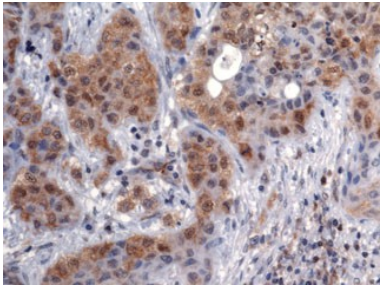
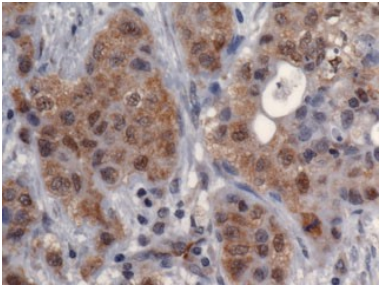
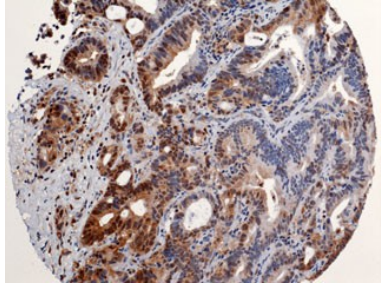
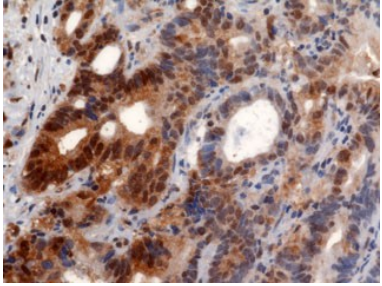
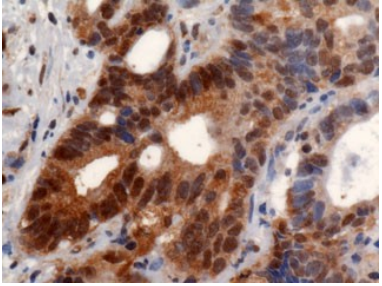
20 × Magnification	40 × Magnification	60 × Magnification
		
Nuclear Intensity 0, Cytoplasmic Intensity 0		
		
Nuclear Intensity 1, Cytoplasmic Intensity 1		
		
Nuclear Intensity 2, Cytoplasmic Intensity 2		
		
Nuclear Intensity 3, Cytoplasmic Intensity 3		

Figure 18: Phospho-Akt in Gallbladder Cancer TMAs

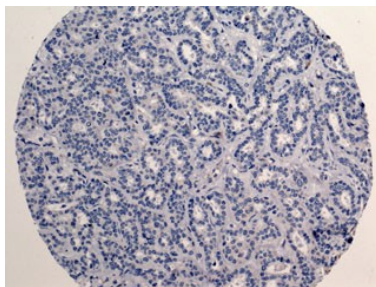
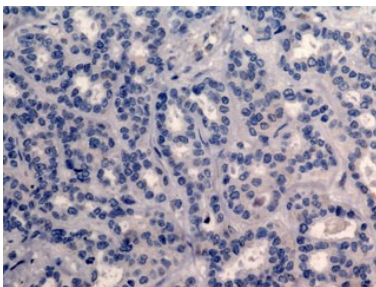
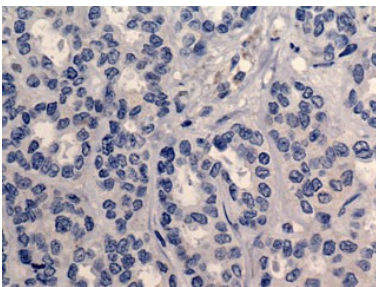
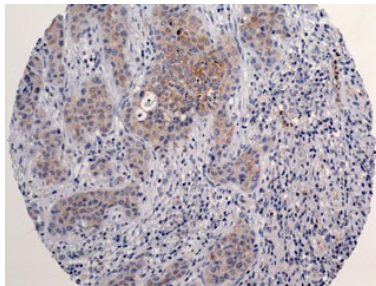
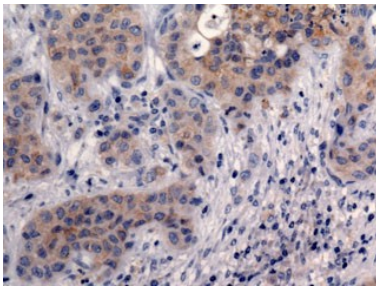
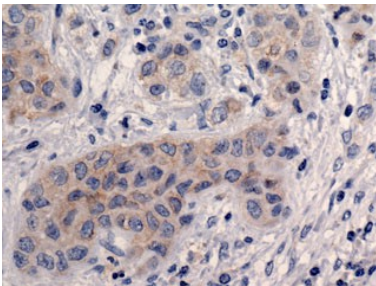
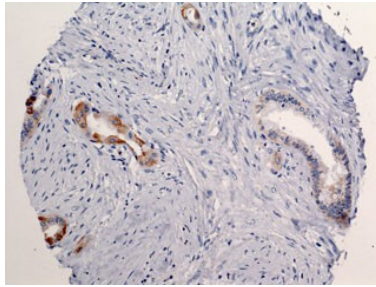
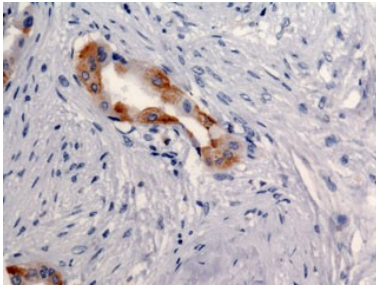
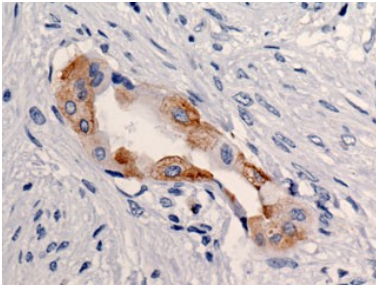
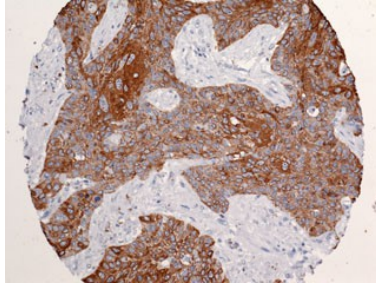
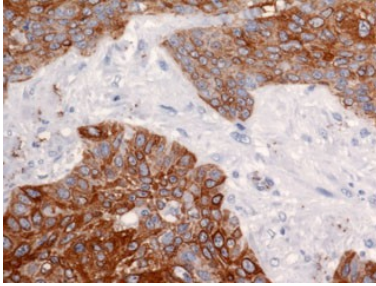
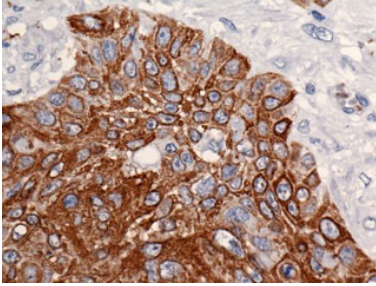
20 × Magnification	40 × Magnification	60 × Magnification
		
Nuclear Intensity 0, Cytoplasmic Intensity 0		
		
Nuclear Intensity 0, Cytoplasmic Intensity 1		
		
Nuclear Intensity 0, Cytoplasmic Intensity 2		
		
Nuclear Intensity 0, Cytoplasmic Intensity 3		

Figure 19: Phospho-mTOR in Gallbladder Cancer TMAs

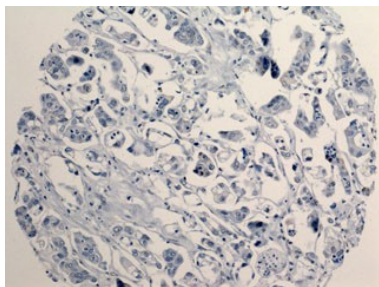
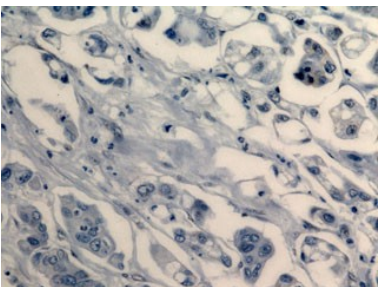
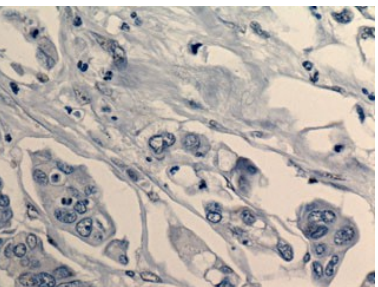
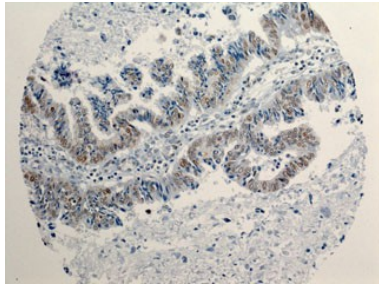
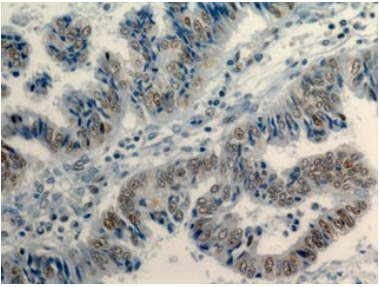
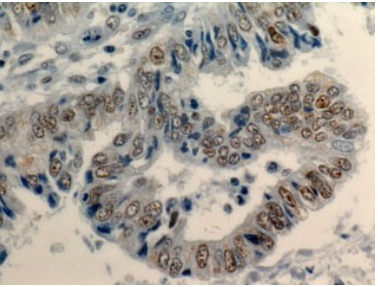
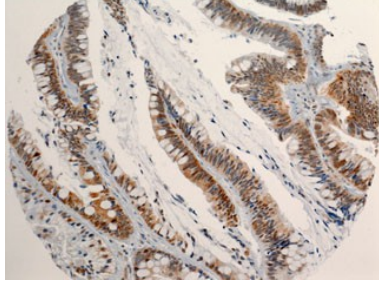
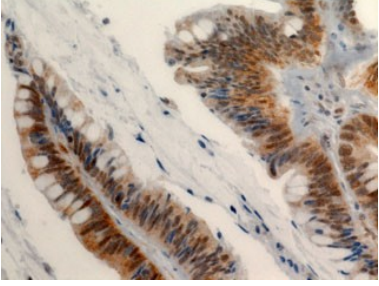
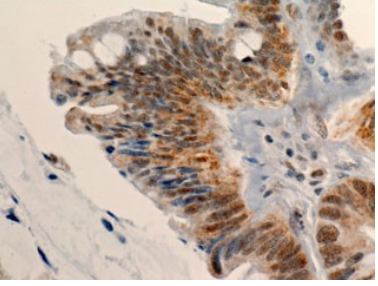
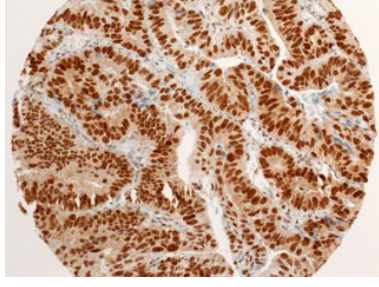
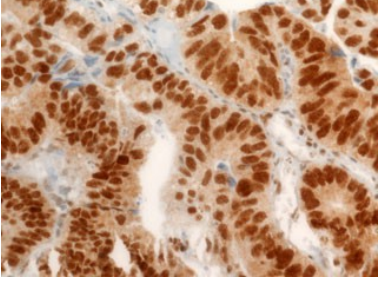
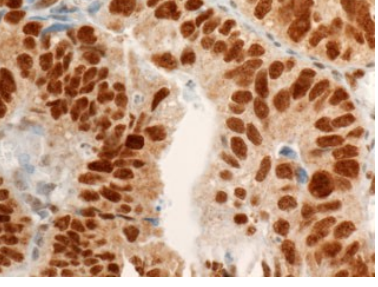
20 × Magnification	40 × Magnification	60 × Magnification
		
Nuclear Intensity 0, Cytoplasmic Intensity 0		
		
Nuclear Intensity 1, Cytoplasmic Intensity 0		
		
Nuclear Intensity 2, Cytoplasmic Intensity 2		
		
Nuclear Intensity 3, Cytoplasmic Intensity 1		

Figure 20: Phospho-p70S6K in Gallbladder Cancer TMAs

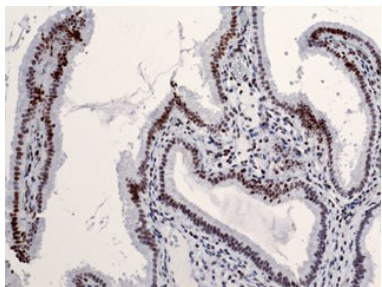
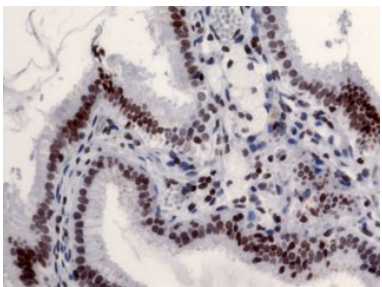
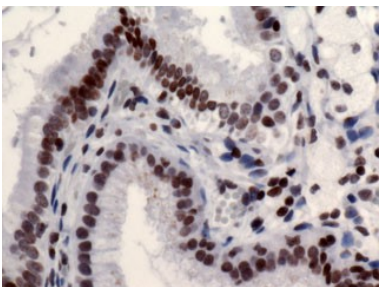
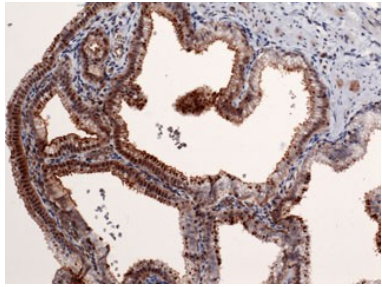
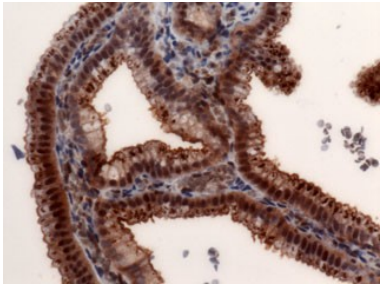
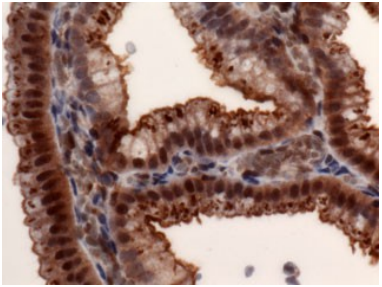
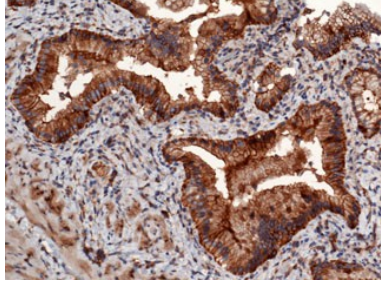
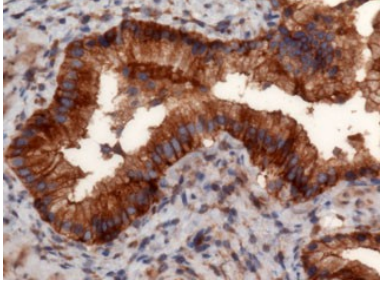
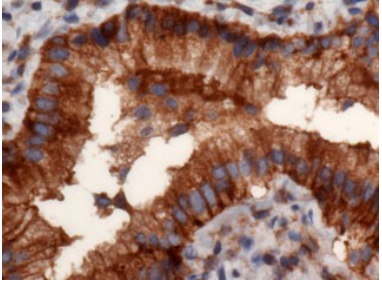
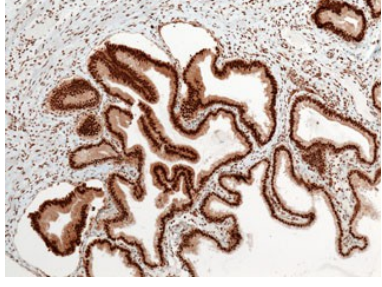
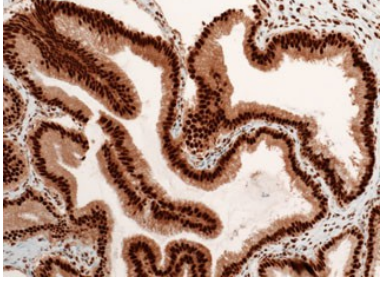
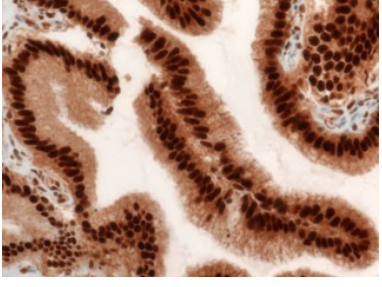
20 × Magnification	40 × Magnification	60 × Magnification
		
PTEN	Nuclear Intensity 3, Cytoplasmic Intensity 0	
		
Phospho-Akt	Nuclear Intensity 3, Cytoplasmic Intensity 3	
		
Phospho-mTOR	Nuclear Intensity 0, Cytoplasmic Intensity 3	
		
Phospho-p70S6K	Nuclear Intensity 3, Cytoplasmic Intensity 2	

Figure 21: Antibody Staining of CC

3.3.2 Kaplan-Meier Survival Analysis

The assessment of immunohistochemical staining was conducted with the help of the intensity and the tissue score (see 2.2.4.2 Evaluation of Immunohistochemical Staining). As the evaluations of intensities and tissue scores were very similar, only the analyses of the intensities are shown hereafter. The number of samples in unevaluable categories (e.g., TX) and the number of unassessable specimens (e.g., tissue cores with only a few tumor cells) are not separately recorded in the following. The latter caused varying numbers of samples between different antibodies. All p-values of the following data and graphs are listed in the appendix (see 6 Appendix).

3.3.2.1 Cohort Description

More females (n = 289) were affected by gallbladder cancer than males (n = 107), although no statistically significant difference was evident regarding the survival time (p = 0.78). Loss of differentiation, tumor growth, and dissemination significantly correlated with poor survival. Only the long-term surveillance of patients with T1 and T2 tumors and the G4 group showed deviations due to the low number of cases (see Figures 22 and 23).

