

Expression changes of BIK in breast cancer tissues of different histological subtypes

Cambios en la expresión de BIK en tejidos de cáncer de mama de diferente subtipo histológico

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Abstract

Objective: The objective of the study was to determine the expression levels of BIK in breast cancer (BC) tissues of different histological subtype and to delve into the participation of BIK in this type of cancer. **Materials and methods:** BIK and p-BIK (the phosphorylated form) protein expressions were tested by immunohistochemistry in BC tissue microarrays (Tumoral [n = 90] and adjacent [n = 40] tissues). **Results:** The data revealed an overexpression of BIK in invasive ductal (Grades I, IIA, and IIB) and in lobular (Grades IIA and IIB) carcinomas compared to their respective adjacent tissues. By contrast, canalicular carcinoma (Grades I and IIB) and phyllodes tumors had very low expression levels of BIK. Only levels of p-BIK were shown to be increased in invasive ductal carcinoma (Grades I, IIA, and IIB). Meanwhile, quantitative polymerase chain reaction analysis showed lower BIK levels in MCF-10A and MCF-7 cells than in MDA-MB-231 and human mammary epithelial cells. In agreement with this, BIK protein was shown to be overexpressed in MDA-MB 231 relative to MCF-7 cells. **Conclusions:** Our results showed an association between BIK expression and the BC tumor subtype under study, which could be related to different BIK functions in the BC subtypes.

Key Words: BIK. Breast cancer. Tissue microarray. Tumor suppressor. Oncogene.

Resumen

Objetivo: Determinar el grado de expresión de BIK en tejidos de cáncer de mama de diferente subtipo histológico para ahondar en la participación de BIK en este tipo de cáncer. **Método:** Por medio de inmunohistoquímica se determinó la expresión de BIK y de su forma fosforilada (p-BIK) en microarreglos de tejidos (tumores [n = 90] y tejidos adyacentes [n = 40]) y líneas celulares. **Resultados:** Los datos mostraron una sobreexpresión de BIK en los carcinomas de tipo ductal invasivo (grados I, IIA y IIB) y lobular (grados IIA y IIB) con respecto a sus tejidos adyacentes respectivos. En contraste, el carcinoma

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canalicular (grados I y IIB) y los tumores filoides mostraron una baja expresión de BIK en relación con sus tejidos adyacentes respectivos. El análisis de la qPCR mostró una menor expresión de BIK en las células MCF-10A y MCF-7 en comparación con las células MDA-MB-231 y HMEC. En concordancia con esto, la expresión proteica de BIK fue mayor en las células MDA-MB 231 que en las células MCF-7. **Conclusión:** Nuestros resultados mostraron una asociación entre la expresión de BIK y el subtipo tumoral en estudio, lo cual sugiere una función diferencial de BIK en el cáncer de mama.

Palabras Clave: BIK. Cáncer de mama. Microarreglos de tejidos. Supresor tumoral. Oncogén.

Introduction

A member of the BH₃-only family¹, BIK is a pro-apoptotic protein located in the endoplasmic reticulum (ER). This protein mediates apoptosis through the mitochondrial way by mobilizing Ca²⁺ from the ER to the mitochondria and by remodeling the mitochondrial cristae²⁻⁴. In different human tissues, BIK plays a role as a tumor suppressor⁵, and its expression in cancer is abolished by chromosomal deletions encompassing the BIK locus or by epigenetic silencing⁵, which results in the evasion of apoptosis and in the induction of cell proliferation. By contrast, BIK overexpression was demonstrated in breast cancer (BC) tissues⁶ and non-small cell lung carcinoma⁷, which correlated with poor prognosis of patients. In addition, the control of autophagy by BIK in the autophagy-dependent BC cell line MDA-MB-231 strongly suggests its involvement in tumorigenesis⁸. Based on this, it seems that both down- and upregulation of BIK is associated with cancer malignancy, which seems to depend on the BC subtype under study.

To better understand the involvement of BIK in BC, its protein expression levels were tested – in four histological subtypes of BC – by immunohistochemistry (IHC) in tissue microarrays (TMAs). Furthermore, BIK expression was determined by quantitative polymerase chain reaction (qPCR) and Western blot in human mammary epithelial cells (HMEC), MCF-7, and MDA-MB-231 cell lines. As expected, data analysis showed differences in BIK expression among the BC tissues tested and in the BC cell lines MDA-MB-231 and MCF-7. The meaning of this must be elucidated with further studies.

Experimental procedures

Samples

Tumoral (n = 90) and adjacent (n = 40) tissues were obtained from women over 45 years old with BC

Table 1. Tumor and adjacent samples employed for breast cancer microarrays

Tumor type	Grade I	Grade IIA	Grade IIB
Invasive ductal carcinoma	Adjacent (n = 3) Tumors (n = 8)	Adjacent (n = 10) Tumor (n = 22)	Adjacent tissues (n = 3) Tumor tissues (n = 18)
Lobular carcinoma	Adjacent (n = 3) Tumors (n = 3)	Adjacent (n = 3) Tumors (n = 11)	Tumors (n = 5)
Canalicular carcinoma	Adjacent (n = 3) Tumors (n = 3)	- -	Adjacent (n = 3) Tumors (n = 3)
Phyllodes	- -	Adjacent (n = 3) Tumors (n = 3)	Adjacent (n = 3) Tumors (n = 3)
Intraductal carcinoma	- -	-	Adjacent (n = 3) Tumors (n = 3)
Mixed ductal and lobular carcinoma	- -	Adjacent (n = 3) Tumors (n = 5)	Tumors (n = 3)

Tumors n = 90; Adjacent tissues: 40.

