Research Article

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Targeting cyclin dependent kinase 5 in hepatocellular carcinoma – A novel therapeutic approach

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Background & Aims: For a long time cyclin dependent kinase 5 (Cdk5) was thought to be exclusively important in neuronal cells. However, increasing evidence recently suggests a function of Cdk5 in cancer progression. In this study, we examined the role of Cdk5 and its therapeutic accessibility in hepatocellular carcinoma (HCC), a highly chemoresistant cancer with poor prognosis and paramount clinical importance in order to develop novel targeted therapies for systemic treatment.

Methods: Expression and activity of Cdk5 was analyzed in a human HCC tissue microarray, human patient samples and HCC cell lines. To characterize Cdk5 functions and signaling pathways in HCC, we applied genetic downregulation and pharmacologic inhibition in various approaches including cell based assays and mouse xenograft models.

Results: Expression and activity of Cdk5 was increased in human HCC tissues as compared to normal liver tissues. Functional ablation of Cdk5 significantly decreased HCC cell proliferation and clonogenic survival. Moreover, genetic and pharmacological inhibition of Cdk5 showed *in vivo* efficacy in HCC xenograft mouse models. Investigating the mechanisms behind these functional effects revealed that Cdk5 is most active in the nucleus of cells in G2/M phase. Cdk5 regulates DNA damage response by phosphorylating ataxia telangiectasia mutated (ATM) kinase and thereby influencing its downstream cascade. Consequently, combination of Cdk5 inhibition with DNA-damage-inducing chemotherapeutics synergistically inhibited HCC tumor progression *in vitro* and *in vivo*.

Conclusions: In summary, we introduce Cdk5 as a novel drugable target for HCC treatment and suggest the combination of Cdk5 inhibition and DNA damaging agents as a novel therapeutic approach.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third leading cause of cancer-related death. It accounts for 695,900 deaths per year and its incidence rate is increasing [1]. Liver transplantation and surgical resection are the major choices of curative treatment for patients with early stage HCC, but in most cases HCC is diagnosed at an advanced stage when surgery is no longer feasible [2]. The only approved systemic therapeutic option for this stage is oral sorafenib treatment [3]. However, the prognosis for these patients is still poor, because the response to sorafenib remains low and the median overall survival is only extended by 2.8 months [4]. Therefore, the development of novel targeted therapies for HCC is of utmost clinical importance.

Cyclin dependent kinase 5 (Cdk5) is an unusual member of the Cdk family. Although Cdk5 is ubiquitously expressed, Cdk5 function is most prominent in cells of neuronal origin. In the central nervous system (CNS), Cdk5 is essential for neuronal development, regulates synaptic plasticity and dendritic spine formation, and is involved in learning and memory. In neurons, Cdk5 regulation is well investigated. In contrast to mitotic Cdks, Cdk5 does not interact with cyclins, but is activated by binding to p35 or p39 [5]. Cdk5 binding to p35 or p39 induces conformational changes of the kinase, resulting in an active conformation of Cdk5. p35 and p39 can get cleaved to the more stable cleavage products p25 and p29 respectively, that cause increased and sustained Cdk5 activation. Moreover, activating phosphorylation of

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Abbreviations: HCC, hepatocellular carcinoma; Cdk5, cyclin dependent kinase 5; ALS, Amyotrophic lateral sclerosis; ATM, ataxia telangiectasia mutated; RNA, ribonucleic acid; shRNA, short hairpin RNA; siRNA, small interfering RNA; TMA, tissue microarray; DNA, deoxyribonucleic acid; DSB, double strand break; PARP, poly(ADP-ribose)-polymerase; ATR, ataxia telangiectasia and Rad3-related; Chk1 or 2, checkpoint kinase 1 or 2; BRCA1, breast cancer 1; cdc2, cyclin dependent kinase 1; TACE, transcatheter arterial chemoembolization.

Cdk5 has been described [5]. Cdk5 has been associated with pathological conditions including neurodegenerative diseases like Alzheimer's disease, Parkinson's disease or amyotrophic lateral sclerosis (ALS), as well as cognitive and psychiatric diseases which is often associated with p25 or p29 generation [5]. Over the past decade, the knowledge on extraneuronal functions of Cdk5 has expanded [6,7]. For instance, some reports link Cdk5 with cancer progression. Cdk5 has been implicated in the regulation of prostate cancer cell motility, proliferation of medullary thyroid carcinoma cells and control of apoptosis in astrocytoma cells [8–11]. In pancreatic cancer, Cdk5 activity is shown to be important for tumor formation, progression and systemic metastasis [12]. Still, the knowledge on Cdk5's function in cancer is incomplete and its role in HCC is completely unknown.

The present study revealed an increased expression of Cdk5 in primary human HCC tissue. Cdk5 is shown to promote progression of HCC *in vitro* and *in vivo* and Cdk5 inhibition has strong chemosensitizing potential. Mechanistic work points to ATM kinase and regulation of DNA damage response are promising Cdk5 driven target circuits in HCC.

Materials and methods

Human HCC microarrays

Tissue microarray containing human HCC samples and matched surrounding non-tumor tissue has been described before [13]. Included HCC patients had been treated with liver transplantation or partial hepatectomy at the University Clinic Munich Großhadern between 1985 and 2008. Statistical analysis of the TMA was performed as already described by using SPSS (version 17, SPSS, Inc) [13].