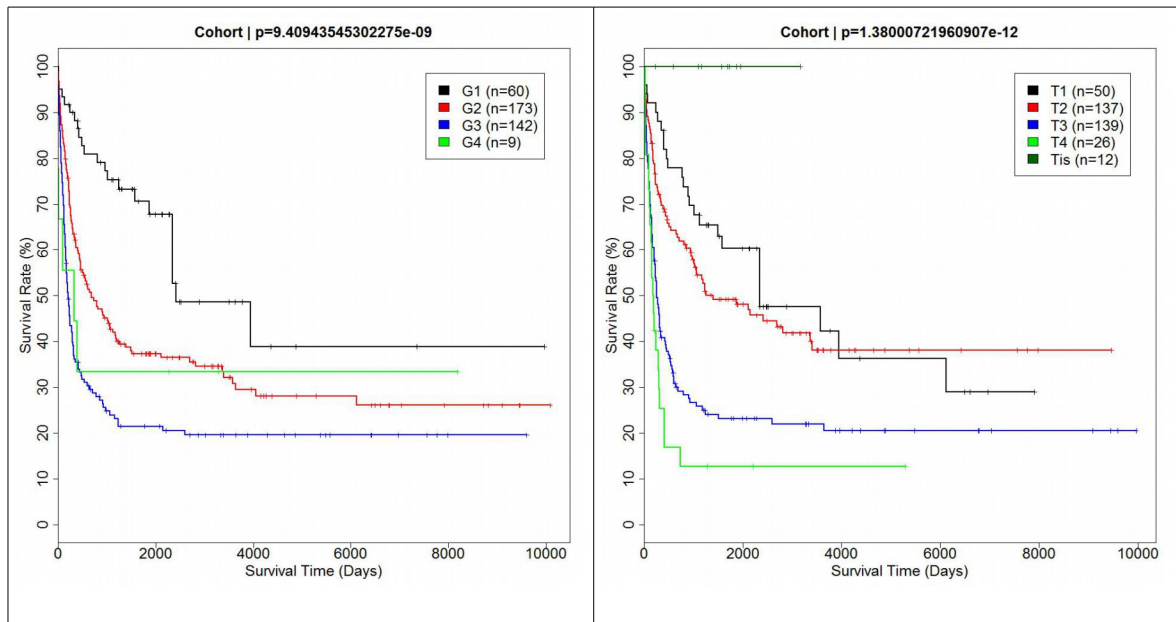


Figure 22: Kaplan-Meier Survival Analysis of the Cohort Part 1

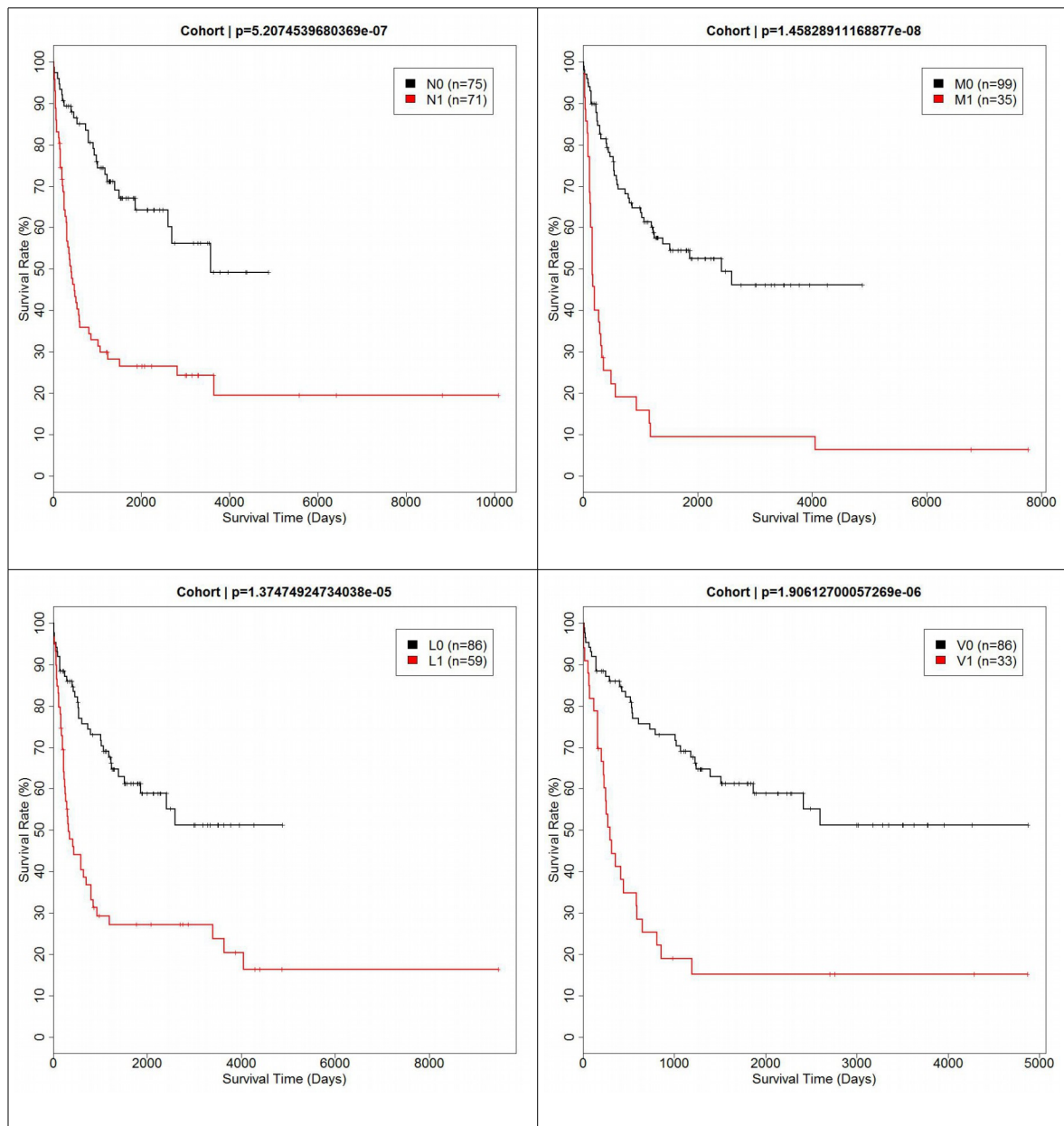


Figure 23: Kaplan-Meier Survival Analysis of the Cohort Part 2

3.3.2.2 Nuclear PTEN in Gallbladder TMAs

PTEN expression was only found in the nucleus. Interestingly, the strongest staining was seen in CC. The staining in Co significantly differed from poorly-differentiated (i.e., G3) and undifferentiated (i.e., G4) tumors as well as L1 cases (each $p < 0.05$). The lower the differentiation, the significantly higher was the expression of PTEN. Accordingly, M1, L1, and V1 cases showed significantly higher PTEN expressions

than M0, L0, and V0 cases, respectively (each $p < 0.05$). However, a similar trend was neither present in tumor extent nor in lymph node involvement (see Table 19).

	Co	CC	Tumor	G1	G2	G3	G4
Intensity	2.05 (n=59)	3.00 (n=3)	2.29 (n=362)	1.98 (n=56)	2.23 (n=158)	2.47 (n=130)	2.75 (n=8)
	Tis	T1	T2	T3	T4	N0	N1
Intensity	2.28 (n=10)	2.44 (n=43)	2.24 (n=128)	2.35 (n=129)	2.23 (n=24)	2.13 (n=71)	2.11 (n=68)
	M0	M1	L0	L1	V0	V1	
Intensity	1.93 (n=95)	2.36 (n=32)	1.86 (n=82)	2.53 (n=56)	1.86 (n=82)	2.51 (n=32)	

Table 19: Mean Intensities of Nuclear PTEN

Tumor corresponds to the summary of all gallbladder cancer samples.

A low intensity of PTEN was associated with an improved survival compared to a high intensity ($p < 0.05$, see Figure 24). Furthermore, patients with T2 tumors and a low intensity had a better prognosis than patients with T2 tumors that showed a high intensity of PTEN ($p < 0.05$). T1 and T3 cases also showed this tendency, but this was not statistically significant (see Figure 25). Survival times of patients with the presence or absence of venous and lymphatic vessel invasion with relation to varied expression levels of PTEN are illustrated in Figure 26.

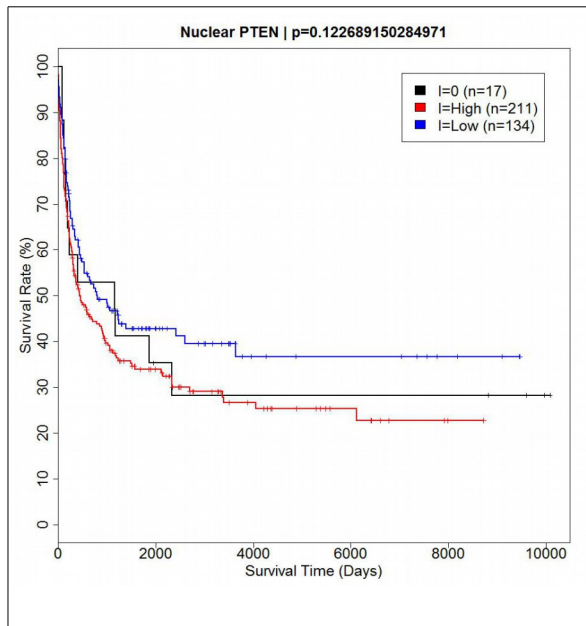


Figure 24: Kaplan-Meier Survival Analysis of Nuclear PTEN Part 1
I (Intensity)

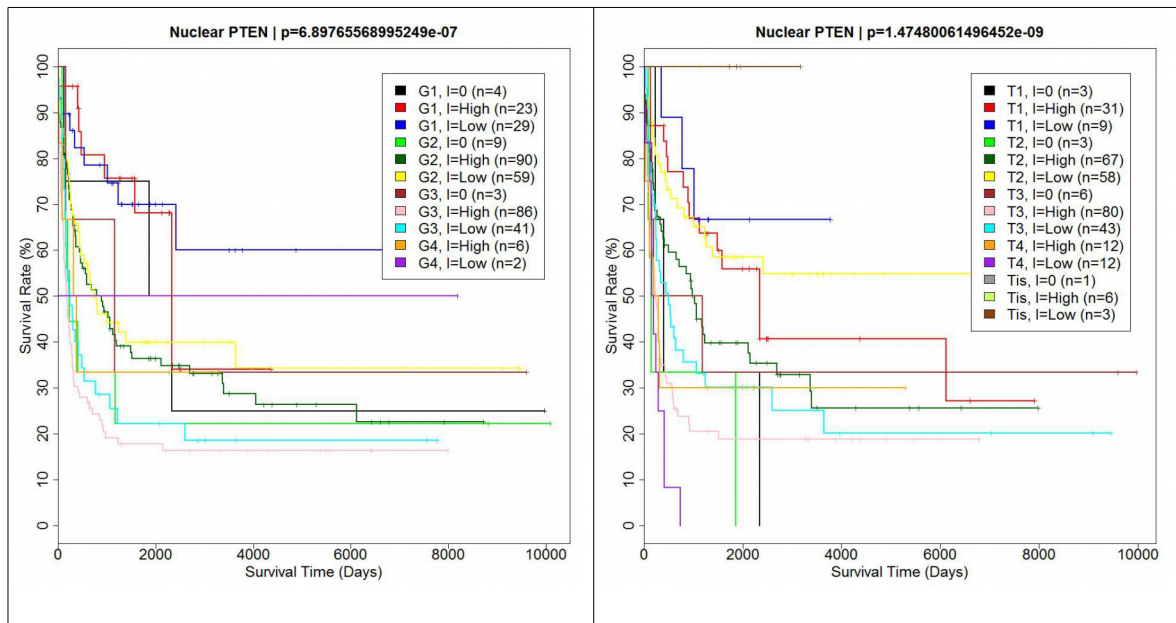


Figure 25: Kaplan-Meier Survival Analysis of Nuclear PTEN Part 2

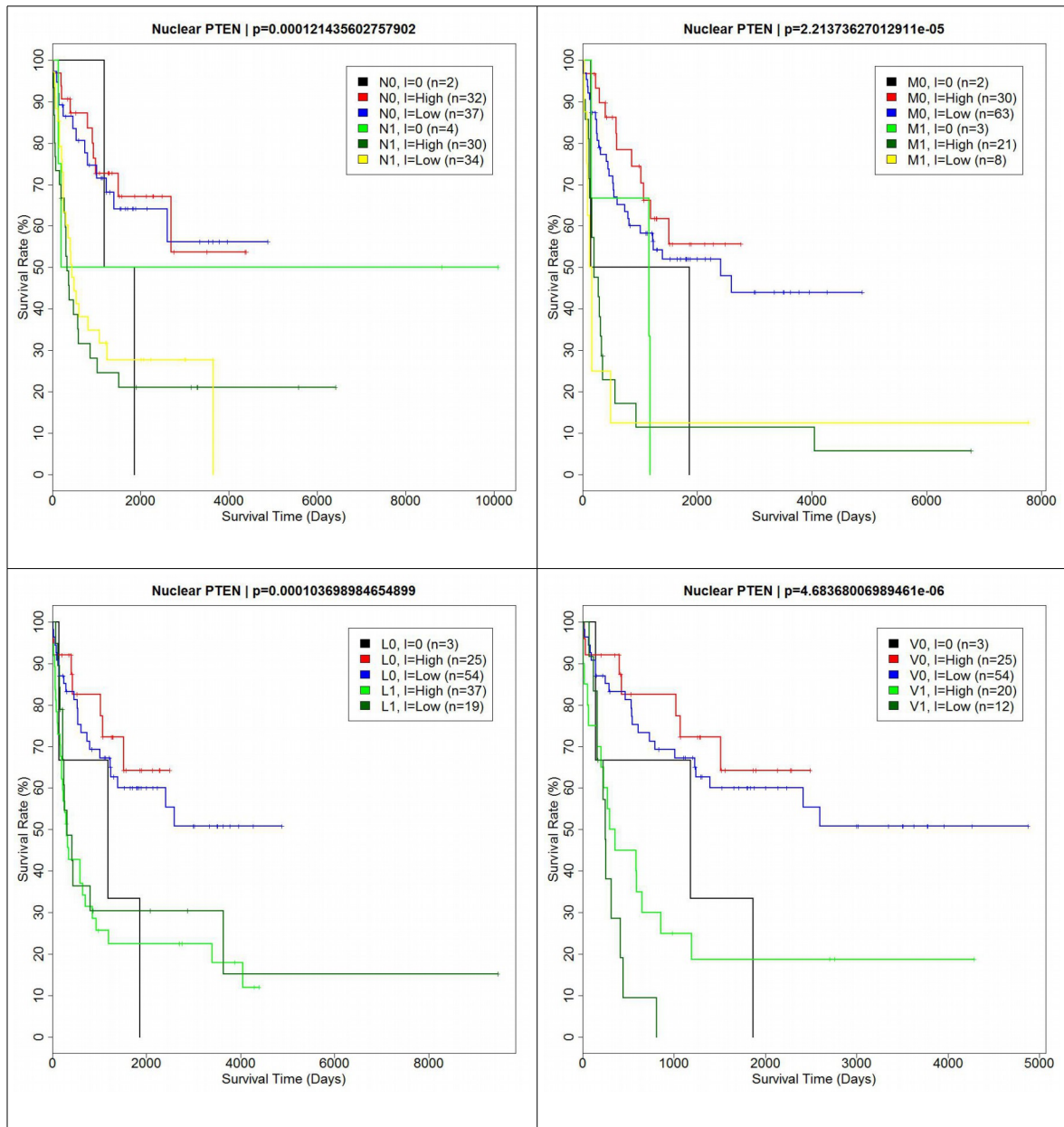


Figure 26: Kaplan-Meier Survival Analysis of Nuclear PTEN Part 3

3.3.2.3 Phospho-Akt in Gallbladder TMAs

Phospho-Akt (Ser473) was expressed in the nucleus and the cytoplasm, and the strongest staining was also seen in CC. CC samples significantly differed from Co, tumor, and all individual tumor categories (e.g., G1) except for Tis cases in both the nucleus and the cytoplasm regarding the expression of phospho-Akt (each $p < 0.05$, see Tables 20 and 21).

	Co	CC	Tumor	G1	G2	G3	G4
Intensity	0.84 (n=55)	3.00 (n=3)	0.74 (n=348)	0.71 (n=55)	0.68 (n=155)	0.79 (n=120)	0.59 (n=9)
	Tis	T1	T2	T3	T4	N0	N1
Intensity	1.52 (n=9)	0.81 (n=42)	0.86 (n=121)	0.65 (n=125)	0.43 (n=24)	0.67 (n=68)	0.63 (n=68)
	M0	M1	L0	L1	V0	V1	
Intensity	0.76 (n=92)	0.61 (n=29)	0.75 (n=79)	0.95 (n=53)	0.75 (n=79)	0.79 (n=30)	

Table 20: Mean Intensities of Nuclear Phospho-Akt

	Co	CC	Tumor	G1	G2	G3	G4
Intensity	0.49 (n=55)	2.33 (n=3)	0.48 (n=348)	0.56 (n=55)	0.48 (n=155)	0.42 (n=120)	0.76 (n=9)
	Tis	T1	T2	T3	T4	N0	N1
Intensity	0.58 (n=9)	0.84 (n=42)	0.48 (n=121)	0.37 (n=125)	0.38 (n=24)	0.69 (n=68)	0.35 (n=68)
	M0	M1	L0	L1	V0	V1	
Intensity	0.52 (n=92)	0.47 (n=29)	0.53 (n=79)	0.45 (n=53)	0.53 (n=79)	0.52 (n=30)	

Table 21: Mean Intensities of Cytoplasmic Phospho-Akt

A high intensity of nuclear phospho-Akt tended to be associated with a better survival rate, however this was not statistically significant (see Figure 27). In accordance with this trend, N1 cases with a high intensity had a better prognosis than N1 cases with no or a low intensity of nuclear phospho-Akt (each $p < 0.05$). By contrast, high intensity in L1 and V1 cases was associated with a reduced survival time compared to no or low intensity in L1 and V1 cases, respectively (each $p < 0.05$, see Figure 29). Survival times of patients with different histological grades and tumor extents with relation to varied expression levels of nuclear phospho-Akt are displayed in Figure 28.

