Abstract

**Purpose:** Serotonin is a well-known neurotransmitter and vasoactive substance. Recent research indicates that serotonin contributes to liver regeneration and promotes tumor growth of human hepatocellular cancer. The aim of this study is to investigate the expression of serotonin receptors in hepatocellular cancer and analyze their potential as a cytotoxic target.

**Experimental Design:** Using a tissue microarray and immunohistochemistry, we analyzed the expression of serotonin receptors in the liver from 176 patients with hepatocellular carcinoma, of which nontumor tissue was available in 109 patients. Relevant clinicopathologic parameters were compared with serotonin receptor expression. Two human hepatocellular cancer cell lines, Huh7 and HepG2, were used to test serotonin antagonists as a possible cytotoxic drug.

**Results:** The serotonin receptors 1B and 2B were expressed, respectively, in 32% and 35% of the patients with hepatocellular cancer. Both receptors were associated with an increased proliferation index, and receptor 1B correlated with the size of the tumor. Serotonin antagonists of receptors 1B and 2B consistently decreased viability and proliferation in Huh7 and HepG2 cell lines.

**Conclusion:** We identified two serotonin receptors that are often overexpressed in human hepatocellular cancer and may serve as a new cytotoxic target.

Introduction

Hepatocellular carcinoma (HCC) is the 5th most frequent cancer (1) and has become the 3rd leading cause of cancer-related death worldwide (2). To face this global health problem, the investigation of new molecular targets is necessary to develop new treatment strategies.

Recent in vitro and in vivo studies have suggested that serotonin (5HT) contributes to the tumor growth in a variety of cancer including cholangiocarcinoma (3, 4), colon cancer (5), and HCC (6). Within the liver, 5HT was found to be implicated in the pathogenesis of liver fibrosis (7, 8), nonalcoholic steatotic hepatitis (9), and viral hepatitis (10); all these conditions are involved in the tumorigenesis of HCC. We have recently proposed that 5HT, through its 5HT2B receptor, may represent a potential target in HCC (6). We observed that the inhibition of 5HT2B receptors leads to cell death of hepatocellular cancer cells in vitro and decreased tumor growth in a subcutaneous tumor model in mice.

At the cellular level, 5HT acts predominantly via G-protein coupled receptors. The availability of 7 receptor classes including 14 subtypes of serotonin receptors reflect the diversity of the serotonergic actions (11). Within these subtypes, 5HT receptors (1A, 1B, 2A, 2B, and 7) are widely distributed in the human gastrointestinal tract and are involved in normal liver function and disease (12, 13). In contrast, 5HT receptors 4 and 5 are not or only poorly expressed in the liver (14, 15).

In this study, the expression of different serotonin receptors was analyzed in specimens obtained from 176 patients with HCC using immunohistochemistry (IHC) on a tissue microarray (TMA). Receptor expression was compared between nontumoral and tumoral tissue in 109 patients, in whom nontumor tissue was available. We tested whether the expression of serotonin receptors in HCC correlated to the phenotype of the tumor and a variety of clinicopathologic parameters. From these data, we conducted experiments in human cell lines of HCC to investigate the cytotoxic effect of antagonizing serotonin receptors.

Patients and Methods

Patients

One hundred seventy-six patients with HCC who underwent surgery between 1992 and 2007 in Zürich, Switzerland
 accumulated evidence suggests a potential impact of serotonin in tumor biology, particularly in primary cancer of the liver. Recently, we have shown that serotonin promotes tumor growth of human hepatocellular cancer. In this study, we analyzed the expression of different serotonin receptors in specimens from a large patient group and tested whether specific serotonin receptor antagonists may serve as drugs to reduce tumor growth.

As human hepatocellular cancer is a disease with a poor prognosis and the incidence of this cancer is expected to increase in the future, the development of new treatment strategies is of utmost importance. Our analysis suggests serotonin receptors as possible molecular targets of human hepatocellular cancer.

**Translational Relevance**

Accumulating evidence suggests a potential impact of serotonin in tumor biology, particularly in primary cancer of the liver. Recently, we have shown that serotonin promotes tumor growth of human hepatocellular cancer. In this study, we analyzed the expression of different serotonin receptors in specimens from a large patient group and tested whether specific serotonin receptor antagonists may serve as drugs to reduce tumor growth.

