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Original Paper

Hepatocellular Carcinoma and Nuclear Paraspeckles: Induction in Chemoresistance and Prediction for Poor Survival

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Key Words
Liver cancer • Chemosensitivity • Chemotherapy • Therapy response • lncRNA • *MALAT1* • *NEAT2* • mRNA stability • mRNA decay • Actinomycin D

Abstract
Background/Aims: Hepatocellular carcinoma (HCC) represents the second most common cause of cancer-related deaths worldwide, not least due to its high chemoresistance. The long non-coding RNA nuclear paraspeckle assembly transcript 1 (*NEAT1*), localised in nuclear paraspeckles, has been shown to enhance chemoresistance in several cancer types. Since data on *NEAT1* in HCC chemosensitivity are completely lacking and chemoresistance is linked to poor prognosis, we aimed to study *NEAT1* expression in HCC chemoresistance and its link to HCC prognosis. ***Methods:*** *NEAT1* expression was determined in either sensitive, or sorafenib, or doxorubicin resistant HepG2, PLC/PRF/5, and Huh7 cells by qPCR. Paraspeckles were detected by immunostaining of paraspeckle component 1 (PSPC1) in cell culture and in a cohort of HCC patients. PSPC1 expression was correlated with clinical data. The expression of transcript

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variants of *NEAT1* and transcripts encoding the paraspeckle-associated proteins was analysed in the TCGA liver cancer data set. ***Results:*** *NEAT1* was overexpressed in all three sorafenib and doxorubicin resistant cell lines. Paraspeckles were present in all chemoresistant cells, whereas no signal was detected in the sensitive cells. Expression of *NEAT1* transcripts as well as transcripts encoding *PSPC1*, *NONO*, and *RBM14* was increased in tumour tissue. Expression of *PSPC1*, *NONO*, and *RBM14* transcripts was significantly associated with poor survival, whereas *NEAT1* expression was not. Immunohistochemical analysis revealed that nuclear and cytoplasmic PSPC1-positivity was significantly associated with shorter overall survival of HCC patients. ***Conclusion:*** Our data show an induction of *NEAT1* in HCC chemoresistance and a high correlation of transcripts encoding paraspeckle-associated proteins with poor survival in HCC. Therefore, *NEAT1, PSPC1, NONO,* and *RBM14* might be promising targets for novel HCC therapies, and the paraspeckle-associated proteins might be clinical markers and predictors for poor survival in HCC.

Introduction

Liver cancer – with its predominant form hepatocellular carcinoma (HCC) – represents the second most common cause of cancer-related death worldwide [1, 2]. Although improvements in the survival of patients with HCC have been achieved in the past two decades, the prognosis of HCC remains one of the worst amongst all cancers [2, 3]. One major issue regarding the therapy of HCC is chemoresistance: no effective conventional systemic chemotherapy for patients with advanced hepatocellular carcinoma has been established until now, resulting in poor prognosis of these patients [4]. The response rate for sorafenib, the only approved drug besides regorafenib for systemic treatment in the late stage of the disease, is below 3.5%. A systemic combination of sorafenib and doxorubicin also leads to poor response rates up to a maximum of 6% [5]. Chemoresistance is closely linked to a poor prognosis, not only in HCC, but also in other cancer types [6-8].

It is known that only 2% of the genome encodes for proteins, whereas the considerably larger part of the transcribed human genome consists of non-coding sequences [9]. One of these sequences is the long non-coding RNA nuclear paraspeckle assembly transcript 1 (*NEAT1*). *NEAT1* is located in nuclear paraspeckles [10], which are found in the nucleus' interchromatin space [11, 12]. The two transcript variants of *NEAT1* described in the literature are the short *NEAT1_v1* (*NEAT1ε*, 3.7 kb in length) and the long *NEAT1_v2* (*NEAT1-202*, *NEAT1β*, 23 kb in length). Ensembl lists four additional transcript variants (*NEAT1-201*, *203 – 205*) (Fig. 1). Although *NEAT1_v1* and *NEAT1-202/v2* transcript variants are essential for the integrity of paraspeckles, the long transcript variant is essential for *de novo* paraspeckle assembly [11, 12]. The biological role of paraspeckles is widely unknown, not least due to the lack of an altered phenotype in *Neat1* knock-out mice [13]. Recently, it has been suggested that *NEAT1* enhances chemoresistance in several cancer cell types [14]. Since data on *NEAT1* in HCC chemosensitivity are completely lacking, we aimed to study *NEAT1* expression as well as paraspeckle formation in HCC and in chemoresistant compared to chemosensitive HCC cell lines.

With the recently described effect of increased HCC cell proliferation induced by *NEAT1_v1/202/v2* in HCC cells [15, 16], we also sought to determine whether its expression might serve as a prognostic clinical marker.

Materials and Methods

Cell culture

HepG2, PLC/PRF/5, and Huh7 cells were cultured in RPMI-1640 medium with 10% fetal calf serum, 1% penicillin/streptomycin and 1% glutamine (Sigma-Aldrich, Taufkirchen, Germany) at 37°C and 5% CO_2. To induce and maintain chemoresistance, cells were regularly treated with either doxorubicin (Sigma-

Aldrich) or sorafenib (Biomol, Hamburg, Germany) as previously described [17]. Chemoresistance was regularly confirmed *via* IC50 determination by MTT assay. All cell lines were tested regularly for mycoplasma contamination and found negative. All cell lines were authenticated by the DSMZ (Braunschweig, Germany).

RNA stability was analysed using the transcriptional inhibitor actinomycin D as previously described [18, 19]. Huh7, PLC/PRF/5, and HepG2 cells treated for 0.5, 1, 2, and 4 hours with actinomycin D (10 µM) or DMSO as vehicle. RNA half-life of *NEAT1_v1/201/202/v2* transcripts analysed by qPCR.

