

## Expression pattern of anti-apoptotic protein survivin in dysplastic nevi

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The anti-apoptotic protein survivin was detected in a panel of 27 dysplastic nevi. From each representative paraffin block 4 mm sections were cut and stained with anti-survivin antibody (DAKO, Clone 12C4). In each section, the labeling intensity, the subcellular location of survivin antigen, the percentage of labeled cells and the degree of dysplasia were assessed. Survivin was present in 23 out of 27 cases (85.2%), but absent in 4/27 cases (14.8%). Positive staining was confined to the cytoplasm (C) of nevus cells only in 18 cases (66.7%), while cytoplasmic as well as nuclear positivity (NC) was found in 5 cases (18.5%). In no case solely nuclear staining could be seen. Furthermore, in 4 out of 5 cases (80%) with NC staining, severe dysplasia was found. Our data point at usefulness of survivin staining, otherwise rarely performed in dysplastic nevi. We confirm the importance of nuclear location of the survivin antigen, which may be helpful for assessing the possible progression to melanoma.

*Key words: survivin, immunohistochemistry, nevi, dysplasia, melanoma*

Apoptosis or programmed cell death is fundamental for the maintenance of normal tissue homeostasis and requires a precisely regulated balance between cell proliferation and death. Apoptosis can be induced by either specific extracellular signals or internal stimuli. The molecular mechanisms involved in the enzymatic cascade of apoptosis were comprehensively reviewed [1]. Briefly, in mammalian cells apoptosis can be triggered by a wide spectrum of stimuli, acting both at intra- and/or extracellular environments. The stimuli such as DNA damage, oxidative stress, hypoxia, cell detachment and growth factor deprivation, are mediated via the intrinsic or mitochondrial pathway; in general, they cause activation of BH3-only (a member of Bcl-2 family proteins), which invariably leads to the release of pro-apoptotic factors into cytoplasm from the inter-membranous space of mitochondria. One of these factors, cytochrome-c, is considered critical for the intrinsic apoptotic pathway [2, 3]. Cytochrome-c induces the formation of a large multimetric complex „apoptosome“, which consists of cytochrome-c, the adapter protein Apaf-1, adenosine triphosphate (ATP) and procaspase-9. The special three-dimensional structure of this complex recruits and mediates the auto-activation of the initiator caspase, caspase -9, which continues to activate caspase-3 and caspase-7, triggering a subsequent cascade of caspase cleavage and activation [4].

The active caspases are subject to inhibition by the inhibitor of apoptosis proteins (IAP). Another mitochondria-derived protein, Smac/DIABLO, physically interacts with multiple IAPs and removes IAP-mediated caspase inhibition in course of apoptosis. Thus mitochondria play an indispensable role in the intrinsic form of apoptotic cascade [5, 6].

The extracellular stimuli (extrinsic pathway), such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), TNF-related apoptosis inducing ligand (TRAIL), fibroblast associated ligand (FasL) and granzyme B, directly activate the death receptors through ligand-induced trimerization and assembly of a large death-inducing signaling complex (DISC) at plasma membrane. One adapter protein of DISC, the Fas-associated death domain or FADD is able to activate the initiator caspase, procaspase-8. The active caspase-8 cleaves and activates effector caspases-3 and -7, which are directly or indirectly responsible for the cleavage and degradation of several crucial cellular proteins, as well as for the execution of cell death [7, 8, 9].

Cells of malignant tumors show a certain degree of resistance to apoptosis, while some types of tumor cells are remarkably resistant. Malignant melanoma, one of the most aggressive tumors, is characterized by high resistance to therapeutic drugs and by elevated capacity to metastasize [10, 11]. Inherited or common acquired nevi can give rise to dysplastic nevi, which may progress into early neoplastic lesions. Dysplastic nevi form, both clinically and histologically, a continuum extending from a common nevus to a superficial spreading

