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CLINICAL RESEARCH

Expression of *c-kit* receptor in human cholangiocarcinoma and *in vivo* treatment with imatinib mesilate in chimeric mice

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Abstract

AIM: To investigate the *c-kit* expression in biliary tract cancer cell lines and histological sections from patients with extrahepatic cholangiocarcinoma (CC) and to evaluate the efficacy of *in vitro* and *in vitro* treatment with imatinib mesilate.

METHODS: The protein expression of *c-kit* in the human biliary tract cancer cell lines Mz-ChA-2 and EGI-1 and histological sections from 19 patients with extrahepatic CC was assessed by immunoblotting, immunocytochemistry, and immunohistochemistry. The anti-proliferative effect of imatinib mesilate on biliary tract cancer cell lines Mz-ChA-2 and EGI-1 was studied *in vitro* by automated cell counting. In addition, immunodeficient NMRI mice (Taconic™) were subcutaneously injected with 5 x 10^6 cells of cell lines MzChA-2 and EGI-1. After having reached a tumour volume of 200 mm^3, daily treatment was started intraperitoneally with imatinib mesilate at a dose of 50 mg/kg or normal saline (NS). Tumor volume was calculated with a Vernier caliper. After 14 d, mice were sacrificed with tumors excised and tumor mass determined.

RESULTS: Immunoblotting revealed presence of *c-kit* in Mz-ChA-2 and absence in EGI-1 cells. Immunocytochemistry with *c-kit* antibodies displayed a cytoplasmatic and membraneous localization of receptor protein in Mz-ChA-2 cells and absence of *c-kit* in EGI-1 cells. *c-kit* was expressed in 7 of 19 (37%) extrahepatic human CC tissue samples, 2 showed a moderate and 5 a rather weak immunostaining. Imatinib mesilate at a low concentration of 5 μmol/L caused a significant growth inhibition in the *c-kit* positive cell line Mz-ChA-2 (31%), but not in the *c-kit* negative cell line EGI-1 (0%) ($P < 0.05$). Imatinib mesilate at an intermediate concentration of 10 μmol/L inhibited cellular growth of both cell lines (51% *vs* 57%). Imatinib mesilate at a higher concentration of 20 μmol/L seemed to have a general toxic effect on both cell lines. The IC_{50} values were 9.7 μmol/L and 11 μmol/L, respectively. After 14 d of *in vitro* treatment with imatinib mesilate, using the chimeric mouse model, *c-kit* positive Mz-ChA-2 tumors had a significantly reduced volume and mass as compared to NS treatment ($P < 0.05$). In contrast to that, treatment of mice bearing *c-kit* negative EGI-1 tumors did not result in any change of tumor volume and mass as compared to NS treatment.

CONCLUSION: *c-kit* expression is detectable at a moderate to low protein level in biliary tract cancer. Imatinib mesilate exerts marked effects on tumor growth *in vitro* and *in vitro* dependent on the level of *c-kit* expression.

Key words: Cholangiocarcinoma; Imatinib; Tyrosine kinase inhibitor; c-kit; Chimeric mice

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INTRODUCTION

Protein kinases are functionally involved in a broad variety of human cancer[1,2]. In tumor cells, it is common that key tyrosine kinases are no longer adequately controlled, and excessive phosphorylation sustains signal transduction pathways in an activated state. Receptor tyrosine kinases (RTKs) are membrane bound proteins, consisting of a ligand-binding domain at the extracellular surface, a single transmembrane segment, and a cytoplasmic part harboring the protein tyrosine kinase activity. Ligand-induced dimerization, resulting in autophosphorylation of their cytoplasmic domains, is the major mode of activation of RTKs[3]. Many oncogenic mutations in RTKs involve either point mutations leading to constitutive dimerization of the receptors or translocations causing fusion of oligomeriza-