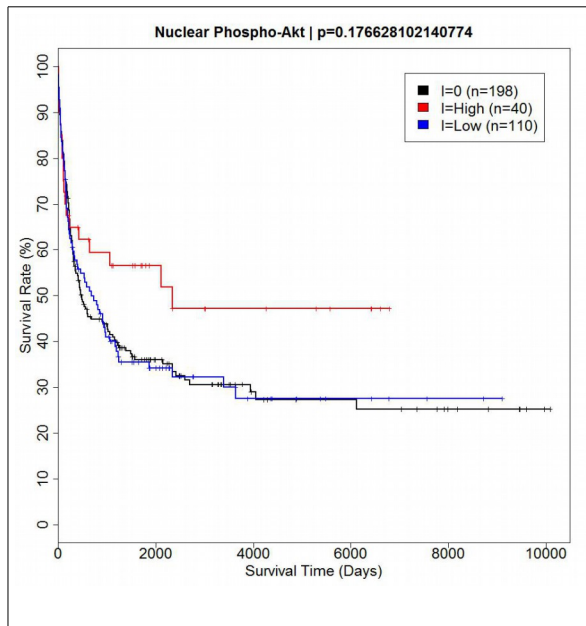


Figure 27: Kaplan-Meier Survival Analysis of Nuclear Phospho-Akt Part 1

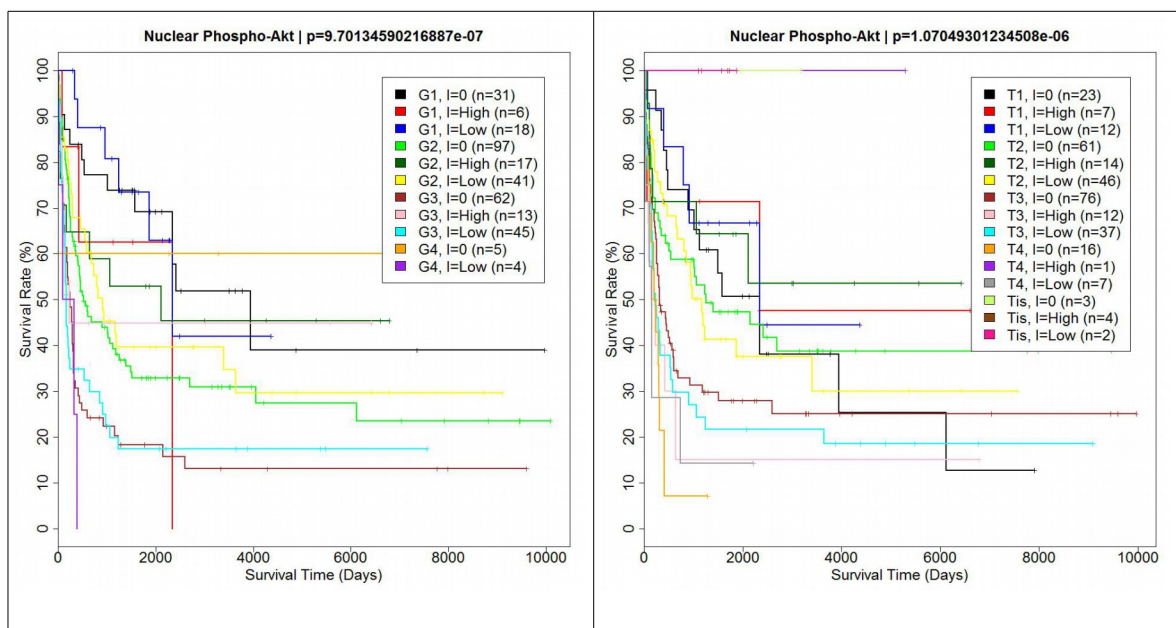


Figure 28: Kaplan-Meier Survival Analysis of Nuclear Phospho-Akt Part 2

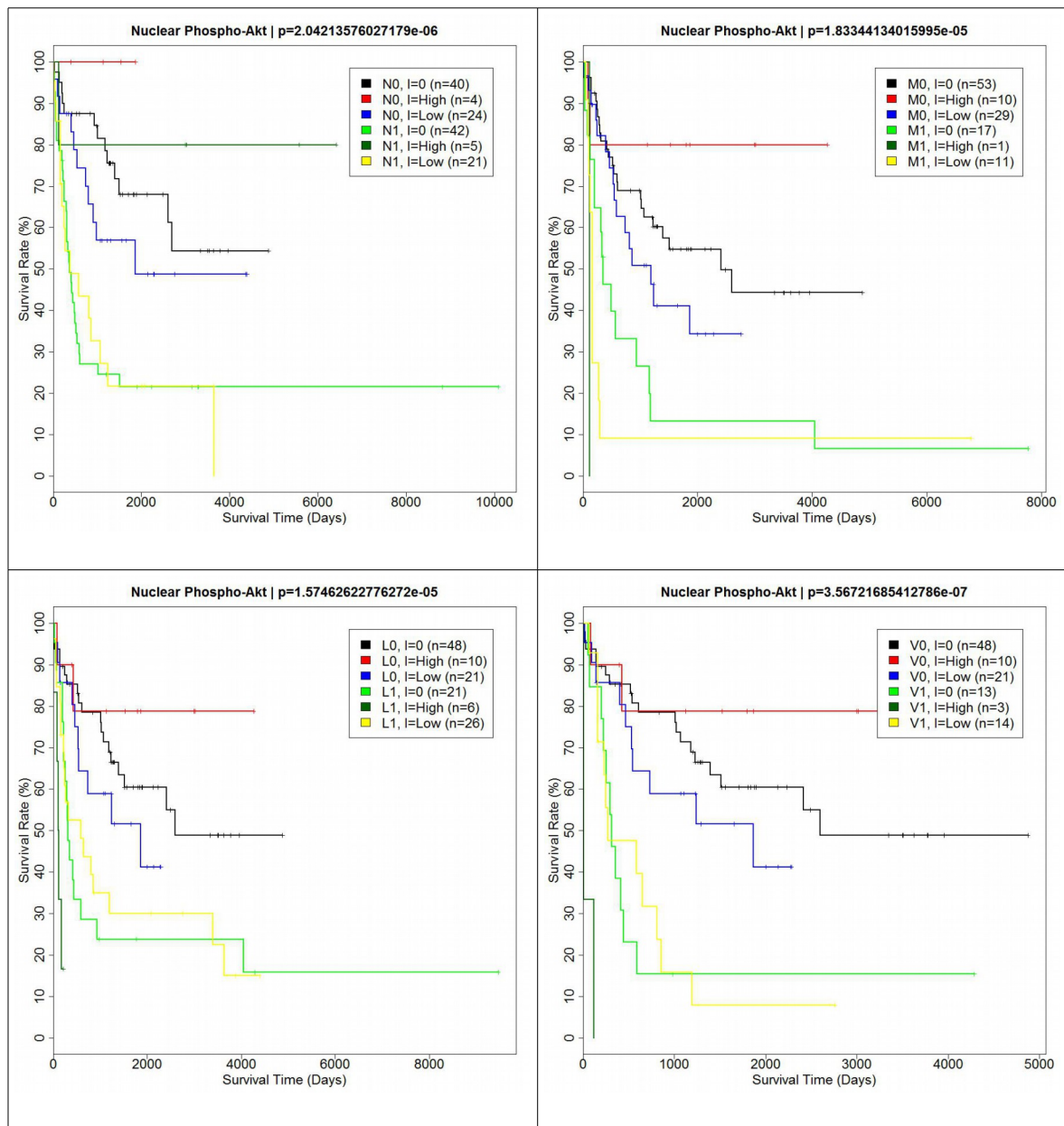


Figure 29: Kaplan-Meier Survival Analysis of Nuclear Phospho-Akt Part 3

A high intensity of cytoplasmic phospho-Akt was associated with an improved outcome in comparison to no or a low intensity (each $p < 0.05$, see Figure 30). The same pattern was seen in G3 cases (each $p < 0.05$). Moreover, patients with stage T1 gallbladder cancer and a high intensity lived longer than patients with a low intensity at stage T1 ($p < 0.05$, see Figure 31). In accordance with these findings, M0 cases with a high intensity had a better prognosis than M0 cases that showed no cytoplasmic phospho-Akt expression ($p < 0.05$, see Figure 32).

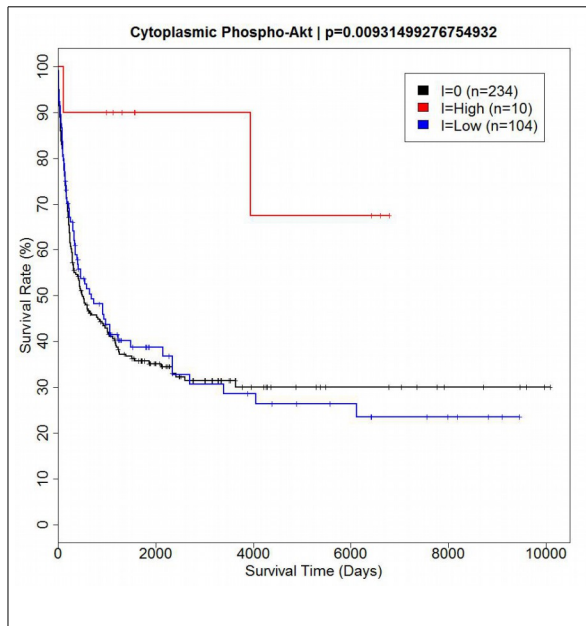


Figure 30: Kaplan-Meier Survival Analysis of Cytoplasmic Phospho-Akt Part 1

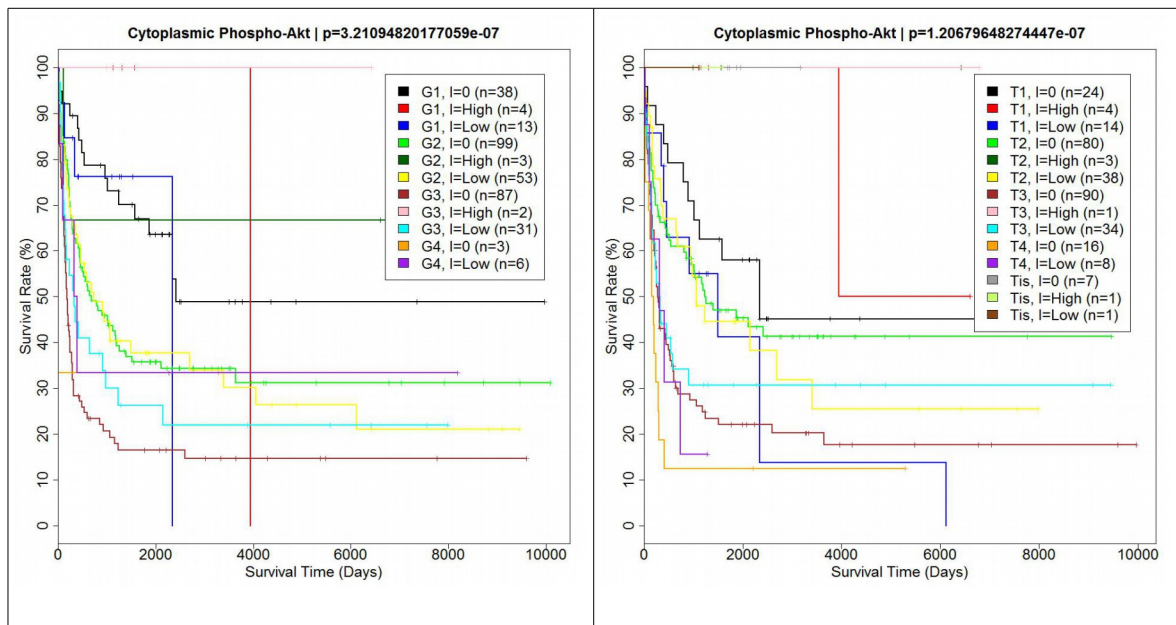


Figure 31: Kaplan-Meier Survival Analysis of Cytoplasmic Phospho-Akt Part 2

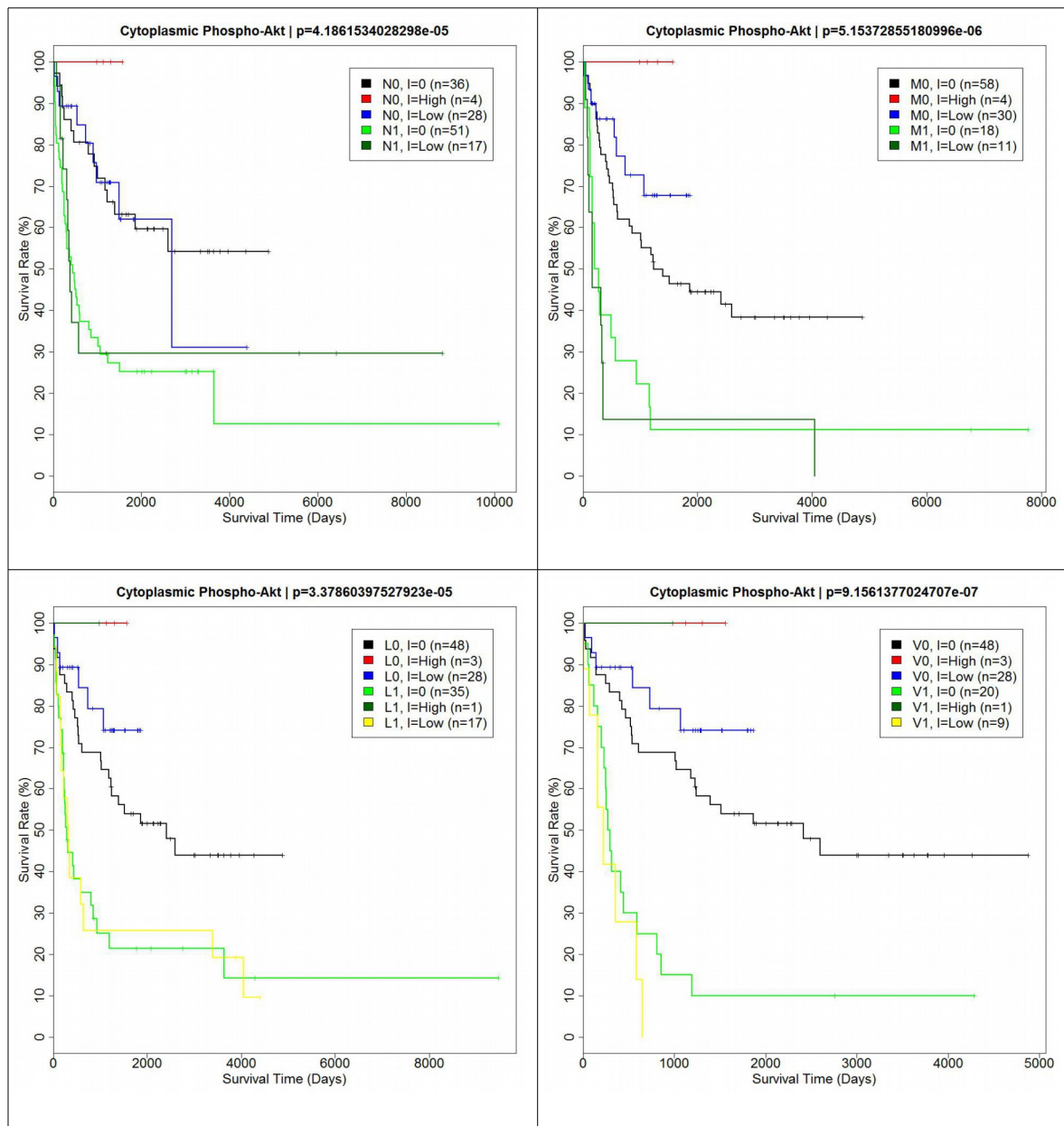


Figure 32: Kaplan-Meier Survival Analysis of Cytoplasmic Phospho-Akt Part 3

3.3.2.4 Phospho-mTOR in Gallbladder TMAs

Phospho-mTOR (Ser2448) expression was exclusively detectable in the cytoplasm. As in PTEN and phospho-Akt, the strongest staining was detectable in CC. CC specimens significantly differed from Co, tumor, and all individual tumor categories except for Tis cases regarding the expression of phospho-mTOR (each $p < 0.05$). By contrast, Co only diverged from well-differentiated (i.e., G1) and low-stage tumors (i.e., Tis, T1, N0, M0, L0, and V0) as well as V1 cases (each $p < 0.05$). The transition

from Tis to an invasive carcinoma as well as the loss of differentiation from G1 to G2 was significantly associated with a decreasing expression of phospho-mTOR (each $p < 0.05$). Furthermore, an increasing loss of differentiation and a growing tumor extent seemed to be related to a decreasing phospho-mTOR expression in general. Accordingly, N1 and V1 cases showed lower expression levels of phospho-mTOR than N0 and V0 cases, but this did not reach statistical significance. However, M1 and L1 cases had significantly lower phospho-mTOR expressions than M0 and L0 cases, respectively (each $p < 0.05$, see Table 22).

	Co	CC	Tumor	G1	G2	G3	G4
Intensity	0.79 (n=56)	2.83 (n=3)	0.95 (n=345)	1.32 (n=56)	0.86 (n=156)	0.83 (n=116)	0.67 (n=9)
	Tis	T1	T2	T3	T4	N0	N1
Intensity	1.99 (n=8)	1.16 (n=44)	1.00 (n=119)	0.86 (n=123)	0.81 (n=24)	1.25 (n=69)	1.06 (n=68)
	M0	M1	L0	L1	V0	V1	
Intensity	1.35 (n=94)	0.56 (n=29)	1.34 (n=81)	0.92 (n=53)	1.34 (n=81)	1.28 (n=30)	

Table 22: Mean Intensities of Cytoplasmic Phospho-mTOR

A low intensity of cytoplasmic phospho-mTOR correlated with an improved survival compared to a lack of any staining ($p < 0.05$, see Figure 33). Patients with T1 tumors and a low intensity had a better prognosis than patients with T1 tumors without any signal ($p < 0.05$). In line with this result was the link between a better survival rate and a high intensity at the T2 stage compared without any expression of phospho-mTOR ($p < 0.05$, see Figure 34). Survival estimates of further categories with relation to phospho-mTOR expression are illustrated in Figure 35.

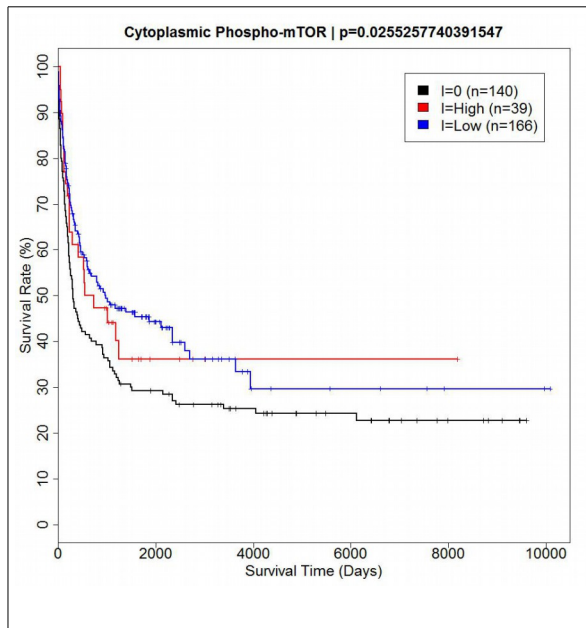


Figure 33: Kaplan-Meier Survival Analysis of Cytoplasmic Phospho-mTOR Part 1

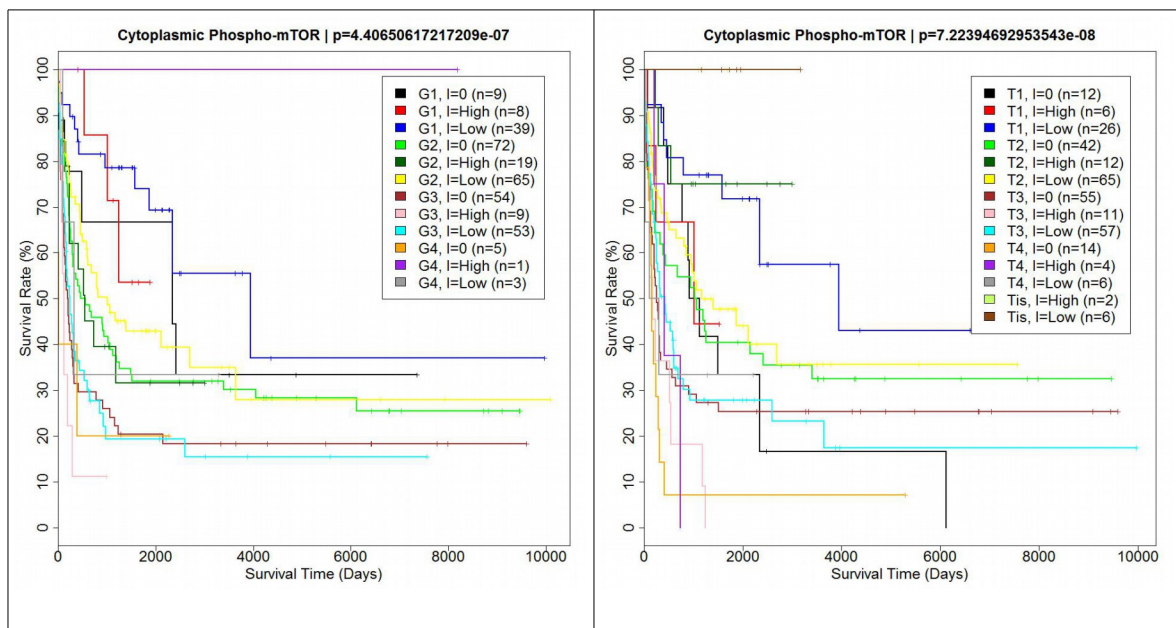


Figure 34: Kaplan-Meier Survival Analysis of Cytoplasmic Phospho-mTOR Part 2

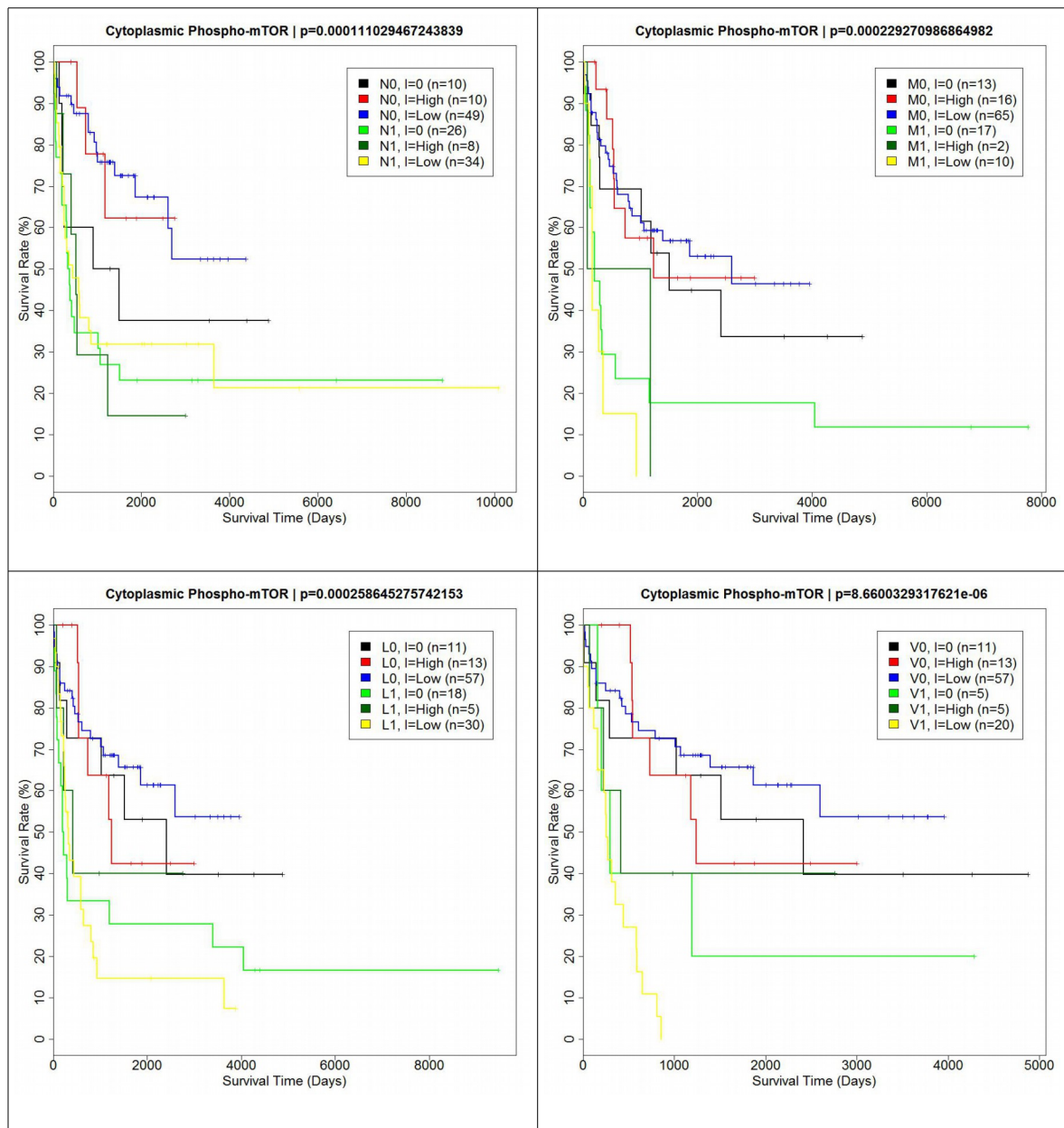


Figure 35: Kaplan-Meier Survival Analysis of Cytoplasmic Phospho-mTOR Part 3

3.3.2.5 Phospho-p70S6K in Gallbladder TMAs

Phospho-p70S6K (Ser411) was expressed in the nucleus and only occasionally expressed in the cytoplasm. A significant difference was detected between nuclear phospho-p70S6K expression in Co and tumor, G2, G3, G4, T3, T4, N1, M1, and L1 cases (each $p < 0.05$). As seen for phospho-mTOR, the transition from Tis to an invasive tumor as well as the loss of differentiation from G1 to G2 was significantly associated with a decreasing expression of nuclear phospho-p70S6K (each $p < 0.05$).

