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Overexpression of survivin in pediatric Hodgkin lymphoma tumor cells: Characterization of protein expression and splice-variants transcription profile

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ABSTRACT

Survivin is abundantly expressed during fetal development but absent in most differentiated adult tissues; an exception being components of the immune system, such as B and T lymphocytes. Beyond acting as a master regulator of the cell cycle, survivin acts as an inhibitor of apoptosis and is overexpressed in almost all carcinoma types; however, its expression in lymphomas is lesser-explored. Survivin's role in carcinogenesis was subjected to its sub-cellular localization and splice transcripts expression, namely wild-type survivin, survivin-ΔEx3 and survivin-2B. To assess survivin's expression and sub-cellular localization in Epstein Barr virus positive and negative biopsies from treatment naïve pediatric patients with Hodgkin lymphoma (HL), samples were stained for survivin protein by immunofluorescence. The proportion of survivin + cells was calculated, survivin sub-cellular localization assessed and its fluorescence intensity quantified. Transcription profile of survivin mRNA variants was studied by RT-qPCR. Survivin was overexpressed in the nucleus of tumor cells, and also in a greater proportion of tumor cells, in comparison with the non-tumoral infiltrating cells. Although a higher expression of survivin was observed in advanced clinical stages, no correlation was found between the expression level of survivin and a proliferation marker, or event-free survival. Instead, survivin was related to apoptosis inhibition in tumor cells. Additionally, survivin's transcriptional variants displayed similar expression levels. Present results suggest that although survivin is overexpressed in Hodgkin's tumor cells, it may not play a central role in the progression of classic HL, or act as a suitable progression biomarker, as suggested for most carcinomas.

1. Introduction

Lymphomas are a group of pathologies with distinctive morphological, clinical and epidemiological characteristics but all derive from a lymphoid precursor (Swerdlow et al., 2016). In Argentina, lymphomas constitute about 16% of all diagnosed tumors in children, of which between the years 2000 and 2009, 42% were Hodgkin lymphoma (HL) (Moreno et al., 2013). This lymphoma is characterized by the disruption of the lymph node architecture due to intrafollicular infiltration by different immune cells, but only scarce malignant Hodgkin Reed-Sternberg cells (HRSc), the pathognomonic tumor cell in HL, or Hodgkin mononuclear cells (HMc) (Ansell, 2015; King et al., 2014). In developed countries, HL displays a bimodal incidence pattern, with a first peak during adolescence or early adulthood and a second peak after 60 years of age (King et al., 2014; Kusminsky et al., 2016). In developing countries, like Argentina, HL shows a deviation of the first incidence peak towards early ages, particularly in children under 10 years (Chabay and Preciado, 2013).

Epstein Barr virus (EBV) has been implicated in the onset of a subset of HL (Young et al., 2016); however, the proportion of EBV-associated HL cases is also discrepant according to the socio-economic development of the involved population (Chang et al., 2009). Argentina shows an association pattern similar to developed countries in adult HL cases (31%), but rises to 53% in children under 10 years, while primary

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infection with EBV also occurs at an early age, typical of developing regions (Balfour Jr et al., 2015; Chabay and Preciado, 2013). This fact, together with the high proportion of EBV+ HL cases in children < 10 years, suggests a possible link between an early infection and the risk of HL development (Campos et al., 2018). One possible mechanism by which EBV may contribute to HL is by rescuing HRSc from apoptosis (Carbone and Gloghini, 2018). Two viral proteins, LMP1 and LMP2A, are well known to be expressed in HRSc and mimic the function of an activated CD40 and B cell receptor, respectively; hence providing for anti-apoptotic signals (Vrzalikova et al., 2018; Young et al., 2016).

Inhibitors of Apoptosis Proteins (IAPs) are cellular proteins that regulate the delicate balance between proliferation and apoptosis (Garg et al., 2016). Of all IAPs, survivin (codified by the *BIRC5* gene) exhibits the most drastic over-expression in almost all types of epithelial tumors and all cancer derived cell lines, where it activates a myriad of anti-apoptotic and proliferation signaling cascades (Chen et al., 2016; Kanwar et al., 2013). Furthermore, a transcriptome analysis proved that survivin was among the most over-expressed transcripts in human cancer cells (Velculescu et al., 1999).

Even though, 5 splice variants were identified, wild-type (wt) survivin, survivin-ΔEx3 and survivin-2B are the most widely studied in carcinoma samples, and the general consensus states that wt-survivin and survivin-ΔEx3 display anti-apoptotic functions, while survivin-2B may be pro-apoptotic (Rodel et al., 2012). However, the over-expression of survivin in virtually every human carcinoma is associated with poor prognosis, a fact that turned survivin into an important target in the quest for cancer therapies (Chen et al., 2016; Garg et al., 2016). An additional level of complexity is added with respect to the sub-cellular localization of survivin, to the nucleus, cytoplasm or the mitochondria (Garg et al., 2016; Kanwar et al., 2013).

In lymphomas, survivin was studied to a lesser extent than in carcinomas and mainly in diffuse large B-cell lymphomas (DLBCL) and other non-Hodgkin lymphomas (He et al., 2015; Zhang et al., 2015). Given the particular incidence pattern of HL in our country and that survivin expression has been studied to a lesser extent in HL, we sought to analyze its expression, cellular localization and the relative expression of its clinically relevant splice variants in a set of pediatric classic HL from our population.