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melanoma [12]. Pre-neoplastic and early neoplastic lesions are characterized by impairment of the cell cycle barrier at the G1/S phase and/or by double strand breaks in their DNA, caused by replication errors during aberrant cell cycles driven by oncogene activation. This breakage leads to prolonged activation of the DNA damage checkpoint that is associated with cell cycle arrest at the dysplastic lesion, and increased proliferation of the primary melanoma [13]. The appearance of aberrant cells enhances genetic instability and together with the suppression of apoptosis, it potentiates the malignant transformation [13, 14]. During tumor progression, melanomas accumulate alterations in several genes that encode proteins involved in apoptotic pathways. The inhibitor of apoptosis protein (IAP) survivin is the only known apoptosis regulator expressed both in nevi and malignant melanoma. Similarly to other inhibitors of the apoptotic process, the survivin mediated protection from entering apoptosis may be conferred by multiple mechanisms, including caspase inhibition [15, 16]. The survivin gene is under p53 control, its expression being cell cycle dependent [17]. Also the activators of pRB/E2F pathway can interact with the survivin promoter when increasing survivin expression [18]. In such way, survivin is undetectable in the G0 phase cells of normal differentiated tissues, but frequently expressed in fetal tissues, in pre-neoplastic and early neoplastic lesions, as well

as in developed malignancies [19]. Survivin has also been demonstrated in common nevi, in malignant melanoma, in epithelial skin tumors and tumor-like lesions; however, its expression was very rarely studied in dysplastic nevi [10, 11, 12, 16, 20].

In present study, we determine the expression pattern of survivin in dysplastic nevi stressing its overall cytoplasmic distribution. We claim that nuclear locations of survivin antigen may be a marker for malignant progression.

### Material and methods

Samples from 27 dysplastic nevi were enrolled onto this study. Hematoxylin and eosin stained slides of all cases were reviewed by two pathologists (LL, MA) to confirm the diagnosis. Each representative paraffin block was cut into four micrometer sections subjected to immunohistochemical staining where three sections from each one have been stained for survivin protein. For greater adherence of tissue sections to glass slides, we used silanized slides (DAKO, Denmark), which were baked for 2 hours in an oven at 56°C. The sections were deparaffinized in xylene for 20 minutes, rehydrated at decreasing ethanol concentrations and washed with phosphate-buffered saline (PBS). The endogenous peroxidase activity was quenched

Table 1. Panel of 27 dysplastic nevi submitted to survivin staining

	S. I.	I	%	Nevus diagnosis	Size of skin lesion	Dysplasia	Age/ Years	Sex
1.	C	+	>10%	compound	6 mm	+	41	F
2.	C	+	>10%	congenital	8 mm	+	16	M
3.	NC	++	>10%	compound	10 mm	+++	18	F
4.	A	-	-	compound	6 mm	+	46	F
5.	C	+++	>10%	compound	6 mm	++	54	F
6.	C	+	<10%	compound	5 mm	++	24	F
7.	C	+	>10%	compound	10 mm	++	39	F
8.	C	++	>10%	compound	5 mm	+++	34	F
9.	C	+++	>10%	compound	6 mm	+	30	F
10.	NC	++	>10%	compound	7 mm	+++	32	F
11.	C	+	>10%	compound	5 mm	++	52	M
12.	A	-	-	congenital	6 mm	++	14	F
13.	A	-	-	intradermal	9 mm	++	50	M
14.	C	++	>10%	compound	7 mm	+	40	F
15.	C	+++	>10%	compound	5 mm	+	17	F
16.	C	+	>10%	compound	5 mm	+	21	F
17.	A	-	-	compound	4 mm	++	29	M
18.	NC	++	>10%	compound	5 mm	+	18	F
19.	NC	++	>10%	compound	6 mm	+++	29	F
20.	C	+	>10%	compound	9 mm	+++	31	F
21.	C	+	<10%	compound	5 mm	+	57	M
22.	C	++	>10%	compound	8 mm	++	49	M
23.	C	++	>10%	compound	10 mm	+	32	M
24.	C	++	>10%	compound	7 mm	++	35	M
25.	C	++	>10%	compound	5 mm	+	68	F
26.	C	++	>10%	compound	10 mm	+	32	F
27.	NC	+++	>10%	compound	8 mm	+++	31	F

