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Expression and functional role of inhibitor-of-apoptosis protein livin (BIRC7) in neuroblastoma

Anindya Dasgupta [a], Carlos S. Alvarado [a], Zhiheng Xu [b], Harry W. Findley [a,*]

[a] Department of Pediatrics, Emory University School of Medicine, Atlanta, GA 30322, USA
[b] Department of Biostatistics and Bioinformatics, Winship Cancer Institute, Rollins School of Public Health, Emory University, Atlanta, GA 30322, USA

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ABSTRACT

We evaluated the expression of the inhibitor-of-apoptosis protein (IAP) livin (BIRC7) in 59 cases of neuroblastoma (NBL) by quantitative RT-PCR. We also examined the role of livin in protecting tumor cells from chemotherapy drugs. Livin expression varied significantly among tumors. High levels of expression were observed in 17 of 39 patients with advanced stages (stages 3 and 4) and 6 of 20 patients with localized stages (stages 1 and 2). Livin-transfected, *MYCN*-amplified NBL cells showed increased resistance to doxorubicin and etoposide. Conversely, livin knockdown with siRNA enhanced spontaneous and drug-induced apoptosis in NBL cells. Multivariate analysis of prognostic factors showed that high livin expression worsened prognosis for patients with *MYCN*-amplified tumors. Our data suggest that (i) livin is frequently expressed in NBL and protects tumor cells *with amplified MYCN oncogene* from genotoxic agents; (ii) the antiapoptotic effect of livin in NBL is blocked by siRNA; (iii) in the sample studied, high livin expression enhanced the adverse prognostic impact of *MYCN* amplification. These findings suggest that livin may contribute to drug resistance in NBL.

1. Introduction

Livin is a 39 kDa inhibitor-of-apoptosis protein (IAP) composed of a single Baculovirus IAP-Repeat (BIR) domain and a zinc-binding RING-domain. The antiapoptotic activity of livin is mediated via inhibition of caspase-3, -7, and -9, as well as by its E3 ubiquitin-ligase-like activity which promotes degradation of Smac/DIABLO, a critical endogenous regulator of all IAP's [1–3]. Splicing of the gene in exon-6 yields two isoforms, α and β, which differ in size and antiapoptotic properties. Both splicing variants are detected in some fetal and adult tissues and in the majority of livin-expressing tumors [4–6].

Aberrant livin expression has been demonstrated in tumor cell lines and human neoplasms, including the neural-crest-derived tumors melanoma and neuroblastoma (NBL) [4,7–14]. In most tumors, the presence or expression level of livin correlated with *in vitro* drug resistance, advanced tumor stages, and poor outcome [4,13,14]. Antisense oligonucleotides and small interference RNA (siRNA) molecules have been shown to downregulate livin expression and its antiapoptotic activity in melanoma and epithelial tumor cells lines [15,16]. Together, these data suggest that livin may be essential for survival of some cancer cells.

NBL is the most common extracranial malignant solid tumor in children, accounting for 7% of all pediatric malignancies. Age, stage and *MYCN* oncogene status at diagnosis are well-established prognostic factors. Children older than 12 months at diagnosis typically present with metastatic, MYCN-amplified tumors which often prove resistant to aggressive, multimodal therapy [17]. However, multicenter NBL studies [18,19] have revealed patients with treatment outcomes inconsistent with those clinical and biological features. While ongoing cooperative trials may shed light on the prognostic impact of additional cytogenetic or molecular characteristics of the tumor [20], an increasing body of evidence linking livin and other IAP's to drug resistance suggests that further studies of livin in this tumor are warranted.

In our previous study [14], we demonstrated livin expression by immunohistochemistry (IHC) in 80% of patients with NBL and all six NBL cell lines studied. Because evaluation of staining intensity by IHC is inherently subjective, the primary aims of this follow-up study were (a) to assess livin expression in NBL tumor tissue by RT-PCR (qRT-PCR) and immunoblot, as these are more quantifiable and reproducible methods; (b) to assess the functional role of livin expression using gene-overexpression and silencing methods, respectively; and (c) to analyze the potential prognostic significance of livin in this childhood tumor.

* Corresponding author. Address: Department of Pediatrics, Emory University School of Medicine, 2015 Uppergate Drive, Atlanta, GA 30322, USA. Fax: +1 770 274 5565.
E-mail address: harry.findley@oz.ped.emory.edu (H.W. Findley).