2. Materials and methods

2.1. Ethics statement

Hospital's ethics committee reviewed and approved this study (CEI N° 12.33), which is in accordance with the human experimentation guidelines of our institution and with the Helsinki Declaration of 1975, as revised in 1983. A written informed consent was obtained from all patient's parents or tutors.

2.2. Patients and samples

A total of 55 pediatric patients were enrolled: i) 35 HL cases, median age of 11 years (age range 3 to 18 years), 60% males, and ii) 20 cases of reactive lymphoid hyperplasia (RLH), median age 9 years (age range, 3 to 15 years), 60% females. Lymph node biopsies from presumptive HL and RLH tonsils were collected for diagnosis and were treatment naïve. Biopsies were sectioned whenever possible; one half was formalin-fixed and paraffin embedded (FFPE) and the other portion stored at −80 °C. All RLH had fresh samples, while 25/35 HL cases had paired fresh and FFPE samples; the remaining 10 cases were only FFPE samples. Hodgkin lymphoma diagnosis and histological classification (Swerdlow et al., 2016) (Supplementary Table 1), as well as RLH diagnosis, were assessed at the Pathology Division.

2.3. EBERs in situ hybridization

EBV presence in HL cases was assessed on FFPE samples with a commercial *in situ* hybridization (ISH) kit for EBERs according to the manufacturer's instructions (Dako). Cases with positive nuclear staining in tumor cells, with or without occasional staining in infiltrating lymphocytes, were considered EBV associated.

2.4. Survivin and CD30 double staining

Three micrometer thick HL slides were deparaffinized and antigen unmasking was performed in 0,01 M pH 6 citrate buffer for 5 min at 2 psi in autoclave. Slides were stained for CD30 in an automated BenchMark GX instrument with an anti-CD30 (clone Ber-H2 ready to use mouse mAb) and amplified with Opti-view Universal DAB detection Kit, avoiding hematoxylin counterstain (All from Ventana-Roche). Slides were then blocked with PBS 1× pH 8, 1% bovine serum albumin, 0,3% Triton X-100 y 1% normal goat serum for 60 min at room temperature and incubated with a 1/800 dilution of anti-survivin, clone 71G4B7 Rabbit mAb (Cell signaling) for 1 h at room temperature and then overnight at 4 °C. After thorough wash, slides were incubated with a 1/400 dilution of Alexa Fluor 568 goat anti-rabbit IgG (Thermo-Fisher Scientific) for 2 h at room temperature in the dark. Finally, slides were counterstained with Hoechst (Sigma-Aldrich). Ten fields per slides were photographed at high magnification (1000×) in bright field for CD30 and under epi-fluorescent excitation for survivin and Hoechst. Conversion of RGB color to false color (green channel) images for CD30 and the merge with survivin (red channel) and Hoechst (blue channel) were performed in Image-Pro Plus v.6.0.0.260 (Media Cybernetics) as previously described (van der Loos, 2008). Survivin positive tumor and non-tumor cells were counted for each case in the merged photographs. The proportion of survivin positive tumor cells over the total number of tumor cells and the proportion of survivin positive non-tumor cells over the total number of non-tumor cells was then calculated.

2.5. Survivin immunofluorescence

Survivin immunofluorescent staining was performed as described for the double-staining method. All slides were stained in the same day and 10 fields containing tumor and infiltrating cells were photographed at high magnification (1000×). For quantitation purposes, a special care was taken to use the same illumination intensity and camera settings for all slides. Images were then analyzed and fluorescent signal quantified with Image-Pro Plus v.6.0.0.260 as previously described (Wang et al., 2009). Briefly, for each of the 10 fields in each HL case, the nucleus of all HRSc and HMc (tumor cells) and 5 random survivin positive infiltrating cells (non-tumor cells) were delimited and 3 parameters were measured (density, area and integrated optical density (IOD)), where IOD represents fluorescence intensity relative to the total area of measurement, which compensates for the difference in fluorescent signal between different cellular types (HRSc and HMc *vs* infiltrating lymphocytes) given their difference in size. Mean values for IOD (tumor and non-tumor) were then obtained for each independent case. Positive and negative controls were included as previously described.

2.6. Ki-67 staining

Cells under active proliferation in HL cases were assessed by immunohistochemistry for Ki-67 with anti-Ki-67, clone 30-9, ready to use rabbit mAb, and amplified with Ultra-view Universal DAB detection Kit in the BenchMark GX instrument (All from Ventana-Roche). Ten high-magnification fields (1000×) were photographed and the proportion of Ki-67 positive tumor cells and the proportion of Ki-67 positive non-tumor cells were calculated as previously described.

2.7. TUNEL assay

Apoptotic cells were assessed in HL biopsies by means of a Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) assay as described in the ApopTag Fluorescein *In Situ* Apoptosis Detection Kit (Millipore-Sigma). Slides were visualized, photographed and counted as previously described.

2.8. RNA extraction and cDNA synthesis

Total RNA was purified from frozen HL and tonsil samples with Master Pure RNA purification kit (Illumina) according to manufacturer's instructions. RNA was then treated with RQ1 RNAsa-free DNAsa (Promega Corporation) for 2 h at 37 °C. Quantification was performed with a NanoDrop One instrument (Thermo-Fisher Scientific) and RNA with a 260/280 relation above 1.8 were considered suitable for qPCR reaction. Reverse transcription was carried out with 2 μg of RNA and Invitrogen SuperScript II Reverse Transcriptase (Thermo-Fisher Scientific) following manufacturer's instructions. Resulting cDNA was diluted by half in pure water and stored at −20 °C until qPCR reaction.