RNA isolation and qPCR

Total RNA was extracted using Qiazol lysis reagent (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Residual genomic DNA was removed by treatment with DNase I (Ambion / Invitrogen, Carlsbad, California, USA). Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California, USA) as recommended by the supplier. Real-time quantitative polymerase chain reaction (qPCR) was performed in a CFX96 cycler (Bio-Rad, München, Germany) with 5× HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne, Tartu, Estonia). All samples were estimated in triplicate. The following primers were used (see also Fig. 1): *NEAT1_v1/202/v2* forward: TGCTACAAGGTGGGGAAGACTG; *NEAT1_v1/202/v2* reverse: CCCACACCCCAAACAAAACAA; *NEAT1_v1/201/202/v2* forward: CCCCTTCTTCCTCCCTTTAAC; *NEAT1_v1/201/202/v2* reverse: CCTCTCTTCCTCCACCATTAC; *NEAT1-202/v2* forward: TTTCAAAGGGAGCAGCAAGGG; *NEAT1-202/v2* reverse: ACGGCACAGGCAAATAAGACAC. Annealing temperature was 60°C for *NEAT1_v1/202/v2* and *NEAT1_v1/201/202/v2,* and 64°C for *NEAT1-202/v2*. Primer concentrations for all three were 0.25 µM. Efficiency for each experiment was determined using a dilution series. Gene expression was normalised to *ACTB* mRNA values.

Immunofluorescence

Cells were grown on coverslips overnight and fixed with 4% paraformaldehyde for 15 min on ice. After permeabilisation with 1% Triton X 100 for 15 min, unspecific binding was blocked using a combination of 2% bovine serum albumin and 10% FCS for 1.5 h. The cells were incubated with paraspeckle component 1 (PSPC1) antibody (1:20 dilution; sc-374181, Santa Cruz, CA, USASA) overnight at 4°C. After washing, the secondary antibody, goat anti-mouse AlexaFluor 488 (Invitrogen, Karlsruhe, Germany), was added for 1.5 h at room temperature. After washing and adding DAPI (Sigma-Aldrich) for nuclear staining, coverslips were mounted with FluorSave™ (Calbiochem, Sandhausen, Germany). Images were obtained and analysed with an Axio Observer Z1 epifluorescence microscope equipped with an AxioCam Mrm (Zeiss, Oberkochen, Germany). All cell images were obtained using either a 63x objective (for Huh7 and PLC/PRF/5 cells), or a 100x objective (for HepG2 cells). Data were obtained and analysed using the AxioVision software (Zeiss).

TCGA data

RNAseq expression data were obtained from The Cancer Genome Atlas pan cancer dataset produced *via* Toil [20]. RSEM [21] reported transcripts per million values were downloaded *via* the UCSC Xena Browser (https://xenabrowser.net) and comprised 369 primary solid tumour as well as 50 matched non-tumour tissue samples for gene expression. Only transcript variants with an average expression rate of log2(TPM + 0.001) > 0 were considered for analysis in the R statistical environment (v. 3.4.2). For survival analysis we considered two groups (split at 50% quantile) and three groups (split at 25% and 75% quantiles), respectively. Significance of differences between survival curves was computed with a log rank test using the survival R package (Therneau and Grambsch, 2000). Further, we determined pairwise Pearson correlation of transcript expression and plotted them using the *corrplot* R package [22].

Hepatocellular carcinoma patients and tissue samples

One hundred sixty hepatocellular carcinomas (HCCs), from which the medical records, the histologic slides, and paraffin tissue blocks were available, were included into this study. The HCC patients underwent radical resection in the Chonbuk National University Hospital between January 1998 and August 2009. The HCC cases were reviewed and classified according to the criteria of the World Health Organization Classification [23] and the staging system of the American Joint Committee on Cancer [24]. This study obtained institutional review board approval from the Chonbuk National University Hospital, and the requirement for informed consent was waived (IRB number, CUH 2018-03-002).

Immunohistochemical staining and scoring of tissue microarrays

Immunohistochemical staining was performed on tissue microarray (TMA) sections with one 3.0 mm core per HCC cases. Antigen retrieval for the tissue sections was performed in a microwave oven for 12 minutes in Dako Target Retrieval Solution (pH 6.0, DAKO, Glostrup, Denmark). A primary antibody for PSPC1 in a 1:200 dilution (ThermoFisher Scientific, Rockford, IL, USA) was used for immunohistochemical detection. Immunohistochemical scoring for PSPC1 was performed by two pathologists (KYJ and KMK) with consensus for the cytoplasmic and nuclear expression of PSPC1. The scoring was performed without information of the clinicopathological factors of HCC patients. The immunohistochemical score was obtained by adding the intensity scores (0, no staining; 1, weak staining; 2, intermediate staining; 3, strong staining) and the area scores (0, no stained cells; 1, 1% positive; 2, 2-10% positive; 3, 11-33% positive; 4, 34-66% positive; 5, 67-100% positive) [25-27]. The sum score ranged from zero to eight. Subsequently, receiver operating characteristic curve analysis was performed to estimate death of HCC patients by determining positivity for cytoplasmic and nuclear expression of PSPC1 [26, 27].

Statistical analysis

Data analysis and statistics were performed with OriginPro 8.6G (OriginLab Corporation, Northampton, USA) and SPSS software (IBM, version 20.0, CA). Values were expressed as box plots with 25th/75th percentile boxes, geometric medians (line), means (square), and 10th/90th percentile as whiskers. Statistical differences were calculated using an independent two-sample t-test or Mann-Whitney test as indicated depending on whether the data were normally distributed. The survival analysis of TMAs was performed for the overall survival (OS) and relapse-free survival (RFS). The follow-up end point was June 2013. In OS analysis, the event was death of patients by HCC, and the patients who were alive at last contact or died from other causes were treated as censored. In RFS analysis, the event was relapse of HCC and death of patients from HCC. The patients who were alive at last contact without relapse of the tumor or died from other causes were treated as censored. Univariate and multivariate Cox proportional hazards regression analyses, Kaplan-Meier survival analysis, and Pearson's chi-square test were used. The *P* values less than 0.05 were considered statistically significant.