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2. Materials and methods

2.1. Patients

The study analyzed livin expression in tumor tissue from 59 children with NBL diagnosed and treated at the Children's Healthcare of Atlanta (Henrietta Egleston and Scottish Rite Hospitals) from November 1993 through February 2008. Forty-seven children (80%) were 12 months of age or older at diagnosis. Clinical stages, determined according to the International Neuroblastoma Staging System [21], were stage 1 in 14 patients, stage 2 in 6 patients, stage 3 in 14 patients, and stage 4 in 25 patients. MYCN oncogene data was available in all cases and tumor histology by Shimada criteria in 44 (75%) cases. *Patients at diagnosis and relapse were treated according to relevant* Pediatric Oncology Group (POG) and Children's Oncology Group (COG) protocols. Median follow-up was 48 months.

2.2. Methods

2.2.1. Neuroblastoma tumor tissue and cells lines

Snap-frozen tumor tissue from 39 patients and archival, paraffin-embedded tumor tissue from 20 patients were available for study. Tumor samples were obtained at the time of diagnosis in all cases. An IRB-approved, informed consent for use of tumor tissue was obtained from parents. All specimens were coded prior to analysis. NBL cells lines IMR32, LA155n, and SH-SY5Y were obtained from American Tissue Type Culture Collection (Rockville, MD). IMR32 and LA155n are *MYCN*-amplified; SH-SY5Y is *MYCN*-non-amplified. LA155n is a doxorubicin-resistant line that expresses high levels of endogenous livin together with amplified *MYCN*.

2.2.2. RNA extraction from primary and archival tumor

RNA from snap-frozen tumor tissue was isolated using RNA extraction reagent (UltraspecRNA, Biotecx, Houston, TX) following the manufacturer's recommendations. In brief, tumor was homogenized in a Rotor–Stator homogenizer (Fisher Scientific, Waltham, MA) with RNA extraction reagent at 4 °C and RNA was purified. RNA from archival, paraffin-embedded tumor was isolated from 25-μm-thick sections following deparaffination in xylene and rehydration in graded ethanol. After evaporating solvent, tumor sections were homogenized in RNA extraction reagent and RNA was purified. All samples for study consisted of highly purified RNA with A260/280 ratios of 1.8–2.1.

2.2.3. Quantitative-reverse transcription-polymerase chain reaction (qRT-PCR)

Reverse transcription was performed using the Quantitect cDNA synthesis system (Qiagen, Valencia, CA) in a final volume of 20 μl containing 1 μg of total RNA and random hexamers according to the manufacturer's recommendation. Quantitative RT-PCR for livin was performed using validated primer sets (Qiagen) and SYBR-Green PCR kit (Qiagen) on a 7500 Real-Time PCR instrument (Applied Biosystems, Foster City, CA). Livin primers did not discriminate between the two livin isoforms. GAPDH was used as an internal control. RNA extracted from IMR32 cells served as the positive control, and reagent without template served as the negative control. PCR amplification reactions included the following steps: enzyme activation at 95 °C for 1.5 min, followed by 40 cycles at 94 °C for 15 s, 55 °C for 30 s with the final extension at 72 °C for 34 s. The extension step was set as the fluorescence-detection step. Analysis of dissociation curves for each of the primers generated a single, sharp peak, confirming amplification of gene-specific amplicons. Gene expression was quantitated using Applied Biosystems software. The mean qRT-PCR value for livin expression was determined using IMR32 as the calibrator. Livin expression was classified as either "high" (i.e. qRT-PCR $\geqslant$ mean value) or "low" (i.e. qRT-PCR < the mean value).

2.2.4. Protein isolation and immunoblotting

We evaluated protein expression by immunoblot using a polyclonal anti-human livin antibody (Imgenex, San Diego, CA). Cells from snap-frozen tumor samples were harvested by trypsinization followed by centrifugation at 300*g* for 10 min (4 °C). The pellets were washed twice with ice-cold PBS and resuspended in lysis buffer [20 mM Tris–HCl (pH 7.4), 200 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulphonyl fluoride, 1 μg/ml of leupeptin, 1 μg/ml aprotinin and 1 μg/ml pepstatin] for 1 h in ice. Cells were sonicated in a Sonic Dismembranator (Fisher Scientific, Pittsburgh, PA) followed by centrifugation at 10,000*g* for 10 min to remove cellular debris. Protein concentrations in cell lysates were determined using the Bradford protein assay (Bio-Rad Laboratories, Richmond, CA). Cell lysates were analyzed by SDS–PAGE at 4 °C and transferred overnight at 4 °C onto a PVDF membrane. Blots were probed at 4 °C overnight with the following primary antibodies: polyclonal rabbit anti-human livin (1:500 dilution), monoclonal rabbit anti-human cleaved caspase-3 (1:1000 dilution, Cell Signaling Technology, Danvers, MA), monoclonal mouse anti-human PARP (1:200 dilutions, BD Biosciences, San Diego, CA) and monoclonal mouse β actin (1:5000 dilutions, Sigma, MO). The primary antibody was detected by chemiluminescence using either secondary anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase. All tumors with adequate protein recovery ($n = 23$) were analyzed.