S. I. – subcellular localization of survivin positivity: A – absent, N – nuclear, C – cytoplasm, NC – nuclear and cytoplasm; I – intensity of immunoreactivity; % – % of labeled cell;

Dysplasia: + mild, ++ moderate, +++ severe

with 3% hydrogen peroxide for 30 minutes. Antigen unmasking was achieved by heating the sections which had been immersed in target solution (DAKO) within a hot water bath (96 °C) for 45 minutes. Immunohistochemical staining was performed using monoclonal mouse anti-survivin antibody (DAKO, Clone12C4, dilution 1:50). After overnight incubation, immunodetection was performed with the LSAB Visualization System (DAKO) using 3, 3'-diaminobenzidine chromogen as substrate, according to manufacturer's instructions. All sections were counterstained with hematoxylin (DAKO). Negative controls were obtained by omitting the primary antibody.

## Results

*Evaluation of immunohistochemical staining.* The survivin antibody stained sections were observed in the light microscope, and the positivity of cytoplasmic (C) versus nuclear (N) staining or both together (NC) was determined. Survivin antigen expression was scored semiquantitatively as follows:

1. *The intensity of staining*
  - a. absent or barely detectable (0)
  - b. weak (+)
  - c. moderate (++)
  - d. strong (+++)
2. *Number of positively stained cells*
  - a. more than 10% ( $\geq 10\%$ ) per field of view
  - b. less than 10% ( $\leq 10\%$ ) per field of view
3. *Subcellular localization of staining*
  - a. nuclear (N) only
  - b. cytoplasmic (C) only
  - c. combined nuclear and cytoplasmic (NC)
4. *The degree of cell atypia*
  - a. mild (+)
  - b. moderate (++)
  - c. severe (+++)

The above mentioned parameters were evaluated by two pathologists separately (LL, MA) and scored using unified and clear cut-off criteria, to achieve good reproducibility.

*Localization of survivin antigen.* Survivin protein expression by immunohistochemistry was carried out on 27

dysplastic nevi. The results of expression profiling are summarized in Table 1 and illustrated in Figure 1 A– C. At higher magnification cellular staining pattern of survivin was for the most part granular and some positive melanocytes showed cytologically nuclear atypia characterized by irregularly shaped, large and hyperchromatic nuclei (Figure 1 B and C) This finding constituted “random” cytological atypia. Nucleoli were often prominent. Dysplastic cells tended to form small nests, which were found both in epidermis and dermis (Figure 1 B). Because the lesions showed mostly heterogenous survivin immunoreactivity, the dominant pattern was used for scoring. The basal epidermal layer in proximity to dysplastic nevus expressed strong cytoplasmic immunopositivity.

Nevertheless, some more significant findings are worth comment: survivin expression was observed as a cytoplasmic staining in 18 cases (66,7%), combined nuclear and cytoplasmic positivity was found in 5 cases (18,5%). No one case showed only nuclear staining. Interestingly, in 4 out of 5 cases with NC staining, severe dysplasia was detected (Table 2). Nucleoli were stained in 2 out of 4 NC cases with severe dysplasia (Figure 1 C).

## Discussion

Survivin is a multifunctional protein that inhibits apoptosis, regulates cell division and enhances angiogenesis. It is rarely expressed in terminally differentiated normal adult tissues. Upregulation of survivin is found in most premalignant lesions and malignant tumors [15, 21]. It was shown, that survivin was the fourth most highly expressed transcript in a number of common malignancies [22]. There is large difference in expression between malignancy and corresponding normal tissue, therefore survivin is an attractive target as tumor marker.

Survivin is a member of the inhibitor of apoptosis protein (IAP) family of molecules, that specifically inhibit caspases-3, -7 and -9 and their activity is blocked by Smac/DIABLO, a protein also released from mitochondria after apoptotic stimuli [15, 21, 23].