In addition, an increasing loss of differentiation and a growing tumor extent seemed to be associated with a decreasing expression of nuclear phospho-p70S6K in general. Tumors with N1, M1, and L1 had significantly lower nuclear phospho-p70S6K expressions than N0, M0, and L0 cases, respectively (each $p < 0.05$). Patients staged with V1 or V0 showed the same tendency, however this was not statistically significant (see Table 23). The strongest staining of cytoplasmic phospho-p70S6K was observable in CC, and the mean cytoplasmic intensity of CC significantly differed from Co, tumor, and each individual tumor category (each $p < 0.05$, see Table 24).

	Co	CC	Tumor	G1	G2	G3	G4
Intensity	2.51 (n=41)	3.00 (n=3)	2.07 (n=365)	2.62 (n=55)	2.03 (n=160)	1.87 (n=130)	1.33 (n=9)
	Tis	T1	T2	T3	T4	N0	N1
Intensity	3.00 (n=11)	2.12 (n=45)	2.22 (n=127)	1.89 (n=131)	1.87 (n=23)	2.32 (n=70)	1.85 (n=68)
	M0	M1	L0	L1	V0	V1	
Intensity	2.53 (n=93)	1.72 (n=31)	2.51 (n=80)	1.77 (n=55)	2.51 (n=80)	2.11 (n=31)	

Table 23: Mean Intensities of Nuclear Phospho-p70S6K

	Co	CC	Tumor	G1	G2	G3	G4
Intensity	0.10 (n=41)	1.67 (n=3)	0.11 (n=365)	0.06 (n=55)	0.13 (n=160)	0.12 (n=130)	0.09 (n=9)
	Tis	T1	T2	T3	T4	N0	N1
Intensity	0.02 (n=11)	0.08 (n=45)	0.12 (n=127)	0.14 (n=131)	0.16 (n=23)	0.16 (n=70)	0.13 (n=68)
	M0	M1	L0	L1	V0	V1	
Intensity	0.14 (n=93)	0.06 (n=31)	0.11 (n=80)	0.17 (n=55)	0.11 (n=80)	0.15 (n=31)	

Table 24: Mean Intensities of Cytoplasmic Phospho-p70S6K

A high intensity of nuclear phospho-p70S6K was associated with a better outcome compared to a low intensity ($p < 0.05$, see Figure 36). Patients with stage T3 gallbladder cancer and a low intensity of nuclear phospho-p70S6K had a poorer prognosis than patients with no or a high intensity at stage T3 (each $p < 0.05$, see

Figure 37). Accordingly, a low intensity in L1 and V1 cases was associated with a reduced overall survival in relation to a high intensity in these cases (each $p < 0.05$, see Figure 38).

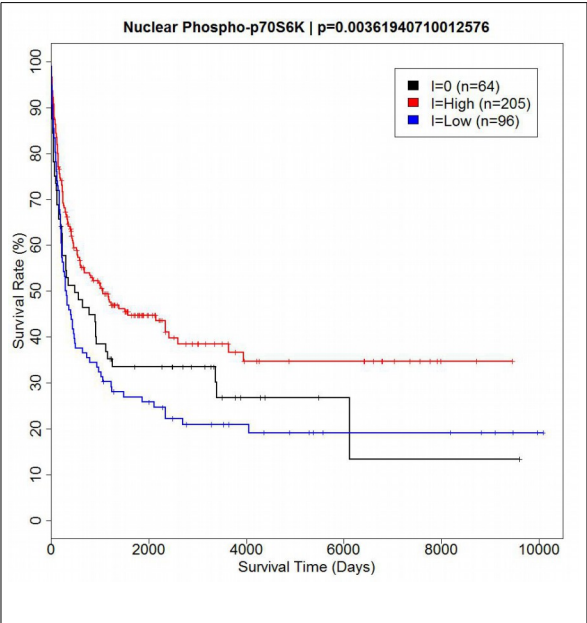


Figure 36: Kaplan-Meier Survival Analysis of Nuclear Phospho-p70S6K Part 1

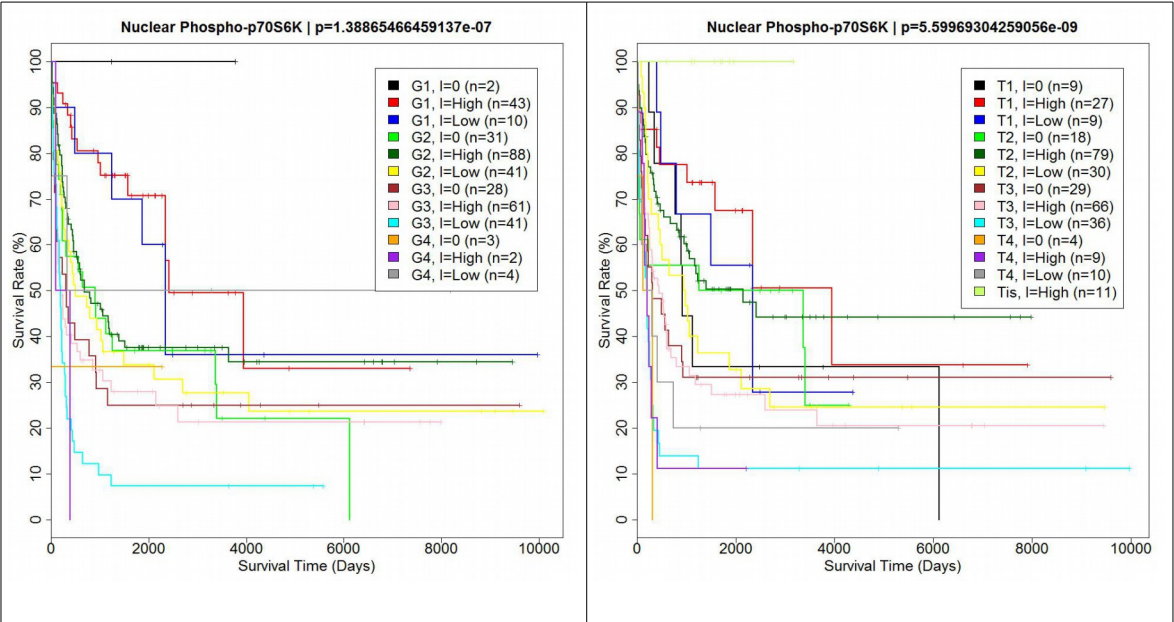


Figure 37: Kaplan-Meier Survival Analysis of Nuclear Phospho-p70S6K Part 2

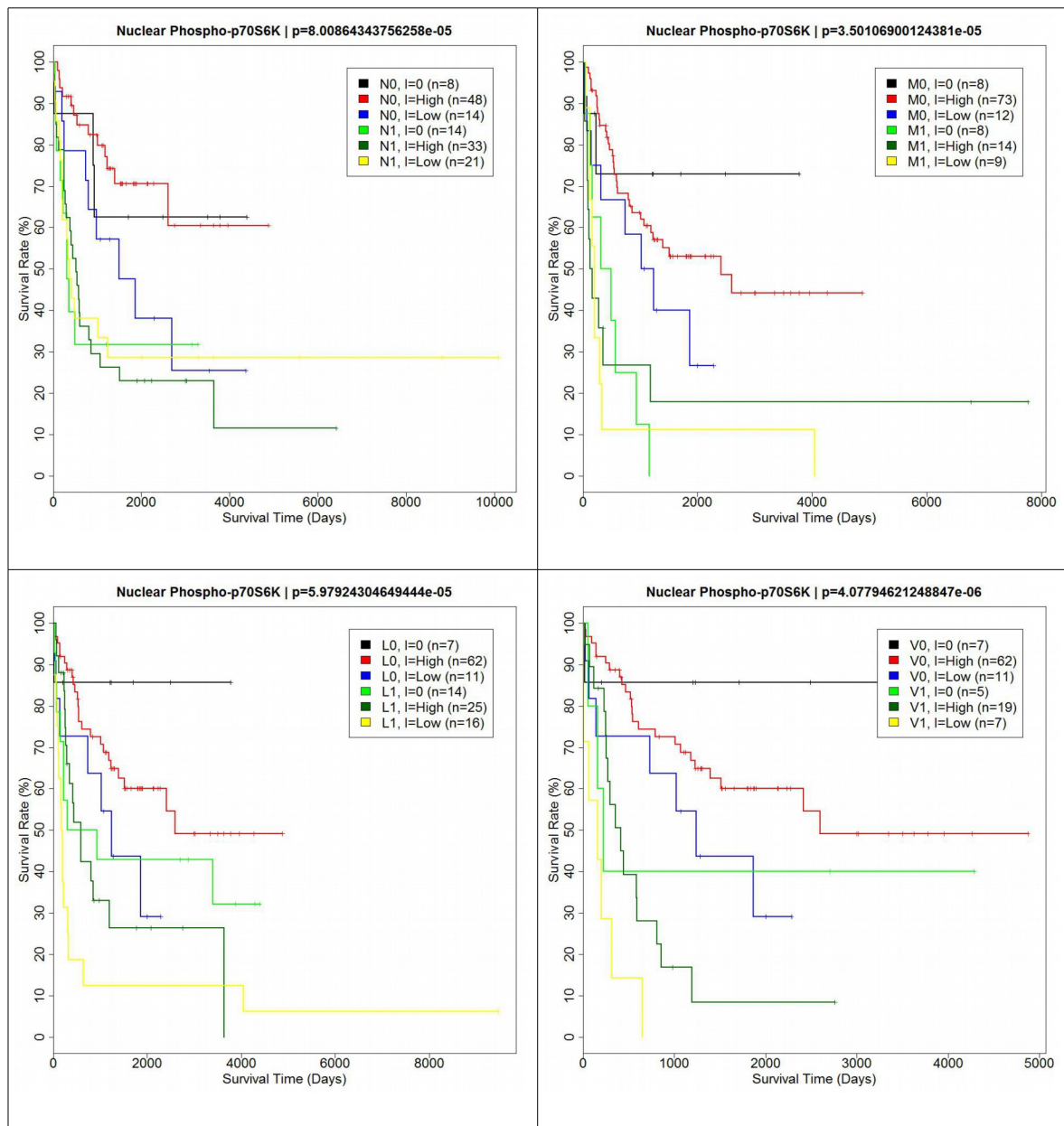


Figure 38: Kaplan-Meier Survival Analysis of Nuclear Phospho-p70S6K Part 3

No correlations were found between the expression of cytoplasmic phospho-p70S6K and survival rates of patients with gallbladder cancer (see Figures 39–41).

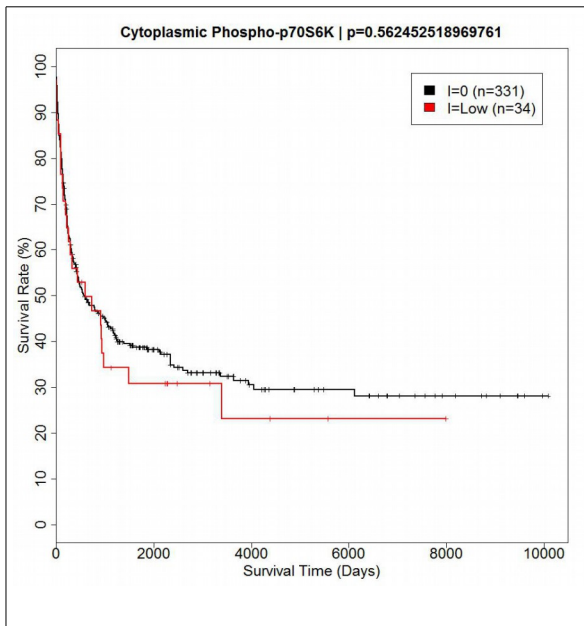


Figure 39: Kaplan-Meier Survival Analysis of Cytoplasmic Phospho-p70S6K Part 1

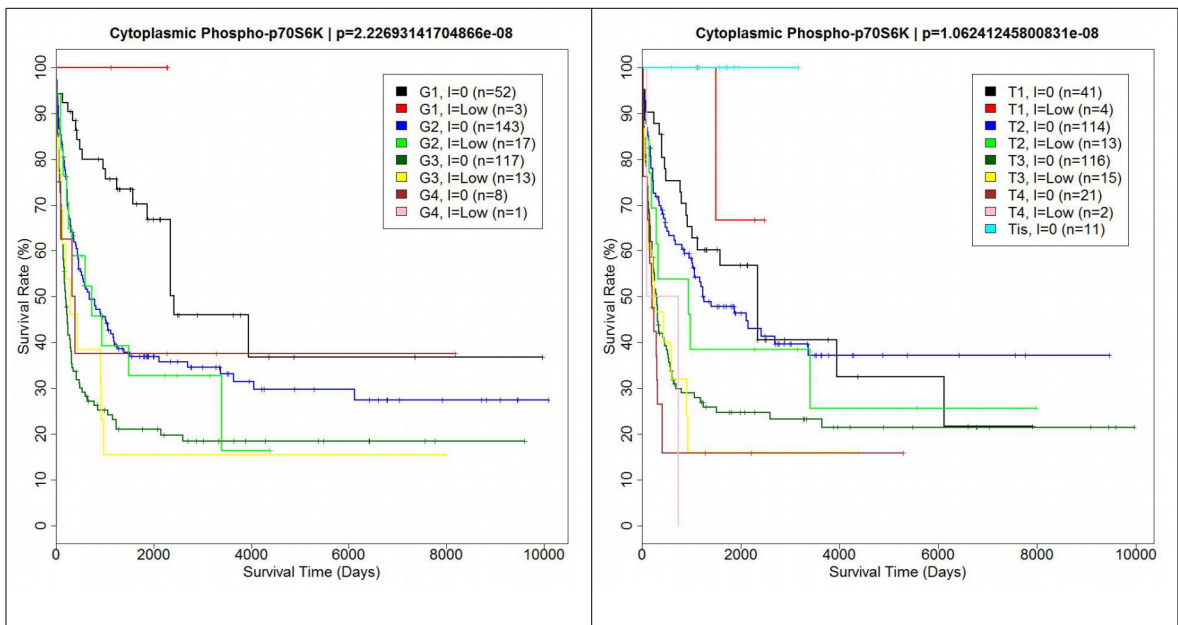


Figure 40: Kaplan-Meier Survival Analysis of Cytoplasmic Phospho-p70S6K Part 2

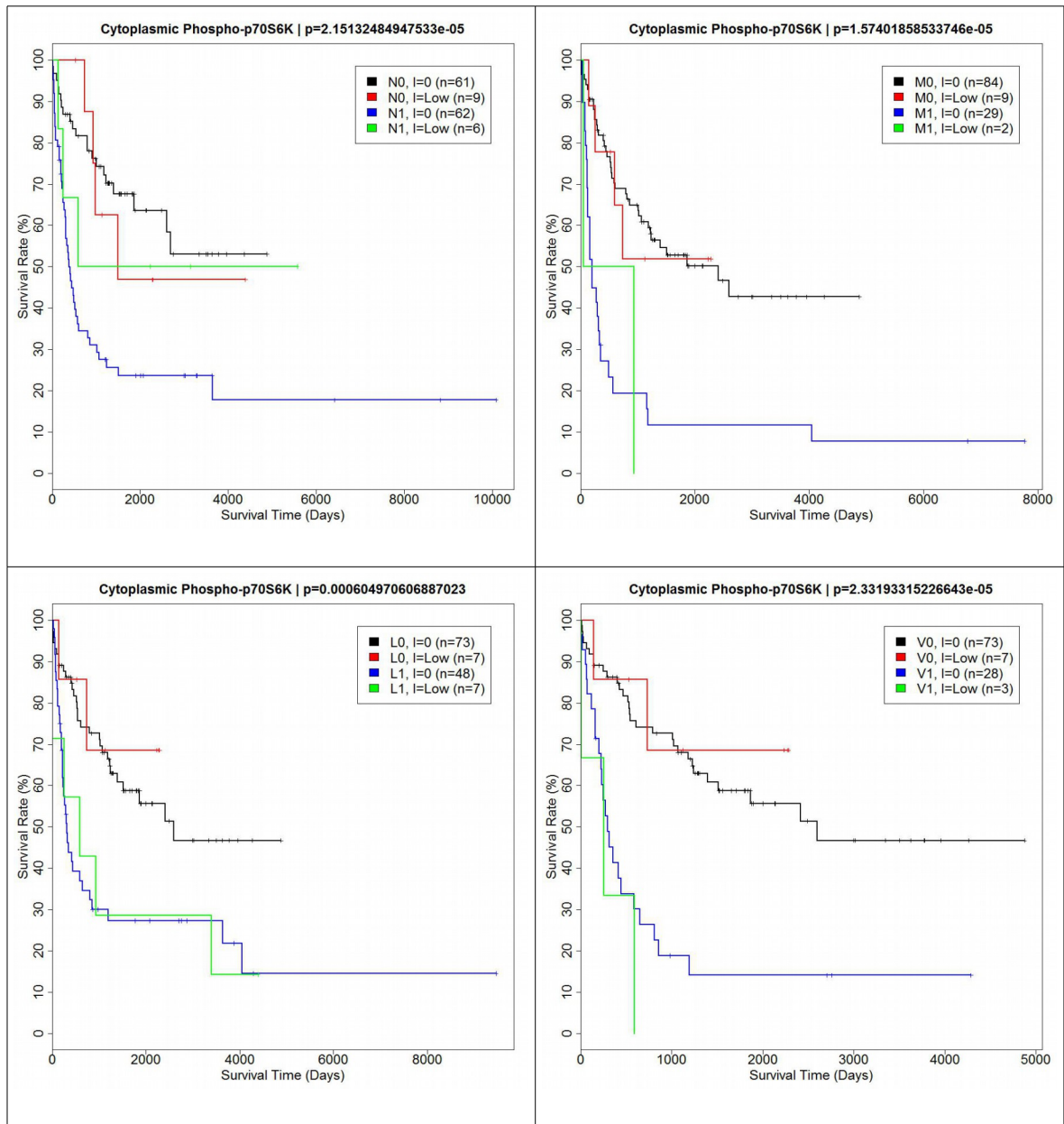


Figure 41: Kaplan-Meier Survival Analysis of Cytoplasmic Phospho-p70S6K Part 3

4 Discussion

4.1 The PI3K/Akt Pathway in Cancer Cell Lines

PTEN was downregulated in all biliary tract cancer cell lines in comparison to HeLa cells. The expression of *PTEN* was even reduced by almost 5-fold and 3-fold in the gallbladder cancer cell lines GBC and Mz-ChA-1, respectively (see Figure 8). Accordingly, phospho-PTEN (Ser380) and PTEN were not detectable in the gallbladder cancer cell lines GBC and Mz-ChA-1 by Western blot analyses (see Figure 12). By contrast, PTEN expression was shown in the GBC cell line by Herberger et al. (143). However, all antibodies used in this work were tested in both negative and positive controls, and the results were confirmed by at least two independent Western blot analyses. Therefore, a downregulation of PTEN in GBC next to Mz-ChA-1 is likely also assumed. Except for GBC, the expression of PTEN was detected in Mz-ChA-2 and TFK-1 in the present study as well as by Herberger et al. (143). Furthermore, all cell lines aside from GBC and Mz-ChA-1 showed both phospho-PTEN (Ser380) and PTEN expression (see Figure 12). Phospho-PTEN (Ser380, Thr382/383) and PTEN expression was also shown in another seven cholangiocarcinoma cell lines (144), and phospho-PTEN (Ser380) was additionally expressed in the intrahepatic biliary tract cancer cell line HuCCT1 (118).

