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ORIGINAL ARTICLE

Markers of Hippo-Pathway Activity in Tumor Forming Liver Lesions

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Abstract Hepatocellular Carcinoma (HCC) is a lethal cancer worldwide. Recently, the hippo signaling pathway has been implicated in tumorigenesis of HCC and other malignant tumors. Aim of the study was therefore to evaluate the hippo signaling pathway activity and its clinico-pathological associations and crosstalk in different tumor forming hepatocellular lesions (HCC, hepatocellular adenoma (HCA), focal nodular hyperplasia (FNH) and cirrhosis). A tissue micro array (TMA) from paired human tumorous and non-tumorous (NT) tissue samples of HCC (n = 92), HCA (n = 25), FNH (n = 28) and cirrhosis (n = 28; no NT) was constructed. The hippo-pathway related proteins of MST1/2, (nuclear(n)/cytoplasmic(c)) YAP and (phospho(p)) TAZ and interactors as Glypican3, RASSF1a, pAKT, pERK and pP70S6K were evaluated by immunohistochemistry (IHC). Proliferation was assessed by Ki67-IHC and apoptosis by TUNEL-technique. MST1/2- and nYAP-immunoreactivity was associated with lymph node status (p = 0.048, p = 0.001), higher grading (p = 0.012, p = 0.24) and unfavorable relapse-free survival (p = 0.004, p = 0.003). MST1/2, c/nYAP and pTAZ were significantly different between HCC/NT (p < 0.001, p = 0.029, p < 0.001, p < 0.001) and mono−/polyclonal hepatocellular lesions (HCC/HCA vs. FNH/cirrhosis; all $p \leq 0.001$). Phospho-TAZ-negativity and nYAP-positivity were almost exclusively and MST1/2 exclusively detected in HCC. MST1/2 correlated with pP70S6K (p = 0.002), pERK (p = 0.042), RASSF1a-IRS (p = 0.002) and GPC3 (p < 0.001) and nYAP with GPC3 (p = 0.025), higher Ki67-indices (p = 0.016) and lower apoptosis rate (p = 0.078). MST1/2 and nYAP are unfavorable prognostic markers associated with an aggressive tumor-phenotype in HCC. Positive nYAP- and negative pTAZ-immunostaining were strong indicators of a monoclonal hepatocellular lesion. The unexpected findings for MST1/2 remain to be elucidated.

Keywords Hippo-pathway · HCC · Hepatocellular adenoma · FNH · Cirrhosis · Immunohistochemistry

Introduction

Hepatocellular carcinoma (HCC) is a common malignant tumor and the second most common cause of cancer related death worldwide [1]. While prognosis is dismal in progressed stages, curative regimens are more likely to be successful in small and/or early HCC [2]. Beside prevention of HCC by diminishing HCC-related agents and conditions, improvement of diagnostic methods and better understanding of the underlying molecular mechanisms in HCC tumorigenesis are needed.

Recently, the hippo signal transduction pathway, first identified in *Drosphila melanogaster*, has been implicated as a vital mechanism in organ size control during developmental growth [3]. Being highly conserved between *Drosophila* and humans, this pathway has been implicated in HCC tumorigenesis and mutations are associated with severe tissue overgrowth. In the sequence of hippo signaling, the most prominent event is nuclear translocation and accumulation of Yes-

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associated protein 1 (YAP), which finally promotes increased cell proliferation and inhibition of apoptosis as well as cellular de-differentiation (reviewed in [4, 5]). Despite the final importance of nuclear YAP translocation, a number of other pathways and cellular mechanisms interact with hippo signaling pathway kinases and the hippo pathway itself is subject to a variety of modifications thus demonstrating it as a central integrative cellular mechanism with complex regulation [4].