2.9. Quantitation of survivin variants by real-time PCR

Relative quantitation of total survivin and its major splice variants was assessed by quantitative PCR with primers described in (Vargas and Vivas-Mejia, 2013) (Supplementary Table 2) and in accordance with MIQE guidelines (Bustin et al., 2009). The EBV negative Ramos (RA 1) lymphoblastoid cell line (ATCC CRL-1596) was used as a calibrator and to construct standard curves for each survivin variant. The β-actin and HPRT genes were assessed as endogenous controls and HPRT was chosen based on a similar expression pattern as the survivin gene. Briefly, amplification of target and endogenous cDNA were assessed by qPCR with a StepOne One instrument (Applied Biosystems) and relative quantification of viral genes were calculated by the ΔΔCt method as described by Pfaffl et al. (Pfaffl, 2001). When no signal was detected in a given sample we assigned an arbitrary Ct value above maximal cycle number (Ct = 41). All reactions were carried out in a final volume of 20 μl with SYBR green PCR master mix (Applied Biosystems), 0.1 μg cDNA and 200 nM of the specific primer set; annealing temperatures were as shown in (Supplementary Table 2).

2.10. Statistical analysis

Statistical analysis was performed with GraphPad Prism v5.01 (GraphPad Software) and Operating Characteristic (ROC) curve analysis, which was performed with MedCalc easy-to-use statistical software v18.10 (*MedCalc Software*).

3. Results

In order to compare survivin's expression pattern in EBV+ and EBV- classic HL biopsies, we first assessed the presence of EBV in HRSc or HMc (occasional normal lymphocytes might also be positive for EBV) by means of EBERs *in situ* hybridization; in this regard, 25/35 HL cases were EBV+.

3.1. Survivin is expressed in the nucleus of tumor cells

Double immune-staining was performed for survivin and CD30. Since survivin was assessed by immunofluorescence and CD30 was assessed by immunohistochemistry in the same tissue slide, false color images for CD30 were then generated and merged with survivin and Hoechst counterstained images (Fig. 1A–F). In all cases, survivin was expressed in the nucleus of tumor and infiltrating cells; no case of cytoplasmic survivin was detected in the tumor cell population. Sub-cellular localization of survivin within infiltrating cells was not evaluated. Given HL tissue heterogeneity, evidenced by the larger size and scarcity of tumor cells compared to non-tumor infiltrating cells, we compared the proportion (survivin positive/total cells) of survivin positive tumor cells compared to the proportion of survivin positive infiltrating cells; the latter considered as a non-tumor cell population within HL biopsies. The proportion of survivin positive tumor cells ranged from 0.68 to 1.0, median 0.97, while the proportion of survivin positive non-tumor infiltrating cells ranged from 0.11 to 0.86, median 0.46. The proportion of survivin positive tumor cells was statistically higher than that of non-tumor cells (t-student test, $P < .0001$) (Fig. 1G). Regarding EBV association, no differences in the proportion of survivin positive cells were found in either cellular populations (Mann-Whitney test, $P > .05$ in both cases); however, significance between tumor and non-tumor cells, remained (Fig. 1H). Interestingly, in all studied cases, survivin expression intensity appeared to be grater in tumor than in non-tumor cells.

3.2. Survivin is expressed at a higher level in tumor cells in HL

In order to quantify survivin protein expression level in tumor and non-tumor infiltrating cells, HL tissue sections were stained for survivin and 10 high-magnification (1000×) fields were photographed under the same illumination and camera settings (Fig. 2A–C). Thereafter, 5 tumor cells (whenever possible) and 5 random non-tumor cells were selected in each of the ten fields for each case. The nuclear area of the selected cells was delimited and the Integrated Optical Density (IOD) was calculated. In tumor cells, IOD ranged between 3.8×10^3 and 4.3×10^6, median 1.10×10^5, while in non-tumor cells it ranged between 2.4×10^3 and 1.9×10^6, median 1.70×10^4. Therefore, survivin expression was significantly higher in tumor cells than in non-tumor cells (Mann-Whitney test, $P = .0005$) (Fig. 2D). Moreover, when IOD was compared against clinical stage (Ann Arbor staging criteria), survivin was expressed at a higher level in advanced clinical stages (III and IV) *vs.* milder clinical stages (I and II) (Mann-Whitney test, $P = .0307$) (Fig. 2E). However, after an appropriate cutoff level determination for survivin's expression in tumor cells, 7.6×10^4 (ROC curve analysis); no differences were found in a 5 year period event-free survival for high *vs* low survivin expression level (Kaplan-Meier test, $P > .05$) (Fig. 2F). Again, there was no difference in survivin's expression intensity either in tumor or non-tumor cell populations when assessing EBV presence (Mann-Whitney test, $P > .05$ in both cases) (Fig. 2G).

