Nuclear localization of X-linked inhibitor of apoptosis protein (XIAP): impact on drug resistance, cell growth and prognosis in breast cancer

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Evasion from apoptosis is one of the hallmarks of cancer. X-linked inhibitor of apoptosis protein (XIAP) is known to modulate apoptosis by inhibiting caspases and ubiquitinating target proteins **(Figure 1)**. XIAP is mainly found at the cytoplasm, but recent data link nuclear XIAP to poor prognosis in breast cancer. Here, we generated a mutant form of XIAP with a nuclear localization signal ($XIAP^{NLS\text{-}C\text{-}term}$) and investigated the oncogenic mechanisms associated with nuclear XIAP in breast cancer. We show that cells overexpressing $XIAP^{\Delta RING}$ (RING deletion) and $XIAP^{NLS\text{-}C\text{-}term}$ exhibited XIAP nuclear localization more abundantly than XIAPwild-type, as analyzed by confocal microscopy, cell fractionation and immunoblotting **(Figure 2)**. Remarkably, overexpression of $XIAP^{NLS\text{-}C\text{-}term}$, but not $XIAP^{\Delta RING}$, induced cell growth **(Figure 3)** and chemoresistance **(Figure 4)**, as assessed by cell counting, flow cytometry, clonogenic, MTT and crystal violet assays. Interestingly, Survivin, c-IAP1, c-Myc expression **(Figure 5)**, as well as NFKB activity, were not associated with RING-mediated XIAP oncogenic effects **(Figure 6)**. However, ubiquitination of K63, but not K48 chains, was increased following $XIAP^{NLS\text{-}C\text{-}term}$ overexpression, pointing to nuclear signaling transduction **(Figure 7)**. Consistently, multivariate analysis found nuclear, but not cytoplasmic XIAP, as an independent prognostic factor in hormone receptor-negative breast cancer patients **(Figure 8 and Table 1)**. Altogether, our findings suggest that nuclear XIAP associates with poor outcome and RING-dependent breast cancer growth and chemoresistance.

Keywords: Breast cancer; Evasion from apoptosis; XIAP subcellular localization; Drug resistance; Prognosis

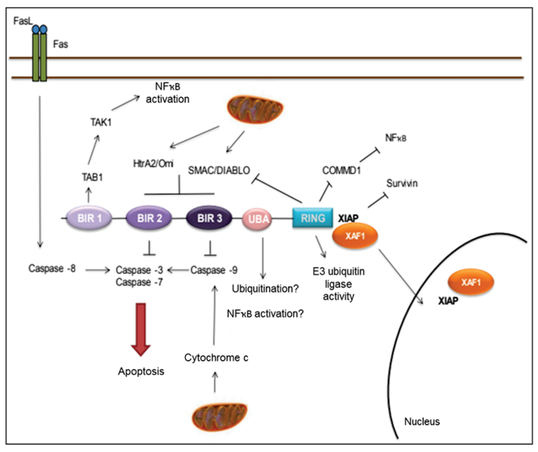

Figure 1: XIAP domains: functions and regulators. Structurally, XIAP protein is composed of a RING, a UBA and BIR1, BIR2 and BIR3 domains. Through the BIR2 and BIR3 domains, XIAP inhibits the apoptotic machinery directly binding to caspases -9, -3 and -7 and blocking the apoptotic pathways. In parallel, XIAP is also involved in the activation of NFkB. The UBA domain is poorly studied and seems to participate in ubiquitination and NFkB activation processes. The RING domain is responsible for ubiquitination and regulation of itself and cellular targets, such as SMAC/DIABLO and COMMD1. XIAP is negatively regulated by SMAC/DIABLO and HtrA2/Omi proteins, which are released from mitochondria after apoptotic stimulus. Additionally, XIAP can be negatively regulated by XAF1, which binds to XIAP and translocates from the cytoplasm to the nucleus, inhibiting the apoptotic function of XIAP. SMAC/DIABLO: Second mitochoncrial-derived activator of caspases/Direct IAP binding with low PI; XAF1: XIAP-associated factor 1; BIR: baculoviral IAP repeat; NFκB: nuclear factor kappa B; RING: really interesting new gene; UBA: ubiquitin associated domain ; TAB1:TAK1-binding protein ; TAK1: transforming growth factor β-activated protein kinase 1.