As the results from hippo pathway activation such as proliferation and inhibition of apoptosis are indispensable mechanisms in malignant tumors, in vitro over-expression of YAP was found to lead to transforming phenotypes and inverse results were detected in cancer-cell lines when YAP was removed [4, 6]. In line with in vitro and murine-model results, YAP over-expression and/or nuclear accumulation has been detected in several human malignant tumors such as prostate, colorectal and lung cancer as well as HCC [4, 7–12]. In the latter, YAP expression also was also found to influence the prognosis and was associated with poorer tumor cell differentiation [13, 14]. These findings and the emerging role of the hippo signaling pathway in other tumors attracted interest for therapeutical intervention with first favorable results in breast cancer cell lines [15].

As research focused on malignant liver tumors, aim of the present study was therefore to gain insights in the hippo pathway activity in broad variety of tumor forming hepatocellular lesions in humans such as hepatocellular adenoma (HCA), focal nodular hyperplasia (FNH) and cirrhosis as well as HCC. Additionally the results were correlated with clinico-pathological and survival data as well as to activity of hippo pathway related regulators.

Material and Methods

Cohort

All formalin-fixed (4 % buffered formalin) and paraffin embedded (FFPE) tissue samples were retrospectively obtained from the archive of the Institute of Pathology at the University Hospital of Essen. Tumor diagnosis was conducted according to current WHO-criteria and re-classified corresponding to the recent TNM-system [16–18]. A tissue micro array (TMA) with three cores per case was constructed with paired tumorous and non-tumorous (NT) liver specimens of HCC (n = 92), HCA (n = 25), FNH (n = 28) and cirrhosis (n = 28; no NT) summing up to a total of 318 tissue samples. Whole mount sections were analyzed in two additional HCC/NT cases to prevent TMA-related misinterpretation. Clinico-pathological data was collected from the reports and patients' files. Details of the cohort and tissue samples have been published previously [19]. The study protocol was in accordance with the ethical guidelines of the Declaration of Helsinki. The local ethics committee approved the study (#15–6230-BO).

Evaluation of (Hippo-) Pathway Related Targets (Immunohistochemistry, IHC)

From the TMA-FFPE-blocks, 1–2 μm thick sections were cut, dewaxed and pre-treated in each case. All IHC staining procedures were conducted on an automated staining device (Dako Autostainer, Dako, Glostrup, Denmark). Detailed antibody and protocol information is given in Table 1. Suitable positive as well as negative controls were included in every run.

The immunoslides of mammalian sterile twenty-like kinases 1/2 (MST1/2), YAP, phospho-Tafazzin phosphorylated at Ser 89 (pTAZ), Glypican 3 (GPC3) and ras association domain-containing protein 1 isoform a (RASSF1a) were assessed using a semi-quantitative score (immunoreactivity score, IRS) as the sum of quantity (0 %: 0 points, 1–5 %: 1 point, 6–10 %: 2 points, 11–50 %: 3 points, 51–100 %: 4 points) and quality (none: 0 points, weak: 1 point, moderate: 2 points, strong: 3 points). Both, the scores of the lesion and NT were assessed using this system. In case of YAP, both the nuclear (nYAP) and the cytoplasmic (cYAP) reactivity were analyzed. All evaluations were carried out twice in a blinded manner (by HAB and HR) on an Olympus BX 51 (HAB) and Nikon Eclipse E800 (HR) (Nikon, Tokyo, Japan). A threshold of 5 % was defined for considering a case as positive. Immunostaining results regarding protein kinase B phosphorylated at Ser 473 (pAKT), p44/42 mitogen-activated protein kinases phosphorylated at Thr202/Tyr204 (pERK) and P70S6-Kinase phosphorylated at Thr421/Ser424 (pP70S6K) were assessed as published previously [20].

Evaluation of Proliferation and Apoptosis

For the assessment of the proliferation fraction, the percentage of positive tumor nuclei was counted in 300 tumor cells [21]. The results were additionally grouped (0 %, 1–5 %, 6–10 %, >11 %). Evaluation of the apoptotic cells was conducted using the *TUNEL* (terminal deoxyribonucleotide transferase (TdT)-mediated dUTP nick end labeling) technique with the ApopTag™ Plus Peroxidase in Situ Apoptosis Detection Kit (Intergen, GA, USA) [21]. Additionally, *TUNEL* results were dichotomized at 10 % level.