2.2.5. Generation of livin-overexpressing IMR32 and SH-SY5Y cells

IMR32 and SH-SY5Y cells in exponential growth were transfected with either pcDNA3.1-Livin expression plasmid (kindly provided by Dr. Bruce Gomes, Astrazeneca Pharmaceuticals) or empty control vector by electroporation at 300 V, 950 μF with Gene Pulsar II system (Bio-Rad, Carlsbad, CA). Cells were seeded 48 h post-transfection into culture dishes for selection of G418-resistant colonies. Colonies were grown in methylcellulose-medium with G418 for 2–3 weeks, and single clones picked and cultured in RPMI with G418 for duration of experiments. Ten clones of IMR32 and seven clones of SH-SY5Y were tested for livin expression. Three clones from each line with the highest livin expression were selected and tested for resistance to doxorubicin and other agents using the WST cytotoxicity assay. The neo-transfected parental IMR32 line was used as a control in these experiments.

2.2.6. Anti-apoptotic role of livin in neuroblastoma

To evaluate whether livin protects neuroblastoma cells from apoptotic stimuli, cells from three livin-transfected clones from both IMR32 (*MYCN*-amplified) and SH-SY5Y (*MYCN*-non-amplified) NBL lines were tested for resistance to different chemotherapeutic agents (data for clone-10 of IMR32 are presented). These agents included two topoisomerase-II inhibitors (doxorubicin and etoposide) and two mitotic inhibitors (vincristine and paclitaxel). Following drug incubation, cell lysis was assessed by WST cytotoxicity assay.

2.2.7. WST cellular cytotoxicity assay

Cells were cultured in phenol-red-free RPMI with 10% FBS in 96-well plates at a density of 10^5 cells/well. The cells were allowed to attach in plates overnight at 37 °C prior to addition of drugs at different concentrations. After 44 h incubation, WST reagent (WST-1, Roche Diagnostics, Mannheim, Germany) was added and plates were incubated for an additional 3 h. Absorbance was measured spectrophotometrically at 450 nm.

2.2.8. Apoptosis assay

Nucleosomes (mono- and oligonucleotides) in the cytoplasmic fraction of cell lysates were quantitated by ELISA assay (Cell Death Detection ELISA PLUS, Roche Diagnostics) following livin siRNA treatment of LA155n cells. In brief, cells were seeded in 96-well plates, transfected with siRNAs, and incubated for 48 h. Cells were then lysed and lysates transferred to a streptavidin-coated plate. A mixture of anti-histone-biotin and anti-DNA-peroxidase monoclonal antibodies was added to the lysates for an additional 2 h at room temperature. After washing-off unbound antibody, nucleosomes were quantitated spectrophotometrically at 405 nm.

2.2.9. Gene knockdown assay

To further evaluate the functional role of livin, gene expression was blocked by small-interfering RNA (siRNA, 25-mer Stealth RNAi duplexes, Invitrogen Corporation, Carlsbad, CA). A set of three non-overlapping siRNA duplexes were individually tested for maximal knockdown of gene expression. Duplex sequences were as follows: *siRNA-livin1*: 1A-GGAGUUGCGUCUGGGCUC CUCUAU and 1B-AUAGAAGGAAGGCCAGACGCAACUCC; *siRNA-livin2*: 2A-G CUGU CAGUUCCUGCUCCGGUCAAA and 2B-UUGACCGGAGCAGGAA CU G ACAGC; and *siRNA-livin3*: 3A-GAAGAGACUUUGUCCACAGUGUGCA and 3B-UGCACACUGUGGACAAAGUCUCUUC. Appropriate control siRNA (with similar G:C content) was selected per manufacturer's recommendations. Cells cultured in 6-well plates were transfected with 100 nM of each of the siRNA duplexes using Lipofectamine 2000 reagent (Invitrogen). After 24–72 h, cells were harvested and analyzed for reduction of livin expression by qRT-PCR and immunoblotting. The siRNA duplex giving maximal knockdown was selected.

Drug sensitivity of livin siRNA-treated cells was assessed by WST assay. Controls consisted of either lipofectamine-treated or control siRNA-treated cells, respectively.

