Expression of apoptosis-inducing factor (AIF) in keratoacanthomas and squamous cell carcinomas of the skin

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Abstract: The complex biological trait ‘susceptibility to apoptosis’ is a nosological feature distinguishing squamous cell carcinomas (SCC) from keratoacanthomas (KA). The purpose of this study was to compare the expression of apoptosis-inducing factor (AIF), a major effector of the caspase-independent apoptosis pathway, in formalin-fixed SCC (N = 23) and KA (N = 29) resection specimens. SCC express statistically significant more AIF than KA both as proportion of AIF+ cells by immunohistochemistry (median: 54% vs 33%; P < 0.01) and as total AIF protein content by western blot quantification (six-fold increased; P < 0.01). However, the contribution of AIF to apoptosis, measured as fraction of apoptotic nuclei with overt DNA fragmentation by the TUNEL method that co-express AIF translocated to nucleus, is significantly less prevalent among SCC (median: 19% vs 48% in KA; P < 0.01). These findings indicate to a distinctive involvement of AIF in the progression of certain epithelial skin tumors that might be exploited as a promising treatment target.

Key words: apoptosis-inducing factor – keratoacanthoma – non-melanoma skin cancer – squamous cell carcinoma

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Introduction

The pathobiological position of keratoacanthomas (KA) within the spectrum of skin tumors of the squamous cell carcinoma (SCC) histophenotype is still disputed. KA are regarded as benign tumors or ‘incomplete’ respectively ‘abortive’ SCC with restricted potential of unlimited growth and metastasis or even as cancers resembling a benign neoplasm (pseudobenignity) (1). To date, there is no single clinical or histomorphological marker (1,2) or genomic alteration to distinguish a KA sufficiently from an SCC, although quantitative differences have been established (3,4), like increased expression of cyclin D1 (5) and decreased somatic p53 mutations (6) in KA. Moreover, accumulating evidence suggests that the pro-apoptotic tissue state is a central distinguishing biological feature of KA compared to SCC (7–10).

Apoptosis-inducing factor (AIF) is a mitochondrial NADH oxidase with redox function acting in normal oxidative phosphorylation and as an endogenous cytoprotector. Upon translocation into the nucleus, it participates in several ‘caspase-independent’ programmed cell death (PCD) scenarios, including apoptosis (11,12). In unstimmed cells, AIF is confined to the mitochondrial intramembrane space. After initiation of the apoptotic pathway, AIF is released to the cytoplasm and is eventually translocated to the nucleus where it participates to PCD-associated DNA fragmentation (13).
In normal epidermis, AIF is constitutively expressed in the keratinocytes of basal and spinal layers (13). Survivin (14) and heat shock proteins (Hsp), particularly HSP-70, (15,16) have emerged as the main regulators of the pro-apoptotic potential of cytosolic AIF in the epidermis by preventing its nuclear translocation.

Leverrier et al. (17) have previously demonstrated that AIF translocation into the nucleus is associated with the induction of apoptosis in SCC cells. In this study, the impact of AIF nuclear translocation on apoptosis was compared in KA and SCC by co-localizing AIF and effected apoptosis in individual nuclei.

**Materials and methods**

The expression of AIF was evaluated in formalin-fixed, paraffin-embedded archival resection specimens of 52 neoplasms (29 KA, 23 SCC) (a) by AIF-immunohistochemistry and Western blot analysis of tumor probes to assess differences in expression pattern of AIF at the cellular and tissue level respectively and (b) by combined AIF immunofluorescence and *in situ* fluorescence TUNEL method to identify cells with nuclear AIF translocation and overt apoptosis at the same time. Moreover, the impact of apoptosis was compared between KA and SCC by the *in situ* immunohistochemistry TUNEL method. Methods are presented in detail in Appendix S1 and the main results are summarized in Table 1 in Appendix S2.