Statistical Analysis

All statistical analyses were performed using SPSS (V21; IBM, Armonk, NY, USA). Pearson and Spearman correlation analyses were performed when appropriate. For comparison of paired non-parametric dichotomous variables the McNemar-test was used. Due to non-normally distribution of variables, the Wilcoxon-signed rank-test was calculated for further analyses of paired samples. In non-paired samples the t-test was used. Survival analyses (overall survival (OS), relapse-free survival (RFS)) were performed using the Kaplan–Meier method and the log-rank test for trends. All

Table 1 Detailed information on employed antibodies and immunohistochemical protocols

Antibody	Company	Clone/#	Clonality	Dilution	Incubation	Antigen retrieval	Detection
GPC3	DCS	G1829c002	monoclonal	1:800	30 min. RT	20 min. 98 °C, pH 9.0	Zytomed HRP, DAB
Ki67	DCS	SP6	monoclonal	1:400	30 min. RT	30 min. 99 °C, pH 6.0	Zytomed HRP, DAB
MST1/2	Abcam	ab87322	polyclonal	1:400	30 min. RT	20 min. 99 °C, pH 9.0	Zytomed HRP, DAB
pAKT	Cell Signaling	cs#9277	polyclonal	1:200	o.n. 4 °C	30 min. 99 °C, pH 6.0	Dako APAAP, NF
pERK	Cell Signaling	cs#9106	monoclonal	1:500	o.n. 4 °C	30 min. 99 °C, pH 6.0	Dako APAAP, NF
pP70S6K	Cell Signaling	cs#9204	polyclonal	1:200	o.n. 4 °C	o.n. 60 °C, pH 7.0	Dako APAAP, NF
pTAZ (S89)	Santa Cruz	sc-17,610-R	polyclonal	1:750	30 min. RT	20 min. 99 °C, pH 9.0	Cell signaling CS boost, DAB
RASSF1a	Abcam	ab23950	monoclonal	1:75	60 min. RT	20 min. 99 °C, pH 9.0	Dako CSA II, DAB
YAP	Cell Signaling	cs#4912	polyclonal	1:30	30 min. RT	20 min. 99 °C, pH 9.0	Zytomed HRP, DAB

Abbreviations: GPC3: Glypican 3, RT: Room temperature, HRP: Horseradish peroxidase, DAB: Diaminobenzidine, MST1/2: Mammalian sterile twenty-like kinases 1/2, pAKT: Protein kinase B phosphorylated at Ser 473, o.n.: over night, APAAP: Alkaline Phosphatase - Anti-Alkaline Phosphatase, NF: New fuchsin, pERK: p44/42 mitogen-activated protein kinases phosphorylated at Thr202/Tyr204, pP70S6K: P70S6-Kinase phosphorylated at Thr421/Ser424, pTAZ (S89): Tafazzin phosphorylated at Ser 89, RASSF1a: Ras association domain-containing protein 1 isoform A, YAP Yes-associated protein 1

p-values <0.05 were regarded statistically significant and a trend was assumed in case of $p < 0.1$.

Results

Clinico-Pathological Correlations and Survival Analyses

Detailed clinico-pathological data is given in Table 2.

Mean follow-up was 990.9d (SE: 90.4d) for OS and 879.4d (SE: 90.7d) for RFS.

Immunoreactivity of MST1/2 and n/cYAP correlated with positive lymph node status and higher tumor grading/anaplasia in HCC as well as older age at diagnosis (Table 2). Phospho-TAZ-immunopositivity correlated with younger age at diagnosis (Table 2). No other associations were noted.

A trend for an unfavorable OS was detected in MST1/2-immunopositive HCCs ($p = 0.079$). No such association was noted for nYAP-positivity. Regarding RFS, MST1/2-immunopositivity was a strong predictor of shorter survival ($p = 0.004$, Fig. 1a). The same was true for nYAP-immunoreactivity (nYAP) in grouped analyses ($p = 0.003$, Fig. 1b). No other significant prognostic associations were noted.