Human HCC tissue samples

Human patient HCC and corresponding healthy liver tissue for immunoblotting (freshly frozen) was provided from Human Tissue and Cell Research HTCR, Hospital of the Ludwig Maximilians University of Munich, Campus Großhadern, Munich, Germany.

In vivo experiments

Experiments were performed according to German legislation for the protection of animals and approved by the local government authorities.

3.3×10^6 HuH7 or nt/Cdk5 shRNA HuH7 cells were injected subcutaneously into the flank of female SCID mice (8–10 weeks).

Roscovitine and irinotecan were injected intraperitoneally (100 µl; solvent: PBS/DMSO/Solutol 17:1:2). Single roscovitine treatment was carried out daily at 150 mg/kg/d for seven days, beginning seven days after implantation. Combinatorial treatment was started ten days after implantation with 150 mg/kg/d roscovitine 3-times/week and 10 mg/kg/d irinotecan 2-times/week for 4 weeks.

Tumor volume (V) was evaluated with tumor length (L), width (W) and height (H) according to the formula $V = \pi/6\ (L \times W \times H)$ and modeled using an exponential non-linear mixed effects modeling technique where the tumor volume at a given time t (N(t)) is a function of the starting volume N(0), the time of growth t and of a growth rate α: $N(t) = N(0) \times \exp\alpha \times t$. Data from all xenograft experiments were combined for modeling purpose. Effect of tumor growth inhibition was linked to the model predicted plasma concentrations of irinotecan and roscovitine respectively. For roscovitine a concentration dependent Emax model on the growth rate α was implemented. The effect of irinotecan was described by an induction of a concentration and tumor dependent apoptosis process. Modeling was performed using the software NONMEM 7.3 and SAS 9.3.

Statistical analysis

All experiments were performed at least three times unless otherwise indicated in the figure legend. Data are expressed as mean ± SEM. Statistical analysis was performed with SigmaPlot® software version 10.0 (Systat Software Inc., Chicago, USA). Statistical tests are indicated in the figure legend. Statistical significance is assumed if $p \leqslant 0.05$.

Additional experimental procedures are provided in the Supplementary Material.

Results

Cdk5 expression and activity is increased in human HCC

Immunohistochemical analysis of a human HCC tissue microarray (TMA) [13] showed prevalent expression of Cdk5 in HCC (Fig. 1A). In detail, 41.3% of analyzed HCC patient tissues showed Cdk5 expression whereas only 26.4% of respective healthy liver tissues showed Cdk5 expression (Fig. 1A upper panels; Supplementary Table 2). Moreover, high Cdk5 expression (staining intensity 2 and 3) was found in 13.4% of HCC tissue *vs.* 4.6% in normal liver tissue.

Expression of p35 (Fig. 1A, lower panels), the neuronal activator of Cdk5, was equally distributed: 89.9% of patients showed p35 expression in HCC and 92.5% showed p35 expression in their healthy liver tissues (Supplementary Tables 3 and 4). Nevertheless, p35 intensity was increased in HCC *vs.* normal liver tissue: 62.5% of HCC tissues showed high expression of p35 (intensity 2 and 3), whereas only 40.2% of normal liver tissues expressed respective high level of p35 (Fig. 1A, lower panels; Supplementary Table 4). However, there was no correlation between expression of Cdk5 and p35 (Supplementary Table 5).

Cdk5 did not correlate with patient survival, tumor metastasis or tumor aggressiveness. This is likely due to the fact that our TMA represents only early stage HCC of patients who underwent surgical resection. Neither did Cdk5 expression correlate with non-tumor specific clinical parameters (Supplementary Table 6). Nevertheless, interestingly, intensities of Cdk5 expression between tumor and non-tumor samples were correlated ($p = 0.016$, Supplementary Table 7).

Results from the TMA were confirmed by immunoblots from fresh human HCC and corresponding non-tumor tissues of five HCC patients from a different collective: HCC samples showed increased expression of Cdk5 and its activator p35 compared to corresponding normal liver tissue of the same patient both on protein (Fig. 1B) as well as mRNA (Fig. 1C) levels.

In line with these findings, Cdk5, phosphorylated Cdk5 and p35 were increased in human HCC/hepatoblastoma cell lines compared to primary human hepatocytes (Fig. 1D). Comparing the different cell lines, levels of total Cdk5 were highest in HepG2, HuH7, and Hep3B cells. p35 expression was similar in HCC/hepatoblastoma cell lines. Phosphorylated Cdk5 was increased in aggressive HCC cell lines HuH7 and Hep3B supported by higher Cdk5 enzyme activity in more aggressive HuH7 cells (Fig. 1E).

This set of data suggests that Cdk5 expression and activity is more prominent in HCC compared to healthy liver.

Inhibition of Cdk5 inhibits HCC cell growth in vitro

Effects of Cdk5 inhibition in HCC were examined in HepG2, HuH7, and Hep3B cell lines. We used pharmacologic inhibition employing roscovitine, a well-established Cdk5 inhibitor [14]. Roscovitine does not selectively inhibit Cdk5, but also blocks other Cdks including Cdk1 and Cdk2 [14,15]. Therefore, in order