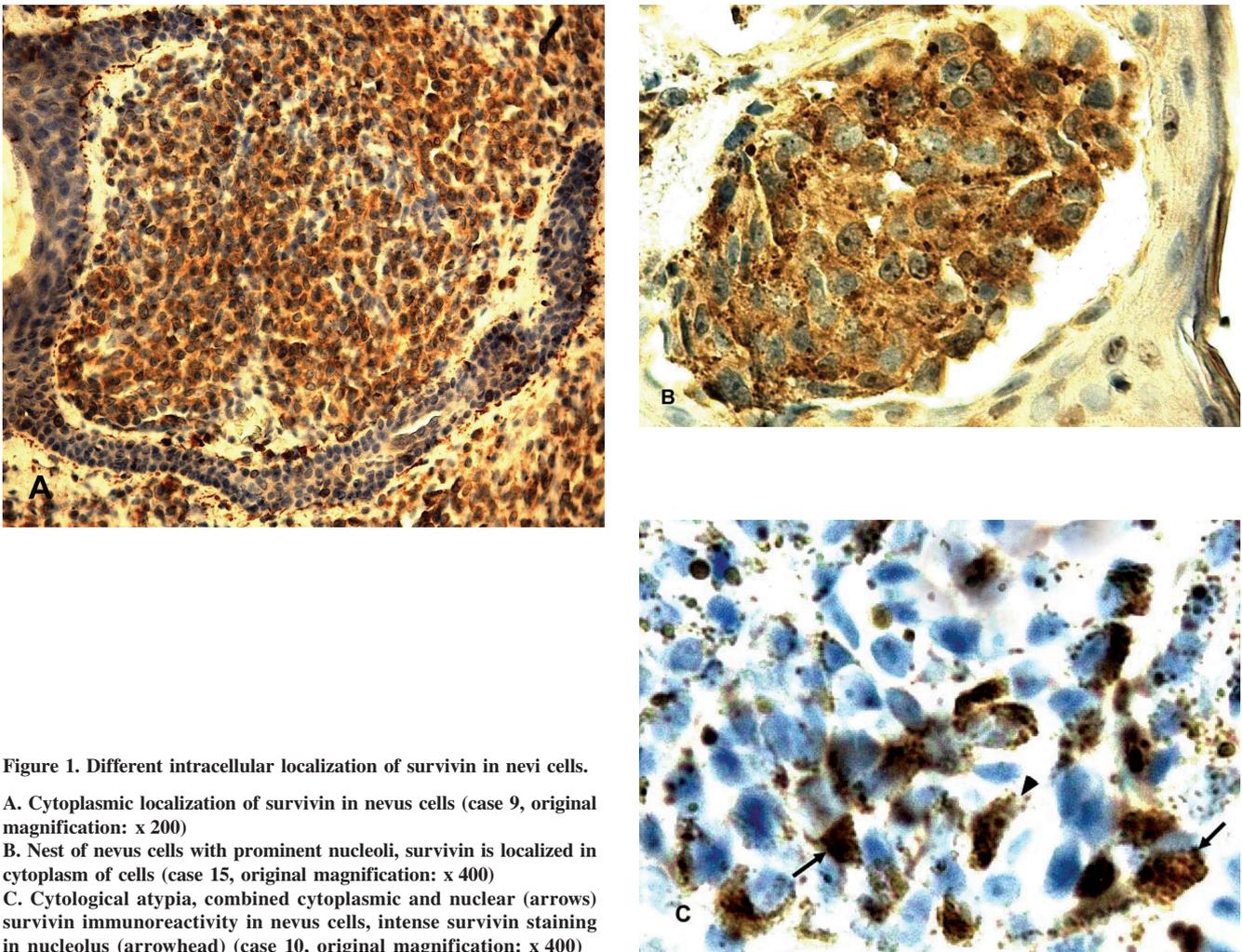
Survivin is not expressed in normal melanocytes, but is detected in dysplastic nevi and malignant melanomas [24]. Functional studies have shown that survivin contributes to increased apoptosis resistance in melanoma cells [25]. Recent studies have indicated that survivin is a prognostic factor for melanomas since melanomas with higher levels of survivin expression are more resistant to therapeutic agents [26].

Survivin can be found in different subcellular localizations. Immunohistochemistry or subcellular fractionation revealed two main pools of survivin: the nucleus and cytoplasm. Recently, a further pool of survivin was identified in mitochondria from tumor cells [27]. Following cell death stimulation, mitochondria survivin pool was released into the cytoplasm, where it was found to inhibit apoptosis. The mitochondrial fraction of survivin appears to play critical role in tumor progression.