Results

To determine a possible role of *NEAT1* in HCC chemoresistance, we established human hepatoma cells (HepG2, PLC/PRF/5 and Huh7) resistant to the chemotherapeutics sorafenib or doxorubicin [17]. We measured *NEAT1* expression in these cells using three primers denoted as *NEAT1_v1/202/v2, NEAT1_v1/201/202/v2*, and *NEAT1-202/v2* (Fig. 1, Supplementary Table S1 - all supplementary material available online at www.cellphysiolbiochem.com). *NEAT1_v1/202/v2* was significantly overexpressed in all three sorafenib and doxorubicin resistant cell lines (Fig. 2A, B). In addition, sorafenib resistant HepG2 and Huh7 cells as well as doxorubicin resistant PLC/PRF/5 and Huh7 cells showed an increased expression of *NEAT1_v1/201/202/v2* and *NEAT1-202/v2* (Fig. 2A, B).

NEAT1-202/v2 was found to be essential for *de novo* paraspeckle assembly [28]. Due to the overexpression of *NEAT1-202/v2,* we hypothesized that chemoresistant hepatoma cell lines have an increased paraspeckle formation. Therefore, cells were stained for paraspeckle component 1 (PSPC1). In fact, positive signals were detected in all chemoresistant cells, whereas no signal could be detected in the control cells (Fig. 3). The most intense paraspeckle staining was observed in Huh7 cells, which showed the highest *NEAT1-202/v2* expression (Fig. 2A, B). Our data suggest that doxorubicin resistance induces paraspeckle formation slightly more intensely compared to sorafenib resistance, which is in accordance with the higher *NEAT1-202/v2* RNA expression in doxorubicin resistant PLC/PRF/5 and Huh7 cells (Fig. 2B, 3). PLC/PRF/5 and Huh7 cells showed no differences regarding this aspect (Fig. 3).

Our data show a distinct connection of chemoresistance, *NEAT1* induction, and paraspeckle formation in three different HCC cell lines. Accordingly, two recently published papers described that *NEAT1_v1/202/v2* knockdown resulted in elevated apoptosis and reduced viability / proliferation in different HCC cell lines [15, 16]. Since our data indicate

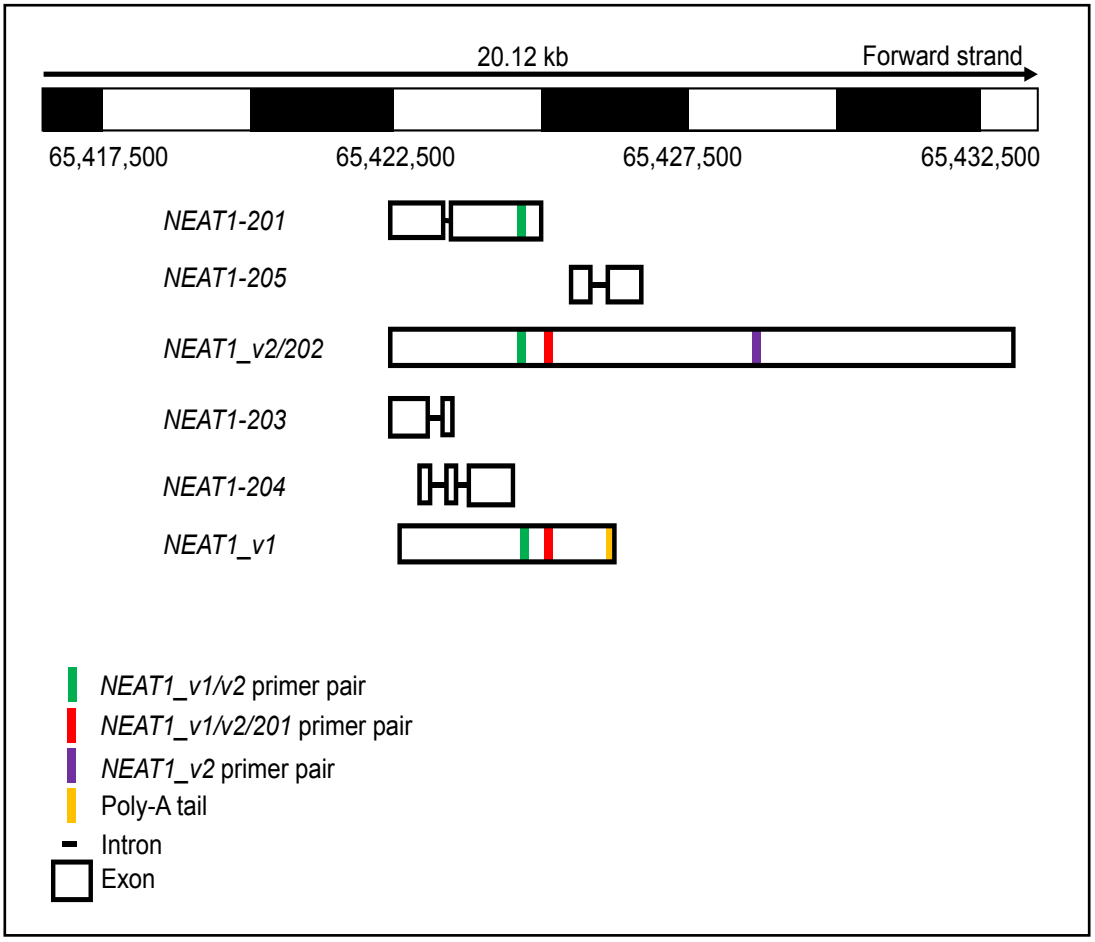

Fig. 1. *NEAT1* gene locus. Size and location of *NEAT1* transcript variants (Supplementary Table S1) on the *NEAT1* gene locus on chromosome 11 (schematic). Coloured areas indicate regions amplified in the respective qPCR reaction. Scheme is not drawn to scale.

that chemosensitive cell lines do not form paraspeckles, one might speculate that *NEAT1* might also act independently of paraspeckles [29].