**Results**

The proportion of TUNEL positive apoptotic nuclei (Fig. 1a,b) is significantly higher in KA (median: 12%) compared with SCC (median: 4%; *P* = 0.039; Fig. 1e, column B). TUNEL negative cells with apoptotic morphology were rarely encountered in this material, as a rule in <1% of apoptotic cells irrespective of tumor type. In both tumors, AIF immunostaining is of a predominantly diffuse cytoplasmic pattern with vast inter- and intra-tumoral vari-

![Figure 1](image1.png)

**Figure 1.** (a) Representative keratoacanthoma (KA) section showing high proportion of TUNEL positive cells. (b) Representative squamous cell carcinoma (SCC) section showing scattered TUNEL positive cells. In situ DNA fragmentation in tissue sections was detected by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labelling technique (TUNEL), using a commercially available apoptosis kit (Apoptag, Oncor, Maryland, IN, USA). Original magnification: ×200. (c) Representative KA section showing a predominantly diffuse cytoplasmic distribution pattern of AIF by immunohistochemistry. (d) Representative SCC section showing AIF detection by immunohistochemistry. Note the stronger staining intensity of SCC compared with KA, both as intensity per cell and as proportion of AIF+ cells. Tissue immunostaining was performed on formalin-fixed, paraflin-embedded 4 μm tissue sections with a monoclonal antibody directed against the C-terminus of AIF(92) using the avidin-biotin-peroxidase method. Original magnification: ×60. (e) Comparison of the proportion of AIF+ cells (column A), TUNEL-positive cells (column B) and proportion of AIF+ among all TUNEL positive cells (column C) between KA and SCC. The proportions of AIF- or TUNEL-positive cells were evaluated in 16 randomly selected fields of ‘viable’ tumor areas per tumor at ×40 objective lens magnification by light microscopy. The impact of nuclear AIF translocation on apoptosis was assayed in each tumor as the proportion of TUNEL-positive cells with concomitant AIF translocation among all TUNEL-positive nuclei. Per tumor 100 consecutive TUNEL-positive nuclei in randomly chosen microscopic fields at ×40 objective lens magnification were evaluated. (f) Comparison of relative AIF protein content in SCC and KA (AIF/β-actin band intensity ratio by western blotting). Insert: representative western-blots of SCC and KA showing AIF and β-actin bands. For protein extraction, 5 mm² x 30 μm thick tissue areas without vast necroses were dissected per tumor specimen from archival, formalin-fixed and paraffin-embedded material. *P*-values, calculated with the Mann-Whitney *U*-test, refer to the statistical comparison between KA and SCC.

![Figure 2](image2.png)

**Figure 2.** Confocal two-colour fluorescence microscopy of colocalization of AIF by immunofluorescence (yellow) and of DNA fragmentation by the fluorescence TUNEL method (red) in squamous cell carcinoma (a,c) and keratoacanthoma (b,d). Detection of AIF by immunofluorescence in a representative section of squamous cell carcinoma (a) and of keratoacanthoma (b). Note that (blue) arrows indicate to apoptotic cells as they have been identified after *in situ* fragmentation identification test. Inserts: magnification of the indicated regions discloses some cells with nuclear AIF localization (yellow coloured nuclear shadow). Immunofluorescence combined with the *in situ* fragmentation detection in a representative section of squamous cell carcinoma (c) and of keratoacanthoma (d). The arrows indicate apoptotic TUNEL-positive nuclei without significant AIF signal (red) or colocalization of effected DNA fragmentation and nuclear AIF (orange). For the visualization of apoptosis, DNA fragments in tissue sections were detected by an *in situ* TUNEL method using a commercial apoptosis kit. For the two-colour combination AIF and *in situ* TUNEL immunofluorescence method, first the enzyme terminal deoxynucleotidyl transferase (TdT) was applied to incorporate digoxigenin-conjugated dUTP to the ends of DNA fragments, which were further detected by the addition of anti-digoxigenin conjugated TRITC antibody. The AIF detection step followed by exposing the slides to the first specific anti-AIF antibody and detected with a FITC conjugated secondary antibody. The slides were evaluated under a Leica TC5-SP scanning confocal microscope (Leica, Wetzlar, Germany) using an integrated camera for digital documentation. Original magnification: ×400.
ability (Fig. 1c,d). The proportion of AIF-positive (AIF+) cells was significantly lower in KA (median: 33%) compared with SCC (median: 54%; P < 0.01; Fig. 1e, column A). Moreover, in contrast to relatively weak cellular immunostaining of most AIF+ KA cells, many SCC cells exhibit comparatively strong immunostaining with the well-differentiated areas being generally stained more intensively than the rest of the tissue. The higher quantities of AIF protein expressed in SCC compared with KA were further confirmed by Western blot analysis of tissue samples prepared from the tumors (about six-fold increased; Fig. 1f).