tion motifs to RTKs. Overexpression, probably caused by mutations in gene regulatory sequences or gene amplification, may lead to constitutive dimerization of RTKs. Moreover, mislocalization of RTKs due to fusion to other proteins may contribute to their oncogenic phenotype. Phosphorylated tyrosine residues in the receptor tails then function as recruitment sites for downstream signalling proteins containing phosphotyrosine-recognition domains, such as the SRC homology 2 (SH2) domain or the phosphotyrosine-binding (PTB) domain. These molecules act as relay points for a complex network of independent signalling molecules that ultimately affect gene transcription within the nucleus. *c-kit* (CD-117) is a transmembrane tyrosine kinase receptor in which the extracellular protein binds a ligand known as stem-cell factor (also known as Steel factor) and the intracellular portion contains the actual kinase enzymatic domain[4-6]. *c-kit* is similar in structure to several other RTKs with oncogenic capabilities, including platelet-derived growth factor receptor (PDGF-R) A and B, colony stimulating factor 1 receptor (CSF1-R), and fms-related tyrosine kinase 3 receptor (FLT3-R). *c-kit* is expressed at high levels in hematopoietic stem cells, mast cells, melanocytic cells, germ cells, and the interstitial cells of Cajal (ICC)[7-10]. The receptor forms homodimers upon ligand binding leading to receptor activation. This triggers activation of critical downstream signalling pathways, such as Ras/Raf/mitogen-activated protein kinase kinase (MAPKK)/mitogen-activated protein kinase (MAPK) (cell proliferation) and phosphatidylinositol 3-kinase (PI3K)/AKT (cell survival)[11,12]. There are receptor activating oncogenic *KIT* mutations which involve the extracellular (exon 9), juxtamembrane (exon 11) and kinase domains (exons 13 and 17). As a consequence, in hematologic neoplasms, the exact signalling pathways activated by the mutant *KIT* differ from those activated by normal *KIT*[13,14].

The clinical development of targeted tyrosine kinase inhibitors represents a breakthrough for cancer treatment. They are designed to take advantage of the molecular differences specific to tumor cells compared with normal tissues. The goal is to achieve tumor responses with better safety profiles than those associated with cytotoxic chemotherapies that exhibit narrow therapeutic indices and little selectivity for cancer cells over normal proliferating cells. Imatinib mesilate (STI-571, Gleevec™, Novartis), a 2-phenylaminopyrimidine, was primarily designed to treat chronic myeloid leukemia (CML) by inhibiting bcr-abl fusion protein, created by the t(9; 22) chromosomal translocation, which generates the distinctive Philadelphia (Ph) chromosome[15]. However, imatinib mesilate is not a specific bcr-abl inhibitor, its action extends to c-abl receptor, PDGF-R, and *c-kit*-R[15]. Moreover, it promotes NK cell activation[16] and downregulates telomerase activity[17]. Therefore, imatinib mesilate has been evaluated for the therapy of gastrointestinal stromal tumors (GISTs), because most of them characteristically show *c-kit* overexpression. For metastatic GISTs, imatinib mesilate is nowadays the therapeutical standard, based on a phase II study, including 147 patients with unresectable GISTs, which reported a partial response rate of 54 % and a rate of stable disease of 28 %[18]. A closer inspection of the data clearly displayed that *KIT* mutational status correlated with response to imatinib mesilate. Thus patients with activating *KIT* mutations in exon 11 had a partial response of close to 80%. In contrast, patients whose tumors expressed wild-type *KIT*, with no mutations, had a response rate of only 18%[19]. However, there are only a few published phase I and II studies investigating the effect of imatinib mesilate on gastrointestinal tumors, others than GISTs. Whereas three studies detected a modest level of biological effect of imatinib in hepatocellular carcinoma (HCC) and carcinoid tumor, there was no effect for the treatment of pancreatic carcinoma in two other studies[20-24]. In this study we investigated *c-kit* protein expression in biliary tract cancer cell lines and histological sections from 19 patients with extrahepatic cholangiocarcinoma (CC) and evaluated the efficacy of *in vitro* and *in vivo* treatment with imatinib mesilate.

MATERIALS AND METHODS

Materials

Human gallbladder cancer cell line Mz-ChA-2[25], human extrahepatic cholangiocarcinoma cell line EGI-1[26], and colorectal carcinoma cell line HT-29[27] were cultured as mono-layers in Dulbecco`s modified Eagle`s medium (DMEM, Invitrogen GmbH Karlsruhe, Germany) supplemented with 100 mL/L fetal bovine serum (FBS, Invitrogen GmbH Karlsruhe, Germany), 100 ku penicillin and 100 g/L streptomycin in humidified atmosphere of 900 mL/L air and 100 mL/L CO_2 at 37 ℃. Small cell lung cancer (SCLC) cell line NCI-H69[28] was kept in RPMI 1640 medium (Invitrogen GmbH Karlsruhe, Germany) containing 100 mL/L FBS and antibiotics in humidified atmosphere of 950mL/L air and 50mL/L CO_2 at 37 ℃. The 2-phenylaminopyrimidine derivative STI-571 (imatinib mesilate, Gleevec™) was provided by Novartis Pharma AG (Basel, Switzerland). Stock solution (10 g/L) was prepared in normal saline (NS) and stored at -20 ℃. Hoechst dye was purchased from Sigma (Sigma-Aldrich Chemie GmbH Munich, Germany), rabbit polyclonal c-kit antibody (CD117 Ab-6) from Neomarkers (Dunn Labortechnik GmbH, Asbach, Germany), and Cy™3-conjugated donkey anti-rabbit antibody from Jackson ImmunoResearch (Jackson ImmunoResearch Europe Ltd., Cambridgeshire, United Kingdom). Six to eight-week-old female athymic NMRI nude mice were supplied from Taconic (Taconic Europe, Ry, Denmark) and held under pathogen-free conditions. Human care was administered, and study protocols complied with the institutional guidelines.