3.3. Proliferation and apoptosis in Hodgkin lymphoma

Cells under proliferation, as assessed by the expression of Ki-67, were also quantified as the proportion of the total number of cells in 10 areas at high magnification in HL cases (Fig. 3A). The proportion of Ki-67 positive cells was higher in the tumor cell population, ranging from 0.5 to 1.0, median 1.00, than in non-tumor cells, 0.11 to 0.64 with a median of 0.25; (Mann-Whitney test, $P < .0001$) (Fig. 3B). No difference was detected in the proportion of Ki-67 positive cells either in tumor or non-tumor cell populations when assessing EBV presence (Mann-Whitney test, $P > .05$ in both cases). When the relation between survivin expression and proliferation was analyzed, we found no correlation between the proportion of Ki-67 positive tumor cells and the proportion of survivin positive tumor cells nor between the proportion of Ki-67 positive cells and survivin IOD in tumor cells, (Spearman correlation coefficient, $P > .05$; in both cases). No correlation between survivin expression and proliferation suggests that survivin may act as a contributing factor, among many others, in tumor cell proliferation.

Additionally, late apoptosis was assessed by the TUNEL assay, and apoptotic cells within both cellular populations were also quantified as proportions. In all cases, non-tumoral infiltrating cells displayed a low proportion of apoptotic cells, (range 0.11 to 0.86, median 0.46) (Fig. 3C–D). Regarding tumor cells, 27/35 cases contained no apoptotic