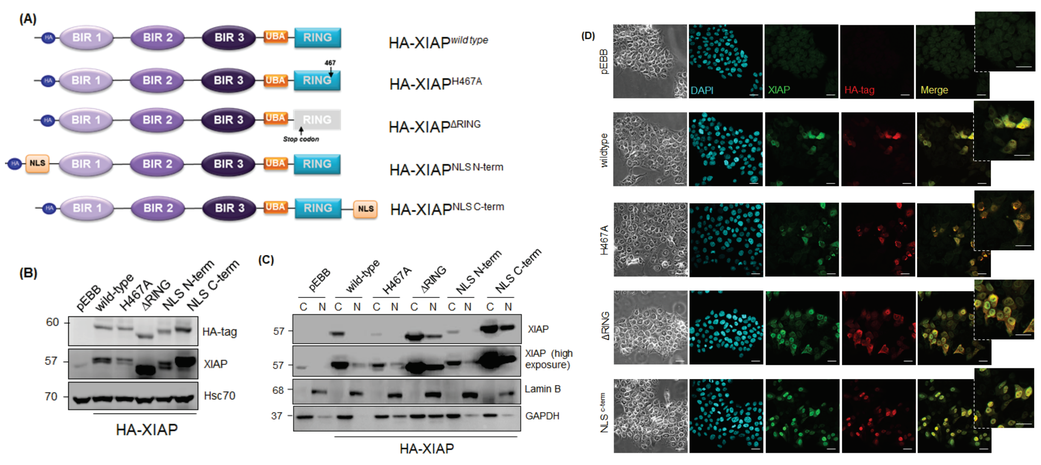

Figure 2: XIAP expression and subcellular localization in MCF-7 cells overexpressing the vectors pEBB, $XIAP^{wild\ type}$, $XIAP^{H467A}$, $XIAP^{\Delta RING}$, $XIAP^{NLS\text{-}N\text{-}term}$ and $XIAP^{NLS\text{-}C\text{-}term}$. MCF-7 cells were left to adhere in petri dishes (10cm) for 24 h and there after, were transfected with the pEBB, $XIAP^{wild\ type}$, $XIAP^{H467A}$, $XIAP^{\Delta RING}$ and $XIAP^{NLS\text{-}C\text{-}term}$ vectors (A), using Lipofectamine 2000. (B) The levels of XIAP expression in XIAP-transfected cells were examined by Western blotting . (C) MCF7 cells had their cytoplasmic and nuclear fractions separated by NE-PER kit (Thermoscientific). (D) Immunofluorescence analysis indicates the subcellular localization in XIAP-transfected cells immunolabeling with XIAP and HA-tag antibodies. Hsc70 was used as constitutive. Lamin B was used as a nuclear constitutive control, while GAPDH, as cytoplasmic constitutive control. C: cytoplasm; N: nucleus.