Since previous studies had reported elevated *NEAT1* in HCC in samples from up to 95 HCC compared to non-tumour liver tissues – as assessed by qPCR [30, 31], we hypothesised that *NEAT1* expression might serve as a prognostic marker for HCC patients. To test this hypothesis, we first analysed the expression of total *NEAT1* (sum of all variants *201-205*) as well as the individual transcript variants (Fig. 1) in the TCGA dataset comprising 369 HCC tissues and 50 non-tumour tissues. We could confirm that the expression of total *NEAT1* was significantly increased in the tumour tissues as were the transcript variants *NEAT1-201, NEAT1-202/v2,* and *NEAT1-205*, while the expression of *NEAT1-203* and *NEAT1-204* was below the threshold (Fig. 4A; Supplementary Table S2).

Interestingly, though, there was no significant association with survival for any of the differentially expressed *NEAT1* transcripts (Table 1; Supplementary Fig. S1).

With *NEAT1* representing a critical component of paraspeckles, we wondered whether the expression of genes encoding paraspeckle proteins was upregulated in HCC. For that, we analysed the expression of transcripts encoding *PSPC1*, non-POU domain containing octamer binding protein (*NONO*), and RNA-binding motif protein 14 (*RBM14*) in the TCGA samples. All transcripts described in Ensembl release 91 [32] were analysed, whereby only transcripts reaching the threshold mean expression of log2(TPM + 0.001) > 0 were taken into consideration (Supplementary Table S2).

We found that the expression of transcripts encoding for all three paraspeckle proteins was significantly elevated in HCC. For *PSPC1*, this was true for both protein-coding transcripts *201* and *206* as well as for the non-coding transcripts *203* and *204* (Fig. 4B). Additionally, the expression of the protein coding *NONO* transcripts *201* and *202* as well as the non-coding processed transcripts *210* and *211* were significantly elevated in HCC (Fig. 4C). For *RBM14*, the two protein-coding transcripts *201* and *202* were significantly elevated (Fig. 4D). Most interestingly, there was a significant association with poor prognosis for transcript variants of all three paraspeckle proteins (Table 1; Supplementary Fig. S2-4).

Except for *RBM14-202*, expression levels of all protein-coding and non-coding paraspeckle associated transcripts showed distinct correlations with each other (Fig. 5).

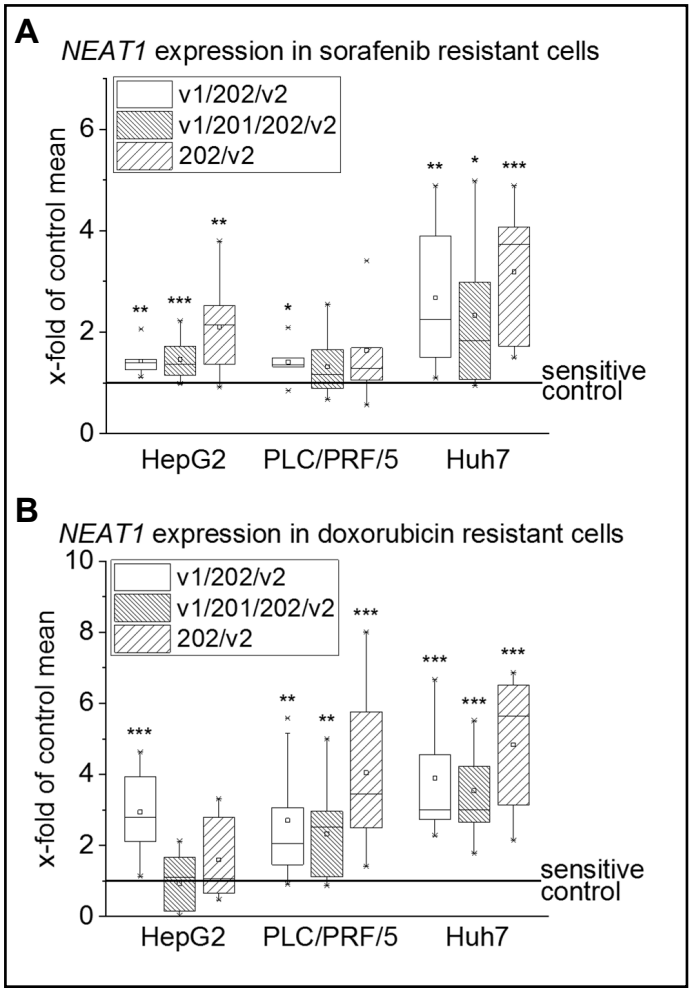

Fig. 2. *NEAT1* expression in chemoresistant hepatoma cell lines. *NEAT1_v1/202/v2*, *NEAT1_v1/201/202/v2*, and *NEAT1-202/v2* expression determined by qPCR in sorafenib resistant (A) and doxorubicin resistant (B) cells (n = 3, triplicates). Data are shown as x-fold with sensitive control cells set to 1. Results of independent two-sample t-test or Mann-Whitney test: *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$.

In order to confirm association of PSPC1 with prognosis on protein level, immunohistochemistry using an additional HCC cohort consisting of 160 patients was performed. In human HCC tissue samples, PSPC1 was expressed in both the nucleus and the cytoplasm of cancer cells (Fig. 6A). The cut-off points of immunohistochemical scores for the cytoplasmic and nuclear expression of PSPC1 were eight and seven, respectively (Fig. 6B, Table 2). Cytoplasmic expression of PSPC1 was considered positive if the score was eight. Nuclear expression of PSPC1 was considered positive if the score was equal or greater than seven (Fig. 6B, Table 2). With these cut-off points, 48% (77/160) of HCCs were classified as nuclear PSPC1 positive and 15% (24/160) of HCCs were classified as cytoplasmic PSPC1 positive. Nuclear and cytoplasmic PSPC1 positivity were significantly associated with shorter OS and relapse-free survival (RFS) of HCC patients (Fig. 6C and Supplementary Table S3).