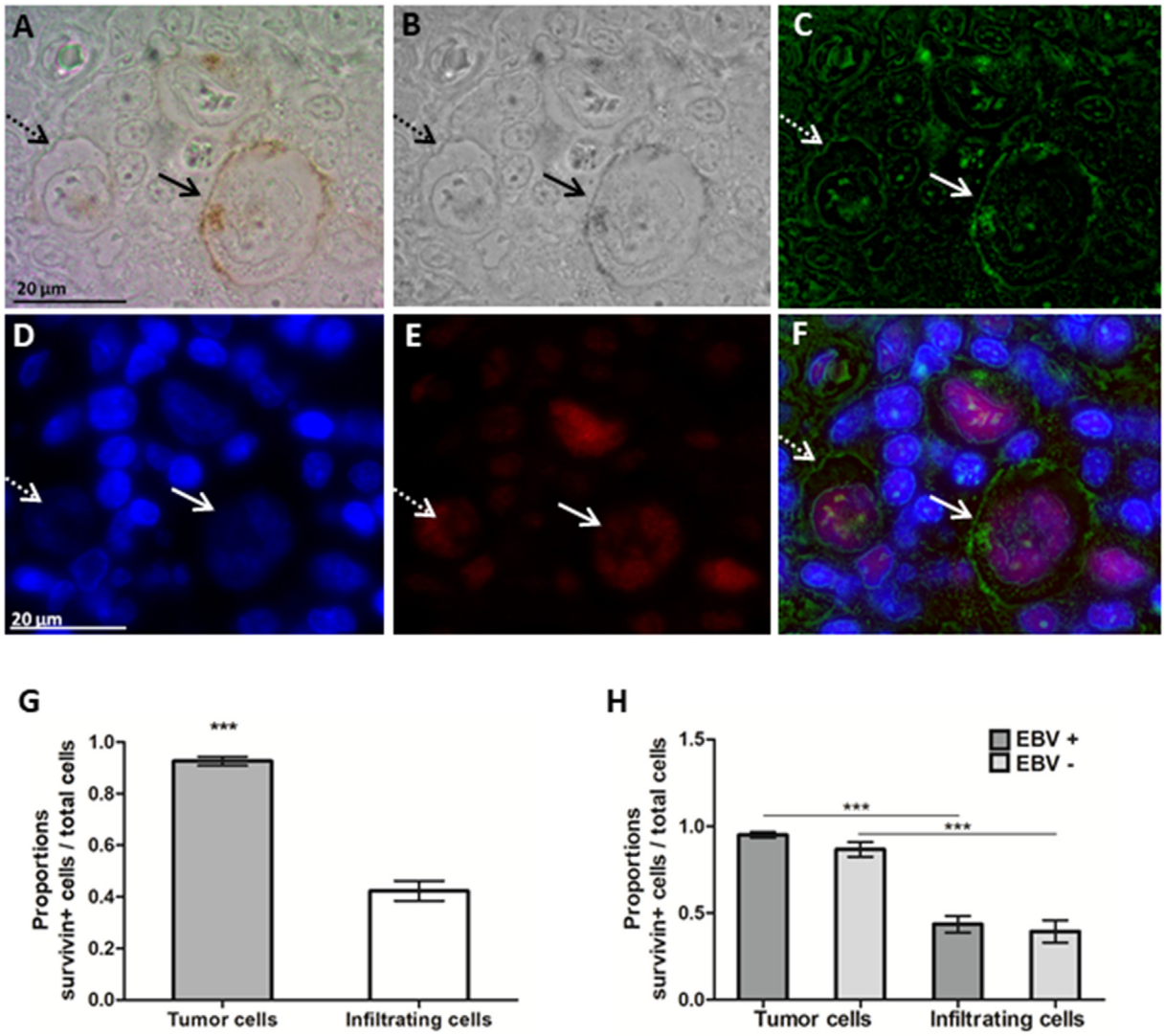

Fig. 1. Survivin and CD-30 double-staining in Hodgkin lymphoma. Bright-field immunohistochemistry for CD-30 in a representative HL case (A). Gray scale conversion of image in A (B). Green false-color image of CD-30 staining (C). Hoechst counter-staining (D). Immunofluorescence for survivin (E). Merge of C, D and E (F). Full arrow shows a HRSc; dotted arrow shows a HMCc, 1000× magnification. Mean comparison of the proportion of survivin + cells; mean ± MSE (0.93 ± 0.01 for tumor cells and 0.42 ± 0.03 for infiltrating cells) (G), *** $P < .001$. Mean comparison of the proportion of survivin + cells in relation to EBV status; mean ± MSE (0.95 ± 0.01 and 0.87 ± 0.04 for tumor cells in EBV+ and EBV- cases; 0.44 ± 0.04 and 0.39 ± 0.06 for infiltrating cells in EBV+ and EBV- cases) (H). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cells whereas the remaining 8 cases only displayed apoptotic tumor cells in a very low proportion of the 10 analyzed fields. Given that the vast majority of cases had no tumor cells in apoptosis, mean comparison was not possible. However, when the occurrence of apoptosis and cell type (tumor *vs.* non-tumor) were considered as discrete variables, a statistical association between tumor cells and absence of apoptosis was found (Fisher's exact test, $P < .0001$).

3.4. Relative expression of survivin variants

In order to characterize the expression of the clinically relevant survivin variants, the relative abundance of wt-survivin, survivin-2B, survivin-ΔEx3 transcripts and the total amount of survivin transcripts were assessed by RT-qPCR. As expected, the total amount of survivin transcripts showed a significantly higher expression than survivin-wt, survivin-2B and survivin-ΔEx3 ($P < .001$); however, no difference was detected between the relative expression levels among the three studied variants (Fig. 4A) (One way ANOVA, $P > .05$). In line with previous results, no difference in survivin variants relative expression was found when discriminating against EBV presence in HL cases (Fig. 4B) (Mann-Whitney test, $P > .05$). Additionally, we sought to check if the similar expression level of survivin transcripts was inherent to HL or a common phenomenon in lymphoid tissues. For this purpose, and seeking a non-tumoral lymphoid tissue, we assessed the expression of survivin variants in twenty reactive lymphoid hyperplasia (RLH) biopsies. The expression profile of survivin transcripts in RLH proved to be similar than in HL samples (Fig. 4C) (One way ANOVA, $P > .05$); although the relative expression of the transcripts was higher in RLH than in HL (Fig. 4D) (Mann-Whitney test, $P < .0001$).

4. Discussion

Inhibitors of apoptosis proteins are a family of multifunction proteins; among which, survivin is related to cell cycle regulation and inhibition of apoptosis. Moreover, survivin overexpression was observed and linked to a worst prognosis in a variety of epithelial tumors and has become of interest as a target for anti-tumoral therapies (Chen et al., 2016; Garg et al., 2016). However, survivin contribution to