As human hepatocellular cancer is a disease with a poor prognosis and the incidence of this cancer is expected to increase in the future, the development of new treatment strategies is of utmost importance. Our analysis suggests serotonin receptors as possible molecular targets of human hepatocellular cancer.

(n = 94) and Regensburg, Germany (n = 82) were included in this study. The patients’ ages ranged from 20 to 85 years (median 61 years). The underlying liver diseases of the HCC were: hepatitis B virus (HBV) infection (27 cases, 15.3%), hepatitis C virus (HCV) infection (37 cases, 21%), alcohol abuse (46 cases, 26.1%), hemochromatosis (9 cases, 5.1%), Alagille’s syndrome (1 case, 0.6%), and unknown etiologies (56 cases, 31.8%). Corresponding nonneoplastic liver tissue of 109 patients with HCC was available. The median follow-up time of all patients was 17 months (range 1–120 months). Patients were treated with resection (n = 136) or liver transplantation (n = 40). Median follow-up time of patients without disease progression was 17 months (range 1–120 months). A total of 35% of patients died during follow-up after a median time of 14 months (range 1–79 months).

The study was approved by the local ethics committee (Kantonale Ethikkommission Zurich, StV 26-2005 and EK-1017). A written informed consent was obtained from the patients.

**TMA**

Formalin-fixed, paraffin-embedded tissues were used to construct 5 TMAs with liver tumor tissues. The TMA was constructed as described previously (16). Two tissue cores per tumor with a diameter of 0.6 mm were punched out of the donor block and transferred to the recipient block.

**IHC**

The TMA blocks were freshly cut (3 μm) and mounted on superfrost slides (Menzel Gläser). IHC was conducted with the Ventana Benchmark automated staining system (Ventana Medical Systems) using Ventana reagents for the entire procedure. The primary antibody was detected using the UltraVIEW DAB detection kit using the benchmarks CC1m heat-induced epitope retrieval. The following primary antibodies were used: HTR1A (LS-B970, Lifespan), HTR1B (ab13514, Abcam), HTR2B (ab32994, Abcam), HTR7 (AHP1344, AbDSerotec), and Ki67 (M7240, Dako). Slides were counterstained with hematoxylin, dehydrated, and mounted. Human tissue from brain, kidneys, smooth muscle, and arteries served as positive control.

**Evaluation of immunohistochemical staining**

The staining intensity of HTR2B, HTR1A, HTR1B, and HTR7 was semiquantitatively evaluated with a 4-tiered system: 0 (negative), 1+ (weak), 2++ (moderate), and 3+++ (strong). Weak immunoreactivity was defined as minute granules projecting to the cytoplasm. Moderate immunoreactivity was diagnosed when a coarser and more intense staining was seen, whereas chunky, dark lumps were scored as strongly positive. A tumor was scored as positive when at least moderate staining (2++) was achieved. Because all of the receptors analyzed showed cytoplasmic staining, only this pattern was used in the evaluation. As a positive control, human brain tissue was used for each antibody and slide separately. When a tumor was positive it was homogenously positive in more than 90% of the tumor cells, therefore only the intensity was relevant in the analysis. For statistical analysis, tumors were grouped into weakly positive (0 and 1) and strongly positive (2 and 3) for HTR2B, HTR1A, HTR1B, and HTR7. The proliferation rate (Ki-67) was defined as percentage of positive nuclei per 100 tumor cells as recently described (17).

**Real-time PCR**

RNA extraction from Huh7, HepG2, and Hep3B was conducted using the RNeasy MinElute Cleanup kit (Qiagen) following the manufacturer’s instructions. Five micrograms of RNA were reverse transcribed using ThermoScript reverse transcription PCR System (Invitrogen) yielding the cDNA template. Quantitative real-time PCR amplification and data analysis were carried out using an ABI Prism 7000 Sequence Detector System (PE Applied Biosystems). Taq-Man gene expression assays (PE Applied Biosystems) for HTR1A (Hs00265014_s1), HTR1B (Hs00265286_s1), HTR2A (Hs00167241_m1), HTR2B (Hs00168362_m1), HTR4 (Hs00168380_m1), HTR5 (Hs00168381_m1), HTR7 (Hs00252002_m1), were used to quantify mRNA expression of the respective genes. Messenger RNA expression levels of each sample were normalized to 18S RNA (TaqMan rRNA control reagents; PE Applied Biosystems).