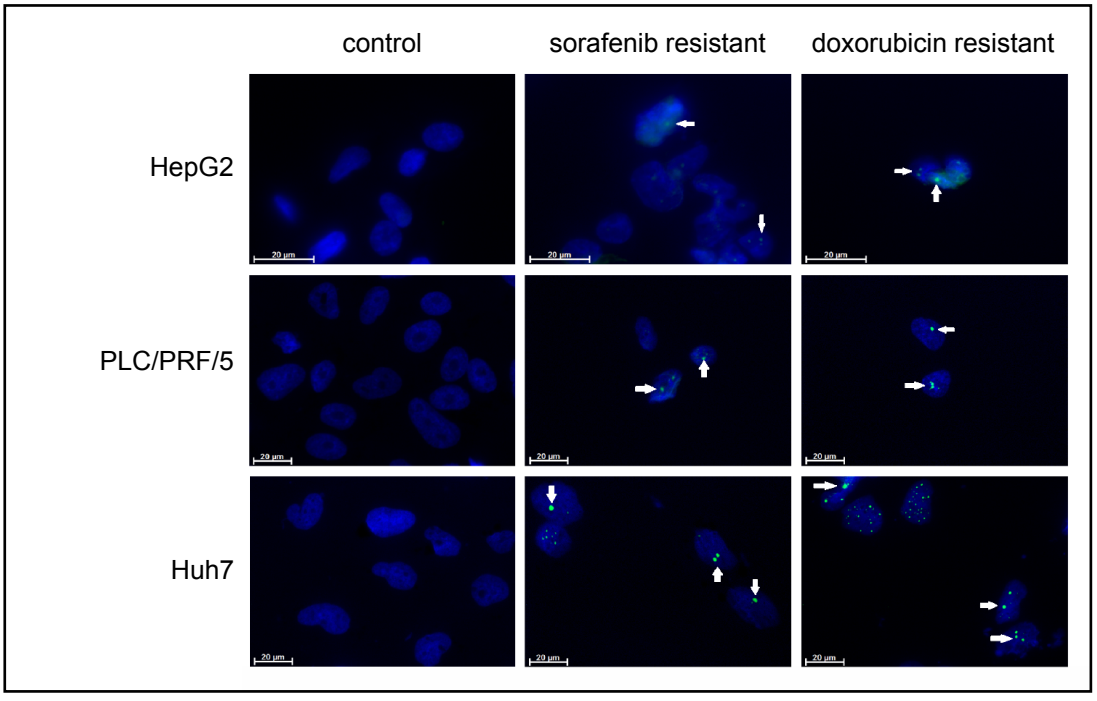

Fig. 3. Paraspeckle formation in chemoresistant human hepatoma cells. Figure shows a representative image of doxorubicin and sorafenib resistant HepG2, PLC/PRF/5 and Huh7 cells. DAPI: blue, PSPC1: green. Arrows show PSPC1 positive signals. Scale bars = 20 µm.

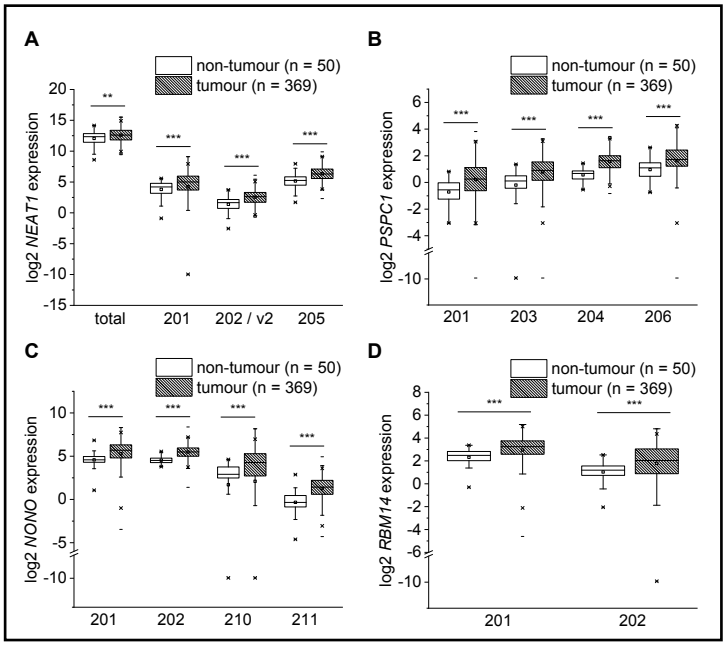

Fig. 4. *NEAT1, PSPC1, NONO,* and *RBM14* expression in human liver tissue. Expression of total *NEAT1* and its transcript variants (A) and the transcript variants of *PSPC1* (B), *NONO* (C), and *RBM14* (D) in human tumour and non-tumour tissue from a TCGA data set. The figure only shows the transcript variants with an average expression rate of log2(TPM + 0.001) > 0. Results of independent two-sample t-test or Mann-Whitney test: **: $p \leq 0.01$; ***: $p \leq 0.001$.

Nuclear PSPC1-positivity further correlated with the absence of liver cirrhosis (Table 3).

Beside parameters, which are commonly known to be associated with survival of HCC patients, such as age, serum AFP levels, and tumor stage, nuclear expression of PSPC1 was significantly associated with survival of HCC patients in a multivariate analysis (Table 4). Nuclear expression of PSPC1 indicated a 1.978-fold (95% confidence interval; 1.252-3.125) greater risk in OS and a 1.566-fold (95% confidence interval; 1.069-2.295) greater risk in RFS (Table 4).

Discussion

An implication of *NEAT1* in the process of chemoresistance was previously described in human breast cancer MCF-7, neuroblastoma NGP, colon carcinoma HCT116, osteosarcoma U2OS cells, and renal cell carcinoma [14, 33]. Our study is the first to implicate *NEAT1* in HCC chemoresistance: We find the variants *NEAT1_v1* and *NEAT1-202/v2* to be significantly overexpressed in HepG2, PLC/PRF/5, and Huh7 cells that have developed resistance against either sorafenib or doxorubicin.