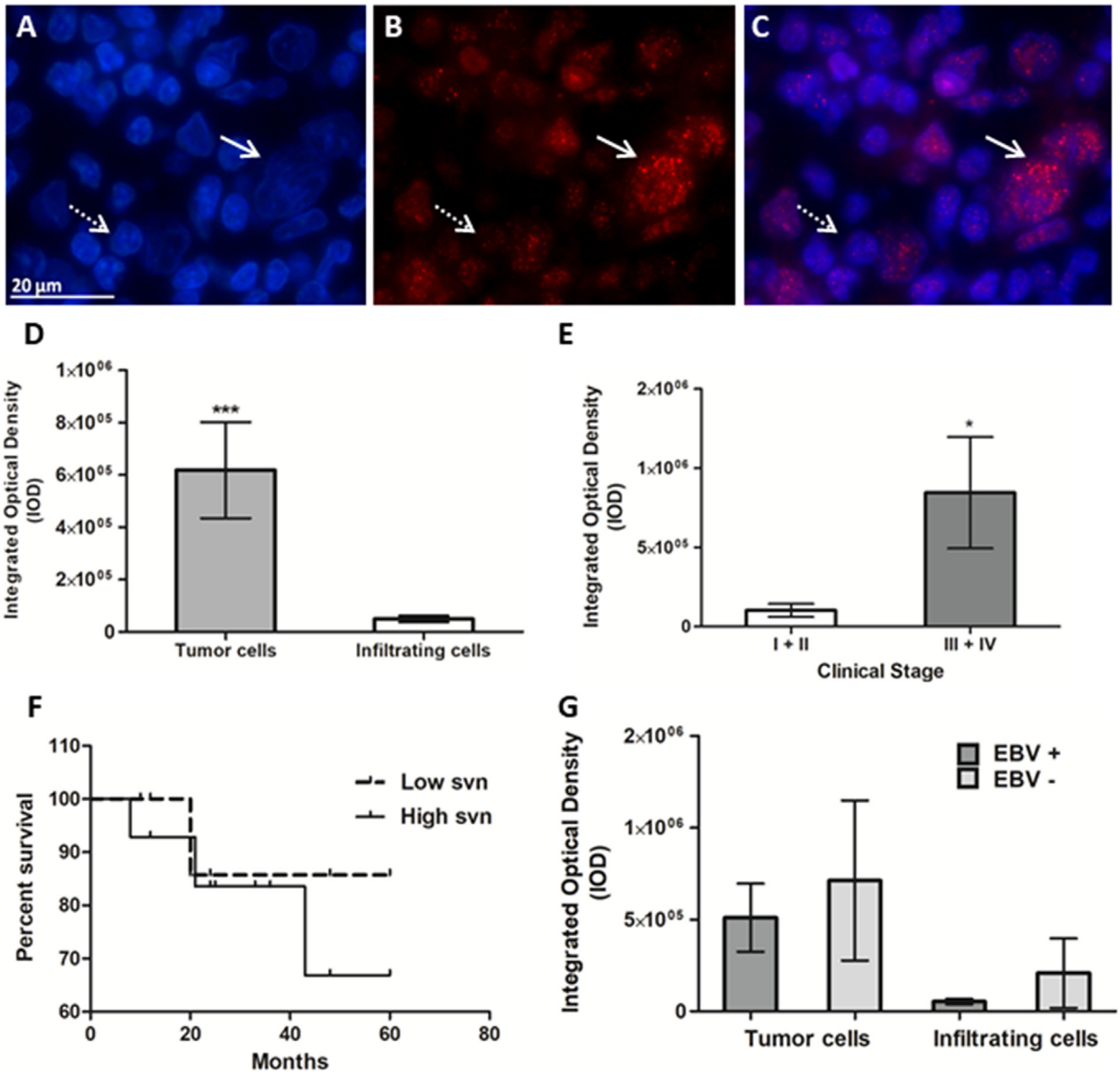

Fig. 2. Survivin expression in Hodgkin lymphoma. Hoechst counter-staining of a representative case (A). Immunofluorescence for survivin (B). Merge of A and B images (C). Full arrow shows a HRSc; dotted arrow shows an infiltrating cell, 1000× magnification. Mean comparison of survivin staining intensity, quantified as the integrated optical density (DOI), in arbitrary units, in tumor and infiltrating cells; mean ± MSE ($5.70 \times 10^5 \pm 1.80 \times 10^5$ for tumor cells; $9.90 \times 10^4 \pm 5.60 \times 10^4$ for infiltrating cells) (D), $^{***}P < .001$. Mean comparison for survivin staining intensity regarding clinical stage, $^{*}P < .05$ (E). Five years event-free survival analysis for patients with low or high survivin expression (F). Mean comparison of survivin staining intensity in tumor and infiltrating cells, in relation to EBV status; mean ± MSE ($5.10 \times 10^5 \pm 1.90 \times 10^5$ and $7.10 \times 10^5 \pm 4.40 \times 10^5$ for tumor cells in EBV+ y EBV- cases; $5.30 \times 10^4 \pm 1.50 \times 10^4$ and $2.10 \times 10^5 \pm 1.90 \times 10^5$ for infiltrating cells in EBV+ y EBV- cases (G).

lymphomagenesis is less understood.

Here we observed nuclear expression of survivin in HRSc and HMc in all (100%) classic HL cases. This percentage, above that previously described in one study involving HL samples (83%) (Aktas et al., 2007) and other reports on non-Hodgkin lymphomas (75% to 87%) (Kalungi et al., 2013; Liu et al., 2008), may be due to the higher sensitivity of immunofluorescence over the immunohistochemistry approach used in those reports to detect survivin.

A further innovation over previous reports is the fact that survivin expression was also assessed in infiltrating cells, which were considered as non-tumor cells. In this regard, the proportion of survivin positive cells, as well as survivin expression level was statistically higher in the tumor cell population, suggesting a possible role for survivin in HRSc and HMc survival. Even though survivin was located in the nucleus of tumor cells, a significant association was observed between tumor cells and apoptosis inhibition, which could mean that the mechanism by which survivin inhibits apoptosis in HL may differ from that described in carcinomas, where cytoplasmic survivin is believed to have anti-apoptotic functions (Khan et al., 2015). Despite appearing only in the nucleus of tumor cells, no correlation was found between survivin expression and proliferation. A possible explanation could be the multifactorial nature that drives tumor cell growth, where once established, tumor cells grow independently of survivin expression level; however, survivin's role in tumor cell survival may be crucial upon cellular transformation. In this way, assessing survivin in biopsies of already established HL may present a possible drawback to establish a causal role for survivin in lymphomagenesis.

Regarding sub-cellular localization, in our HL series it differed from a previous report on diffuse large B cell lymphomas where survivin was detected in both, nucleus and cytoplasm of tumor cells (Zhang et al., 2015); however, its sub-cellular localization was not explored in previous HL studies (Aktas et al., 2007; Garcia et al., 2003). Altogether,