2.2.10. Statistical methods

For *in vitro* drug-sensitivity assays, differences among means were analyzed by Student's *t*-test. To analyze the prognostic significance of livin expression, the Kruskal–Wallis test was used to test the association between livin expression level (i.e. "high" vs. "low") and other prognostic factors. The Cox proportional hazard model was used to analyze data for livin expression against survival time (in months) and patient status (deceased vs. alive). Covariates included livin expression level (as a continuous variable), MYCN oncogene status (amplified vs. non-amplified), age at diagnosis (<12 months vs. > 12 months), tumor stage (low stages 1 and 2 vs. advanced stages 3 and 4), and histology (favorable vs. unfavorable). Kaplan–Meier survival curves were constructed to assess the relationship between livin expression and duration of survival after controlling for age and *MYCN* status, using log-rank statistics. In the Kaplan–Meier analysis, the subset of patients with amplified *MYCN* was divided into high- and low-level livin expression groups, using as the stratification point the mean qRT-PCR value for livin expression among all 59 tumors studied.

3. Results

3.1. Livin mRNA and protein expression in neuroblastoma

Forty-eight (81%) of 59 NBL tumors were positive for livin RNA by qRT-PCR (values greater than 0.05). The mean livin RNA value among the 48 positive cases was 1.08 (Fig. 1A). High expression levels (defined as values greater than the mean tumor level of 1.08) were observed in 23 (39%) tumors. These included 17/39 (44%) patients with advanced stage (3 and 4) disease, 6/20 (30%) patients with localized stage (1 and 2) disease, 16/47 (39%) patients older than 12 months at diagnosis, and 11/21 (59%) patients with MYCN-amplified tumors. Data for livin expression in individ-

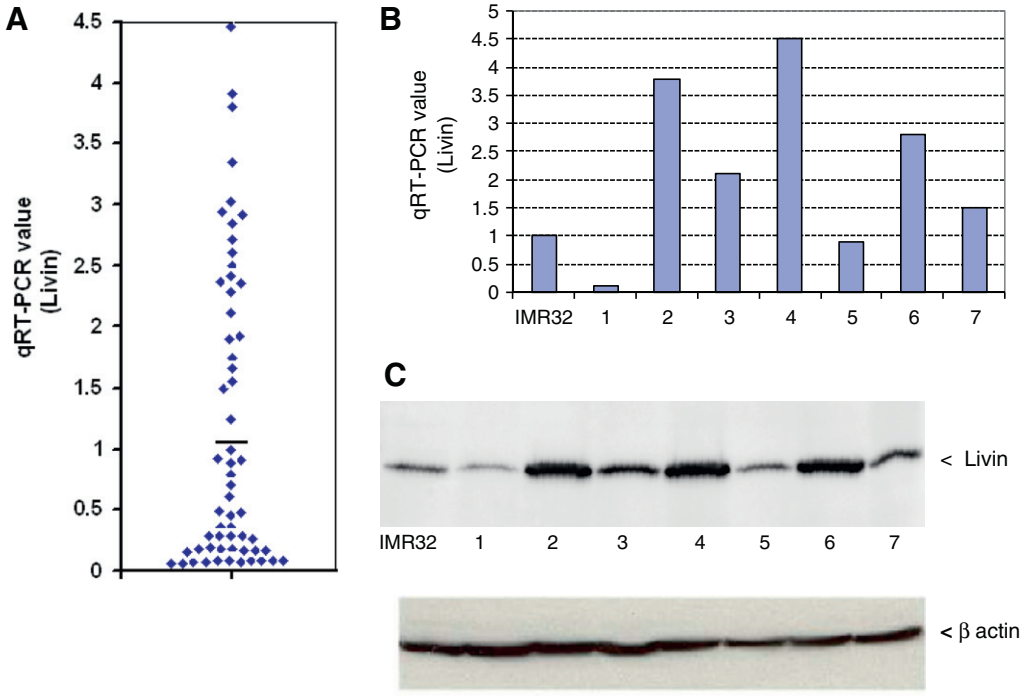

Fig. 1. (A) Expression of livin in NBL tumor tissue: qRT-PCR values for livin expression in 59 tumors. Data are plotted using the IMR32 NBL line as calibrator (livin qRT-PCR value = 1.0). Bar shows mean value (1.08) for livin expression in primary tumors. (B) Expression of livin in primary NBL tumors: upper panel shows representative livin expression in seven primary tumors, as detected by qRT-PCR; five tumors showed high levels of livin expression (i.e. greater than the mean value for all 59 primary NBL tumors studied) and two showed low-livin expression. Lower panel (C) shows livin and actin (control) immunoblots for these specimens.