However, apoptosis-related nuclear AIF translocation was more frequent in KA compared with SCC (Fig. 2): Confocal two-colour fluorescence microscopy confirmed colocalization of trans-located nuclear AIF and DNA fragmentation in statistically more apoptotic KA nuclei (median: 48% of all TUNEL+ nuclei) than corresponding apoptotic SCC nuclei (median: 19%; P < 0.01; Fig. 1e, column C).

Discussion

Our results support a distinctive role of AIF in the pathobiological behaviour of KA and SCC. A similar range of AIF+ cells (0–100%) and cells with nuclear AIF translocation (about one-third of parenchymal tumor cells) has been previously reported for SCC (17). Furthermore, our findings (a) of more prominent pro-apoptotic nuclear AIF localization in KA and (b) of increased quantities of this protein in SCC tissues are in accordance with the proposed in the literature ‘pro-apoptotic’ tissue state in KA and the ‘state of comparably inhibited apoptosis’ in SCC respectively (7–10). To date, there are no data regarding AIF mutations in SCC or KA; however, the stronger expression of AIF in SCC is consistent with reports of overexpression of mutated AIF in other cancer types (18).

In addition, our findings are in agreement with evidence indicating an important role of AIF in skin carcinogenesis. In normal skin, UV irradiation induces pro-apoptotic nuclear AIF translocation in damaged epidermal keratinocytes (17), a pathway that is the main regulatory target of survivin, a key apoptosis protective factor in UVB-exposed keratinocytes (14). The role of AIF in removing UV-damaged keratinocytes from the epidermis, and thus counteracting carcinogenesis, seems to be more important in cases of concomitant insufficiency of the ‘classic’ caspase pathway of apoptosis. Such scenarios include UV-irradiation of keratinocytes that carry p53 mutations or are infected by HPV(17) or when irradiation takes place in NO-(19) or H2O2(20)-enriched environment. Moreover, AIF deficiency promotes epidermal carcinogenesis(21) as it makes cells resistant to UV radiation (22), and also protects them from apoptosis induced through pharmacological inhibitor of kappa kinase (IKK) inhibition (23).

Meanwhile, accumulating evidence indicates that AIF is also a promising antineoplastic therapeutic target, especially under apoptosis refractory conditions due to defects of caspase activation (24,25). In many tumor types, the efficacy of different antineoplastic modalities in inducing apoptosis has been associated with the release of AIF from mitochondria (25). Synthetic low molecular compounds and specific biologics that target the pro-apoptotic nuclear translocation of AIF become increasingly available (26–28). To date, efforts targeting apoptosis for skin cancer therapy focused on the caspase-dependent pathway (29). The recently reported key role of AIF as mediator of lactic acid cytotoxicity in HaCaT cells(30), in addition to our present results, suggests that future studies should also evaluate AIF targeting modalities for their therapeutic potential against keratinocyte-derived skin cancers.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:
Appendix S1. Materials and Methods.
Appendix S2. Table 1.
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