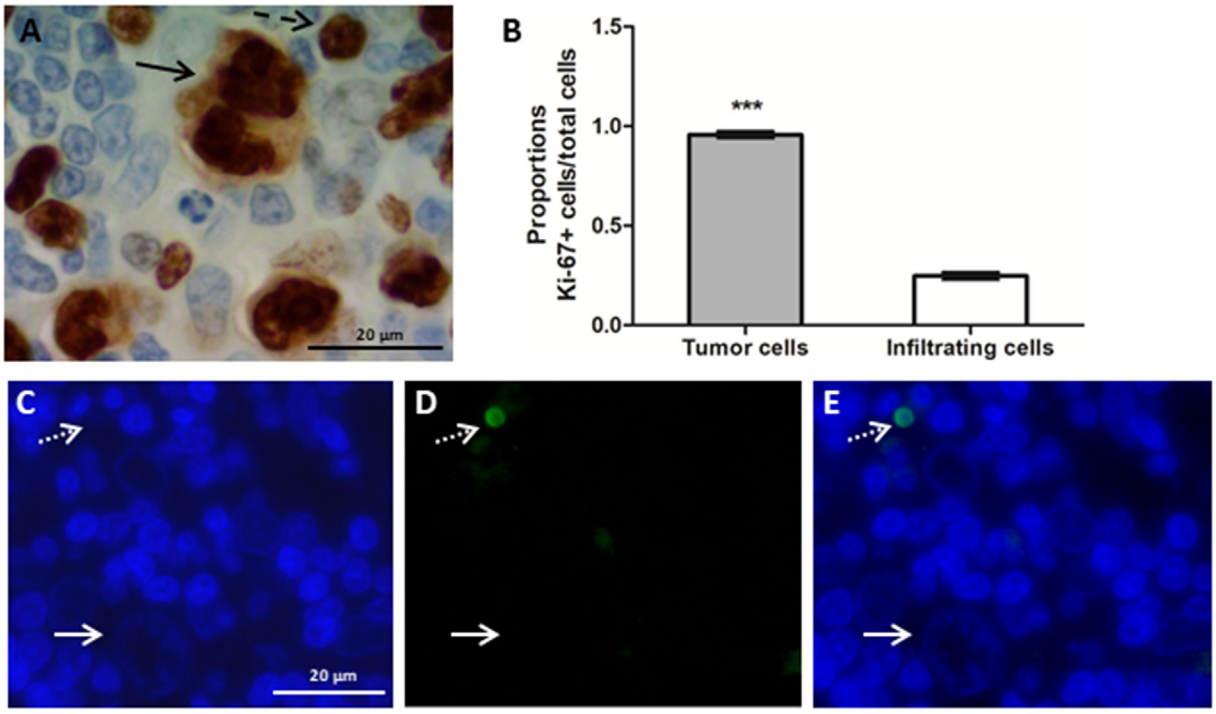

Fig. 3. Proliferation and apoptosis in Hodgkin lymphoma. Immunohistochemistry for Ki-67 in a representative HL case. Full-arrow shows a HRSc; dotted arrow shows an infiltrating cell, 1000× magnification (A). Mean comparison of the proportion of Ki-67 + cells; mean ± MSE (0.96 ± 0.01 for tumor cells; 0.25 ± 0.01 for infiltrating cells) (B), ***P < .001. Hoechst counter-staining of a representative case (C). TUNEL reaction staining (D). Merge of C and D images (E). Full arrow shows a TUNEL negative HRSc; dotted arrow shows a TUNEL positive infiltrating cell, 1000× magnification.

overexpression of survivin in tumor cells together with minor apoptosis in the infiltrating cells was not surprising, since it is compatible with the notion that HRSc inhibit reactive infiltrating cells, causing an exhaustion in their immune function with consequent quiescence state and decreased cellular turnover, which ultimately allows tumor cells to evade immune surveillance and prevail (Aldinucci et al., 2016).

Another factor associated with HL is EBV, an oncogenic virus that expresses latency proteins assumed to up-regulate proliferation signaling cascades (Vrzalikova et al., 2018). Additionally, EBNA1, LMP1 and LMP2, which are expressed during viral latency in HL (Carbone and Gloghini, 2018; Vrzalikova et al., 2018), were also described to up-regulate survivin in *in vitro* studies (Hino et al., 2008; Lu et al., 2011; Sun et al., 2015). However, and in line with another study in HL biopsies (Aktas et al., 2007), we found no changes in the expression of survivin in our pediatric HL series in relation to EBV. Moreover, a similar lack of association between EBV and survivin expression was observed in non-HL (Aktas et al., 2009). These observations in clinical biopsies could reflect the fact that *in vitro* results are not always reproduced in *ex vivo* samples. It could also mean that EBV may act as a co-factor during the early onset of some HL cases, either by rescuing defective cells from apoptosis and/or by enhancing survivin expression and hence, proliferation.

Finally, and regarding the expression of the alternative survivin isoforms, which arise from alternative splicing of RNA transcripts, definitive conclusions to whether they correlate to a favorable or adverse survival are still controversial; in particular regarding survivin-2B, which expression negatively correlates with clinical stage in some of carcinomas (Necochea-Campion et al., 2013). However, despite conflicting results, the general consensus is that all variants correlate with poorer survival outcomes in carcinomas, most likely through apoptosis inhibition in cancer cells (Kanwar et al., 2013; Necochea-Campion et al., 2013). Moreover, disparities in the transcription of survivin variants have been described in thyroid and lung cancers, where survivin-2B and survivin-ΔEx3 were transcribed at a higher ratio than wild-type survivin (Nakano et al., 2008; Waligorska-Stachura et al., 2017). On the contrary, in colorectal adenocarcinoma samples, wild-type survivin was the most prevalent transcript (Pavlidou et al., 2011). In our series of cHL biopsies, no differences were detected in the relative expression of survivin's alternative transcripts. However, it should be mentioned that this observation was not completely unexpected since RT-qPCR cannot discriminate between the transcription profile provided by tumor cells and that of the infiltrating cells; hence, any possible differences in variant's transcript expression from tumor cells could be masked by that of non-tumor cells. This fact may be particularly so in the case of cHL, since non-tumor cells usually outnumber tumor HRSc and HMc cells by > 100:1 (Tan et al., 2018). Additionally, RLH tonsils showed a very similar, although increased, transcription profile which could be due to the nature of the actively-proliferating non-tumor cells within the germinal center reactions of these tonsils, in contrast to the quiescent infiltrating non-tumor cells in HL biopsies.