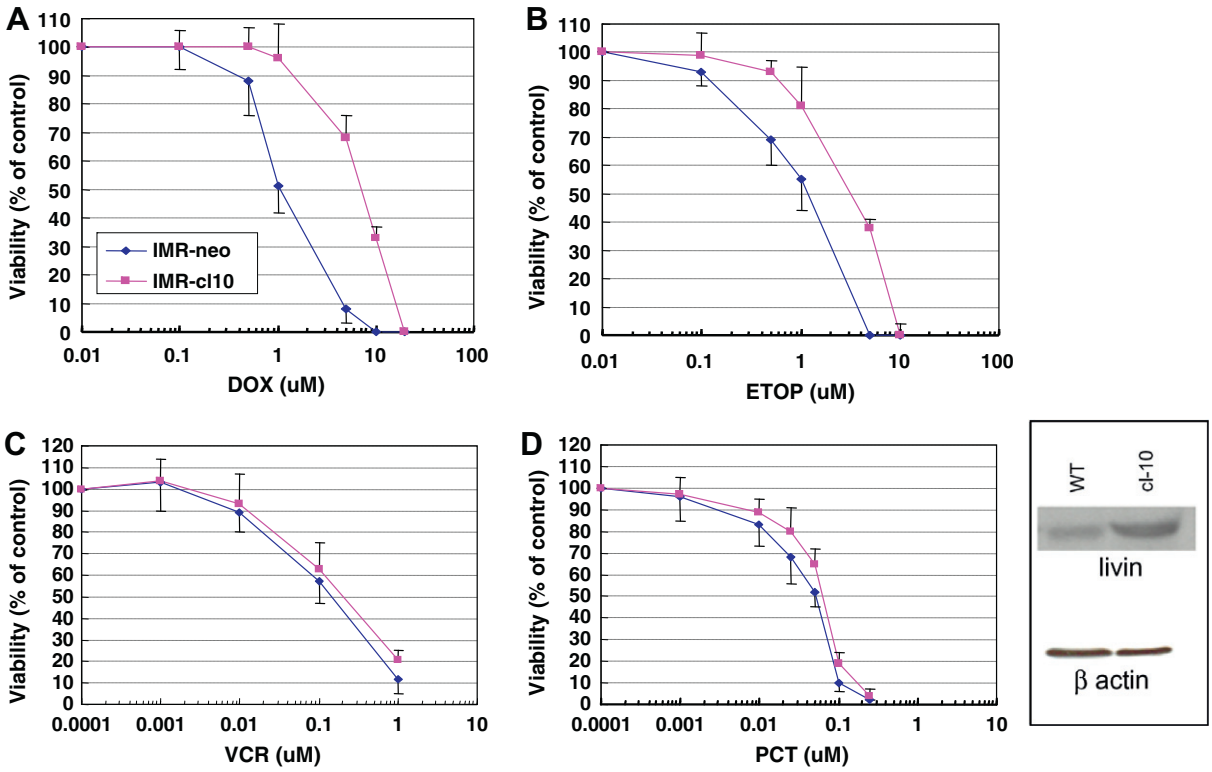

Fig. 2. Ectopically overexpressed livin protects NBL cells from genotoxic agents but not from mitotic inhibitors: NBL cells expressing high levels of ectopic livin (IMR32 clone-10) show increased resistance to both doxorubicin and etoposide (A and B) but not to vincristine or paclitaxel (C and D). IMR32 cells transfected with the empty vector (IMR32-neo) served as the control. Data are from 48 h WST cytotoxicity test; each point represents mean ± SD for three independent experiments. Inset in (D) contains immunoblot showing overexpression of livin in IMR32 clone-10 cells.

ual tumors as detected by qRT-PCR and immunoblot is shown in Fig. 1B and C, respectively. In general, levels of livin mRNA and protein were comparable. Of the 23 tumors analyzed by immunoblot, 18 (78%) were positive for livin protein; all of these tumors were also positive by qRT-PCR. Conversely, all five tumors negative for livin protein were also negative by qRT-PCR.

3.2. Effects of ectopically induced overexpression of livin

A significant increase in resistance of livin-transfected IMR32 (*MYCN*-amplified) clone-10 cells to doxorubicin and etoposide was observed (Fig. 2A and B). However, these cells did not exhibit increased resistance to vincristine and paclitaxel (Fig. 2C and D). Similar results were seen for two other livin-transfected clones of IMR32 (data not shown). In contrast, livin-transfected SH-SY5Y (MYCN-non-amplified) clones did not show increased resistance to either doxorubicin or etoposide (Supplementary data).