Table 1. Kaplan Meier survival analysis according to the expression level of the respective transcript. Column 3: Patients were stratified into two groups, i.e. expression was above or below the median expression (≤ 50% vs > 50% quantile). Column 4: Patients were stratified into three groups, i.e. expression was low (below 25% expression quantile), high (above 75% expression quantile), or intermediate (>25% and <75% expression quantile). p-values ≤ 0.05 were considered as significant and are printed in bold letters. (TPM + 0.001) > 0. p-values ≤ 0.05 were considered as significant and are printed in bold letters

Gene	Transcript variant	p-value ≤ 50% vs > 50% quantile	p-value > 25% & ≤ 75% vs ≤ 25% vs > 75% quantile
NEAT1	201	0.1	0.25
	202 / v2	0.53	0.19
	205	0.88	0.25
PSPC1	201	0.4	0.4
	203	0.0022	0.0092
	204	0.075	0.16
	206	0.026	0.071
NONO	201	0.055	0.099
	202	0.00019	0.00072
	210	0.065	0.18
	211	0.022	0.068
RBM14	201	0.0025	0.007
	202	0.16	0.2

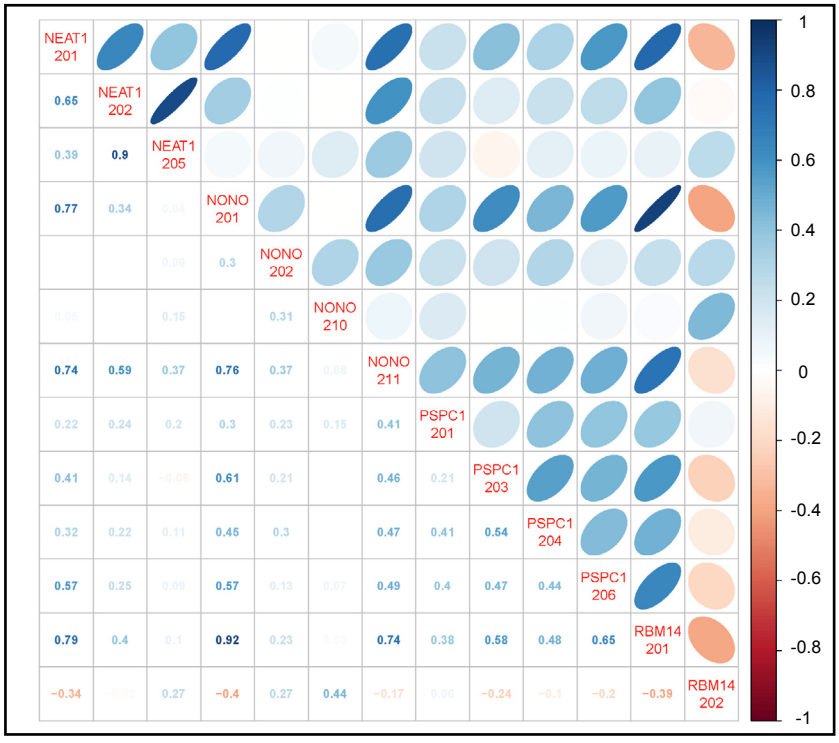

Fig. 5. Pairwise correlation of transcript variants with an average expression rate of log2(TPM + 0.001) > 0 of *NEAT1, PSPC1, NONO*, and *RBM14*. The lower triangle indicates correlation coefficients numerically, whereas the upper triangle depicts them as ellipsoids. Blue indicates positive and red negative correlation as reflected by the colour legend on the right-hand side.