Immunoblotting

Cell culture mono-layers were washed twice with ice-cold PBS and lysed with buffer containing Tris-HCl (20 mmol/L, pH 7.5), NP-40 (10 g/L), Triton-X (5 g/L), NaCl (250 mmol/L), EDTA (1 mmol/L), 100 ml/L glycerol, and one tablet of complete mini-EDTA-free protease inhibitor cocktail (Boehringer, Mannheim, Germany) (in 10 mL buffer). Protein concentration was determined by the Bradford protein assay (Bio-Rad, Munich, Germany). 30 μg of cell lysates were separated on SDS-polyacrylamide gels and electroblotted onto poly-

vinylidene difluoride membranes (Amersham Pharmacia Biotech, Freiburg, Germany). Membranes were then incubated in blocking solution (50 g/L BSA in 10 mmol/L Tris-HCl, 140 mmol/L NaCl, 1 g/L Tween-20) (TBS-T), followed by incubation with the primary antibody at 4 ℃ overnight. The membranes were then washed in TBS-T and incubated with HRPO-conjugated secondary antibodies for 1 h at room temperature. Antibody detection was performed with an enhanced chemoluminescence reaction.

Immunocytochemistry

For immunocytochemistry 1 x 10^5 tumor cells were seeded on coverslips of 12-well plates and incubated for one day. Then, medium was removed, cells were washed with PBS and fixed with 8 g/L formaldehyde in PBS for 2 h, washed again, and then permeabilized in 3 g/L Triton-X and 10 g/L DMSO in PBS for another 10 min. After nonspecific antibody binding was blocked by incubation with 50 ml/L donkey serum in 3 g/L Triton-X and 10 g/L DMSO in PBS at room temperature for 2 h, the primary antibody was added, followed by an incubation at 4 ℃ overnight. The omission of primary antibody served as negative control. The next day, cells were washed again and probed with Cy^{Tm}3-conjugated donkey anti-rabbit antibody at room temperature for 2 h. Then, extensive washing and nuclear staining was performed using Hoechst dye according to manufacturers instructions. Finally, coverslips were mounted on slides with 900 ml/L glycerol and slides were analyzed with a Zeiss LSM 510 laser scanning microscope (Carl Zeiss Jena GmbH, Jena, Germany).

Immunohistochemical staining

Paraffin-embedded tissue sections from resection specimens of 19 patients with extrahepatic CC were de-waxed, hydrated through graded alcohol, and immunostained with antibodies to c-kit using the avidin-biotin horseradish peroxidase method as previously described[29]. Immunoreactivity was revealed using True Blue^{TM} peroxidase substrate (KPL, Gaithersburg, USA). The sections were counterstained with hematoxilin and mounted with Permount (Fisher, Pittsburg, USA). For the tumor tissues, the percentage of positive cells was estimated and the staining intensity was semiquantitatively recorded as 1+, 2+, or 3+. For statistical analyses, the staining results were categorized into four groups according to Went *et al*[30]. Tumors without any staining were considered negative. Tumors with 1+ staining intensity in less than 60% of cells and 2+ intensity in less than 30% of cells were considered weakly positive. Tumors with 1+ staining intensity in ⩾ 60% of cells, 2+ intensity in 30% to 79%, or 3+ intensity in less than 30% were considered moderately positive. Tumors with 2+ intensity in ⩾ 80% or 3+ intensity in ⩾ 30% of cells were considered strongly positive. Only membranous or membranous plus cytoplasmic staining was considered for analysis because cytoplasmic staining alone proved to be false-positive in all preabsorption control experiments.