3.3. Silencing of livin-gene expression by siRNA

A livin siRNA construct (siRNA-livin1) transfected into LA155n cells repressed endogenous livin mRNA expression to less than 10% of that in control cells (i.e. cells transfected with control siRNA) within 24 h post-transfection; inhibition persisted for up to 72 h post-transfection (Fig. 3A, upper panel). To correlate decreases in livin mRNA expression with livin protein levels, immunoblot analysis was performed at 48 h after siRNA treatment (Fig. 3A, lower panel). siRNA treatment reduced livin at the protein level, thereby confirming efficient knockdown. A second livin siRNA duplex (siRNA-livin2) also resulted in comparable repression. Livin knockdown was associated with increased sensitivity to doxorubicin as measured by WST analysis (Fig. 3B).

3.4. Induction of spontaneous apoptosis in neuroblastoma cells by livin siRNA

Treatment of LA155n cells with anti-livin siRNA increased proteolytic cleavage of caspase-3 and poly(ADP-ribose) polymerase (PARP) (Fig. 3C, upper and middle panels, respectively). Further, the nucleosome-release apoptosis assay showed a significant ($p < 0.05$) increase in apoptosis in livin siRNA-treated cells as compared to control cells (Fig. 3C, lower panel).

3.5. Relationship between livin-gene expression, other prognostic factors, and outcome

There was no correlation between livin-gene expression and other prognostic factors, i.e. MYCN status, age, stage, and histology. Livin expression alone was not a significant prognostic factor for survival in the overall patient cohort. However, among patients with *MYCN*-amplified tumors, those with high livin expression ($n = 11$) had a median survival time of 24.9 months, which was significantly shorter ($p = 0.01$) than 53.2 months recorded for patients with low-livin expression ($n = 10$) (Fig. 4). The adverse impact of high livin expression was independent of both age at diagnosis (<12 months vs. ⩾12 months) and tumor stage (localized stages 1 and 2 vs. advanced stages 3 and 4) (Cox proportional hazards, $p = 0.015$, hazard ratio = 2.316).

4. Discussion

In this study, livin-gene expression was demonstrated by qRT-PCR in 81% of NBL tumors studied. This finding confirms and extends our previous work using IHC [14], which detected livin expression in the majority of NBL tumor tissues and all six NBL cell