**In vitro experiments**

Cell lines were purchased from American Type Culture Collection and Huh7, HepG2, and Hep3B cultured in Dulbecco’s Minimal Essential Medium with 4.5 g/L glucose, sodium pyruvate, GlutaMAX (Invitrogen), and 10% fetal calf serum (FCS; PAA Laboratories), with the addition of 100 units/mL of penicillin and 100 μg/mL of streptomycin (Invitrogen). Cells were maintained at 37°C in a 5% CO2 atmosphere.

All antagonists were purchased from Tocris. The following selective antagonists were used: for HTR1A (S)-WAY 100135 dihydrochloride (S-WAY 100), for HTR2B LY 272015 hydrochloride (LY272), for HTR7 SB269970 (SB269), and for HTR1B SB 216641 hydrochloride (SB216).
Human hepatocellular cell lines, Huh7 and HepG2, were seeded into 24-well plates at a density of approximately 25% corresponding to 2.5 × 10^3 cells per well and allowed to adhere overnight before the medium was changed to the specified conditions, containing 100 μmol/L 5HT creatinine complex (Sigma Aldrich) and serotonin antagonists. Dosages of 5HT and antagonists were taken from response curves of previous experiments (6).

Time-dependent experiments were conducted with subsequent stimulation after serum withdrawal. In antagonist experiments, cells were incubated with the antagonist for 20 minutes before addition of the 5HT or FCS.

The number of viable cells was quantified by the addition of 25 μL of a 0.5% tetrazolium salt solution MTT (Sigma Aldrich). After 45 minutes of incubation, the formation of the formazan product was monitored by measuring absorbance at 570 nm after solubilization in acidic isopropranol (5% formic acid in isopropranol). Values were calculated as percentage of untreated controls.

**Western blotting**

Cells were seeded overnight in 10 cm dishes (1 × 10^6 /well). After treatment as indicated cells were homogenized in lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 5 mmol/L EDTA, 0.5% NP40, containing protease, and phosphatase inhibitor cocktail (Roche Diagnostics)]. Cell extracts were diluted in sample buffer [187.5 mmol/L Tris-HCl (pH 6.8), 6% SDS, 30% glycerol, 150 mmol/L DTT, and 0.3% bromophenol blue] and heated for 10 minutes at 90°C and cooled for 2 minutes on ice. Forty micrograms of protein were loaded, SDS-PAGE was conducted and samples were blotted onto a polyvinylidene difluoride membrane. Primary antibodies were phospho-p44/42 MAPK(ERK1/2) (#4370, Cell Signaling) and Anti-beta-Tubulin (ab6046; Abcam). Secondary staining and detection was conducted according to standard protocols with the enhanced chemiluminescence detection reagent (GE Healthcare Ltd). Results were displayed as fold induction to untreated controls.

**Statistics**

Data are given as mean and SD. Statistical analysis was conducted using SPSS, version 18.0 (SPSS Inc.) and Prism 4.0 (GraphPad, Inc.). Correlations were calculated according to Spearman ρ. Fisher exact test was applied to assess the statistical significance of the associations between the expression of 5HT receptors and various clinicopathologic parameters. Univariate survival analysis was carried out according to Kaplan–Meier, and differences in survival curves were assessed with the Log-rank test. Multivariate analyses were calculated according to the Cox regression model. P values <0.05 were considered significant. For cell culture experiments differences between the groups were assessed by 1-way or 2-way ANOVA using an appropriate posttest, including Dunnett and Bonferroni post hoc test.

**Results**

**5HT receptors in human hepatocellular cancer cell lines**

First, we screened 3 human hepatocellular cancer cell lines (Huh7, HepG2, and Hep3B) for transcription of 7 different 5HT receptors (HTR1A, HTR1B, HTR2A, HTR2B, HTR4, HTR5, and HTR7). In all 3 cell lines, PCR products were identified for HTR1A, HTR1B, HTR2B, and HTR7 (Fig. 1). Therefore, we focused on the expression of these four 5HT receptors using TMA in the available specimens of HCC.