Inhibition of cell growth

The effect of imatinib treatment on cellular proliferation was assessed by automated cell counting (Schaerfe Casy 2.0 Cell Counter, Reutlingen, Germany) according to the manufacturers instructions. Briefly, 2 × 10^5 cells were seeded in T-25 cell culture flasks. Twenty-four hours after incubation, cells were treated with imatinib mesilate at 5 different concentrations (0, 1, 5, 10, and 20 μmol/L, respectively). After 6 d of incubation, cells were trypsinized, washed, and analyzed in triplicates by automated cell counting.

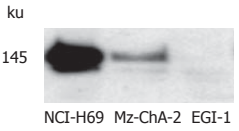

Figure 1 c-kit protein expression in biliary tract cancer cell lines.

Animal studies

Tumors were induced by injecting 5×10^6 Mz-ChA-2 or EGI-1 cells in 200 μL PBS sc into the flank region of NMRI nude mice (n = 40). Treatment was started when an average tumor volume of 200 mm³ was reached (usually after 2 wk). The verum group (n = 2×10) received imatinib mesilate, dissolved in normal saline (NS), ip once a day at 50 mg/kg, whereas the control group (n = 2×10) received NS only. Treatment was continued for 14 consecutive days, tumors were daily measured with a Vernier caliper and tumor volumes were calculated using the formula tumor volume = 0.5 × L × W^2, where L represents the length and W the width of the tumor. When treatment was finished, animals were sacrificed with tumors excised and weighed.

Statistical analysis

Statistical calculations were performed using SPSS, version 10.0 (SPSS Inc., Chicago, USA). Numeric data were presented as mean value with standard deviation (SD). Inter-group comparisons were performed with the Student t test. P< 0.05 was considered significant.

RESULTS

c-kit protein expression in biliary tract cancer cell lines

The expression of *c-kit* in both human biliary tract cancer cell lines Mz-ChA-2 and EGI-1 was assessed by immunoblotting. Cell lysate of SCLC cell line NCI-H69, which has been reported previously to express *c-kit*[28], served as positive control and showed an intense band at about 145 kDa, corresponding to *c-kit* receptor (Figure 1). In accordance with our previous study of *c-kit* m-RNA expression[31], immunoblotting of Mz-ChA-2 cell lysate demonstrated *c-kit* protein expression in this cell line as well. In contrast, *c-kit* expression was not detected in the m-RNA negative cell line EGI-1 (Figure 1). To further analyze *c-kit* expression in these cell lines, immunocytochemistry was performed. In this experiment, colorectal cancer cell line HT-29, which is known to express *c-kit*, served as positive control[27]. Immunocytochemical analysis showed a strong membraneous staining of the receptor protein in this cell line (Figure 2). In the tested biliary tract cancer cell lines, staining of *c-kit* revealed both cytoplasmatic and membraneous localization of receptor protein in Mz-

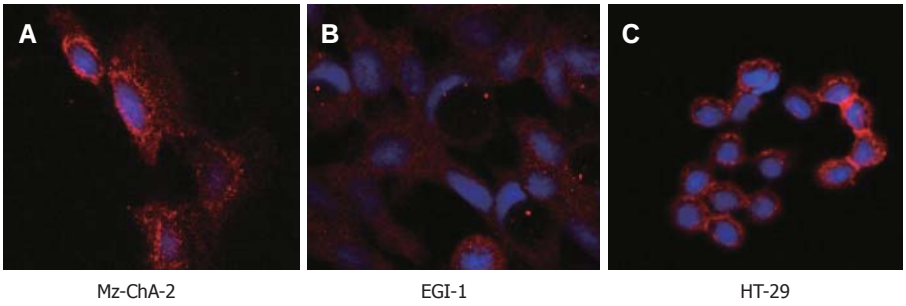

Figure 2 Immunocytochemistry in biliary tract cancer cell lines of Mz-ChA-2 (**A**), EGI-1 (**B**) and HT-29 (**C**).

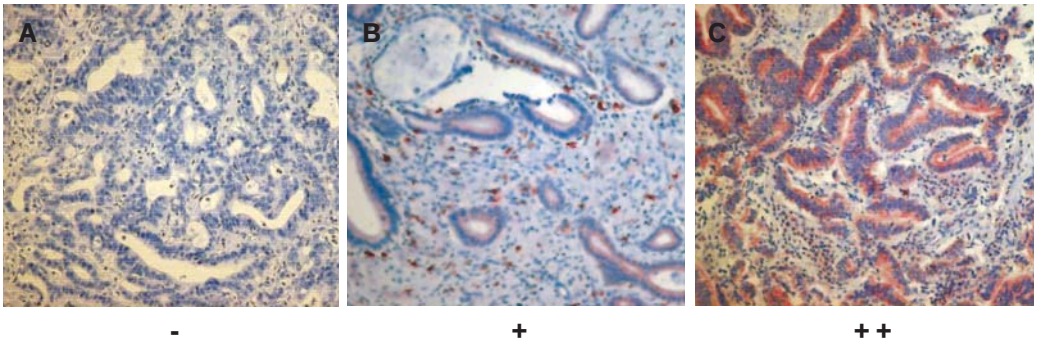

Figure 3 c-kit protein expression in 12 (**A**), 5 (**B**) and 2 (**C**) human biliary tract cancer tissue samples (SABC x 200).