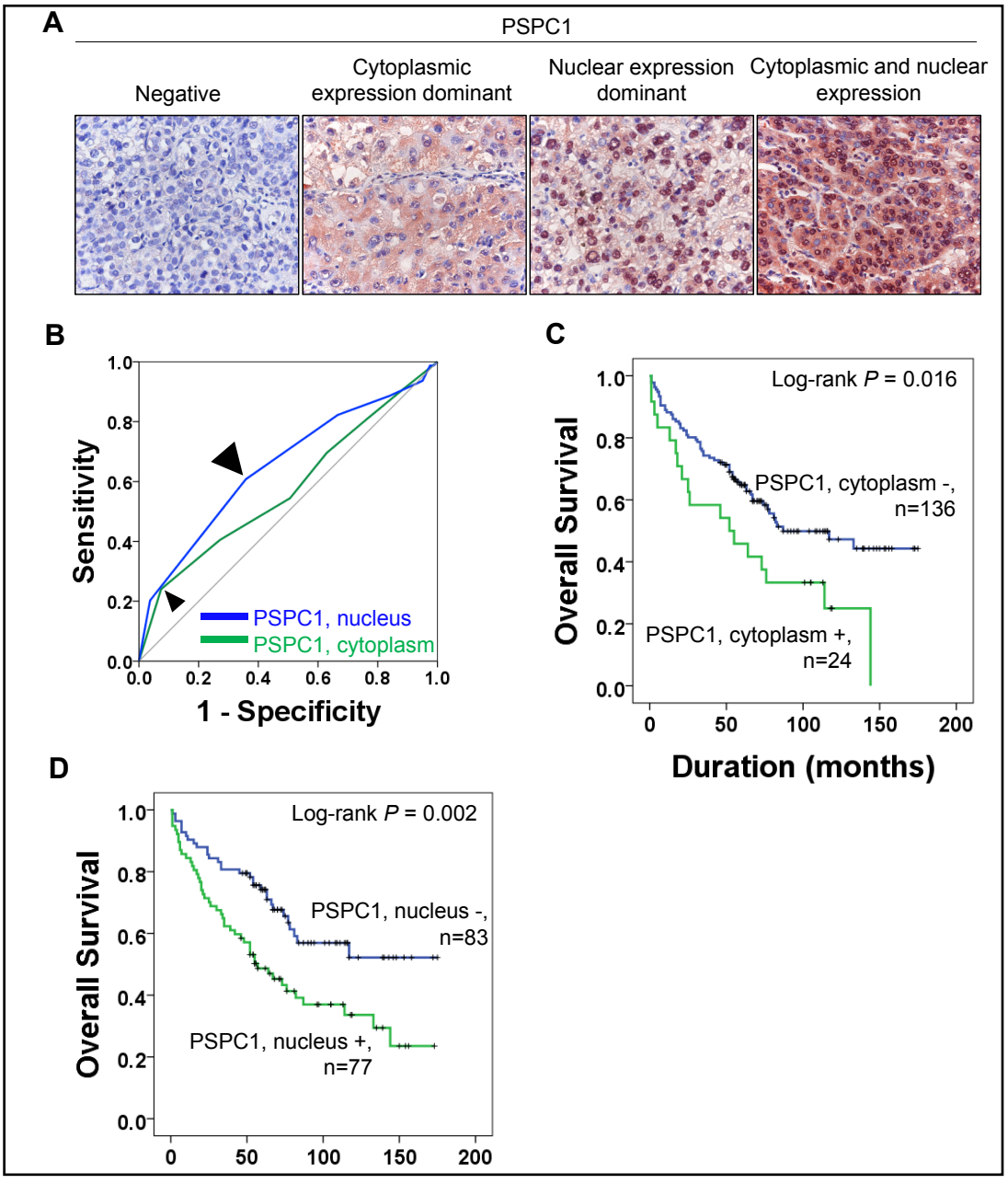

Fig. 6. Immunohistochemical detection of PSPC1 and survival analysis in 160 hepatocellular carcinoma samples. A. Representative pictures of immunohistochemical staining of PSPC1 regarding cytoplasmatic and nuclear staining of tumor cells. B. Statistical analysis to determine cut-off points for cytoplasmic and nuclear expression of PSPC1 with receiver operator characteristic curve analysis. The points with the highest area under the curve (AUC) were cut-off points for cytoplasmic and nuclear PSPC1 expression. C. Kaplan-Meier survival analysis according to cytoplasmic PSPC1 expression and nuclear PSPC1 expression for the overall survival and relapse-free survival.

Although it is well established that *NEAT1* plays an important role in paraspeckle formation [28], details on the function of paraspeckles are as yet unknown. However, it is assumed that they locate RNAs inside the nucleus and are implicated in the reprogramming of a cell that takes place with differentiation. This may happen due to the inhibition of the expression of key proteins *via* nuclear RNA retention [34]. Paraspeckles were also reported to contribute to tumourigenesis by inhibiting DNA damage-induced cell death [35]. Although paraspeckles have been described in diverse cell lines [13], to our knowledge, our report is the first one showing paraspeckles and their induction in liver cells. *NEAT1-202/v2* – which constitutes paraspeckles – was elevated in nearly all of the analysed chemoresistant hepatoma cells. For cell lines showing induction of *NEAT1_v1/202/v2* but not of *NEAT1-202/v2* and *NEAT1_v1/201/202/v2* RNA, an elevated expression of *NEAT1_v1* seems probable. However, this assumption cannot be ultimately confirmed due to the lack of a unique sequence of *NEAT1_v1* (Fig. 1). Although *NEAT1_v1* cannot induce nuclear body formation by itself [28], it was suggested to increase the number of paraspeckles [11]. Another hypothesis is that *NEAT1_v1* localises in non-paraspeckle foci (so called 'microspeckles'), which may carry paraspeckle-independent functions [35].

Our findings indicate an elevated expression of *NEAT1_v1* in doxorubicin resistant HepG2 cells compared to sorafenib resistant HepG2 cells. Since paraspeckles were also detected in doxorubicin resistant HepG2 cells, which showed no increased expression of *NEAT1-202/v2*, a positive association between *NEAT1_v1* and the number of paraspeckles - as suggested previously [13] – seems to be likely. These results are consistent with previous findings on a link between *NEAT1_v1/202/v2* and *NEAT1-202/v2* expression and chemoresistance in several other tumour types [14] and extend them towards chemoresistance in HCC.

lncRNA *MALAT1/NEAT2*, which is located adjacent to the gene locus of *NEAT1,* was reported to have a similar role in chemoresistance [36-38]. Thus, a similar mechanism between the two lncRNAs has been suggested [39]. Interestingly, we observed strong correlations between *NEAT1* and *MALAT1* transcripts in the TCGA data set (Supplementary Fig. S5), suggesting further research in the connection of those genes.

Table 2. Cut-off points for cytoplasmic and nuclear expression of PSPC1. Statistical analysis to determine cut-off points with receiver operator characteristic curve analysis. The points with the highest area under the curve (AUC) were cut-off points for cytoplasmic and nuclear PSPC1 expression (Fig. 6B)

Protein staining	Cut-off	AUC	*P*
PSPC1, cytoplasm	All	0.575	0.100
	≥ 8	0.583	0.069
PSPC1, nucleus	All	0.652	< 0.001
	≥ 7	0.625	0.006

Table 3. The association between clinicopathologic variables and the expression of PSPC1. AFP, a-fetoprotein; HBV, hepatitis B virus; HCV, hepatitis C virus

Characteristics		No.	PSPC1, nuclear		PSPC1, cytoplasm	
			Positive	P	Positive	P
Sex	Male	139	71 (51%)	0.054	23 (17%)	0.159
	Female	21	6 (29%)		1 (5%)	
Age, y	< 55	63	32 (51%)	0.586	7 (11%)	0.267
	≥ 55	97	45 (46%)		17 (18%)	
AFP, ng/mL	< 100	105	49 (47%)	0.61	15 (14%)	0.727
	≥ 100	55	28 (51%)		9 (16%)	
HBV	Negative	42	20 (48%)	0.939	10 (24%)	0.063
	Positive	118	57 (48%)		14 (12%)	
HCV	Negative	150	71 (47%)	0.438	20 (13%)	0.022
	Positive	10	6 (60%)		4 (40%)	
Liver cirrhosis	Absence	84	47 (56%)	0.037	15 (18%)	0.287
	Presence	76	30 (39%)		9 (12%)	
Bilirubin, mg/dl	< 0.7	73	35 (48%)	0.967	7 (10%)	0.079
	≥ 0.7	87	42 (48%)		17 (20%)	
Albumin, ng/dl	< 3.5	21	10 (48%)	0.96	4 (19%)	0.577
	≥ 3.5	139	67 (48%)		20 (14%)	
Tumor stage	I	59	23 (39%)	0.165	9 (15%)	0.155
	II	63	32 (51%)		6 (10%)	
	III & IV	38	22 (58%)		9 (24%)	
Histologic grade	Low	101	46 946%)	0.393	16 (16%)	0.696
	High	59	31 (53%)		8 (14%)	
PSPC1, cytoplasm	Negative	136	55 (40%)	< 0.001		
	Positive	24	22 (92%)			