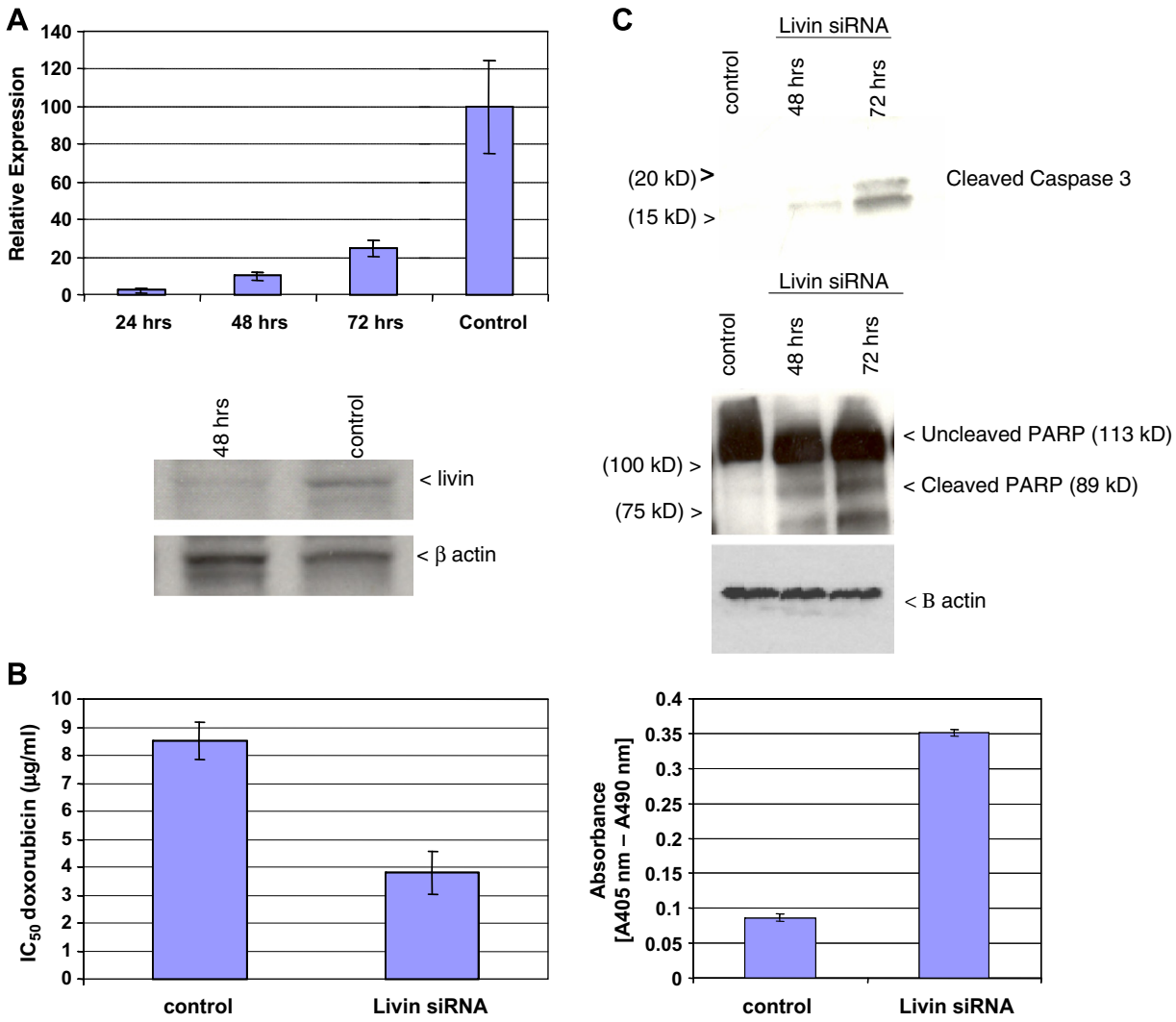

Fig. 3. (A) Repression of livin by siRNA sensitizes NBL cells to doxorubicin: livin siRNA-mediated efficient knockdown of endogenous livin expression, as measured by qRT-PCR and immunoblot (A) in LA155n, a high livin-expressing, doxorubicin-resistant neuroblastoma line. Knockdown of livin expression was monitored by qRT-PCR (upper panel) and immunoblot (lower panel) after livin siRNA treatment. (B) Silencing of livin expression was associated with increased sensitivity to doxorubicin: cells treated with siRNA had a significantly lower IC_{50} for doxorubicin compared to untreated (control) cells. WST assays were performed at 48 h after livin siRNA treatments; each data point represents mean ± SD for three independent experiments. (C) Livin knockdown induces spontaneous apoptosis in livin-expressing NBL cells: treatment of LA155n cells with livin siRNA for 48 h resulted in proteolytic activation of caspase-3 (upper panel) and poly(ADP-ribose) polymerase (middle panel) and secondary spontaneous apoptosis as measured by nucleosome-release assay (lower panel).

lines studied. Although a wide range of livin expression at both mRNA and protein level was noted, there was general agreement between results from both methods; in particular, tumors with livin expression by RT-PCR were positive for protein expression, while those negative by RT-PCR were also negative by immunoblot. Variability in livin expression has been detected by qRT-PCR in archival tissue samples from other solid tumors as well, i.e. non-small cell lung cancer [9], melanoma [10], osteogenic sarcoma [12], and renal cell cancer [13].

In functional studies, ectopically overexpressed livin protected MYCN-amplified IMR32 cells from the topoisomerase-inhibitors doxorubicin and etoposide; these agents induce apoptosis via activation of the intrinsic mitochondrial pathway. However, overexpressed livin did not protect *MYCN*-non-amplified SH-SY5Y cells from either of these drugs, supporting the notion that high levels of livin may cooperate with *MYCN* to protect neuroblastoma cells from genotoxic agents. Furthermore, overexpressed livin did not protect MYCN-amplified cells from the mitotic inhibitors vincristine and paclitaxel, drugs that cause cell death by interfering with formation of spindle fibers during metaphase. The reason for the differential protective effect of these two classes of drugs is not clear. Other studies [4,7,13] have reported *in vitro*, livin-mediated drug resistance to topoisomerase-inhibitors in T-cell leukemia and melanoma. Notably, Nachmias et al. [13] observed a direct correlation between livin expression levels in cultured primary melanoma cells, *in vitro* drug resistance, and patient response to chemotherapy.

In gene-silencing studies, targeted inhibition of endogenous livin expression in LA155n cells by siRNA resulted in downregulation of livin (at both mRNA and protein levels) and increased apoptosis, as shown by activation of caspase-3 and poly(ADP-ribose) polymerase (PARP). Similarly, siRNA-mediated livin knockdown in the doxorubicin-resistant LA155n cells increased doxorubicin-induced apoptosis by greater than 50%. These findings are similar to those reported by Crnkovic-Mertens et al. for melanoma [16], non-small cell lung cancer [22], and renal cell carcinoma [23]. Our findings also agree with data of Wang et al. [24] in human colorectal and lung carcinoma lines. In these studies, siRNA downregulation of livin-gene expression (as measured by qRT-PCR and immunoblot) was accompanied by cell cycle arrest and