Activity of Hippo-Pathway Elements in Different Entities

In HCC, immunopositivity-rates were differing significantly between HCC and NT in case of MST1/2 ($p < 0.001$), c/nYAP ($p = 0.029$, $p < 0.001$) and pTAZ ($p < 0.001$) (Table 3). While MST1/2-positivity was detected exclusively in HCC and not in NT, nYAP-immunopositivity was also found in NT of HCC (Table 3). However, it was detected only in a low number ($n = 6$, 6.8 %) of cases with mostly minor percentages of positive nuclei ($n = 4$: 6–10 % tumor cell reactivity, $n = 2$: 11–50 %). Cytoplasmic YAP-immunoreactivity was additionally detected significantly more often in HCC compared to NT, with higher rates of positivity in NT compared to nYAP (Table 3). Contrarily, pTAZ-immunopositivity was detected more often in NT compared to HCC-tumorous tissue (Table 3).

In HCA and FNH, correlation analyses were limited as all cases were negative for MST1/2 in both entities and in FNH additionally for nYAP. No significant differences regarding any of the variables and HCA or FNH and NT were detected (Table 3). In cirrhosis, no comparison of lesional and NT was possible, as by definition NT does not exist.

When analyzing the distribution of the immunoreactivity rates in the total cohort, in HCC versus all other cases and in monoclonal (HCC/HCA) versus polyclonal (FNH/cirrhosis) lesions, all analyzed hippo pathway targets exhibited strong and statistically significant differences between the groups, i.e. MST1/2 (all $p < 0.001$), nYAP (all $p < 0.001$), cYAP (all $p < 0.001$) and pTAZ (all $p \leq 0.001$) (Table 3). Regarding the differences in NT, no analysis was feasible for MST1/2, as all cases were found negative. In NT, nYAP-immunoreactivity was significantly differing only in analyses of HCC versus all other cases ($p = 0.004$) and cYAP in all groups (all $p \leq 0.005$) (Table 3). MST1/2- and nYAP-immunopositivity exhibited a considerable overlap as 12 of the 17 MST1/2 positive cases (70.6 %) were detected in nYAP-positive HCCs ($p = 0.009$). No significant differences were noted for pTAZ in NT.

Crosstalk of Hippo-Pathway Elements and Regulatory/Effector Mechanisms

In HCC, MST1/2 positivity was positively correlated with expression of pP70S6K ($p = 0.002$), pERK ($p = 0.042$), RASSF1a-

Table 2 Clinicopathological data in the studied cohort. *P*-values for MST1/2, (n/c) YAP and pTAZ regarding association with age at diagnosis were calculated at the median of the total cohort as a cut-off

		HCC	HCA	FNH	Cirrhosis	Total	MST1/2 *P*	YAP (n/c) *P*	pTAZ *P*
Sex	Male	70 (74.5 %)	6 (24 %)	4 (14.3 %)	17 (60.7 %)	97 (55.4 %)	*n.s.*	*n.s.*	*n.s.*
	Female	24 (25.5 %)	19 (76 %)	24 (85.7 %)	11 (39.3 %)	78 (44.6 %)			
	Total	94	25	28	28	175			
Age	median (SD)	68 (11)	34 (12)	37 (12)	48 (18)	37 (19)	*<.001*	*<.001 / <.001*	*.003*
T	pT1	43 (46.2 %)	-	-	-		*n.s.*	*n.s.*	*n.s.*
	pT2	32 (34.4 %)							
	pT3	15 (16.1 %)							
	pT4	3 (3.2 %)							
N	pN0	77 (82.8 %)	-	-	-		*.048*	*.001 / .003*	*n.s.*
	pN1	5 (5.4 %)							
	pNx	11 (11.8 %)							
G	G1	14 (15.1 %)	-	-	-		*.012*	*.024 / .028*	*n.s.*
	G2	49 (52.7 %)							
	G3/4	30 (32.3 %)							
L	L0	92 (98.9 %)	-	-	-		*n.s.*	*n.s.*	*n.s.*
	L1	1 (1.1 %)							
V	V0	56 (60.2 %)	-	-	-		*n.s.*	*n.s.*	*n.s.*
	V1	37 (39.8 %)							
R	R0	79 (84.9 %)	-	-	-		*n.s.*	*n.s.*	*n.s.*
	R1/2	14 (15.1 %)							