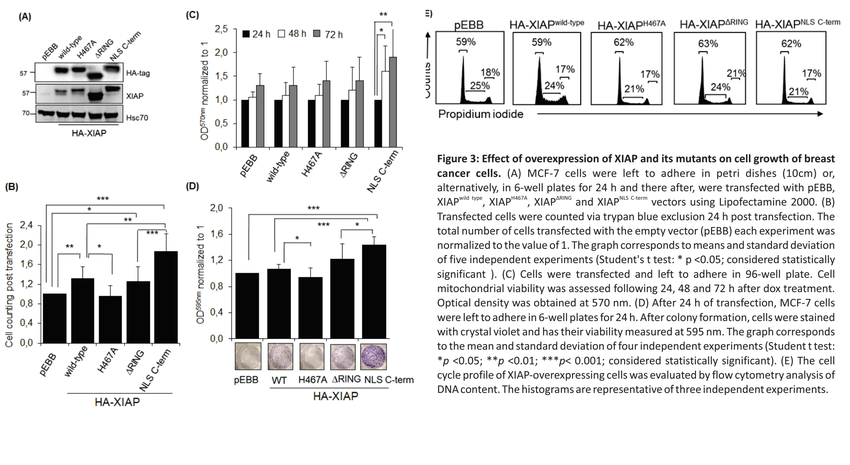

Figure 3: Effect of overexpression of XIAP and its mutants on cell growth of breast cancer cells. (A) MCF-7 cells were left to adhere in petri dishes (10cm) or, alternatively, in 6-well plates for 24 h and there after, were transfected with pEBB, $XIAP^{wild\ type}$, $XIAP^{H467A}$, $XIAP^{\Delta RING}$ and $XIAP^{NLS\ C\text{-}term}$ vectors using Lipofectamine 2000. (B) Transfected cells were counted via trypan blue exclusion 24 h post transfection. The total number of cells transfected with the empty vector (pEBB) each experiment was normalized to the value of 1. The graph corresponds to means and standard deviation of five independent experiments (Student's t test: * p <0.05; considered statistically significant). (C) Cells were transfected and left to adhere in 96-well plate. Cell mitochondrial viability was assessed following 24, 48 and 72 h after dox treatment. Optical density was obtained at 570 nm. (D) After 24 h of transfection, MCF-7 cells were left to adhere in 6-well plates for 24 h. After colony formation, cells were stained with crystal violet and has their viability measured at 595 nm. The graph corresponds to the mean and standard deviation of four independent experiments (Student t test: *p <0.05; **p <0.01; ***p< 0.001; considered statistically significant). (E) The cell cycle profile of XIAP-overexpressing cells was evaluated by flow cytometry analysis of DNA content. The histograms are representative of three independent experiments.

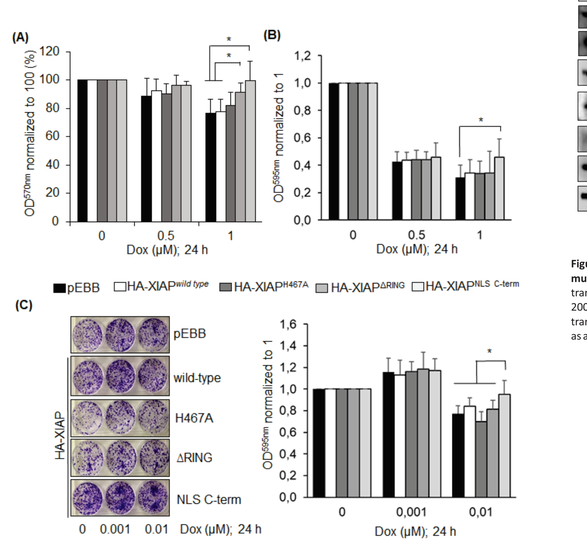

Figure 4: Effect of overexpression of XIAP and its mutants on doxorubicin (dox) resistance in breast cancer cells. MCF-7 cells were left to adhere in petri dishes (10cm) or, alternatively, in 6-well plates for 24 h and there after, were transfected with the vectors pEBB, $XIAP^{wild\ type}$, $XIAP^{H467A}$, $XIAP^{\Delta RING}$, $XIAP^{NLS\text{-}N\text{-}term}$ and $XIAP^{NLS\text{-}C\text{-}term}$, using Lipofectamine 2000. (A) After 24 h of transfection, MCF-7 cells were left to adhere in 96-well plates for 24 h. Subsequently, dox was added at 0,5 e 1 μM concentrations and the cells were incubated for 24 and 72 h for MTT and crystal violet assays. Cell viability was measured at 570nm and 595nm, respectively. For each dox concentration, the cell lines transfected with the different XIAP-encoding plasmids were compared to the XIAP wild-type transfected cells and to the empty vector pEBB. Graph A corresponds to means and standard deviation of three independent experiments (Student t test: * p <0.05, considered statistically significant). (B) Cells were transfected and left to adhere in 96-well plate. After 24, 48 and 72 h of drug exposure, they were fixed and stained with crystal violet and has their viability measured at 595nm (n=1). (C) After 24 h of transfection, MCF-7 cells were left to adhere in 6-well plates for 24 h. The cells were treated with doxorubicin for 24 h and left for 14 days in the CO_2 incubator. After colony formation, cells were stained with crystal violet and has their viability measured at 595nm.