Several research groups described the inactivation of PTEN through phosphorylation (70–72). Moreover, an increased expression of phospho-PTEN (Ser380) was demonstrated in gastric cancer, and this thereby supports the hypothesis of PTEN inactivation by phosphorylation (145). In line with this hypothesis, PTEN was inactivated in one gallbladder cell line (Mz-ChA-2) and considerably downregulated in another two gallbladder cancer cell lines (GBC, Mz-ChA-1), whereas PTEN was inactivated by phosphorylation in all extra- and intrahepatic biliary tract cancer cell lines (see Figure 12). These results show the importance of PTEN inactivation in carcinogenesis and notably emphasize the differences between gallbladder cancer and other biliary tract cancer types.

The PTEN-deficient gallbladder cancer cell lines GBC and Mz-ChA-1 showed highly activated Akt (i.e., phospho-Akt, Ser473) expression, whereas Akt was predominantly inactivated in other cancer cell lines (see Figure 12). In line with this result, Kobayashi et al. also found total and activated Akt (Ser473) expressed in Mz-ChA-1 (114). Four studies reported the expression of phospho-Akt (Ser473) in TFK-1 (113,146–148), although two of them only showed a weak expression (113,146), and one also detected phospho-Akt expression in SK-ChA-1 (148). For some unknown reason, none of them were reproducible in this work. However, downregulation of PTEN expression seems to lead to active Akt, while inactivation of PTEN by phosphorylation causes no or only a slight activation of Akt.

Interestingly, cell lines without phospho-Akt expression showed equal expressions of phospho-mTOR (Ser2448) and phospho-p70S6K (Thr421/Ser424) exactly as cell lines with activated Akt expression (see Figure 12). Therefore, mTOR activation in an Akt-independent manner has to be considered. Although the results with the mTOR antibodies should be considered with caution (see 3.1.2 Western Blots of Cancer Cell Lines and 3.2.2 Western Blots of Frozen Gallbladder Tissue), the expression levels of phospho-mTOR (Ser2448) and mTOR were in line with previous studies (146,148). Problems with the level of specificity of mTOR antibodies also became obvious in another study (143). Hence, the development of specific mTOR antibodies should be pushed ahead in the future.

As Akt, mTOR, and p70S6K were present in all biliary tract cancer cell lines (see Figure 12) and also expressed and mainly activated in numerous other biliary tract cancer cell lines (143,144,147,149–151), the PI3K/Akt pathway apparently plays a major role in the carcinogenesis of biliary tract cancer. The fact that the proto-oncogenic PI3K/Akt pathway members *Akt*, *mTOR*, *p70S6K*, *PI3K*, *XIAP*, *eNOS*, and *cyclin D1* were mostly higher expressed in biliary tract cancer cell lines than in HeLa cells supports this theory (see Figures 8, 9, and 11).

Phospho-p53 (Ser15) was highly expressed in the PTEN-deficient gallbladder cancer cell lines GBC and Mz-ChA-1 (see Figure 12). The phosphorylation of p53 at Ser15

can be caused by DNA damage (152,153). Phosphorylated p53 prevents its inhibition by MDM2 (152) and is crucial for apoptotic signaling (154). Interestingly, genome integrity depends on PTEN activity (56). Therefore, a connection between the downregulation of PTEN and p53 activation by phosphorylation may be considered. In almost all cholangiocarcinoma cell lines phospho-p53 was not detectable, while PTEN was inactivated by phosphorylation (see Figure 12). As PTEN and p53 interact in a complex manner, a downregulation of phospho-p53 seems to be possible due to an inactivation of PTEN.

These results are consistent with the observations by Teh et al. and Washington and Gottfried, who reported that an overexpression of p53 was more frequently present in gallbladder cancer compared with cholangiocarcinoma (155,156). As mutations of *p53* are associated with its overexpression (123), *p53* mutations could be more frequent in gallbladder cancer than in cholangiocarcinoma. However, Jarnagin et al. could not show a stronger expression of p53 in gallbladder cancer compared to cholangiocarcinoma in their study (157). Besides, the results shown in this thesis confirm the expression levels of total p53 in EGI-1, GBC, Mz-ChA-1, Mz-ChA-2, SK-ChA-1, and TFK-1 published by Caca et al. (158).

Cyclin D1 was clearly more highly expressed in biliary tract cancer cell lines in comparison to HeLa cells. The expression of *cyclin D1* was even increased by almost 600-fold in Mz-ChA-2 (see Figure 11). These results are in line with a previous study that reported the important role of gene amplification and overexpression of cyclin D1 in the carcinogenesis of intrahepatic cholangiocarcinoma (159). Other research groups found a correlation between cyclin D1 overexpression and poor prognosis (160) or metastases in gallbladder cancer (161,162). This thesis supports the hypothesis that the overexpression of cyclin D1 is essentially generated by gene amplification (161).

4.2 The PI3K/Akt Pathway in HeLa cells

The gene expressions of proto-oncogenic PI3K/Akt pathway members were mainly downregulated in HeLa cells in comparison to biliary tract cancer cell lines (see

Figures 8–11). In accordance with these results, phospho-Akt (Ser473), phospho-mTOR (Ser2448), and phospho-p70S6K (Thr421/Ser424) were not detectable in HeLa cells, although phospho-PTEN (Ser380) was present (see Figure 12). As already mentioned, the phosphorylation of PTEN leads to its own inactivation (70–72). The inactivation of PTEN obviously does not lead to the upregulation of the PI3K/Akt pathway in HeLa cells. PTEN inactivation might be connected with lacking p53 expression in HeLa Cells, the latter was first published by Benchimol et al. (163) and was replicable in this study (see Figure 12).

4.3 Western Blot Loading Controls for Gallbladder Tissue

GAPDH was used as housekeeping gene in all qRT-PCR experiments and showed the same expression patterns as the other well-established reference genes *Beta-Actin*, *Cyclophilin A*, and *Lamin A/C* (see Figures 6, 7, 13, and 14). Furthermore, *GAPDH* confirmed an equal protein loading in the Western blots of cancer cell lines (see Figure 12). By contrast, *GAPDH* did not seem to be suitable as a loading control in the Western blots of frozen gallbladder tissue; *Beta-Actin* showed a much more homogeneous protein loading in these cases (see Figure 16). Whereas Wu et al. termed *GAPDH* as a universal loading control for Western blot (164), limits for the usage of *GAPDH* were proven in kidney tissue (165). This thesis challenges *GAPDH* as an appropriate loading control in gallbladder tissue for the first time. However, in contrast to *GAPDH*, *Beta-Actin* could not discriminate different protein loadings originating from MDA-MB-231 cells (166). Hence, it would also be conceivable that *Beta-Actin* disguised differences between the protein amounts of gallbladder samples, which *GAPDH* recognized.

Seen in whole, further studies are required to determine reliable housekeeping proteins for gallbladder tissue. In addition, these results have shown that more attention must be generally paid to use the appropriate loading controls in Western blot experiments, depending on the investigated cells or tissue. Otherwise, wrong conclusions could be made due to unexpected expression patterns of internal controls.

4.4 The PI3K/Akt Pathway in Frozen Gallbladder Tissue

Except for *PTEN* and *eNOS*, the gene expressions of PI3K/Akt pathway members did not show notable differences between gallbladder cancer samples and non-tumorous gallbladder tissue (see Figure 15). Nevertheless, there can be changes in these genes (e.g., point mutations and deletions), which are naturally not detectable by qRT-PCR. Moreover, the lacking differences in gene expressions could indicate that the PI3K/Akt pathway is predominantly post-translationally regulated, e.g. by acetylation, oxidation, phosphorylation, or ubiquitylation. Furthermore, protein-protein interactions could play a major role in the carcinogenesis of gallbladder cancer. However, differences between the protein expressions of gallbladder cancer samples and non-tumorous gallbladder tissue were also not detectable (see Figure 16). Several facts have to be taken into consideration as possible causes of these results.

Firstly, the number of samples could be too small to identify relevant distinctions between gallbladder cancer and tumor-free gallbladder tissue.

Secondly, the used tumor-free gallbladder samples could represent an unsuitable control group because one acute cholecystitis and two samples with residues after cholecystitis were included. Furthermore, all tumor-free samples were obtained from gallbladders that were affected by cancer. In 2014, Sanz-Pamplona et al. reported multiple abnormalities in tumor-adjacent tissue compared to tissue from healthy donors (167). Tumor-adjacent tissue, which can present itself histologically normal, forms the environment of the tumor, is thereby strongly influenced by the latter and shows considerable biological changes (167,168). In accordance with these observations, no convincing differences of phospho-Akt (Ser473) and Akt could be demonstrated between cholangiocarcinoma and tumor-adjacent tissue by Dokduang et al. (169).

Thirdly, there are no differences in the PI3K/Akt pathway between gallbladder cancer and normal gallbladder tissue. The further results of this study as well as published reports definitely disagree with the third supposition. For instance, numerous research groups described the upregulation of p53 in gallbladder cancer compared with normal

gallbladder tissue (125,170,171). Ewald et al. also reported an Akt expression in all investigated cholangiocarcinoma cases by Western blot analysis (148). Accordingly, Akt was expressed in all Western blots of gallbladder cancer samples in this work (see Figure 16). Furthermore, this thesis showed similar expression patterns of PTEN, phospho-Akt, phospho-p70S6K, phospho-mTOR, and mTOR in gallbladder cancer samples compared to cholangiocarcinoma samples (148). Consequently, the PI3K/Akt pathway obviously plays a role in the carcinogenesis of both gallbladder cancer as well as cholangiocarcinoma.

4.5 The PI3K/Akt Pathway in Gallbladder TMAs

The investigated gallbladder cohort showed a significant correlation between tumor progression (i.e., loss of differentiation, tumor growth, and dissemination) and poor survival (see Figures 22 and 23). The female-male ratio was 2.7 and corresponds to the generally observed ratio (15). These results demonstrate that an appropriate study cohort was collected to investigate the PI3K/Akt pathway in gallbladder cancer. The fact that the results of intensities and tissue scores were very similar shows that both evaluation tools are most likely of the same value and confirms the reliability of this study.

Significant higher PTEN expressions were seen in poorly-differentiated and undifferentiated tumors compared to normal gallbladder tissue ($p < 0.05$, see Table 19). Whereas Chung et al. could not detect differences between PTEN expression in normal biliary epithelium and gallbladder cancer (172), Pignochino et al. also reported a stronger staining of PTEN in biliary tract cancer samples compared to normal cholangiocytes (146). In 2009, Li and Yang reported that the expression of PI3K, the antagonist of PTEN, was significantly higher in poorly-differentiated gallbladder adenocarcinomas and larger tumors with lymph node metastasis and invasion (128). In this study, PTEN was significantly higher expressed in less-differentiated tumors, and the expression positively correlated with distant metastasis, lymphatic vessel invasion, and venous invasion (each $p < 0.05$, see Table 19). An increased expression of PI3K was linked with a worse prognosis ($p = 0.062$) (128), and a high intensity of PTEN was associated with a reduced overall

survival compared to a low intensity in this study ($p < 0.05$, see Figure 24). In summary, increasing expressions of both PI3K and PTEN were related to tumor progression and decreased overall survival. Interestingly, PTEN was highly expressed in chronic cholecystitis in this (see Table 19) and another study (126), whereas PI3K was only weakly present (128). These results could indicate that it is not the absolute expression levels that are important, but rather the ratio between PI3K and PTEN. More precisely, a disturbed balance between PI3K and PTEN might lead to gallbladder cancer.

In contrast to the results of this thesis, loss of PTEN expression significantly correlated with poorly-differentiated gallbladder adenocarcinomas, invasion, and a worse outcome in a previous study (126). However, PTEN was mainly expressed in the cytoplasm (126), whereas PTEN was exclusively detected in the nucleus in this study (see Figure 17). Loss of PTEN expression was also associated with a reduced overall survival and predominantly detected in the cytoplasm in intrahepatic (116) and extrahepatic biliary tract cancer (172) and ampullary cancer (173) in previously published studies. As outlined in the introduction (see 1.3.1 Functions of PTEN), the tumor suppressor activity of PTEN is possibly dependent on its cytoplasmic as well as on its nuclear presence (62). Therefore, cytoplasmic and nuclear PTEN are obviously differently regulated in gallbladder carcinogenesis. In 2013, Yothaisong et al. reported that 89 % of PTEN positive cholangiocarcinomas were inactivated by phosphorylation (144). In another study, phospho-PTEN was detected in 71 % of cholangiocarcinoma cases (112). Thus, the phosphorylation of PTEN could explain the fact that PTEN expression was positively correlated with poor prognosis in this study. To confirm this theory, gallbladder cancer samples should be stained for phospho-PTEN in the future.

The strongest staining of phospho-Akt (Ser473) was detected in chronic cholecystitis, and these specimens significantly differed from normal biliary tissue as well as gallbladder cancer cases excepted from Tis samples (each $p < 0.05$, see Tables 20 and 21). Interestingly, an enhanced expression of Akt was reported in PSC (114). Taken together, Akt is obviously upregulated in chronic inflammation and

could therefore be responsible for supporting or even initiating carcinogenesis in biliary tract cancer (114). Significantly higher phospho-Akt expressions were detected in cholangiocarcinoma when compared to normal biliary tissue (118,172), which could not be shown between gallbladder cancer and normal gallbladder specimens in this study (see Tables 20 and 21). However, a phospho-Akt antibody that only detects Akt when phosphorylated at Ser473 was used in this thesis, whereas phospho-Akt (Thr308) or phospho-Akt with an unknown target were used in the above-named studies.

No differences regarding phospho-Akt (Ser473) expressions were detectable between different gradings or tumor extents in this (see Tables 20 and 21) and a previous study (174). Furthermore, no clear tendency could be derived from the effect of phospho-Akt (Ser473) on the survival time in gallbladder cancer. Although high intensities differed from no or low intensities in some categories, there were some contradictory results most likely due to the low number of cases that show a high intensity (see Figures 27–32). In accordance with these results was the fact that phospho-Akt (Ser473 or unknown phosphorylated target) showed neither prognostic relevance in intrahepatic (175) nor in extrahepatic biliary tract cancer (172) in previous studies. However, further studies reported that Akt and phospho-Akt (unknown phosphorylated target) were associated with an improved outcome in cholangiocarcinoma (116,117).

Leal et al. reported that phospho-mTOR (Ser2448) was significantly stronger expressed in advanced gallbladder cancer than in chronic cholecystitis (129), whereas the exact contrary was shown in this study ($p < 0.05$, see Table 22). While only three cases of mild chronic cholecystitis were examined in this thesis, Leal et al. investigated 99 cases of chronic cholecystitis (degree of inflammation not specified) (129). However, 345 gallbladder samples were investigated in this study, and a loss of phospho-mTOR (Ser2448) expression correlated with a poorer prognosis compared to a weak expression ($p < 0.05$, see Figure 33). By contrast, high expression of phospho-mTOR (Ser2448) was significantly associated with a reduced overall survival in comparison to low expression in a study of 114 gallbladder cancer

samples (129). Although these results seem to be mutually incompatible, it has to be considered that only advanced gallbladder cancer cases were investigated in the above-mentioned study, whereas all types of tumor extent were included in this study (see Figure 22).

In addition, it would be imaginable that a low or rather normal expression of phospho-mTOR is more favorable than both a loss and an overexpression of phospho-mTOR. However, different interpretations of immunohistochemical staining were used, which is a principal problem in comparing immunohistochemical studies. Whereas one study found an overexpression of phospho-mTOR (Ser2448) associated with an improved outcome in intrahepatic biliary tract cancer (116), another study reported a correlation between positive expression and reduced overall survival in biliary tract cancer (115), while a third study could not show any significant prognostic value for phospho-mTOR (unknown phosphorylated target) in extrahepatic cholangiocarcinoma (172). Apart from the difficulty in comparing different immunohistochemical studies, phospho-mTOR could play variant roles in different types of biliary tract cancer.

The transition from Tis to an invasive tumor as well as the loss of differentiation from G1 to G2 was significantly associated with a decreasing expression of phospho-mTOR (each $p < 0.05$, see Table 22). In addition, patients with M1 and L1 had significantly lower phospho-mTOR expressions than patients staged with M0 and L0, respectively (each $p < 0.05$, see Table 22). Accordingly, phospho-mTOR (Ser2448) expression was significantly stronger in well- to moderately-differentiated as well as in N0 staged intrahepatic cholangiocarcinomas (116). N0 staged cancer samples also showed higher phospho-mTOR (Ser2448) expressions in this study, but this was not statistically significant (see Table 22). By contrast, Herberger et al. did not find any significant relation between phospho-mTOR (Ser2448) expression and histological differentiation or lymph node status in biliary tract cancer (115), probably because both extrahepatic and intrahepatic biliary tract cancer and gallbladder cancer were combined in this study.

A higher phospho-mTOR (unknown phosphorylated target) expression was shown in extrahepatic biliary tract cancer compared to normal biliary tissue (172). This relation was also detectable in gallbladder cancer, but only in comparison with well-differentiated (i.e., G1) and low-stage tumors (i.e., Tis, T1, N0, M0, L0, and V0) as well as V1 cases (each $p < 0.05$, see Table 22). However, Wu et al. detected higher expression levels of phospho-Akt (Ser473) and phospho-mTOR (Ser2448) by immunofluorescence staining in gallbladder cancer samples ($n = 27$) compared to normal gallbladder specimens ($n = 7$) (176).

Strong expression of nuclear phospho-p70S6K was associated with a better survival compared to a weak expression ($p < 0.05$, see Figure 36). In line with this relation was the possible association between a positive expression of phospho-p70S6K-beta and an improved outcome in intrahepatic biliary tract cancer (116). As seen for phospho-mTOR, the transition from Tis to an invasive carcinoma and the loss of differentiation from G1 to G2 were significantly associated with a decreasing expression of nuclear phospho-p70S6K (Ser411, each $p < 0.05$, see Table 23). In addition, an increasing loss of differentiation, a growing tumor extent, and invasion were related to lower expressions of phospho-mTOR and nuclear phospho-p70S6K (see Tables 22 and 23).

Phospho-mTOR expression was significantly lower in tumor-free gallbladder tissue compared to N0 and M0 tumors (each $p < 0.05$, see Table 22), and nuclear phospho-p70S6K expression was significantly higher in tumor-free tissue than in N1 and M1 tumors ($p < 0.05$, see Table 23). Thus, these two phosphoproteins could be used to estimate the likelihood of metastasis by comparing the expression levels in cancer and tumor-free tissue of patients who suffer from gallbladder cancer. This information might help to choose the best therapy option in these patients and could therefore improve the outcome in the future.

4.6 Conclusion

PTEN was significantly downregulated in two gallbladder cancer cell lines and inactivated in one gallbladder and all cholangiocarcinoma cell lines by

phosphorylation. The downregulation of PTEN correlated with the activation of Akt, while the inactivation of PTEN led to no or only a slight activation of Akt. All cell lines had expressions of phospho-mTOR and phospho-p70S6K. Therefore, mTOR activation occurred in an Akt-independent manner. The PTEN-deficient cell lines showed a high expression of phospho-p53, which could be caused by DNA damage (152,153). Phospho-p53 was absent in almost all cholangiocarcinoma cell lines, which was possibly caused by the inactivation of PTEN.

The overexpression of PTEN was possibly accompanied by PTEN phosphorylation, which would explain why this tumor suppressor was upregulated in less-differentiated, invasive gallbladder cancer and correlated with a reduced overall survival. Similar findings were reported for PI3K, which was only weakly present in chronic cholecystitis (128) in contrast to PTEN. The alteration of the ratio between PI3K and PTEN may lead to the activation of Akt, mTOR as well as p70S6K. It may also promote gallbladder carcinogenesis in chronic cholecystitis. Decreasing levels of nuclear phospho-p70S6K and phospho-mTOR correlated with the loss of differentiation, a growing tumor extent, invasion, and poor prognosis. Furthermore, these two markers could be used to estimate the likelihood of metastasis by comparing the expression levels in cancer and tumor-free tissue of patients with gallbladder cancer.

With respect to the advanced hypotheses, PTEN was downregulated or inactivated in biliary tract cancer cell lines, whereas Akt, mTOR, and p70S6K were mostly activated. The transition from normal gallbladder epithelium to gallbladder cancer as well as an increasing invasive capacity and a poor differentiation of gallbladder cancer was not characterized by the loss or downregulation of PTEN, but by its upregulation and possible inactivation through phosphorylation. This was accompanied by an enhanced expression of activated Akt, mTOR, and p70S6K in chronic cholecystitis and decreasing expressions of phospho-mTOR and phospho-p70S6K associated with tumor progression.