Table 4. Multivariate Cox regression survival analysis in 160 hepatocellular carcinoma patients. HBV, hepatitis B virus; HCV, hepatitis C virus; AFP, α-fetoprotein; HR, hazard ratio. * Variables considered in multivariate analysis were age of the patients, AFP levels, albumin levels, TNM stage, histologic grade, nuclear expression of PSPC1, and cytoplasmic expression of PSPC1

Characteristics	OS		RFS	
	HR (95% CI)	P	HR (95% CI)	P
Age, ≥ 55 (vs < 55)	1.650 (1.014-2.685)	0.044	1.645 (1.093-2.476)	0.017
AFP ≥ 100 ng/ml (vs < 100 ng/ml)	1.574 (0.990-2.502)	0.055	1.795 (1.208-2.668)	0.004
Tumor stage, I	1	0.001	1	< 0.001
II	1.413 (0.801-2.494)	0.232	1.336 (0.838-2.131)	0.224
III & IV	2.760 (1.553-4.904)	< 0.001	2.524 (1.537-4.145)	< 0.001
PSPC1 nuclear positive (vs negative)	1.978 (1.252-3.125)	0.003	1.566 (1.069-2.295)	0.021

Beside the effect of *NEAT1* on chemoresistance, it was recently reported that knockdown of *NEAT1* increased apoptosis and reduced both viability and proliferation in different HCC cell lines [15, 16]. Since we could not detect any paraspeckles in untreated, chemosensitive HCC cells, *NEAT1* may also have additional, paraspeckle-independent functions related to cell viability [29]. Recent reports suggest *NEAT1* to promote proliferation and metastasis by different mechanisms: targeting microRNAs as a competing endogenous RNA (ceRNA) / molecular sponge [40-42] and interacting with the transcription factor CDC5L [43] or the histone methyltransferase EZH2 [44].

NEAT1 expression has been shown to be upregulated in different human malignancies, including lung cancer, colorectal cancer, prostate cancer, breast cancer, and HCC [45]. However, the differentiation between the transcript variants of *NEAT1* to our knowledge was never reported. We therefore decided to analyse the expression of the transcript variants of *NEAT1* in human HCC tissue and were able to demonstrate that the transcript variants *NEAT1-201*, *NEAT1-202/v2,* and *NEAT1-205* are elevated in HCC tissue in several hundred tumour samples compared to normal tissues. We were also able to confirm previous findings, in which an increased expression of *NEAT1* for up to 95 HCC *vs*. non-cancerous liver tissues – as assessed by qPCR and primers detecting *NEAT1_v1/202/v2* – was reported [30, 31]. Although *NEAT1_v1/202/v2* expression has been suggested to be associated with poor prognosis in breast cancer, lung cancer, oesophageal squamous cell carcinoma, colorectal cancer, and HCC [16, 46-48], we could not confirm a significant association of the expression of *NEAT1* transcripts with survival in HCC in this study. Interestingly, though, there are also reports that *NEAT1* is downregulated in acute promyelocytic leukaemia [49].

Although the elevated expression of *NEAT1* transcripts suggests *NEAT1* as a clinical marker for HCC, there is one major issue regarding this aspect: the stability of *NEAT1*. In fact, a study performing a genome-wide analysis of long non-coding RNA stability found *Neat1* to be "one of the least stable lncRNAs" [50]. We could confirm instability of *NEAT1* in HepG2, Huh7, and PLC/PRF/5 cells by employing the transcriptional inhibitor actinomycin D resulting in a maximum half-life of 4 hours (Supplementary Fig. S6), which is substantially lower than that of other lncRNAs, such as *H19*, the half-life of which is over 10 hours [51]. Hence, expression analysis of *NEAT1* in clinical practice may lead to incorrect results, while our data show that PSPC1 as a marker for *NEAT1*-dependent paraspeckle formation [28] may be a more valid marker for HCC prognosis. In addition, histological stainings represent an established diagnostic and prognostic technique in clinical practice worldwide. PSPC1 staining intensity was recently correlated with poor survival in different tumor types [52]. In the latter study the authors did not distinguish between nuclear and cytoplasmatic staining, though. In our HCC cohort, nuclear PSPC1 positivity was associated with the absence of liver cirrhosis and cytoplasmatic positivity was associated with HCV infection. Interestingly, hepatitis *delta* virus (HDV) replication was shown to induce translocation of PSPC1 protein from the nucleus to the cytoplasm. To our knowledge, no respective studies have been performed for HCV. Translocation of PSPC1 to the cytoplasm has been reported to coincide with enhanced RNA export during adipocyte differentiation [53]. Other members of the

Drosophila behavior/human splicing (DBHS) protein family have been described to function cytoplasmatically [54, 55], thereby affecting chemosensitivity [55]. In summary, differences in nuclear and cytoplasmatic PSPC1 expression seem to be related to the cause of HCC and should be investigated in more detail in future studies.

In addition to *NEAT1* and PSPC1 protein, we also found transcripts of the paraspeckle-associated proteins *PSPC1*, *NONO*, and *RBM14* to be upregulated in HCC compared to non-cancerous liver tissue. All of these proteins have been reported to be associated with chemoresistance in different cancer cells [35, 56, 57].

Conclusion

In conclusion, a detailed analysis of paraspeckle function in HCC might reveal *NEAT1* and paraspeckle-related proteins as as clinical prognostic markers for HCC and potentially also as promising targets for the development of novel HCC therapies.

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Disclosure Statement

No conflicts of interest, financial or otherwise, are declared by the authors.

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