ChA-2 cells and absence of *c-kit* in EGI-1 cells (Figure 2).

c-kit protein expression in human biliary tract cancer tissue

In order to analyze the expression of c-kit in human biliary tract cancer, paraffin-embedded tissue sections from 19 patients suffering from extrahepatic hilar CC were immunostained with c-kit-antibodies using the avidin-biotin horseradish peroxidase method. The clinical characteristics of the study population were as follows: median age of patients was 61 years (range, 39-74 years); UICC IA, IB, IIA, IIB, III, and IV tumor stage (UICC 2002)[32] had 1, 3, 7, 6, 1, and 1 patients, respectively; Bismuth-Corlette[33] types I, II, IIIA, IIIB, and IV were found in 1, 1, 8, 2, and 7 patients, respectively; and 16 patients had well or moderately differentiated tumors. As a result, 2 of the 19 tissues samples displayed a moderate immunostaining (++), 5 a rather weak immunostaining (+), and 12 were negative (-). Thus, 7 of 19 (37%) of the histological sections of extrahepatic hilar CC tested showed moderate to weak expression of c-kit. The immunoreactivity of c-kit was found mainly in the cell membrane (Figure 3). Highly-differentiated tumors seemed to show a higher level of c-kit expression than low-differentiated tumors.

Inhibition of cellular growth by imatinib mesilate

To assess the effect of imatinib mesilate treatment on cellular proliferation of both biliary tract cancer cell lines, automated cell counting was performed, using the colorectal carcinoma cell line HT-29 as a positive control[27]. After 6 d of incubation, imatinib mesilate at a low concentration of 5 μmol/L caused a significant growth inhibition in *c-kit* positive cell line Mz-ChA-2 (31%±7%), a moderate growth inhibition in *c-kit* positive cell line HT-29 (7%±9%), and no growth inhibition in *c-kit* negative cell line EGI-1 (0%±10%) ($P<0.05$, Mz-ChA-2 *vs* EGI-1). Imatinib mesilate at an intermediate concentration of 10 μmol/L inhibited cellular growth of both biliary tract cancer cell lines significantly (51%±4% *vs* 57%±4%, Mz-ChA-2 *vs* EGI-1). However, this effect was even stronger for HT-29 (91%±19%) ($P<0.05$, HT-29 *vs* Mz-ChA-2 and EGI-1). Imatinib mesilate at a higher concentration of 20 μmol/L seemed to have a general toxic effect in all cell lines (99%±1% *vs* 99%±1% *vs* 100%, Mz-ChA-2 *vs* EGI-1 *vs* HT-29) (Figure 4). The resulting IC_{50} values were 9.7 μmol/L, 11 μmol/L, and 9.5 μmol/L, respectively.

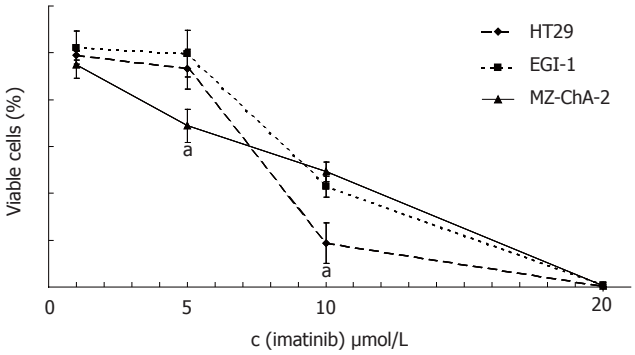

Figure 4 Inhibition of imatinib on cell growth ($^aP<0.05$).

Inhibition of tumor cell growth by imatinib mesilate in nude mice

To assess the antitumor activity of imatinib mesilate *in vivo*, tumors were induced in nude mice by subcutaneous injection of either Mz-ChA-2 or EGI-1 cells into the flanks of the animals ($n=20$ for each cell line). Treatment of mice consisted of daily ip injections of 50 mg/kg BW imatinib mesilate, and injection of NS served as control.