This thesis dealt with the proto-oncogenic PI3K/Akt pathway in biliary tract cancer based on the fact that the belonging cancer types partially share common pathways in carcinogenesis (30). However, the results of this thesis and previous reports showed that there are considerable differences, and it is therefore not recommended to summarize these cancer types in further studies. The exploration of pathways in the carcinogenesis of biliary tract malignancies also contributes to the development of prognostic biomarkers. Some potential markers have already been used in clinical trials. The inactivation of PTEN due to phosphorylation could be a promising objective for targeted therapies in the future. Furthermore, high sample sizes are needed to gain better insight into the PI3K/Akt pathway and the carcinogenesis in gallbladder cancer. The consideration of nuclear and cytoplasmic proteins, different phospho-specific antibodies, and reliable controls as well as the standardization of immunohistochemical analysis will be of fundamental importance for the success of research studies in the future.

5 References

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6 Appendix

Significant p-values are highlighted in yellow.

6.1 P-Values of Kaplan-Meier Survival Analysis of the Cohort

	G1	G2	G3	G4
G1	X	0.00025	1.16E-009	0.05797
G2	0.00025	X	0.00019	0.52509
G3	1.16E-009	0.00019	X	0.65657
G4	0.05797	0.52509	0.65657	X

Table 25: P-Values of the Cohort Part 1

	T1	T2	T3	T4	Tis
T1	X	0.29792	7.38E-006	2.56E-006	0.00229
T2	0.29792	X	4.09E-006	6.09E-005	0.00013
T3	7.38E-006	4.09E-006	X	0.13939	5.94E-008
T4	2.56E-006	6.09E-005	0.13939	X	8.27E-008
Tis	0.00229	0.00013	5.94E-008	8.27E-008	X

Table 26: P-Values of the Cohort Part 2

	N0	N1		M0	M1
N0	X	5.21E-007	M0	X	1.46E-008
N1	5.21E-007	X	M1	1.46E-008	X

Table 27: P-Values of the Cohort Part 3

	L0	L1		V1	V1
L0	X	1.37E-005	V0	X	1.91E-006
L1	1.37E-005	X	V1	1.91E-006	X

Table 28: P-Values of the Cohort Part 4

6.2 P-Values of Nuclear PTEN

6.2.1 P-Values of Mean Intensities

	Tumor	CC	Co
Tumor	X	0.07584	0.12717
CC	0.07584	X	0.07033
Co	0.12717	0.07033	X

Table 29: P-Values of Nuclear PTEN Part 1

	G1	G2	G3	G4	CC	Co
G1	X	0.03230	5.19E-005	0.01104	0.02415	0.46510
G2	0.03230	X	0.00991	0.06723	0.06254	0.34227
G3	5.19E-005	0.00991	X	0.26929	0.13705	0.00680
G4	0.01104	0.06723	0.26929	X	0.44910	0.04995
CC	0.02415	0.06254	0.13705	0.44910	X	0.07033
Co	0.46510	0.34227	0.00680	0.04995	0.07033	X

Table 30: P-Values of Nuclear PTEN Part 2

	T1	T2	T3	T4	Tis	CC	Co
T1	X	0.11034	0.87448	0.13220	0.93308	0.09209	0.05390
T2	0.11034	X	0.11838	0.79201	0.63585	0.06222	0.38847
T3	0.87448	0.11838	X	0.19500	0.93746	0.11151	0.06430
T4	0.13220	0.79201	0.19500	X	0.54654	0.04636	0.59753
Tis	0.93308	0.63585	0.93746	0.54654	X	0.17654	0.49400
CC	0.09209	0.06222	0.11151	0.04636	0.17654	X	0.07033
Co	0.05390	0.38847	0.06430	0.59753	0.49400	0.07033	X

Table 31: P-Values of Nuclear PTEN Part 3

	N0	N1	CC	Co
N0	X	0.95205	0.03011	0.99231
N1	0.95205	X	0.04017	0.91844
CC	0.03011	0.04017	X	0.07033
Co	0.99231	0.91844	0.07033	X

Table 32: P-Values of Nuclear PTEN Part 4

	M0	M1	CC	Co
M0	X	0.00055	0.01109	0.15358
M1	0.00055	X	0.11251	0.14577
CC	0.01109	0.11251	X	0.07033
Co	0.15358	0.14577	0.07033	X

Table 33: P-Values of Nuclear PTEN Part 5

	L0	L1	CC	Co
L0	X	2.85E-007	0.01017	0.08190
L1	2.85E-007	X	0.13116	0.01658
CC	0.01017	0.13116	X	0.07033
Co	0.08190	0.01658	0.07033	X

Table 34: P-Values of Nuclear PTEN Part 6

	V0	V1	CC	Co
V0	X	4.35E-005	0.01017	0.08190
V1	4.35E-005	X	0.09549	0.07647
CC	0.01017	0.09549	X	0.07033
Co	0.08190	0.07647	0.07033	X

Table 35: P-Values of Nuclear PTEN Part 7

6.2.2 P-Values of Kaplan-Meier Survival Analysis

	I=0	I=Low	I=High
I=0	X	0.59302	0.59420
I=Low	0.59302	X	0.04153
I=High	0.59420	0.04153	X

Table 36: P-Values of Nuclear PTEN Part 8

	I=0, N0	I=Low, N0	I=High, N0	I=0, N1	I=Low, N1	I=High, N1
I=0, N0	X	0.29319	0.26088	0.65780	0.77437	0.56760
I=Low, N0	0.29319	X	0.79904	0.57144	0.00089	0.00031
I=High, N0	0.26088	0.79904	X	0.50274	0.00052	0.00014
I=0, N1	0.65780	0.57144	0.50274	X	0.34789	0.36829
I=Low, N1	0.77437	0.00089	0.00052	0.34789	X	0.56532
I=High, N1	0.56760	0.00031	0.00014	0.36829	0.56532	X

Table 37: P-Values of Nuclear PTEN Part 9

	I=0, M0	I=Low, M0	I=High, M0	I=0, M1	I=Low, M1	I=High, M1
I=0, M0	X	0.25874	0.22196	0.60013	0.65766	0.71870
I=Low, M0	0.25874	X	0.38599	0.18540	0.00470	8.72E-005
I=High, M0	0.22196	0.38599	X	0.09317	0.00100	1.10E-005
I=0, M1	0.60013	0.18540	0.09317	X	0.45551	0.42939
I=Low, M1	0.65766	0.00470	0.00100	0.45551	X	0.60383
I=High, M1	0.71870	8.72E-005	1.10E-005	0.42939	0.60383	X

Table 38: P-Values of Nuclear PTEN Part 10

	I=0, L0	I=Low, L0	I=High, L0	I=Low, L1	I=High, L1
I=0, L0	X	0.12691	0.11595	0.80254	0.91629
I=Low, L0	0.12691	X	0.55826	0.01370	0.00013
I=High, L0	0.11595	0.55826	X	0.00796	0.00017
I=Low, L1	0.80254	0.01370	0.00796	X	0.57442
I=High, L1	0.91629	0.00013	0.00017	0.57442	X

Table 39: P-Values of Nuclear PTEN Part 11

	I=0, V0	I=Low, V0	I=High, V0	I=Low, V1	I=High, V1
I=0, V0	X	0.12691	0.11595	0.05071	0.82403
I=Low, V0	0.12691	X	0.55826	2.81E-005	0.00126
I=High, V0	0.11595	0.55826	X	8.64E-006	0.00053
I=Low, V1	0.05071	2.81E-005	8.64E-006	X	0.13653
I=High, V1	0.82403	0.00126	0.00053	0.13653	X

Table 40: P-Values of Nuclear PTEN Part 12

	I=0, G1	I=Low, G1	I=High, G1	I=0, G2	I=Low, G2	I=High, G2	I=0, G3	I=Low, G3	I=High, G3	I=Low, G4	I=High, G4
I=0, G1	X	0.32659	0.65151	0.48610	0.92043	0.69368	0.93455	0.34772	0.19130	0.89454	0.67793
I=Low, G1	0.32659	X	0.64493	0.01828	0.01466	0.00225	0.35514	7.94E-005	2.15E-007	0.54145	0.09970
I=High, G1	0.65151	0.64493	X	0.03618	0.06698	0.02246	0.52623	0.00075	5.32E-006	0.87218	0.12780
I=0, G2	0.48610	0.01828	0.03618	X	0.26148	0.52172	0.61068	0.87142	0.44285	0.78048	0.79026
I=Low, G2	0.92043	0.01466	0.06698	0.26148	X	0.52724	0.96823	0.02049	0.00019	0.95795	0.52509
I=High, G2	0.69368	0.00225	0.02246	0.52172	0.52724	X	0.72495	0.04697	0.00033	0.70419	0.69307
I=0, G3	0.93455	0.35514	0.52623	0.61068	0.96823	0.72495	X	0.41541	0.26453	0.98353	0.75301
I=Low, G3	0.34772	7.94E-005	0.00075	0.87142	0.02049	0.04697	0.41541	X	0.27788	0.60771	0.65306
I=High, G3	0.19130	2.15E-007	5.32E-006	0.44285	0.00019	0.00033	0.26453	0.27788	X	0.51205	0.33380
I=Low, G4	0.89454	0.54145	0.87218	0.78048	0.95795	0.70419	0.98353	0.60771	0.51205	X	0.99147
I=High, G4	0.67793	0.09970	0.12780	0.79026	0.52509	0.69307	0.75301	0.65306	0.33380	0.99147	X

Table 41: P-Values of Nuclear PTEN Part 13

	I=0, T1	I=Low, T1	I=High, T1	I=0, T2	I=Low, T2	I=High, T2	I=0, T3	I=Low, T3	I=High, T3	I=Low, T4	I=High, T4	I=0, Tis	I=Low, Tis	I=High, Tis
I=0, T1	X	0.12575	0.17437	0.34958	0.12883	0.47161	0.76598	0.67099	0.81233	0.15064	0.81471	0.23903	0.03188	0.02579
I=Low, T1	0.12575	X	0.63431	0.07083	0.59440	0.07859	0.18847	0.01794	0.00094	1.47E-005	0.03088	0.39833	0.16524	0.07972
I=High, T1	0.17437	0.63431	X	0.09224	0.53726	0.13757	0.62238	0.02789	0.00015	6.32E-006	0.05124	0.29194	0.05390	0.02980
I=0, T2	0.34958	0.07083	0.09224	X	0.05686	0.22610	0.32941	0.32679	0.74334	0.51339	0.61805	0.09589	0.01995	0.02579
I=Low, T2	0.12883	0.59440	0.53726	0.05686	X	0.01213	0.25350	0.00203	4.18E-007	1.40E-005	0.02565	0.30624	0.07427	0.02498
I=High, T2	0.47161	0.07859	0.13757	0.22610	0.01213	X	0.91611	0.34028	0.00370	0.00113	0.32268	0.18027	0.01779	0.00293
I=0, T3	0.76598	0.18847	0.62238	0.32941	0.25350	0.91611	X	0.68509	0.32253	0.05354	0.62553	0.19300	0.03910	0.01587
I=Low, T3	0.67099	0.01794	0.02789	0.32679	0.00203	0.34028	0.68509	X	0.09524	0.00842	0.51809	0.13028	0.00868	0.00070
I=High, T3	0.81233	0.00094	0.00015	0.74334	4.18E-007	0.00370	0.32253	0.09524	X	0.17400	0.80208	0.07270	0.00222	3.85E-005
I=Low, T4	0.15064	1.47E-005	6.32E-006	0.51339	1.40E-005	0.00113	0.05354	0.00842	0.17400	X	0.28643	0.02352	0.00047	1.09E-005
I=High, T4	0.81471	0.03088	0.05124	0.61805	0.02565	0.32268	0.62553	0.51809	0.80208	0.28643	X	0.15371	0.02011	0.00223
I=0, Tis	0.23903	0.39833	0.29194	0.09589	0.30624	0.18027	0.19300	0.13028	0.07270	0.02352	0.15371	X	1	1
I=Low, Tis	0.03188	0.16524	0.05390	0.01995	0.07427	0.01779	0.03910	0.00868	0.00222	0.00047	0.02011	1	X	1
I=High, Tis	0.02579	0.07972	0.02980	0.02579	0.02498	0.00293	0.01587	0.00070	3.85E-005	1.09E-005	0.00223	1	1	X

Table 42: P-Values of Nuclear PTEN Part 14

6.3 P-Values of Nuclear Phospho-Akt

6.3.1 P-Values of Mean Intensities

	Tumor	CC	Co
Tumor	X	0.00262	0.67302
CC	0.00262	X	0.00653
Co	0.67302	0.00653	X

Table 43: P-Values of Nuclear Phospho-Akt Part 1

	G1	G2	G3	G4	CC	Co
G1	X	0.72121	0.56554	0.87404	0.00474	0.78146
G2	0.72121	X	0.23590	0.96790	0.00218	1
G3	0.56554	0.23590	X	0.63434	0.00291	0.50004
G4	0.87404	0.96790	0.63434	X	0.01186	0.93701
CC	0.00474	0.00218	0.00291	0.01186	X	0.00653
Co	0.78146	1	0.50004	0.93701	0.00653	X

Table 44: P-Values of Nuclear Phospho-Akt Part 2

	T1	T2	T3	T4	Tis	CC	Co
T1	X	0.70751	0.43036	0.24203	0.12588	0.00607	0.58128
T2	0.70751	X	0.09383	0.08005	0.14450	0.00421	0.35704
T3	0.43036	0.09383	X	0.43545	0.03981	0.00158	0.86847
T4	0.24203	0.08005	0.43545	X	0.04140	0.00383	0.59948
Tis	0.12588	0.14450	0.03981	0.04140	X	0.14539	0.08051
CC	0.00607	0.00421	0.00158	0.00383	0.14539	X	0.00653
Co	0.58128	0.35704	0.86847	0.59948	0.08051	0.00653	X

Table 45: P-Values of Nuclear Phospho-Akt Part 3

	N0	N1	CC	Co
N0	X	0.65594	0.00272	0.88839
N1	0.65594	X	0.00299	0.86806
CC	0.00272	0.00299	X	0.00653
Co	0.88839	0.86806	0.00653	X

Table 46: P-Values of Nuclear Phospho-Akt Part 4

	M0	M1	CC	Co
M0	X	0.79724	0.00340	0.82977
M1	0.79724	X	0.00451	0.87661
CC	0.00340	0.00451	X	0.00653
Co	0.82977	0.87661	0.00653	X

Table 47: P-Values of Nuclear Phospho-Akt Part 5

	L0	L1	CC	Co
L0	X	0.09885	0.00314	0.96895
L1	0.09885	X	0.00454	0.15167
CC	0.00314	0.00454	X	0.00653
Co	0.96895	0.15167	0.00653	X

Table 48: P-Values of Nuclear Phospho-Akt Part 6

	V0	V1	CC	Co
V0	X	0.44312	0.00314	0.96895
V1	0.44312	X	0.00705	0.43577
CC	0.00314	0.00705	X	0.00653
Co	0.96895	0.43577	0.00653	X

Table 49: P-Values of Nuclear Phospho-Akt Part 7

6.3.2 P-Values of Kaplan-Meier Survival Analysis

	I=0	I=Low	I=High
I=0	X	0.99045	0.07185
I=Low	0.99045	X	0.10303
I=High	0.07185	0.10303	X

Table 50: P-Values of Nuclear Phospho-Akt Part 8

	I=0, N0	I=Low, N0	I=High, N0	I=0, N1	I=Low, N1	I=High, N1
I=0, N0	X	0.21273	0.14942	1.28E-005	0.00018	0.37814
I=Low, N0	0.21273	X	0.07074	0.00374	0.01896	0.26129
I=High, N0	0.14942	0.07074	X	0.00227	0.00407	0.27826
I=0, N1	1.28E-005	0.00374	0.00227	X	0.97186	0.01640
I=Low, N1	0.00018	0.01896	0.00407	0.97186	X	0.01192
I=High, N1	0.37814	0.26129	0.27826	0.01640	0.01192	X

Table 51: P-Values of Nuclear Phospho-Akt Part 9

	I=0, M0	I=Low, M0	I=High, M0	I=0, M1	I=Low, M1	I=High, M1
I=0, M0	X	0.20711	0.17137	0.00192	9.14E-005	0.04049
I=Low, M0	0.20711	X	0.07163	0.03525	0.00145	0.08051
I=High, M0	0.17137	0.07163	X	0.00242	0.00284	0.14471
I=0, M1	0.00192	0.03525	0.00242	X	0.12513	0.22090
I=Low, M1	9.14E-005	0.00145	0.00284	0.12513	X	0.37403
I=High, M1	0.04049	0.08051	0.14471	0.22090	0.37403	X

Table 52: P-Values of Nuclear Phospho-Akt Part 10

	I=0, L0	I=Low, L0	I=High, L0	I=0, L1	I=Low, L1	I=High, L1
I=0, L0	X	0.26550	0.26010	0.00123	0.00383	0.00035
I=Low, L0	0.26550	X	0.16618	0.04166	0.16797	0.00287
I=High, L0	0.26010	0.16618	X	0.00348	0.00840	0.00612
I=0, L1	0.00123	0.04166	0.00348	X	0.81984	0.00991
I=Low, L1	0.00383	0.16797	0.00840	0.81984	X	0.01854
I=High, L1	0.00035	0.00287	0.00612	0.00991	0.01854	X

Table 53: P-Values of Nuclear Phospho-Akt Part 11

	I=0, V0	I=Low, V0	I=High, V0	I=0, V1	I=Low, V1	I=High, V1
I=0, V0	X	0.26550	0.26010	0.00051	0.00040	0.00030
I=Low, V0	0.26550	X	0.16618	0.01283	0.01933	0.00181
I=High, V0	0.26010	0.16618	X	0.00286	0.00167	0.00578
I=0, V1	0.00051	0.01283	0.00286	X	0.86088	0.00733
I=Low, V1	0.00040	0.01933	0.00167	0.86088	X	0.00225
I=High, V1	0.00030	0.00181	0.00578	0.00733	0.00225	X

Table 54: P-Values of Nuclear Phospho-Akt Part 12

	I=0, G1	I=Low, G1	I=High, G1	I=0, G2	I=Low, G2	I=High, G2	I=0, G3	I=Low, G3	I=High, G3	I=0, G4	I=Low, G4
I=0, G1	X	0.97738	0.34595	0.00551	0.05886	0.45939	4.46E-006	5.70E-005	0.29636	0.91743	0.00318
I=Low, G1	0.97738	X	0.38677	0.00644	0.04383	0.29618	1.16E-005	5.16E-005	0.12772	0.88239	0.00026
I=High, G1	0.34595	0.38677	X	0.52644	0.80288	0.98640	0.07125	0.09424	0.74030	0.79321	0.02174
I=0, G2	0.00551	0.00644	0.52644	X	0.45381	0.27977	0.00210	0.00803	0.89655	0.35865	0.04114
I=Low, G2	0.05886	0.04383	0.80288	0.45381	X	0.57630	0.00231	0.00932	0.80904	0.57230	0.02804
I=High, G2	0.45939	0.29618	0.98640	0.27977	0.57630	X	0.01283	0.03282	0.78266	0.88200	0.07333
I=0, G3	4.46E-006	1.16E-005	0.07125	0.00210	0.00231	0.01283	X	0.97611	0.15346	0.09294	0.43777
I=Low, G3	5.70E-005	5.16E-005	0.09424	0.00803	0.00932	0.03282	0.97611	X	0.20870	0.15745	0.38000
I=High, G3	0.29636	0.12772	0.74030	0.89655	0.80904	0.78266	0.15346	0.20870	X	0.87379	0.26397
I=0, G4	0.91743	0.88239	0.79321	0.35865	0.57230	0.88200	0.09294	0.15745	0.87379	X	0.26209
I=Low, G4	0.00318	0.00026	0.02174	0.04114	0.02804	0.07333	0.43777	0.38000	0.26397	0.26209	X