The fact that survivin expression level did not correlate with cellular proliferation, nor did have an impact on event free survival, as also reported by Aktas et al. in different lymphomas (Aktas et al., 2007; Aktas et al., 2009), suggests that, as opposed to that described in most carcinomas, survivin may not have a central role in lymphomas, and more specifically in classic HL progression, but may act as an additional co-factor in the very early stages of this multi-factorial disease. Whichever the case, survivin may not be considered a promising biomarker in classic Hodgkin lymphoma, as it is in carcinomas.

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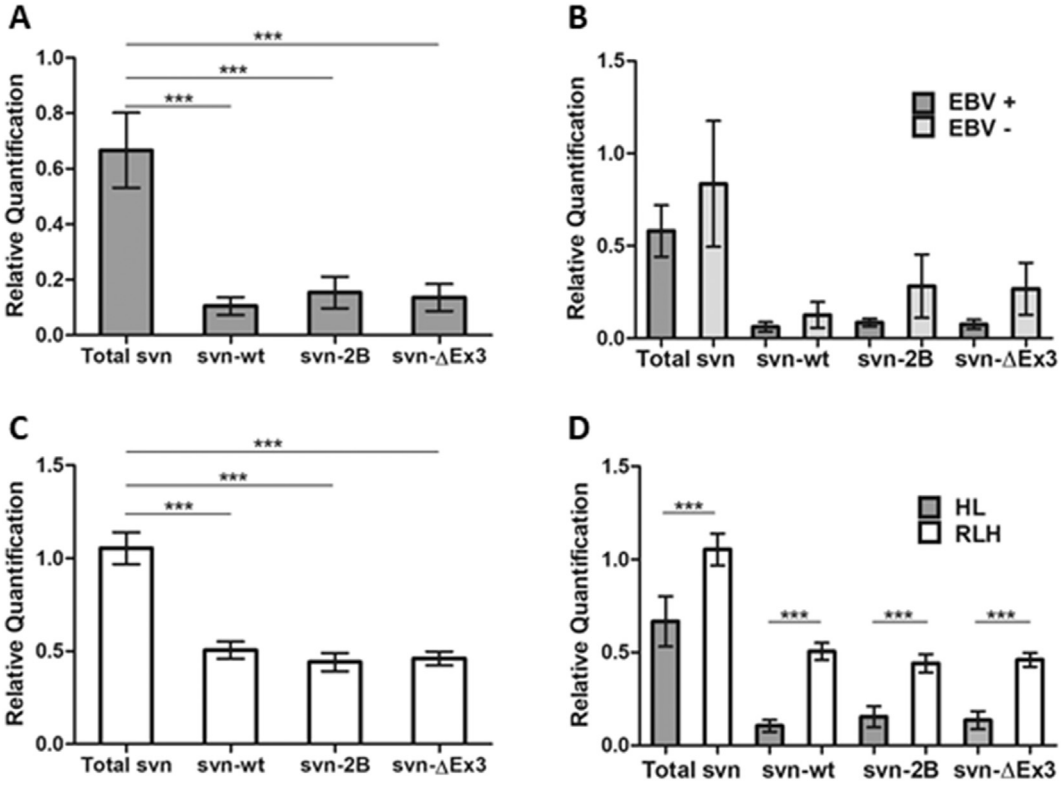

Fig. 4. Relative quantitation of survivin transcriptional variants. Relative quantification of total survivin transcripts and transcripts from wt, 2B and ΔEx3 variants in HL samples; mean ± MSE (0.67 ± 0.14 for total survivin; 0.11 ± 0.03 for survivin-wt; 0.15 ± 0.05 for svn-2B and 0.14 ± 0.04 for svn-ΔEx3). ***P < .001 (A). Relative quantification of survivin transcriptional variants in EBV+ and EBV- HL samples; mean ± MSE (0.59 ± 0.12 and 0.83 ± 0.34 for total survivin in EBV+ and EBV- cases; 0.09 ± 0.03 and 0.13 ± 0.07 for svn-wt in EBV+ and EBV- cases; 0.09 ± 0.02 and 0.28 ± 0.17 for svn-2B in EBV+ and EBV- cases; 0.07 ± 0.02 and 0.27 ± 0.14 for svn-ΔEx3 in EBV+ and EBV- cases); $P > .05$ in all cases (B). Relative quantification of survivin transcriptional variants in RLH samples; mean ± MSE (1.10 ± 0.08 for total survivin; 0.51 ± 0.04 for svn-wt; 0.44 ± 0.04 for svn−2B; 0.46 ± 0.03 for svn-ΔEx3), ***P < .001 (C). Relative quantification of survivin transcriptional variants in HL samples *vs* RLH samples; mean ± MSE (0.66 ± 0.13 for total survivin in HL and 1.05 ± 0.08 in RLH; 0.10 ± 0.50 for svn-wt in HL and 0.50 ± 0.04 in RLH; 0.15 ± 0.05 for svn-2B in HL and 0.44 ± 0.04 in RLH; 0.13 ± 0.04 for svn-ΔEx3 in HL and 0.46 ± 0.03 in RLH, ***$p < 0{,}001$ (D).

Promotion (PICT 2016 N° 0548).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yexmp.2019.03.005.

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