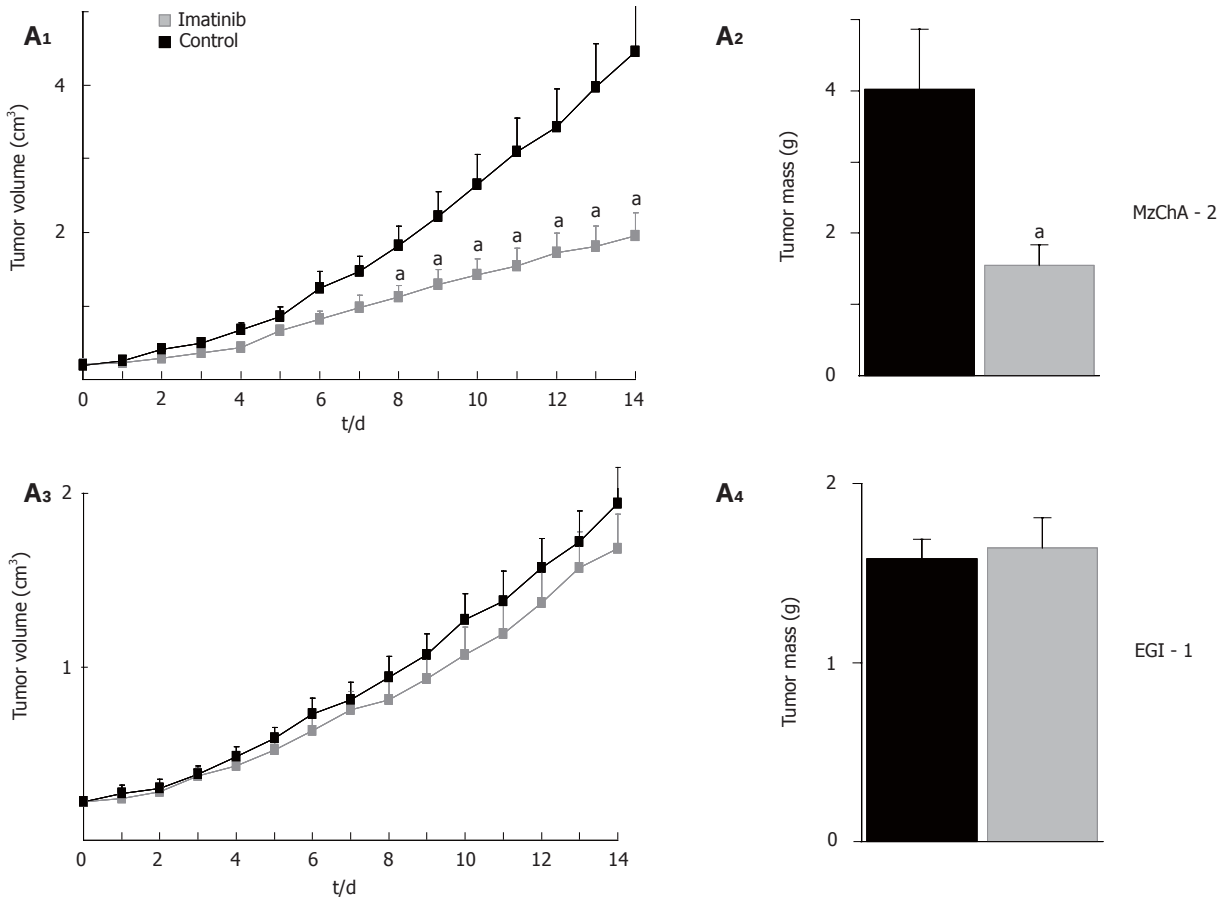

Figure 5 *In vivo* treatment with imatinib mesilate in chimeric mice; **A**: effect on tumor volume and tumor mass ($^aP<0.05$, imatinib *vs* control); **B**: hematoxilin-eosin stained paraffin-embedded tissue sections.

Eight to 14 d after beginning of treatment, Mz-ChA-2 cell tumors had a significantly reduced volume in comparison to control ($P<0.05$). At the end of the experiment (d 14) tumor mass was significantly diminished as compared to control ($P<0.05$). In contrast to that, imatinib mesilate treatment of mice bearing EGI-1 cell tumors did not result in any change of tumor growth compared to control (Figure 5A). Hematoxilin-eosin stained paraffin-embedded tissue sections from mouse tumors showed the typical features of low-differentiated adenocarcinoma (Figure 5B).