**Table 2.** NC localization of survivin and the degree of dysplasia

	Subcellular survivin localization	Dysplasia	Size of skin	Sex lesion	Age/Years
1.	NC	+++	10 mm	F	18
2.	NC	+++	7 mm	F	32
3.	NC	+	5 mm	F	18
4.	NC	+++	6 mm	F	29
5.	NC	+++	8 mm	F	31

NC: nuclear and cytoplasmic localization; **Dysplasia:** + mild, ++ moderate, +++ severe



**Figure 1.** Different intracellular localization of survivin in nevus cells.

**A.** Cytoplasmic localization of survivin in nevus cells (case 9, original magnification: x 200)

**B.** Nest of nevus cells with prominent nucleoli, survivin is localized in cytoplasm of cells (case 15, original magnification: x 400)

**C.** Cytological atypia, combined cytoplasmic and nuclear (arrows) survivin immunoreactivity in nevus cells, intense survivin staining in nucleolus (arrowhead) (case 10, original magnification: x 400)

Moreover, the subcellular localization of survivin may also change during malignant transformation from premalignant lesion into developed malignancy. Ding et al. [28] recently reported, that survivin is variably expressed in the cytoplasm in the entire spectrum of melanocytic lesions, with nuclear expression detectable only in malignant melanomas. These findings may underscore the importance of nuclear survivin in progression to melanoma. Alonso et al. [29] demonstrated nuclear survivin expression in 61% of malignant melanomas, showing notably increased expression with melanoma progression, and nuclear expression was negative in all nevi. Similarly, many other research groups studied cytoplasmic and nuclear expression of survivin in benign and malignant melanocytic lesions [30, 31, 32].

We found in this study, that survivin was variably expressed in panel of 27 dysplastic nevi. Survivin immunoreactivity was absent in 4/27 cases (14,8%). Cytoplasmic localization of immunoreactivity for survivin was detected in 18/27 cases (66,7%). Nuclear localization only was not expressed. These

results are in agreement with the literature, many studies have revealed similar findings [27, 28]. In both localizations, nuclear and cytoplasmic, survivin expression was detected in 5/27 cases (18,5%), with predominance in cytoplasmic compartment. Nuclear staining pattern was diffuse finely granular. There are only very rare data which describe nuclear and cytoplasmic survivin expression in melanocytic nevi. Vetter et al. [31] demonstrated survivin in both localizations in dermal and congenital nevi. Most studies presented cytoplasmic subcellular positivity [28, 32]. Interestingly, 4/5 NC cases (80%) are associated with severe dysplastic changes, while 2/18 C cases (11,1%) only present severe dysplasia. These findings suggest that nuclear survivin expression may be an important in early step in the transformation from nevus to its malignant counterpart. Dysplastic nevi tend to occupy an intermediate position between nevi and malignant melanomas. For nevus growth and progression, cell proliferation should be predominated over apoptotic process. Nevomelanocytes are to be more resist-

ent to apoptosis than normal melanocytes. Increased resistance to apoptosis in nevi is likely mediated by the expression of inhibitor of apoptosis proteins [33] and acquisition of survivin expression in melanocytes may contribute to apoptosis resistance in these skin lesions [16].

The ideal diagnostic tumor marker should be negative in normal tissues or benign lesions but should be expressed in malignancies, especially in early or small ones [34]. Survivin is absent in most normal adult tissues. Survivin belongs to group of attractive tumor diagnostic markers because it is upregulated in wide spectrum of human malignancies. Moreover, increased survivin expression is detected in a number of preinvasive or premalignant lesions with a high predisposition of developing into malignant tumors. For development of malignant melanoma, the strongest single risk factor is the presence and number of dysplastic nevi [35].

For the first time, this study calls attention to the association of nuclear and cytoplasmic survivin immunoreactivity with severe dysplasia in melanocytic nevi. Nevertheless, this data represent a further confirmation of the role of survivin in the progression of dysplastic nevi to melanoma.

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