Abbreviations: HCC: Hepatocellular carcinoma, HCA: Hepatocellular adenoma, FNH: Focal nodular hyperplasia, MST1/2: Mammalian sterile twenty-like kinases 1/2, YAP: Yes-associated protein 1, pTAZ: Tafazzin phosphorylated at Ser 89, c/n: Cytoplasmatic/nuclear immunoreactivity, n.s.: $p > .05$, T: Tumor stage, N: Regional lymph node status, G: Grading, L: Lymph vessel status, V: Blood vessel status, R: Status of resection margins

IRS ($p = 0.002$) and GPC3 ($p < 0.001$). No association between MST1/2 and pAKT was noted. However, reactivity rates of pAKT positively correlated with pERK ($p < 0.001$) and pP70S6K ($p = 0.012$). Nuclear YAP-immunopositivity was associated with GPC3-immunopositivity ($p = 0.025$), higher Ki67-indices in grouped analyses ($p = 0.016$) and a trend to lower rates of apoptosis was additionally detected ($p = 0.078$; grouped variables). Cytoplasmatic YAP- and pTAZ-immunoreactivities exhibited no associations.

Analyses in HCA, FNH and cirrhosis did not yield any associations in feasible calculations.

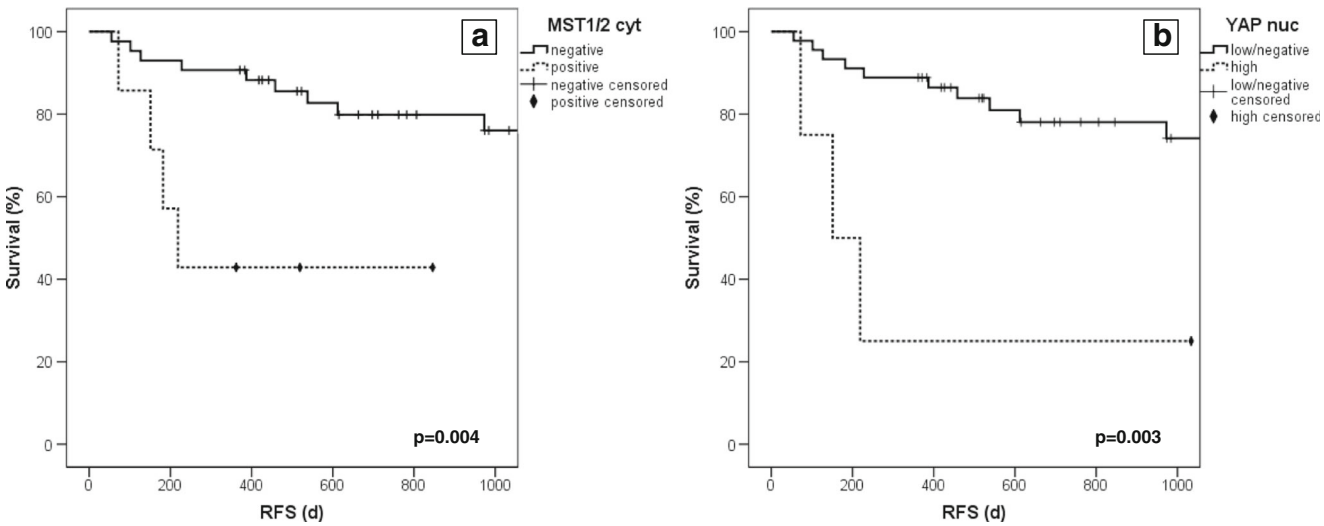

Fig. 1 Relapse-free survival (RFS) regarding cytoplasmatic MST1/2- and nuclear YAP-immunoreactivity. Immunohistochemical detection of MST1/2 is associated with unfavorable RFS in HCC (**a**). High (grouped Immunoreactive-score (IRS) values 6/7) vs. low (negative and IRS-values <= 5) nuclear. YAP- immunoreactivity is a predictor of unfavorable RFS in HCC (**b**)