Table 55: P-Values of Nuclear Phospho-Akt Part 13

	I=0, T1	I=Low, T1	I=High, T1	I=0, T2	I=Low, T2	I=High, T2	I=0, T3	I=Low, T3	I=High, T3	I=0, T4	I=Low, T4	I=High, T4	I=0, Tis	I=Low, Tis	I=High, Tis
I=0, T1	X	0.53630	0.58283	0.83393	0.50269	0.38648	0.04454	0.01703	0.02353	1.99E-005	0.01178	0.12767	0.05138	0.14103	0.04125
I=Low, T1	0.53630	X	0.91420	0.31385	0.20461	0.92772	0.01635	0.00712	0.01129	0.00021	0.01033	0.25225	0.10659	0.23532	0.10659
I=High, T1	0.58283	0.91420	X	0.66835	0.51249	0.90179	0.19111	0.10847	0.12430	0.01575	0.09241	0.28340	0.14733	0.29915	0.16524
I=0, T2	0.83393	0.31385	0.66835	X	0.77509	0.33825	0.03424	0.01394	0.04585	0.00206	0.04406	0.17603	0.03524	0.10256	0.01988
I=Low, T2	0.50269	0.20461	0.51249	0.77509	X	0.22255	0.04468	0.01980	0.02245	0.00057	0.04201	0.13488	0.02669	0.07550	0.01343
I=High, T2	0.38648	0.92772	0.90179	0.33825	0.22255	X	0.02790	0.01320	0.02826	0.00296	0.03593	0.29008	0.10924	0.21162	0.08815
I=0, T3	0.04454	0.01635	0.19111	0.03424	0.04468	0.02790	X	0.35146	0.26949	0.03117	0.23071	0.10218	0.00714	0.03040	0.00223
I=Low, T3	0.01703	0.00712	0.10847	0.01394	0.01980	0.01320	0.35146	X	0.60555	0.23064	0.39584	0.07603	0.00465	0.02000	0.00121
I=High, T3	0.02353	0.01129	0.12430	0.04585	0.02245	0.02826	0.26949	0.60555	X	0.58464	0.96369	0.09081	0.00701	0.02223	0.00259
I=0, T4	1.99E-005	0.00021	0.01575	0.00206	0.00057	0.00296	0.03117	0.23064	0.58464	X	0.98492	0.04431	0.00130	0.00646	0.00032
I=Low, T4	0.01178	0.01033	0.09241	0.04406	0.04201	0.03593	0.23071	0.39584	0.96369	0.98492	X	0.09840	0.00962	0.02690	0.00405
I=High, T4	0.12767	0.25225	0.28340	0.17603	0.13488	0.29008	0.10218	0.07603	0.09081	0.04431	0.09840	X	1	1	1
I=0, Tis	0.05138	0.10659	0.14733	0.03524	0.02669	0.10924	0.00714	0.00465	0.00701	0.00130	0.00962	1	X	1	1
I=Low, Tis	0.14103	0.23532	0.29915	0.10256	0.07550	0.21162	0.03040	0.02000	0.02223	0.00646	0.02690	1	1	X	1
I=High, Tis	0.04125	0.10659	0.16524	0.01988	0.01343	0.08815	0.00223	0.00121	0.00259	0.00032	0.00405	1	1	1	X

Table 56: P-Values of Nuclear Phospho-Akt Part 14

6.4 P-Values of Cytoplasmic Phospho-Akt

6.4.1 P-Values of Mean Intensities

	Tumor	CC	Co
Tumor	X	0.00196	0.86508
CC	0.00196	X	0.00278
Co	0.86508	0.00278	X

Table 57: P-Values of Cytoplasmic Phospho-Akt Part 1

	G1	G2	G3	G4	CC	Co
G1	X	0.66446	0.56505	0.18396	0.00480	0.91345
G2	0.66446	X	0.13837	0.12568	0.00247	0.60329
G3	0.56505	0.13837	X	0.04312	0.00120	0.59849
G4	0.18396	0.12568	0.04312	X	0.02340	0.12590
CC	0.00480	0.00247	0.00120	0.02340	X	0.00278
Co	0.91345	0.60329	0.59849	0.12590	0.00278	X

Table 58: P-Values of Cytoplasmic Phospho-Akt Part 2

	T1	T2	T3	T4	Tis	CC	Co
T1	X	0.08085	0.01530	0.23612	0.41274	0.02676	0.10303
T2	0.08085	X	0.31607	0.96062	0.72117	0.00219	0.87139
T3	0.01530	0.31607	X	0.46808	0.91247	0.00090	0.55050
T4	0.23612	0.96062	0.46808	X	0.62208	0.00405	0.85615
Tis	0.41274	0.72117	0.91247	0.62208	X	0.07837	0.80449
CC	0.02676	0.00219	0.00090	0.00405	0.07837	X	0.00278
Co	0.10303	0.87139	0.55050	0.85615	0.80449	0.00278	X

Table 59: P-Values of Cytoplasmic Phospho-Akt Part 3

	N0	N1	CC	Co
N0	X	0.01448	0.00938	0.13807
N1	0.01448	X	0.00096	0.44705
CC	0.00938	0.00096	X	0.00278
Co	0.13807	0.44705	0.00278	X

Table 60: P-Values of Cytoplasmic Phospho-Akt Part 4

	M0	M1	CC	Co
M0	X	0.88368	0.00322	0.71612
M1	0.88368	X	0.00479	0.71987
CC	0.00322	0.00479	X	0.00278
Co	0.71612	0.71987	0.00278	X

Table 61: P-Values of Cytoplasmic Phospho-Akt Part 5

	L0	L1	CC	Co
L0	X	0.71648	0.00351	0.58868
L1	0.71648	X	0.00343	0.87073
CC	0.00351	0.00343	X	0.00278
Co	0.58868	0.87073	0.00278	X

Table 62: P-Values of Cytoplasmic Phospho-Akt Part 6

	V0	V1	CC	Co
V0	X	0.76671	0.00351	0.58868
V1	0.76671	X	0.00669	0.93398
CC	0.00351	0.00669	X	0.00278
Co	0.58868	0.93398	0.00278	X

Table 63: P-Values of Cytoplasmic Phospho-Akt Part 7

6.4.2 P-Values of Kaplan-Meier Survival Analysis

	I=0	I=Low	I=High
I=0	X	0.84310	0.00282
I=Low	0.84310	X	0.00274
I=High	0.00282	0.00274	X

Table 64: P-Values of Cytoplasmic Phospho-Akt Part 8

	I=0, N0	I=Low, N0	I=High, N0	I=0, N1	I=Low, N1
I=0, N0	X	0.87827	0.09594	0.00023	0.02433
I=Low, N0	0.87827	X	0.10157	0.00059	0.03398
I=High, N0	0.09594	0.10157	X	0.00246	0.00675
I=0, N1	0.00023	0.00059	0.00246	X	0.60164
I=Low, N1	0.02433	0.03398	0.00675	0.60164	X

Table 65: P-Values of Cytoplasmic Phospho-Akt Part 9

	I=0, M0	I=Low, M0	I=High, M0	I=0, M1	I=Low, M1
I=0, M0	X	0.13214	0.02669	0.00189	0.00163
I=Low, M0	0.13214	X	0.10324	0.00014	0.00049
I=High, M0	0.02669	0.10324	X	0.00086	0.00203
I=0, M1	0.00189	0.00014	0.00086	X	0.42282
I=Low, M1	0.00163	0.00049	0.00203	0.42282	X

Table 66: P-Values of Cytoplasmic Phospho-Akt Part 10

	I=0, L0	I=Low, L0	I=High, L0	I=0, L1	I=Low, L1	I=High, L1
I=0, L0	X	0.17426	0.08151	0.00084	0.01380	0.39259
I=Low, L0	0.17426	X	0.20161	4.87E-005	0.00133	0.50671
I=High, L0	0.08151	0.20161	X	0.00507	0.01066	1
I=0, L1	0.00084	4.87E-005	0.00507	X	0.97085	0.10723
I=Low, L1	0.01380	0.00133	0.01066	0.97085	X	0.12120
I=High, L1	0.39259	0.50671	1	0.10723	0.12120	X

Table 67: P-Values of Cytoplasmic Phospho-Akt Part 11

	I=0, V0	I=Low, V0	I=High, V0	I=0, V1	I=Low, V1	I=High, V1
I=0, V0	X	0.17426	0.08151	0.00021	0.00062	0.39259
I=Low, V0	0.17426	X	0.20161	1.19E-005	0.00011	0.50671
I=High, V0	0.08151	0.20161	X	0.00157	0.00170	1
I=0, V1	0.00021	1.19E-005	0.00157	X	0.27119	0.06859
I=Low, V1	0.00062	0.00011	0.00170	0.27119	X	0.03947
I=High, V1	0.39259	0.50671	1	0.06859	0.03947	X

Table 68: P-Values of Cytoplasmic Phospho-Akt Part 12

	I=0, G1	I=Low, G1	I=High, G1	I=0, G2	I=Low, G2	I=High, G2	I=0, G3	I=Low, G3	I=High, G3	I=0, G4	I=Low, G4
I=0, G1	X	0.50933	0.52819	0.00518	0.00829	0.70866	4.08E-008	0.00087	0.17190	0.23109	0.12186
I=Low, G1	0.50933	X	0.06493	0.17822	0.24939	0.48066	0.00120	0.04686	0.10815	0.45120	0.31034
I=High, G1	0.52819	0.06493	X	0.08050	0.09183	0.88941	0.00557	0.03696	0.23903	0.04852	0.18549
I=0, G2	0.00518	0.17822	0.08050	X	0.79609	0.29875	4.15E-005	0.15556	0.05180	0.42862	0.72427
I=Low, G2	0.00829	0.24939	0.09183	0.79609	X	0.18361	0.00142	0.29119	0.03577	0.44736	0.82932
I=High, G2	0.70866	0.48066	0.88941	0.29875	0.18361	X	0.07401	0.17669	0.31213	0.30962	0.39797
I=0, G3	4.08E-008	0.00120	0.00557	4.15E-005	0.00142	0.07401	X	0.15874	0.00981	0.95617	0.30073
I=Low, G3	0.00087	0.04686	0.03696	0.15556	0.29119	0.17669	0.15874	X	0.02649	0.68321	0.77285
I=High, G3	0.17190	0.10815	0.23903	0.05180	0.03577	0.31213	0.00981	0.02649	X	0.12072	0.07922
I=0, G4	0.23109	0.45120	0.04852	0.42862	0.44736	0.30962	0.95617	0.68321	0.12072	X	0.53433
I=Low, G4	0.12186	0.31034	0.18549	0.72427	0.82932	0.39797	0.30073	0.77285	0.07922	0.53433	X

Table 69: P-Values of Cytoplasmic Phospho-Akt Part 13

	I=0, T1	I=Low, T1	I=High, T1	I=0, T2	I=Low, T2	I=High, T2	I=0, T3	I=Low, T3	I=High, T3	I=0, T4	I=Low, T4	I=0, Tis	I=Low, Tis	I=High, Tis
I=0, T1	X	0.14010	0.23850	0.31972	0.19325	0.08847	0.00054	0.02115	0.22821	0.00038	0.01262	0.01077	0.37981	0.34478
I=Low, T1	0.14010	X	0.03409	0.50209	0.68017	0.01184	0.18263	0.50156	0.04374	0.01739	0.08180	0.00296	0.29798	0.21619
I=High, T1	0.23850	0.03409	X	0.14848	0.07138	0.36785	0.01086	0.04901	0.36785	0.00698	0.00464	1	1	1
I=0, T2	0.31972	0.50209	0.14848	X	0.67770	0.03916	0.00074	0.09018	0.18848	0.00287	0.11218	0.00183	0.27232	0.22360
I=Low, T2	0.19325	0.68017	0.07138	0.67770	X	0.02466	0.01047	0.16897	0.11506	0.00483	0.09479	0.00158	0.23584	0.21414
I=High, T2	0.08847	0.01184	0.36785	0.03916	0.02466	X	0.00333	0.01175	1	0.00258	0.01079	1	1	1
I=0, T3	0.00054	0.18263	0.01086	0.00074	0.01047	0.00333	X	0.46907	0.06776	0.11434	0.83317	1.47E-005	0.10548	0.08637
I=Low, T3	0.02115	0.50156	0.04901	0.09018	0.16897	0.01175	0.46907	X	0.13551	0.09542	0.68912	0.00023	0.13551	0.13551
I=High, T3	0.22821	0.04374	0.36785	0.18848	0.11506	1	0.06776	0.13551	X	0.06346	0.10397	1	1	1
I=0, T4	0.00038	0.01739	0.00698	0.00287	0.00483	0.00258	0.11434	0.09542	0.06346	X	0.29691	2.83E-005	0.06346	0.06346
I=Low, T4	0.01262	0.08180	0.00464	0.11218	0.09479	0.01079	0.83317	0.68912	0.10397	0.29691	X	0.00065	0.10397	0.10397
I=0, Tis	0.01077	0.00296	1	0.00183	0.00158	1	1.47E-005	0.00023	1	2.83E-005	0.00065	X	1	1
I=Low, Tis	0.37981	0.29798	1	0.27232	0.23584	1	0.10548	0.13551	1	0.06346	0.10397	1	X	1
I=High, Tis	0.34478	0.21619	1	0.22360	0.21414	1	0.08637	0.13551	1	0.06346	0.10397	1	1	X

Table 70: P-Values of Cytoplasmic Phospho-Akt Part 14

6.5 P-Values of Cytoplasmic Phospho-mTOR

6.5.1 P-Values of Mean Intensities

	Tumor	CC	Co
Tumor	X	0.00690	0.20293
CC	0.00690	X	0.00414
Co	0.20293	0.00414	X

Table 71: P-Values of Cytoplasmic Phospho-mTOR Part 1

	G1	G2	G3	G4	CC	Co
G1	X	0.00056	0.00058	0.02997	0.01364	0.00195
G2	0.00056	X	0.93050	0.62285	0.00704	0.57941
G3	0.00058	0.93050	X	0.63135	0.00508	0.65591
G4	0.02997	0.62285	0.63135	X	0.03667	0.83753
CC	0.01364	0.00704	0.00508	0.03667	X	0.00414
Co	0.00195	0.57941	0.65591	0.83753	0.00414	X

Table 72: P-Values of Cytoplasmic Phospho-mTOR Part 2

	T1	T2	T3	T4	Tis	CC	Co
T1	X	0.33680	0.04500	0.06967	0.02311	0.01535	0.03309
T2	0.33680	X	0.17896	0.17429	0.00425	0.00744	0.11247
T3	0.04500	0.17896	X	0.45960	0.00176	0.00554	0.52752
T4	0.06967	0.17429	0.45960	X	0.00764	0.01544	0.83184
Tis	0.02311	0.00425	0.00176	0.00764	X	0.07715	0.00203
CC	0.01535	0.00744	0.00554	0.01544	0.07715	X	0.00414
Co	0.03309	0.11247	0.52752	0.83184	0.00203	0.00414	X

Table 73: P-Values of Cytoplasmic Phospho-mTOR Part 3

	N0	N1	CC	Co
N0	X	0.17699	0.01034	0.00225
N1	0.17699	X	0.00942	0.12265
CC	0.01034	0.00942	X	0.00414
Co	0.00225	0.12265	0.00414	X

Table 74: P-Values of Cytoplasmic Phospho-mTOR Part 4

	M0	M1	CC	Co
M0	X	2.90E-005	0.01383	0.00033
M1	2.90E-005	X	0.00505	0.36408
CC	0.01383	0.00505	X	0.00414
Co	0.00033	0.36408	0.00414	X

Table 75: P-Values of Cytoplasmic Phospho-mTOR Part 5

	L0	L1	CC	Co
L0	X	0.00526	0.01292	0.00056
L1	0.00526	X	0.00746	0.34530
CC	0.01292	0.00746	X	0.00414
Co	0.00056	0.34530	0.00414	X

Table 76: P-Values of Cytoplasmic Phospho-mTOR Part 6

	V0	V1	CC	Co
V0	X	0.71679	0.01292	0.00056
V1	0.71679	X	0.01772	0.01632
CC	0.01292	0.01772	X	0.00414
Co	0.00056	0.01632	0.00414	X

Table 77: P-Values of Cytoplasmic Phospho-mTOR Part 7

6.5.2 P-Values of Kaplan-Meier Survival Analysis

	I=0	I=Low	I=High
I=0	X	0.00790	0.18014
I=Low	0.00790	X	0.63643
I=High	0.18014	0.63643	X

Table 78: P-Values of Cytoplasmic Phospho-mTOR Part 8

	I=0, N0	I=Low, N0	I=High, N0	I=0, N1	I=Low, N1	I=High, N1
I=0, N0	X	0.14726	0.20586	0.30876	0.38754	0.36691
I=Low, N0	0.14726	X	0.94639	0.00015	0.00034	0.01030
I=High, N0	0.20586	0.94639	X	0.00679	0.02008	0.02277
I=0, N1	0.30876	0.00015	0.00679	X	0.65444	0.79134
I=Low, N1	0.38754	0.00034	0.02008	0.65444	X	0.75947
I=High, N1	0.36691	0.01030	0.02277	0.79134	0.75947	X

Table 79: P-Values of Cytoplasmic Phospho-mTOR Part 9

	I=0, M0	I=Low, M0	I=High, M0	I=0, M1	I=Low, M1	I=High, M1
I=0, M0	X	0.55751	0.68002	0.05438	0.00429	0.18598
I=Low, M0	0.55751	X	0.95378	0.00166	0.00029	0.15342
I=High, M0	0.68002	0.95378	X	0.00719	0.00035	0.19108
I=0, M1	0.05438	0.00166	0.00719	X	0.42306	0.92643
I=Low, M1	0.00429	0.00029	0.00035	0.42306	X	0.36153
I=High, M1	0.18598	0.15342	0.19108	0.92643	0.36153	X

Table 80: P-Values of Cytoplasmic Phospho-mTOR Part 10

	I=0, L0	I=Low, L0	I=High, L0	I=0, L1	I=Low, L1	I=High, L1
I=0, L0	X	0.50850	0.96293	0.11826	0.01340	0.55116
I=Low, L0	0.50850	X	0.53114	0.00351	1.98E-005	0.24732
I=High, L0	0.96293	0.53114	X	0.05805	0.00547	0.32494
I=0, L1	0.11826	0.00351	0.05805	X	0.86049	0.49628
I=Low, L1	0.01340	1.98E-005	0.00547	0.86049	X	0.40795
I=High, L1	0.55116	0.24732	0.32494	0.49628	0.40795	X

Table 81: P-Values of Cytoplasmic Phospho-mTOR Part 11

	I=0, V0	I=Low, V0	I=High, V0	I=0, V1	I=Low, V1	I=High, V1
I=0, V0	X	0.50850	0.96293	0.32839	0.00013	0.55116
I=Low, V0	0.50850	X	0.53114	0.09932	1.58E-007	0.24732
I=High, V0	0.96293	0.53114	X	0.17888	2.13E-005	0.32494
I=0, V1	0.32839	0.09932	0.17888	X	0.10211	0.73028
I=Low, V1	0.00013	1.58E-007	2.13E-005	0.10211	X	0.09219
I=High, V1	0.55116	0.24732	0.32494	0.73028	0.09219	X

Table 82: P-Values of Cytoplasmic Phospho-mTOR Part 12

	I=0, G1	I=Low, G1	I=High, G1	I=0, G2	I=Low, G2	I=High, G2	I=0, G3	I=Low, G3	I=High, G3	I=0, G4	I=Low, G4	I=High, G4
I=0, G1	X	0.50418	0.94057	0.43970	0.62095	0.48794	0.07481	0.06289	0.01215	0.05219	0.69829	0.18261
I=Low, G1	0.50418	X	0.52764	0.00053	0.00830	0.01143	1.15E-006	1.09E-006	4.93E-005	0.00664	0.25383	0.19762
I=High, G1	0.94057	0.52764	X	0.07503	0.25119	0.10421	0.00609	0.00427	0.00053	0.05176	0.26753	0.31213
I=0, G2	0.43970	0.00053	0.07503	X	0.29500	0.92451	0.03270	0.02926	0.01432	0.17891	0.93377	0.10401
I=Low, G2	0.62095	0.00830	0.25119	0.29500	X	0.50875	0.00222	0.00228	0.00365	0.07563	0.70667	0.12275
I=High, G2	0.48794	0.01143	0.10421	0.92451	0.50875	X	0.08070	0.09940	0.01878	0.18167	0.81176	0.15040
I=0, G3	0.07481	1.15E-006	0.00609	0.03270	0.00222	0.08070	X	0.83046	0.26107	0.57994	0.50931	0.07218
I=Low, G3	0.06289	1.09E-006	0.00427	0.02926	0.00228	0.09940	0.83046	X	0.18218	0.48897	0.56056	0.06258
I=High, G3	0.01215	4.93E-005	0.00053	0.01432	0.00365	0.01878	0.26107	0.18218	X	0.97682	0.25501	0.07279
I=0, G4	0.05219	0.00664	0.05176	0.17891	0.07563	0.18167	0.57994	0.48897	0.97682	X	0.53433	0.13826
I=Low, G4	0.69829	0.25383	0.26753	0.93377	0.70667	0.81176	0.50931	0.56056	0.25501	0.53433	X	0.23903
I=High, G4	0.18261	0.19762	0.31213	0.10401	0.12275	0.15040	0.07218	0.06258	0.07279	0.13826	0.23903	X