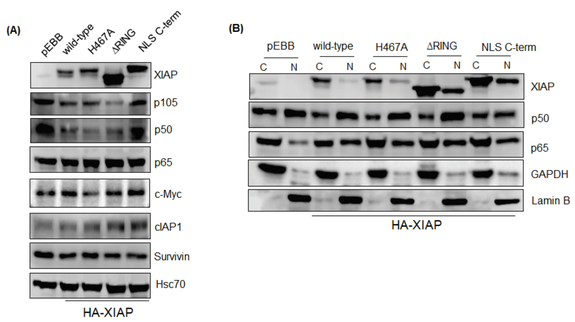

Figure 5: Expression pattern of NFkB subunits, c-Myc, cIAP1 and Survivin following overexpression of XIAP and its mutants in breast cancer cells. MCF-7 cells were left to adhere in petri dishes (10cm) for 24 h and there after, were transfected with the pEBB, $XIAP^{wild\ type}$, $XIAP^{H467A}$, $XIAP^{\Delta RING}$, $XIAP^{NLS\ N\text{-}term}$ and $XIAP^{NLS\ C\text{-}term}$ vectors, using Lipofectamine 2000. The expression levels of XIAP, NFkB subunits (p50, p105 and p65), c-Myc, cIAP1 and Survivin in XIAP-transfected cells were examined in whole cell (A) and fractioned lysates (B) by Western blotting . Lamin B was used as a nuclear constitutive control, while GAPDH, as cytoplasmic constitutive control. C: cytoplasm; N: nucleus

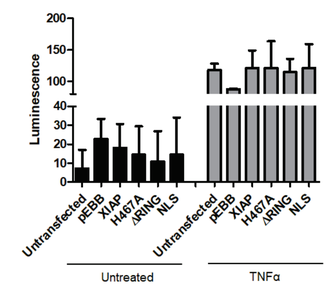

Figure 6: Overexpression of XIAP mutants do not affect NF-kB transcriptional activity. HEK293 cells expressing pBIIx-luc were transfected with 200ng of indicated plasmids with Lipofectamine 2000, according to manufacturer instructions. Following 24h of transfection, cells were treated with 10 ng/mL of TNF-α (Sigma Aldrich) during 24h. Cere were then lysed and luminescence of total extracts was measured with a SpectraMax i3 luminometer (Molecular Devices), where NFkB transcriptional activity was assessed by the NF-κB luciferase reporter assay.

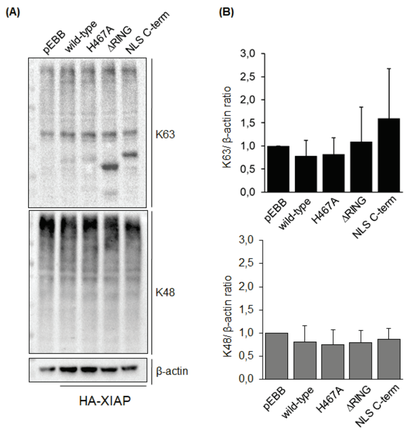

Figure 7: Overexpression of XIAP mutants is associated with an increase in K63, but not K48-linked ubiquitination. MCF-7 cells were left to adhere in petri dishes (10cm) for 24 h and there after, were transfected with the pEBB, $XIAP^{wild\ type}$, $XIAP^{H467A}$, $XIAP^{\Delta RING}$, $XIAP^{NLS\text{-}N\text{-}term}$ and $XIAP^{NLS\text{-}C\text{-}term}$ vectors, using Lipofectamine 2000. The expression pattern of K63 and K48 ubiquitin chains were measured by Western blotting using K63 and K48-specific antibodies (A) and quantification was performed following normalization against β-actin levels (B).