DISCUSSION

Non-resectable biliary tract cancer is associated with a poor prognosis due to wide resistance to chemotherapeutic agents and radiotherapy. It is therefore essential to search for new therapeutical approaches. Imatinib mesilate (STI571, Gleevec™), which was found to inhibit the *bcr-abl* tyrosine-kinase resulting from the translocation t(9;22) in chronic myeloid leukemia (CML), as well as *c-kit* and PDGF-R tyrosine kinases, may be an alternative[34]. We have previously shown expression of *c-kit* and PDGF-R m-RNA in 50% and 75% of 12 biliary tract cancer cell lines[31]. Activating mutations of *KIT* were not found, which is consistent with the data of Sihto *et al*[35]. This group screened 334 human cancers (32 histologic tumor types) for mutations with sensitive denaturing high-performance liquid chromatography (DHPLC) and found *KIT* mutations only in GIST tumors. In our study, *c-kit* receptor ligand SCF was present in all cells lines, respectively. We reasoned that the proliferation of these cells might be stimulated by an autocrine mechanism. In order

to test this hypothesis, we treated cell lines with different concentrations of imatinib mesilate. Imatinib mesilate at an intermediate concentration of 10 μmol/L and a higher concentration of 20 μmol/L reduced survival in all cell lines examined by manual cell counting after staining with trypan blue. The reduction was statistically significant higher in *c-kit* positive cell lines ($P < 0.02$) and was independent from PDGF-R status. At 50 μmol/L, a general non-specific toxic effect occurred in all cell lines. The average IC_{50} for growth inhibition of *c-kit* positive cell lines was estimated to be 10 μmol/L. 5-Fluorouracil (5-FU) alone (0.1 μg/mL) reduced cell growth by 40 % - 50 %, while the combination with imatinib mesilate reduced cell growth by another 20% (1 μmol/L) ($P = 0.008$) and 30% (10 μmol/L) ($P = 0.0001$), respectively. Additionally, treatment of cells with imatinib mesilate ± 5-FU had no modulating effects on S-phase fraction, but was associated with a significant induction of apoptosis[31].

However, at that time it was unclear whether *c-kit* could be detected at the protein level and how its distribution whithin the tumor cells was. Moreover, while this work was in progress, Chiorean *et al* were unable to detect *c-kit* at protein levels in human cholangiocarcinoma cell lines KMCH-1 and Mz-CHA-1[36]. In the present study, immunoblotting confirmed the results of our previous m-RNA analysis for Mz-ChA-2 and EGI-1 cells. Immunocytochemistry with *c-kit* antibodies displayed a cytoplasmatic and membraneous localization of receptor protein in *c-kit* positive Mz-ChA-2 cells, indicating a steady receptor turnover. Cell lysates of SCLC cell line NCI-H69 and colorectal cancer cell line HT-29 served as positive controls for immunoblotting and immunocytochemistry. Both cell lines displayed a stronger protein expression that might correlate with a higher sensitivity for imatinib mesilate treatment as shown in previous studies[27,28,37,38]. In order to verify that *c-kit* protein is not only expressed in cholangiocarcinoma cell lines, but also in human biliary tract cancer tissue, paraffin-embedded tissue sections from 19 patients suffering from extrahepatic hilar CC were immunostained with *c-kit*-antibodies using avidin-biotin horseradish peroxidase method. *c-kit* was expressed in 7 of 19 (37%) extrahepatic human CC tissue samples, 2 showed a moderate and 5 a rather weak immunostaining. This result is comparable to a previous analysis of 13 patients with intrahepatic cholangiocarcinoma which demonstrated an expression level of 31%[39]. In contrast, a study from Argentina detected only 6% *c-kit* expression in 50 specimens from gallbladder carcinoma[40]. Moreover, a weak to moderate *c-kit* expression was shown in 7 of 12 (58%) bile duct and gallbladder adenocarcinomas by Aswad *et al*[41]. Finally, a recent multitumor tissue microarray (TMA) analysis by Went *et al*, containing more than 3500 paraffin-embedded tumor samples representing more than 120 tumor types and subtypes, and a recent TMA analysis by Sihto *et al* showed negative results for gallbladder carcinoma ($n = 27$) and cholangiocarcinoma ($n = 3$)[30,35]. These controversial data could be explained by different antibodies used or different tissue selections. Using automated cell counting, we tried to confirm our manual cell counting data for the *c-kit* positive cell line Mz-ChA-2 and the *c-kit* negative cell line EGI-1 after treatment with imatinib mesilate. Imatinib mesilate at a low concentration of 5 μmol/L caused a significant growth inhibition in *c-kit* positive cell line Mz-ChA-2 (31%), but not in *c-kit* negative cell line EGI-1 (0%) ($P < 0.05$). Imatinib mesilate at an intermediate concentration of 10 μmol/L inhibited cellular growth of both cell lines (51% *vs* 57%), but was most effective in colorectal carcinoma cell line HT-29 (91%). Imatinib mesilate at a higher concentration of 20 μmol/L seemed to have a general toxic effect in both cell lines. The results for these particular cell lines indicate that: (1) at a concentration of 5 μmol/L imatinib mesilate seems to exhibit a *c-kit* receptor dependent cell growth inhibition. The different IC_{50} values of 9.5 μmol/L for HT-29, 9.7 μmol/L for Mz-ChA-2, and 11 μmol/L for EGI-1 may therefore be influenced by the different level *of c-kit* expression; (2) at a concentration of 10 and 20 μmol/L the inhibition by imatinib mesilate seems to be no longer *c-kit* receptor dependent. A possible explanation may be a significant decrease in epidermal growth factor receptor (EGF-R) and focal adhesion kinase (FAK) phosphorylation at these concentrations, as observed by Chiorean *et al* for cholangiocarcinoma cell line KMCH-1 which was associated with a reduction in Akt activity resulting in loss of Mcl-1, a potent anti-apoptotic Bcl-2 family member[36]. Whether an additional dose dependent inhibition of telomerase activity, as observed for *c-kit* positive Ewing sarcoma, melanoma, myeloma, breast cancer, Fanconi anemia, and *c-kit* negative murine pro-B cells, plays a role in cholangiocarcinoma has not been evaluated yet[17]; (3) the assumed general toxic effect of imatinib mesilate at a concentration of 20 μmol/L indicates that even a *c-kit* and PDGF-R negative cell line like EGI-1 can sometimes be destroyed at this concentration whereas our previous meta-analysis of 6 *c-kit* m-RNA negative biliary tract cancer cell lines demonstrated a general toxic effect only at 50 μmol/L with a potential inhibition at 20 μmol/L[31].