Table 83: P-Values of Cytoplasmic Phospho-mTOR Part 13

	I=0, T1	I=Low, T1	I=High, T1	I=0, T2	I=Low, T2	I=High, T2	I=0, T3	I=Low, T3	I=High, T3	I=0, T4	I=Low, T4	I=High, T4	I=Low, Tis	I=High, Tis
I=0, T1	X	0.02316	0.91773	0.51654	0.40338	0.04447	0.47069	0.39683	0.00960	0.00465	0.38682	0.05965	0.00188	0.08173
I=Low, T1	0.02316	X	0.18105	0.03158	0.04504	0.71497	0.00044	0.00025	2.77E-005	3.68E-005	0.04230	0.02878	0.05798	0.29121
I=High, T1	0.91773	0.18105	X	0.81322	0.91182	0.27802	0.23387	0.34303	0.08111	0.03235	0.44791	0.26168	0.02311	0.13190
I=0, T2	0.51654	0.03158	0.81322	X	0.49423	0.03170	0.17622	0.15398	0.02072	0.01007	0.54997	0.43512	0.00194	0.07696
I=Low, T2	0.40338	0.04504	0.91182	0.49423	X	0.05912	0.00920	0.01447	0.00193	0.00056	0.27277	0.23133	0.00299	0.10074
I=High, T2	0.04447	0.71497	0.27802	0.03170	0.05912	X	0.00103	0.00195	0.00023	7.21E-005	0.06016	0.04195	0.10542	0.31561
I=0, T3	0.47069	0.00044	0.23387	0.17622	0.00920	0.00103	X	0.68466	0.19831	0.09210	0.96154	0.93747	0.00014	0.02455
I=Low, T3	0.39683	0.00025	0.34303	0.15398	0.01447	0.00195	0.68466	X	0.09452	0.02424	0.78883	0.79584	0.00020	0.02801
I=High, T3	0.00960	2.77E-005	0.08111	0.02072	0.00193	0.00023	0.19831	0.09452	X	0.62694	0.36200	0.61811	2.02E-005	0.00696
I=0, T4	0.00465	3.68E-005	0.03235	0.01007	0.00056	7.21E-005	0.09210	0.02424	0.62694	X	0.39199	0.16780	3.38E-005	0.00667
I=Low, T4	0.38682	0.04230	0.44791	0.54997	0.27277	0.06016	0.96154	0.78883	0.36200	0.39199	X	0.90859	0.00818	0.07922
I=High, T4	0.05965	0.02878	0.26168	0.43512	0.23133	0.04195	0.93747	0.79584	0.61811	0.16780	0.90859	X	0.00356	0.03606
I=Low, Tis	0.00188	0.05798	0.02311	0.00194	0.00299	0.10542	0.00014	0.00020	2.02E-005	3.38E-005	0.00818	0.00356	X	1
I=High, Tis	0.08173	0.29121	0.13190	0.07696	0.10074	0.31561	0.02455	0.02801	0.00696	0.00667	0.07922	0.03606	1	X

Table 84: P-Values of Cytoplasmic Phospho-mTOR Part 14

6.6 P-Values of Nuclear Phospho-p70S6K

6.6.1 P-Values of Mean Intensities

	Tumor	CC	Co
Tumor	X	0.11534	0.00466
CC	0.11534	X	0.38966
Co	0.00466	0.38966	X

Table 85: P-Values of Nuclear Phospho-p70S6K Part 1

	G1	G2	G3	G4	CC	Co
G1	X	0.00112	5.88E-005	0.00079	0.29462	0.67959
G2	0.00112	X	0.25558	0.07758	0.10536	0.00369
G3	5.88E-005	0.25558	X	0.18336	0.07856	0.00065
G4	0.00079	0.07758	0.18336	X	0.05247	0.00210
CC	0.29462	0.10536	0.07856	0.05247	X	0.38966
Co	0.67959	0.00369	0.00065	0.00210	0.38966	X

Table 86: P-Values of Nuclear Phospho-p70S6K Part 2

	T1	T2	T3	T4	Tis	CC	Co
T1	X	0.65995	0.18240	0.26330	0.00835	0.15989	0.05439
T2	0.65995	X	0.01032	0.10561	0.00982	0.17393	0.05509
T3	0.18240	0.01032	X	0.86575	0.00075	0.07382	0.00054
T4	0.26330	0.10561	0.86575	X	0.00155	0.08144	0.00545
Tis	0.00835	0.00982	0.00075	0.00155	X	1	0.09546
CC	0.15989	0.17393	0.07382	0.08144	1	X	0.38966
Co	0.05439	0.05509	0.00054	0.00545	0.09546	0.38966	X

Table 87: P-Values of Nuclear Phospho-p70S6K Part 3

	N0	N1	CC	Co
N0	X	0.02074	0.19885	0.13728
N1	0.02074	X	0.08713	0.00162
CC	0.19885	0.08713	X	0.38966
Co	0.13728	0.00162	0.38966	X

Table 88: P-Values of Nuclear Phospho-p70S6K Part 4

	M0	M1	CC	Co
M0	X	0.00078	0.28761	0.56037
M1	0.00078	X	0.08708	0.00314
CC	0.28761	0.08708	X	0.38966
Co	0.56037	0.00314	0.38966	X

Table 89: P-Values of Nuclear Phospho-p70S6K Part 5

	L0	L1	CC	Co
L0	X	0.00044	0.27876	0.50004
L1	0.00044	X	0.08676	0.00160
CC	0.27876	0.08676	X	0.38966
Co	0.50004	0.00160	0.38966	X

Table 90: P-Values of Nuclear Phospho-p70S6K Part 6

	V0	V1	CC	Co
V0	X	0.12410	0.27876	0.50004
V1	0.12410	X	0.18572	0.08943
CC	0.27876	0.18572	X	0.38966
Co	0.50004	0.08943	0.38966	X

Table 91: P-Values of Nuclear Phospho-p70S6K Part 7

6.6.2 P-Values of Kaplan-Meier Survival Analysis

	I=0	I=Low	I=High
I=0	X	0.43214	0.06668
I=Low	0.43214	X	0.00131
I=High	0.06668	0.00131	X

Table 92: P-Values of Nuclear Phospho-p70S6K Part 8

	I=0, N0	I=Low, N0	I=High, N0	I=0, N1	I=Low, N1	I=High, N1
I=0, N0	X	0.26940	0.76376	0.11890	0.08383	0.01681
I=Low, N0	0.26940	X	0.06404	0.32372	0.34001	0.13822
I=High, N0	0.76376	0.06404	X	0.00335	0.00065	1.14E-005
I=0, N1	0.11890	0.32372	0.00335	X	0.96135	0.92232
I=Low, N1	0.08383	0.34001	0.00065	0.96135	X	0.80623
I=High, N1	0.01681	0.13822	1.14E-005	0.92232	0.80623	X

Table 93: P-Values of Nuclear Phospho-p70S6K Part 9

	I=0, M0	I=Low, M0	I=High, M0	I=0, M1	I=Low, M1	I=High, M1
I=0, M0	X	0.19583	0.43943	0.01173	0.01766	0.02405
I=Low, M0	0.19583	X	0.19636	0.04096	0.09155	0.19307
I=High, M0	0.43943	0.19636	X	0.00145	0.00021	0.00110
I=0, M1	0.01173	0.04096	0.00145	X	0.55407	0.85996
I=Low, M1	0.01766	0.09155	0.00021	0.55407	X	0.60321
I=High, M1	0.02405	0.19307	0.00110	0.85996	0.60321	X

Table 94: P-Values of Nuclear Phospho-p70S6K Part 10

	I=0, L0	I=Low, L0	I=High, L0	I=0, L1	I=Low, L1	I=High, L1
I=0, L0	X	0.09918	0.25077	0.08261	0.00253	0.01124
I=Low, L0	0.09918	X	0.14030	0.94274	0.04997	0.43213
I=High, L0	0.25077	0.14030	X	0.13525	1.07E-005	0.00219
I=0, L1	0.08261	0.94274	0.13525	X	0.06578	0.59407
I=Low, L1	0.00253	0.04997	1.07E-005	0.06578	X	0.03797
I=High, L1	0.01124	0.43213	0.00219	0.59407	0.03797	X

Table 95: P-Values of Nuclear Phospho-p70S6K Part 11

	I=0, V0	I=Low, V0	I=High, V0	I=0, V1	I=Low, V1	I=High, V1
I=0, V0	X	0.09918	0.25077	0.16861	0.00293	0.00214
I=Low, V0	0.09918	X	0.14030	0.97399	0.00631	0.04581
I=High, V0	0.25077	0.14030	X	0.32427	3.02E-005	4.49E-005
I=0, V1	0.16861	0.97399	0.32427	X	0.19214	0.56005
I=Low, V1	0.00293	0.00631	3.02E-005	0.19214	X	0.03959
I=High, V1	0.00214	0.04581	4.49E-005	0.56005	0.03959	X

Table 96: P-Values of Nuclear Phospho-p70S6K Part 12

	I=0, G1	I=Low, G1	I=High, G1	I=0, G2	I=Low, G2	I=High, G2	I=0, G3	I=Low, G3	I=High, G3	I=0, G4	I=Low, G4	I=High, G4
I=0, G1	X	0.15752	0.17663	0.03750	0.03825	0.05069	0.02559	0.00331	0.02128	0.12072	0.17582	0.05836
I=Low, G1	0.15752	X	0.70737	0.20123	0.17310	0.35089	0.10235	0.00035	0.05300	0.21743	0.96180	0.04101
I=High, G1	0.17663	0.70737	X	0.00725	0.00291	0.00485	0.00093	7.86E-010	1.63E-005	0.07474	0.78740	0.01861
I=0, G2	0.03750	0.20123	0.00725	X	0.89478	0.35882	0.52727	0.00293	0.30748	0.42386	0.37453	0.26325
I=Low, G2	0.03825	0.17310	0.00291	0.89478	X	0.34451	0.44692	0.00099	0.20288	0.50267	0.50689	0.21738
I=High, G2	0.05069	0.35089	0.00485	0.35882	0.34451	X	0.09189	2.23E-006	0.01158	0.37544	0.78713	0.14381
I=0, G3	0.02559	0.10235	0.00093	0.52727	0.44692	0.09189	X	0.05290	0.82964	0.64748	0.41991	0.51373
I=Low, G3	0.00331	0.00035	7.86E-010	0.00293	0.00099	2.23E-006	0.05290	X	0.07717	0.79220	0.06133	0.90428
I=High, G3	0.02128	0.05300	1.63E-005	0.30748	0.20288	0.01158	0.82964	0.07717	X	0.64803	0.30144	0.52327
I=0, G4	0.12072	0.21743	0.07474	0.42386	0.50267	0.37544	0.64748	0.79220	0.64803	X	0.40211	0.94583
I=Low, G4	0.17582	0.96180	0.78740	0.37453	0.50689	0.78713	0.41991	0.06133	0.30144	0.40211	X	0.47798
I=High, G4	0.05836	0.04101	0.01861	0.26325	0.21738	0.14381	0.51373	0.90428	0.52327	0.94583	0.47798	X

Table 97: P-Values of Nuclear Phospho-p70S6K Part 13

	I=0, T1	I=Low, T1	I=High, T1	I=0, T2	I=Low, T2	I=High, T2	I=0, T3	I=Low, T3	I=High, T3	I=0, T4	I=Low, T4	I=High, T4	I=High, Tis
I=0, T1	X	0.71906	0.18357	0.84667	0.77145	0.44047	0.48636	0.01584	0.34392	0.01225	0.11807	0.02569	0.00065
I=Low, T1	0.71906	X	0.54288	0.87268	0.34838	0.97622	0.25548	0.00264	0.13336	0.00181	0.05962	0.00447	0.00603
I=High, T1	0.18357	0.54288	X	0.23436	0.04113	0.31652	0.02527	1.28E-005	0.00249	0.03095	0.01862	0.00185	0.01009
I=0, T2	0.84667	0.87268	0.23436	X	0.72362	0.41250	0.70781	0.05122	0.45356	0.43425	0.39849	0.19310	0.00138
I=Low, T2	0.77145	0.34838	0.04113	0.72362	X	0.14423	0.50469	0.00203	0.30344	0.06259	0.16080	0.03302	3.23E-005
I=High, T2	0.44047	0.97622	0.31652	0.41250	0.14423	X	0.04339	1.63E-006	0.00276	0.04466	0.02829	0.00716	0.00036
I=0, T3	0.48636	0.25548	0.02527	0.70781	0.50469	0.04339	X	0.03486	0.85347	0.32216	0.42209	0.21563	1.47E-005
I=Low, T3	0.01584	0.00264	1.28E-005	0.05122	0.00203	1.63E-006	0.03486	X	0.00914	0.82222	0.48251	0.95148	1.53E-008
I=High, T3	0.34392	0.13336	0.00249	0.45356	0.30344	0.00276	0.85347	0.00914	X	0.21236	0.41699	0.13069	1.61E-006
I=0, T4	0.01225	0.00181	0.03095	0.43425	0.06259	0.04466	0.32216	0.82222	0.21236	X	0.62539	0.84852	0.00107
I=Low, T4	0.11807	0.05962	0.01862	0.39849	0.16080	0.02829	0.42209	0.48251	0.41699	0.62539	X	0.57269	4.41E-005
I=High, T4	0.02569	0.00447	0.00185	0.19310	0.03302	0.00716	0.21563	0.95148	0.13069	0.84852	0.57269	X	1.24E-005
I=High, Tis	0.00065	0.00603	0.01009	0.00138	3.23E-005	0.00036	1.47E-005	1.53E-008	1.61E-006	0.00107	4.41E-005	1.24E-005	X

Table 98: P-Values of Nuclear Phospho-p70S6K Part 14

6.7 P-Values of Cytoplasmic Phospho-p70S6K

6.7.1 P-Values of Mean Intensities

	Tumor	CC	Co
Tumor	X	1.90E-006	0.33060
CC	1.90E-006	X	5.90E-006
Co	0.33060	5.90E-006	X

Table 99: P-Values of Cytoplasmic Phospho-p70S6K Part 1

	G1	G2	G3	G4	CC	Co
G1	X	0.23868	0.14596	0.19405	5.81E-007	0.96067
G2	0.23868	X	0.65871	0.54126	5.68E-006	0.34011
G3	0.14596	0.65871	X	0.67609	1.69E-005	0.23045
G4	0.19405	0.54126	0.67609	X	0.00723	0.24437
CC	5.81E-007	5.68E-006	1.69E-005	0.00723	X	5.90E-006
Co	0.96067	0.34011	0.23045	0.24437	5.90E-006	X

Table 100: P-Values of Cytoplasmic Phospho-p70S6K Part 2

	T1	T2	T3	T4	Tis	CC	Co
T1	X	0.84123	0.36631	0.86340	0.78802	1.48E-005	0.61871
T2	0.84123	X	0.30634	0.75444	0.71308	2.75E-006	0.45744
T3	0.36631	0.30634	X	0.40805	0.43221	3.64E-005	0.16726
T4	0.86340	0.75444	0.40805	X	1	0.00029	0.82158
Tis	0.78802	0.71308	0.43221	1	X	0.00177	0.92274
CC	1.48E-005	2.75E-006	3.64E-005	0.00029	0.00177	X	5.90E-006
Co	0.61871	0.45744	0.16726	0.82158	0.92274	5.90E-006	X

Table 101: P-Values of Cytoplasmic Phospho-p70S6K Part 3

	N0	N1	CC	Co
N0	X	0.65965	4.76E-005	0.29575
N1	0.65965	X	1.79E-005	0.48012
CC	4.76E-005	1.79E-005	X	5.90E-006
Co	0.29575	0.48012	5.90E-006	X

Table 102: P-Values of Cytoplasmic Phospho-p70S6K Part 4

	M0	M1	CC	Co
M0	X	0.90596	2.46E-006	0.65376
M1	0.90596	X	3.21E-005	0.79408
CC	2.46E-006	3.21E-005	X	5.90E-006
Co	0.65376	0.79408	5.90E-006	X

Table 103: P-Values of Cytoplasmic Phospho-p70S6K Part 5

	L0	L1	CC	Co
L0	X	0.13362	8.55E-007	0.80073
L1	0.13362	X	0.00028	0.15023
CC	8.55E-007	0.00028	X	5.90E-006
Co	0.80073	0.15023	5.90E-006	X

Table 104: P-Values of Cytoplasmic Phospho-p70S6K Part 6

	V0	V1	CC	Co
V0	X	0.85298	8.55E-007	0.80073
V1	0.85298	X	0.00018	0.71305
CC	8.55E-007	0.00018	X	5.90E-006
Co	0.80073	0.71305	5.90E-006	X

Table 105: P-Values of Cytoplasmic Phospho-p70S6K Part 7

6.7.2 P-Values of Kaplan-Meier Survival Analysis

	I=0	I=Low
I=0	X	0.56245
I=Low	0.56245	X

Table 106: P-Values of Cytoplasmic Phospho-p70S6K Part 8

	I=0, N0	I=Low, N0	I=0, N1	I=Low, N1
I=0, N0	X	0.66918	2.08E-006	0.55515
I=Low, N0	0.66918	X	0.01745	0.65115
I=0, N1	2.08E-006	0.01745	X	0.17969
I=Low, N1	0.55515	0.65115	0.17969	X

Table 107: P-Values of Cytoplasmic Phospho-p70S6K Part 9

	I=0, M0	I=Low, M0	I=0, M1	I=Low, M1
I=0, M0	X	0.91895	1.83E-006	0.10555
I=Low, M0	0.91895	X	0.00798	0.26167
I=0, M1	1.83E-006	0.00798	X	0.86392
I=Low, M1	0.10555	0.26167	0.86392	X

Table 108: P-Values of Cytoplasmic Phospho-p70S6K Part 10

	I=0, L0	I=Low, L0	I=0, L1	I=Low, L1
I=0, L0	X	0.65796	0.00014	0.05958
I=Low, L0	0.65796	X	0.03273	0.14136
I=0, L1	0.00014	0.03273	X	0.99798
I=Low, L1	0.05958	0.14136	0.99798	X

Table 109: P-Values of Cytoplasmic Phospho-p70S6K Part 11

	I=0, V0	I=Low, V0	I=0, V1	I=Low, V1
I=0, V0	X	0.65796	1.51E-005	0.01255
I=Low, V0	0.65796	X	0.00799	0.02826
I=0, V1	1.51E-005	0.00799	X	0.43568
I=Low, V1	0.01255	0.02826	0.43568	X

Table 110: P-Values of Cytoplasmic Phospho-p70S6K Part 12

	I=0, G1	I=Low, G1	I=0, G2	I=Low, G2	I=0, G3	I=Low, G3	I=0, G4	I=Low, G4
I=0, G1	X	0.15086	0.00125	0.02844	1.41E-008	0.00201	0.16337	0.00483
I=Low, G1	0.15086	X	0.01717	0.02174	0.00280	0.00455	0.03994	0.09589
I=0, G2	0.00125	0.01717	X	0.72290	0.00015	0.12372	0.79969	0.01939
I=Low, G2	0.02844	0.02174	0.72290	X	0.11747	0.32960	0.94280	0.07983
I=0, G3	1.41E-008	0.00280	0.00015	0.11747	X	0.92457	0.36626	0.04094
I=Low, G3	0.00201	0.00455	0.12372	0.32960	0.92457	X	0.56715	0.10790
I=0, G4	0.16337	0.03994	0.79969	0.94280	0.36626	0.56715	X	0.35354
I=Low, G4	0.00483	0.09589	0.01939	0.07983	0.04094	0.10790	0.35354	X

Table 111: P-Values of Cytoplasmic Phospho-p70S6K Part 13

	I=0, T1	I=Low, T1	I=0, T2	I=Low, T2	I=0, T3	I=Low, T3	I=0, T4	I=Low, T4	I=0, Tis
I=0, T1	X	0.32898	0.53237	0.34450	0.00056	0.00596	0.00023	0.07808	0.00142
I=Low, T1	0.32898	X	0.17734	0.14964	0.02246	0.01563	0.00943	0.01995	0.12072
I=0, T2	0.53237	0.17734	X	0.47450	0.00016	0.02027	0.00135	0.17611	0.00015
I=Low, T2	0.34450	0.14964	0.47450	X	0.35069	0.22544	0.13690	0.29998	0.00042
I=0, T3	0.00056	0.02246	0.00016	0.35069	X	0.71646	0.20203	0.69282	2.38E-007
I=Low, T3	0.00596	0.01563	0.02027	0.22544	0.71646	X	0.55131	0.70750	5.94E-006
I=0, T4	0.00023	0.00943	0.00135	0.13690	0.20203	0.55131	X	0.94492	9.80E-007
I=Low, T4	0.07808	0.01995	0.17611	0.29998	0.69282	0.70750	0.94492	X	0.00348
I=0, Tis	0.00142	0.12072	0.00015	0.00042	2.38E-007	5.94E-006	9.80E-007	0.00348	X

Table 112: P-Values of Cytoplasmic Phospho-p70S6K Part 14