Table 3 Hippo-pathway element immunoreactivity (positive vs. negative) in different diagnoses

		HCC n (%)	*p*	*HCA n (%)*	*p*	FNH n (%)	*p*	Cirrhosis n (%)	*p total cohort*	*p HCC* vs. *rest*	*p HCC/HCA* vs. *FNH/cirrhosis*
MST1/2 Tu	−	75 (81.5 %)	<.001	*25 (100 %)*	x	28 (100 %)	x	28 (100 %)	*<.001*	*<.001*	*<.001*
	+	17 (18.5 %)		*0 (0 %)*		0 (0 %)		0 (0 %)			
MST1/2 NT	−	90 (100 %)		*25 (100 %)*		17 (100 %)		x	*x*	*x*	*x*
	+	0 (0 %)		*0 (0 %)*		0 (0 %)		x			
nYAP Tu	−	51 (57.3 %)	<.001	*22 (88 %)*	ns	28 (100 %)	x	26 (96.3 %)	*<.001*	*<.001*	*<.001*
	+	38 (42.7 %)		*3 (12 %)*		0 (0 %)		1 (3.7 %)			
nYAP NT	−	82 (93.2 %)		*23 (92 %)*		17 (100 %)		x	*ns*	*.004*	*ns*
	+	6 (6.8 %)		*2 (8 %)*		0 (0 %)		x			
cYAP Tu	−	50 (56.2 %)	.029	*22 (88 %)*	ns	26 (92.9 %)	ns	23 (82.1 %)	*<.001*	*<.001*	*<.001*
	+	39 (42.8 %)		*3 (12 %)*		2 (7.1 %)		5 (17.9 %)			
cYAP NT	−	64 (72.7 %)		*22 (88 %)*		17 (100 %)		x	*.002*	*<.001*	*.005*
	+	24 (27.3 %)		*3 (12 %)*		0 (0 %)		x			
pTAZ Tu	−	19 (21.1 %)	<.001	*1 (4 %)*	ns	1 (3.8 %)	ns	0 (0 %)	*<.001*	*<.001*	*.001*
	+	71 (78.9 %)		*24 (96 %)*		25 (96.2 %)		26 (100 %)			
pTAZ NT	−	3 (3.5 %)		*0 (0 %)*		1 (5.9 %)		x	*ns*	*ns*	*ns*
	+	83 (96.5 %)		*24 (100 %)*		16 (94.1 %)		x			

Abbreviations: Tu: Lesional ('tumorous') reactivity, NT: Non-lesional ('non-tumorous') reactivity, x: No data (in case of statistical analyses due to constancy of at least variable; in cirrhosis no non-tumorous tissue is available by definition), ns: Not statistically significant

Discussion

HCC is a common and lethal cancer worldwide [1]. While therapeutic regimes have advanced, cure is still most likely to be achieved in early stage disease [2]. Better understanding of the underlying molecular mechanisms of hepatocellular tumorigenesis is therefore needed.

One hallmark mechanism in organ size control and HCC-formation, the hippo signaling pathway, has recently been reported [3]. Originally described in *Drosphila melanogaster*, it was named after its key signaling component - the protein kinase Hippo. Being highly conserved between different species, in humans the upstream kinases MST1/2 (orthologs of Hippo), large tumor suppressor kinase 1/2 (LATS1/2), salvador family WW domain containing protein 1 (SAV1) and MOB kinase activator 1 A/B (MOB1A/B) function as a complex phosphorylating YAP and TAZ. This leads to YAP and TAZ sequestration to the cytoplasm in a 14–3-3-dependend manner thus inactivating the complex and preventing localization to the nucleus. When non-phosphorylated, YAP is able to translocate to the nucleus and act as a co-activator for DNA-binding transcription factors such as TEA domain family members 1–4 (TEAD1–4) or members of the SMAD-protein family. This mechanism leads to increased cell proliferation and inhibition of apoptosis as well as cellular de-differentiation (reviewed in [4]). In line with these findings we detected a significant higher fraction of nuclear YAP-positivity in HCC compared to NT and an inverse association for pTAZ (Table 2).