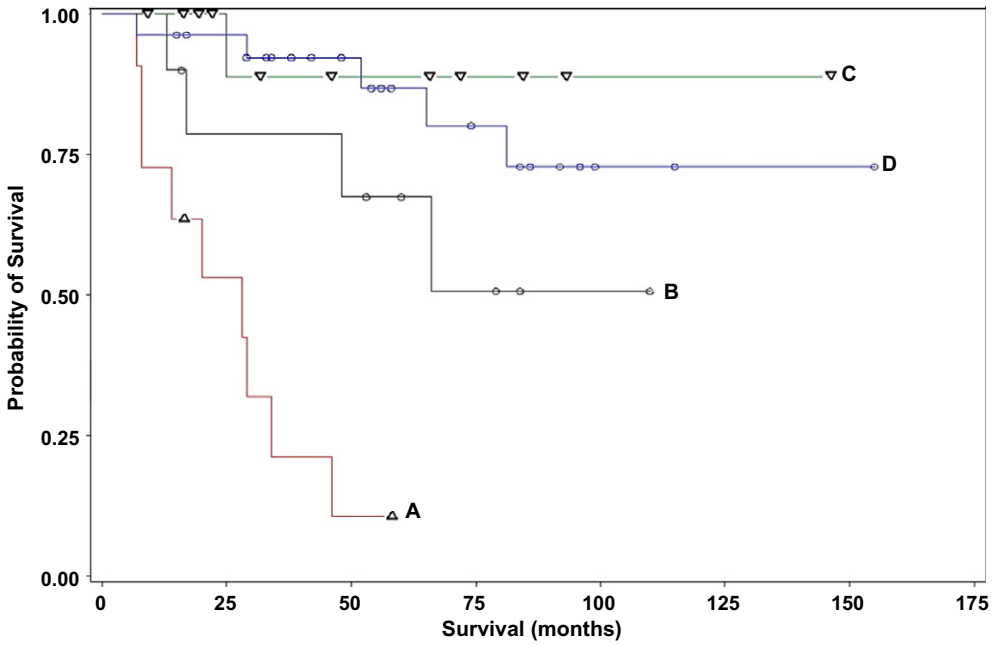

Fig. 4. High levels of livin expression in combination with amplified *MYCN* is associated with shortened overall survival: Kaplan–Meier survival curves for all 59 NBL patients were constructed according to MYCN oncogene status (amplified or non-amplified) combined with level of livin expression (high or low; high was defined as greater than mean value for all tumors analyzed). Patients whose tumors showed high livin expression and amplified *MYCN* (curve A, $n = 11$) experienced a significantly shorter survival than patients whose tumors showed low-livin expression and MYCN amplification (curve B, $n = 10$) ($p = 0.01$). Survival of patients with high livin and MYCN non-amplified tumors (curve C, $n = 12$) was comparable to that of patients with low livin and *MYCN* non-amplified tumors (curve D, $n = 26$) ($p = 0.02$).

decreased tumor cell proliferation. Enhanced *in vivo* sensitivity of siRNA-transfected tumor cells to 5-fluorouracil was also observed, as suggested by decreased tumor growth and increased survival of xenografted mice.

High livin expression had an adverse impact on survival of patients with MYCN-amplified NBL, as these patients experienced significantly shorter survival than those with low-livin expression; overall survival duration for the former group was only 47% of that for the latter group. This is consistent with our previous findings using IHC [14] and suggests that detection of high livin-gene expression by qRT-PCR may be a useful prognostic indicator in *MYCN*-amplified cases. Livin has been reported to be an unfavorable prognostic indicator for superficial bladder carcinoma [8] and high-grade osteogenic sarcoma [12].

The mechanism of interaction between livin and the *MYCN* oncogene leading to tumor drug resistance and poor prognosis is not clear. *MYCN* amplification in NBL cells is typically associated with MycN protein overexpression, increased tumor cell proliferation and, paradoxically, enhanced chemotherapy-induced apoptosis [25,26]. Our recent observation [27] that MycN protein may act as a transcriptional activator of livin expression supports the notion that, in cells co-expressing these genes, the antiapoptotic effect of livin may counteract the apoptotic effects associated with *MYCN* amplification, thus enabling the enhanced tumor-proliferative effects of this oncogene.

Livin has been proposed as a target for tumor therapy [28,29]. Taken together, our findings indicate that this IAP may play a role in drug-resistant NBL, particularly in aggressive, *MYCN*-amplified tumors; this strongly supports the therapeutic targeting of livin to block its antiapoptotic effect.

Conflict of interest statement

The authors have no financial disclosures or other conflicts of interest to report. The funding agencies had no role in this study other than provision of funding.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.08.001.

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