**5HT-receptor expression in HCC and corresponding nontumor liver tissue**

The expression of HTR1A, HTR1B, HTR2B, and HTR7 was compared on 109 TMA tissue cores of HCC’s and corresponding nontumor liver tissue of the same patient (Fig. 2). A similar expression rate was found for HTR1A and HTR7 in nontumoral tissue and in tumoral tissue. In contrast, the expression of HTR1B and HTR2B were significantly increased in HCC (HTR1B: P < 0.0001; HTR2B: P = 0.001). A total of 32 (29.3%) and 38 (34.9%) of the 109 HCC specimens evaluated displayed a strong expression for HTR1B and HTR2B, respectively (Table 1).

**Comparison of 5HT-receptor expression with clinicopathologic parameters**

First, we asked whether HTR1B and HTR2B were concomitant expressed to assure that statistical analyses are not confounded by coexpression of these receptors. We compared all specimens of 176 patients and only 21 HCC specimens were double-positive for HTR1B and HTR2B (Fig. 3).

Next, we analyzed a potential association of 5HT receptors expression with a number of clinicopathologic parameters including age and gender of the patient, tumor stage, histology grade, size, proliferation rate, type...
of underlying liver disease, and patient survival (Table 2). Significant associations were identified for HTR1B and tumor size above 5 cm \((P = 0.002)\). Interestingly, the expression of HTR1B was also associated with a higher proliferation rate, as assessed by Ki-67 staining \((P = 0.036)\). HTR1B correlated with higher proliferation and size in HCC (Spearman \(\rho\); Fig. 4). Consistent with our previous observation, we found that HTR2B correlated to a higher proliferation rate (Spearman \(\rho\); \(n = 176\), \(r = 0.214\), \(P = 0.004\); ref. 6). HTR1A and HTR7 were not associated with any of the evaluated clinicopathologic parameters.

To test whether the expression of 5HT receptors may impact on patient’s outcome, we evaluated a possible correlation between receptor expression and survival. An association could not be identified for any of the receptors studied (Table 2). These findings were similar even by excluding the patients who received transplantation or who had a noncurative resection. A univariate survival analysis of 171 patients showed that transplantation and R0-resection was significantly associated with prolonged overall survival (data not shown).

**Effect of 5HT antagonists on proliferation**

In the light of the significant correlation between the expression of HTR1B and HTR2B markers of tumor cell proliferation, we asked whether 5HT-receptor antagonists might block proliferation *in vitro*. Therefore, we quantified the proliferation rate, using MTT...
Table 2. 5HT-receptor expression in HCC

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*TMA cores of some carcinomas were not evaluable because of insufficient number of carcinoma cells.

assays, of 2 human HCC cell lines (HuH7 and HepG2) exposed to specific antagonists S-WAY (HTR1A), SB216 (HTR1B), LV272 (HTR2B), and SB269 (HTR7). SB216 and LY216 significantly decreased proliferation in the presence of FCS, whereas antagonists against HTR1A attenuated proliferation only under serum-free conditions, and HTR7 had no impact on proliferation (Fig. 5).

Figure 4. Correlation of HTR1B with (A) Ki67 (n = 157, r = 0.279, P < 0.0001) and (B) tumor size (n = 175, r = 0.214, P < 0.004).
5HT activates the mitogen-activated protein kinases (MAPK) or extracellular signal-regulated kinases (ERK) through the stimulation of HTR1B and HTR2B (8, 18–21). The MAPK/ERK pathway is involved in the activation of transcription factors that regulate proliferation and cell-cycle progression (22). To test whether 5HT and specific receptor antagonists change the activation of the MAPK/ERK pathway, we measured the phosphorylation of ERK (pERK) with immunoblots. Stimulation with 5HT of HepG2 cells lead to a temporary phosphorylation after 5 minutes, which decreased again after 30 minutes. In the presence of the HTR1B-antagonist SB216 and the HTR2B-antagonist LY272, no phosphorylation of ERK could be detected (Fig. 6). These findings suggest (1) that proliferation of HCC cell lines is mediated by 5HT receptors and that (2) specific antagonists inhibit HCC cell growth in vitro.