The functional relevance of nuclear YAP-immunopositivity in our cohort of HCC is supported by an increase in proliferation, decrease of apoptosis and positive correlation with GPC3 as a YAP target [22] as well as significantly higher rates of lymph node positive cases and higher tumor grade/anaplasia (Table 2).

Given the importance of these cellular mechanisms for tumor survival, nuclear YAP accumulation and/or overexpression have been implicated in several human carcinomas including HCC [10–12]. In HCC, YAP-expression additionally proved to be an independent prognostic marker [13, 14]. In line with this, we detected a strong unfavorable influence of nuclear YAP-immunoreactivity on RFS (Fig. 1b) thus confirming existing data and the validity of our cohort (Fig. 2).

In addition to significant differences in immunoreactivity rates of all analyzed hippo-pathway elements in HCC versus NT (Table 3), an important finding is the distribution of nuclear YAP-immunopositivity in the other hepatocellular lesions. Although nuclear YAP-immunopositivity was not exclusively detected in HCC, only minor rates were found in HCA and cirrhosis (Table 3). In these entities, nuclear YAP-immunoreactivity was additionally detected in less than half of the cells and staining intensity did not exceed a moderate level. Beside the findings in nYAP reactivity rates in the different entities, the distribution of pTAZ-immunopositivity rates in the different hepatocellular lesions is of importance. Congruent with the hippo signaling pathway sequence, immunopositivity rates of pTAZ exhibited an inverse pattern compared to nYAP. As phosphorylation of TAZ parallels the phosphorylation of YAP, it is part of the mechanism to preclude YAP-translocation to the nucleus and thereby limiting tumor promoting effects such as proliferation. In line with this concept, we detected lower rates of pTAZ-immunopositive cases in HCC compared to HCA, FNH and cirrhosis

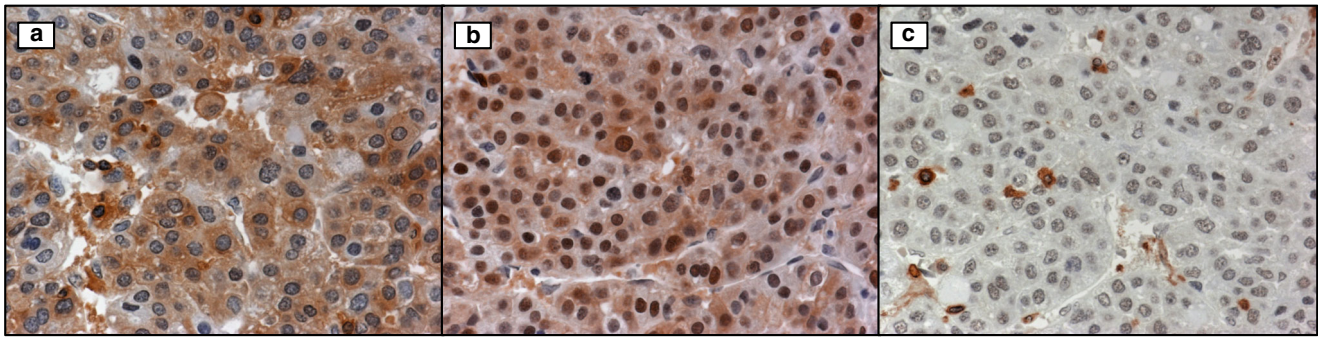

Fig. 2 Hippo-pathway target immunoreactivity in a case of HCC (Reg. #20). The tissue exhibits finely granular cytoplasmic MST1/2-reactivity (**a**), while a strong nuclear and moderate cytoplasmic YAP-immunopositivity is observed (**b**). The same case displays absent cytoplasmic pTAZ-reactivity (**c**). Note the distinct pTAZ-immunopositivity of some tumorous bystander cells. All images taken at 200x magnification

(Table 3). In fact, negative pTAZ immunostaining was a rare or absent event in HCA and FNH (one positive case each, 4 %) and in cirrhosis (0 %). Taken together, the tandem of a positive nuclear YAP-staining and negative immunoreaction for pTAZ strongly argues for an HCC and secondary for an HCA, i.e. it favors a monoclonal over a polyclonal hepatocellular lesion such as a FNH or cirrhosis-nodule.