Even more important are therefore the results from our chimeric mouse model since so far there has been no animal model published for *in vivo* treatment with imatinib mesilate in biliary tract cancer. At the end of a 14 d treatment period with imatinib mesilate, *c-kit* positive Mz-ChA-2 tumors had a significantly reduced volume and mass as compared to NS treatment ($P < 0.05$). In contrast to that, treatment of mice bearing *c-kit* negative EGI-1 tumors did not result in any change of tumor volume and mass as compared to NS treatment. These results support the notion of a *c-kit* receptor expression dependency of sensitivity to imatinib mesilate *in vivo*. At the selected dose of 50 mg/kg, which was chosen according to a study by Druker *et al*[42], no toxic side effects occurred. As discussed by Druker *et al*[43], at steady state, a once-daily administration of 400 mg imatinib mesilate, the commonly used oral dose, translates into a mean maximal serum concentration of 4.6 μmol/L. Therefore, a daily oral dose of 600 mg may be reasonable for a study with patients suffering from biliary tract cancer. Drug-related adverse effects of imatinib mesilate include nausea, vomiting, myalgias, edema, diarrhea, and, less commonly, anemia, thrombocytopenia, neutropenia, and myelosuppression[43-45]. Moreover, there are concerns regarding the drugs liver toxicity in biliary tract cancer patients since it is primarily metabolized in the liver and tumor infiltration of the liver may reduce liver

function. Therefore, Eckel *et al* studied pharmacokinetics of imatinib mesilate in 17 patients with hepatocellular carcinoma and Child A-liver cirrhosis and compared the data with the data of 6 patients with CML and normal liver function[46]. As a result, they found no changes in pharmacokinetics in the first group of patients, thus letting assume the drugs safety in patients with hepatocellular carcinoma and Child A-liver cirrhosis. It is likely that these data can be transferred to patients with invasive biliary tract cancer. However, a phase I/II study is now required to further evaluate our preliminary in vitro and in vivo data. Therefore, our group has designed a clinical study for patients with biliary tract cancer that is now in progress.

In conclusion, *c-kit* expression is detectable by immunoblotting, immunocytochemistry, and immunohistochemistry at a moderate to low protein level in biliary tract cancer. Imatinib mesilate exerts marked effects on tumor growth *in vitro* and *in vivo* dependent on the level of *c-kit* expression. A phase I/II study has been designed to further study the role of imatinib mesilate treatment in patients with biliary tract cancer.

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