Discussion

In this study, we identified that serotonin receptors 1A, 1B, 2B, and 7 are expressed in human HCC tissue as well as in corresponding nontumoral liver tissue. HTR1B and HTR2B are overexpressed in tumor tissue, however, compared with the adjacent liver, and the expression of these receptors correlated with a higher proliferation index. Specific antagonists against HTR1B and HTR2B inhibited the proliferation of cells in 2 human HCC cell lines suggesting a new putative target for therapy.

These findings are consistent with our previous observation, that almost one-third of HCCs are positive for HTR2B, whereas 20 normal livers used as internal control, were negative (6). In the current study, we extended our analysis in nontumoral tissue, as control of the corresponding HCC tumoral tissue. The expression rate...
The involvement of HTR1B in HCC is novel and needs further investigation. Within the liver, HTR1B is involved in the growth of the biliary tree by autocrine and paracrine regulation of 5HT (3, 13). In relation to cancer the role of HTR1B is less defined. A few in vitro studies suggest apoptotic and antiproliferative properties of HTR1B antagonists (28, 29).

Many 5HT-receptor agonist and antagonists are available for experimental research. Most of them are not approved for human use. In the current study, we used a new chemical antagonist against HTR2B [LY272 instead of SB204741, used in recent experiments (6)]. This water-soluble antagonist avoids complicated solubilization protocols such as SB204741. Interestingly, LY272 exhibited the same effect on survival in Huh7 and HepG2 as SB204741, excluding a general toxic effect of SB204741 and suggesting that specific inhibition of HTR2B impairs growth of HCC cells.

Proliferation assays were conducted without (serum-free medium) and with FCS, as standard growth medium. 5HT stimulated HCC cell growth as strong as FCS. Only antagonists against HTR2B and HTR1B inhibited proliferation in the presence of FCS. These experiments suggest 5HT as a serum factor needed for optimal growth of HCC cells mediated by 2 different types of 5HT receptors. The proliferative effect of 5HT is cell-type specific because 5HT did not stimulate proliferation in non-HCC cell lines, as shown in our previous publication (6). 5HT-mediated cell growth may be at least partially explained by the activation of classical MAP kinases such as ERK1/2, a pathway that is frequently activated in cancer including HCC (30–32). The involvement of HTR1B in HCC is novel and needs further investigation. Within the liver, HTR1B is involved in the growth of the biliary tree by autocrine and paracrine regulation of 5HT (3, 13). In relation to cancer the role of HTR1B is less defined. A few in vitro studies suggest apoptotic and antiproliferative properties of HTR1B antagonists (28, 29).

The first, and still only clinically available success of a molecular targeted therapeutic strategy was the use of the multikinase inhibitor sorafenib, leading to survival benefit in patients with advanced HCC (35). This success encourages a molecular classification of HCC to promote a personalized treatment (36). The detection of 5HT receptors in HCC may have a clinical relevance, as a considerable number of patients overexpress HTR1B and HTR2B and many different specific 5HT antagonists are available on the market.

A large amount of work has been done to identify prognostic molecular markers in HCC, for example, to predict survival or help in deciding a specific therapy. A particular difficulty is the presence of a variety of underlying diseases, such as various etiologies for the liver cirrhosis, which may exert differential effects on involved...
molecular pathways leading to HCC. For example, a molecular marker for HBV-related HCC may not be relevant for HCV-related disease. In addition, the majority of patients are likely to have been exposed to a combination of etiologic factors. Therefore, our cohort of 176 patients may still be too small to reveal 5HT-receptors as a predictor of survival or an association with a distinct etiology. It is also possible that not the expression of 5HT receptors itself serves as a marker, for example, predicting survival, but downstream molecules activated by 5HT receptors.

In conclusion, the 5HT receptor 1B and 2B are involved in tumor growth of human HCC. Therefore, 5HT-mediated signaling pathways in the liver may represent a target for new molecular treatment strategies in a subset of patients.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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