In addition to the hippo signaling pathway's relevance in tumor promoting mechanisms, Yimlamai and colleagues recently reported of its acute in-vivo inactivation being sufficient for de-differentiating adult hepatocytes into cells with progenitor aspects [11]. For these and its further important biological effects, the hippo pathway and particularly YAP attracted interest as potential therapeutical targets with first favorable results for an inhibitor of YAP/TEAD-association ('verteporfin') in breast cancer cell lines [15].

However, although YAP is the final endpoint of all hippo-related pathway signaling, a number of different cellular inputs and pathways such as mTOR and RAS interact with hippo pathway kinases [4]. While in our cohort of HCC, YAP expression exhibited an association with GPC3 as a YAP target gene, additional associations were noted for MST1/2. For this kinase, associations with the mTOR-pathway, as expressed by pP70KS6, the MAPK/ERK pathway, as expressed by pERK and RASSF1a were noted. However, these associations are to be interpreted descriptively and no mechanistical conclusions can be drawn from these data. Additionally, the hippo pathway is subject to a variety of modifications including copy number variations (CNV), translocations and epigenetic silencing thus further complicating the interpretation of the data [4].

Another finding from our study is the association of MST1/2-immunopositivity with unfavorable clinicopathological parameters in HCC such as positive lymph node status, higher tumor cell grade (Table 2) and especially worse RFS (Fig. 1a). As we detected positivity of MST1/2 exclusively in HCC and neither in NT nor in HCA, FNH or cirrhosis, the effect seems to be of biologic relevance. Adherent to the logic of the hippo signaling pathway, high levels of MST1/2 should exert an inhibitory impact on nuclear YAP-translocation and thus a tumor attenuating effect [4, 5] – in contrast to clinico-pathological effects we detected. As we found MST1/2 to be significantly and positively associated with immunoreactivity of nuclear YAP ($p = 0.009$) and 70.6 % of MST1/2-positive HCC to exhibit nYAP-positivity, it might well be a quantitative effect or a functional impairment of MST1/2 has to be taken into consideration. However, recent results from Zheng and colleagues [23] indicate Wts (LATS1/2) but not Hpo (MST1/2) being required for intra-cellular cytoskeleton-mediated localization of Yki (YAP/Taz) and MAP4Ks as alternative MST1/2-like kinases in hippo-signaling. Additionally, prior to Zheng's publication, Li and colleagues did not detect any change in amount and activity of MST2 in human HCC versus normal liver tissue [22] and concluded that MST2 might not play a critical role in YAP-inactivation. Another example of hippo pathway independent MST signaling was recently described in T cells with MST1 deficiency leading to a loss of naïve T cells [24]. These new findings and the ongoing discussion about regulation of MST [25] point out the current ambiguity about MST1/2's significance and regulation and calls for further mechanistical studies.

In summary, we conducted an evaluation of hippo pathway signaling related proteins in different tumor forming hepatocellular liver lesions under consideration of clinco-pathological parameters and crosstalk to interacting signaling pathways. We detected adverse clinico-pathological parameters indicating an aggressive tumor-phenotype and dismal prognostic courses in MST1/2- and/or nuclear YAP-immunopositive cases with unfavorable RFS. Positive nuclear YAP-staining and negative pTAZ-immunoreactivity were strong indicators of a monoclonal hepatocellular lesion, primarily for an HCC and secondary for an HCA. The unexpected findings for MST1/2, exhibiting associations with unfavorable clinico-pathological characteristics, remain to be elucidated.

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