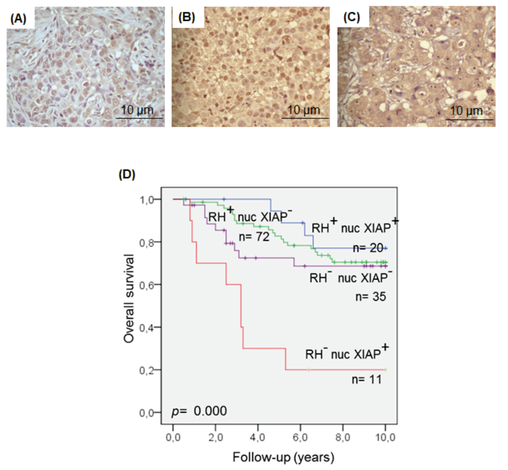

Figure 8: Overall survival of patients with infiltrating ductal breast carcinoma grouped according to XIAP localization and expression of hormone receptors. Representative staining from immunohistochemical analysis of XIAP expression: (A) Nuclear XIAP staining scored 8; (B) Both nuclear and cytoplasmic XIAP staining scored 6 and 8, respectively; (C) Cytoplasmic XIAP staining scored 8. Original magnificátion 40×. (D) The impact of XIAP subcellular localization in the total population was analyzed according to expression of nuclear XIAP and hormone receptors. The Kaplan-Meier curves, were compared by the log-rank test, where the value of p <0.05 was considered statistically significant. HR: Hormone Receptors; Cyt: cytoplasmic; Nuc: Nuclear

Table 1. Multivariate analysis of XIAP expression and localization in patients with infiltrating ductal breast cancer carcinoma according to clinical-biological parameters.

	Total population			Hormone receptor-positive patients			Hormone receptor-negative patients		
	Multivariate analysis								
Characteristics	***p***	**HR**	**(95% CI)**	***p***	**HR**	**(95% CI)**	***p***	**HR**	**(95% CI)**
Age at diagnosis	0.814	0.055	(0.964 - 1.048)	0.569	0.569	(0.933 -1.039)	0.409	0.682	(0.957 - 1.114)
Tumor size	0.631	0.231	(0.583 - 1.386)	0.767	0.088	(0.640 - 1.389)	0.206	1.600	(0.181 - 1.446)
Tumor grade	0.340	0.912	(0.686 - 2.987)	0.498	0.459	(0.504 - 4.089)	0.061	3.497	(0.910 - 55.629)
Her2 expression	0.161	1.964	(0.556 - 34.298)	0.986	0.000	(0.000 -)	0.560	0.340	(0.184 - 22.867)
Hormone receptors	0.023 *	5.167	(1.140 - 5.878)	-	-	-	-	-	-
Total XIAP expression	0.756	0.097	(0.264 - 6.333)	0.406	0.691	(0.024 - 4.503)	0.326	0.966	(0.223 - 92.968)
Cytoplasmic XIAP	0.720	0.128	(0.334 - 4.894)	0.554	0.351	(0.198 - 20.574)	0.669	0.183	(0.174 - 15.216)
Nuclear XIAP	0.358	0.846	(0.175 - 1.875)	0.567	0.328	(0.231 - 14.459)	0.011 *	6.504	(0.004 - 0.483)

Stage III invasive ductal breast carcinoma patients samples, at diagnosis
N=138

CONCLUSIONS

- Nuclear XIAP confers poor clinical outcome in hormone receptor-negative patients.
- Overexpression of nuclear XIAP associates with cell growth and drug resistance in vitro.
- Nuclear XIAP might contributing towards an aggressive phenotype in breast cancer.

NEXT STEPS

- Which mechanism underlies XIAP translocation from the cytoplasm to the nucleus?
- Which partners interact with XIAP to promote cell growth and chemoresistance in the nucleus?
- Nuclear XIAP as an independent prognostic factor in a larger cohort of breast cancer patients?

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