(Table 1). All women included in the present study (>45 years old; patients without treatment) were born in Mexico with grandparents also born in Mexico and from families with no history of cancer (*de novo* tumors). All tumor cases were histologically reviewed by at least two pathologists, one of them selected and marked representative tumor and adjacent areas for the preparation of TMA. The ethical committee of the National Research Council of Instituto Mexicano del Seguro Social approved the study and thus meets the standards of the Declaration of Helsinki in its revised version of 1975 and its amendments of 1983, 1989, and 1996 (JAMA 1997;277:925-926). Patients were informed about the study and ask to sign an informed consent letter.

BC TMA and IHC

TMAs were prepared from blocks of formalin-fixed paraffin, embedded BC specimens obtained from two

Mexican hospitals (Luis Castelazo Gynecology Hospital and Oncology Hospital, CMNSXXI, IMSS). Tissue (2 μm thick) was cored from representative areas of tumor and adjacent tissues for TMA. Clinical and pathologic data were obtained from medical and pathology records. Sections 3 μm thick in size were cut for routine hematoxylin and eosin (H&E) staining and IHC. Microarrays were processed in a Ventana Benchmark system (Roche). TMAs were dehydrated using ascending alcohol solutions, cleared in a xylol solution, and mounted in a synthetic resin. The primary antibodies used were anti-BIK (Abcam, ab52182) or its phosphorylated form (anti-p-BIK) (Abcam, ab55179). A set of microarrays was reacted with normal rabbit IgG, as a negative control. Then, microarrays were observed in a DM750 Leica microscope (Wetzlar, Germany), and digital images obtained using the LEZ software. Two independent observers evaluated the arrays. Densitometry was performed using the Image-Pro software (Media Cybernetics, Rockville, USA) and graded as high (~10,000-15,000 pixels), intermediate (~2000 pixels), and low signal (10-50 pixels), according to the intensity of staining present in tissues.

Densitometry analysis

Densitometry was performed using the Image-Pro software (Media Cybernetics, Rockville, USA) selecting: measure --- Count/Size --- Measure --- Select Measurements --- Density (mean). According to the signal present in tissues, density was divided into high (~10,000-15,000 pixels), intermediate (~2,000 pixels), and low (10-50 pixels) signal; it is important to mention that low signal was not considered and it was taken as background. The background signal was the one obtained from the negative controls (tissue incubation without the specific antibody [anti-BIK]), and it was subtracted from the specific signal for BIK. Furthermore, when the signal was very close to zero (low signal: ~10-50 pixels), it was subtracted from the specific one.

Data represent mean \pm SEM from three experiments in triplicated; statistical significance was determined by the Student's t-test ($p < 0.05$).

Cell culture

Cell lines HMEC, MCF-7, MCF-10A, and MDA-MB-231 (American Type Culture Collection, ATTC, USA) were maintained in Dulbecco's Modified Eagles Medium F:12 (DMEM; Invitrogen, Carlsbad, CA, USA)

at 37° C in 5% CO₂ atmosphere. For HMEC and MCF-10A cells, the medium was supplemented with 5% (v/v) horse serum + 10 $\mu\text{g/mL}$ human insulin + 20 ng/mL hEGF + 100 ng/mL cholera toxin + 0.5 $\mu\text{g/mL}$ hydrocortisone. Meanwhile, MCF-7 and MDA-MB-231 cell lines were maintained in the presence of 10% (v/v) fetal bovine serum-containing penicillin (100 U/mL) and streptomycin (100 $\mu\text{g/mL}$).

qPCR

qPCR for BIK was done in non-tumorigenic cell lines HMEC and MCF10A, and in the BC cells MCF-7 (receptor positive) and MDA-MB-231 (triple negative, claudin-low), by following the conditions established by Pfaffl⁹. Gene 18S was amplified, purified, and quantified. Serial dilutions (1:200; 1:20,000; 1:2,000,000; 1:20,000,000; and 1:20,000,000,000) were used to construct the standard curve. BIK amplification was always carried out in the presence of the standard curve. Primers used for 18S amplification: forward: 5'-TAC-CACATCCAAGGAAGGCAGCA-3'; reverse: 5'-TGG AATTACCGCGGCTTGCTGGCA-3'. Primers used for BIK: forward: 5'-CCCCGAGATAGTGCTGGAAC-3'; reverse: 5'-GCCGAGGGCATCACATATCA-3'. Statistics analysis ($p < 0.05$; Student's t-test) and graphical visualization were carried out with GraphPad Prism 5 (San Diego, CA).

Western blot

Western blot was performed as previously described¹⁰. Cell cultures lysed in RIPA-Tris buffer (mM: EGTA 2; NaCl 316; NaF 50; Tris-HCl 20; Na₃VO₄ 100, PMSF 100, and EDTA 100; 0.1% of leupeptin and aprotinin; sodium dodecyl sulfate [SDS] 0.2% and Triton-X 100 2%) under constant shaking for 2 h at 4°C. After that, the sample was centrifuged for 5 min at 20,800 g and the supernatant (30 μg of protein) was denatured in Laemmli sample buffer, resolved through 15% SDS-polyacrylamide gels and electroblotted to polyvinylidene difluoride membranes. Membranes were incubated for 90 min in Tris-buffered saline (TBS) containing 5% dried skimmed milk and 0.1% Tween 20 (TBS-Tween milk) to block the non-specific protein-binding sites. Subsequently, membranes were incubated for 14 h at 4°C with primary antibodies (anti-BIK [Abcam ab52182]; anti-pBIK [Abcam ab55179]; or anti-GAPDH [EMD Millipore ABS16]) and subsequently with the secondary antibody (ECL-anti-rabbit [Abcam ab6721]) diluted in TBS-Tween milk. The protein was detected

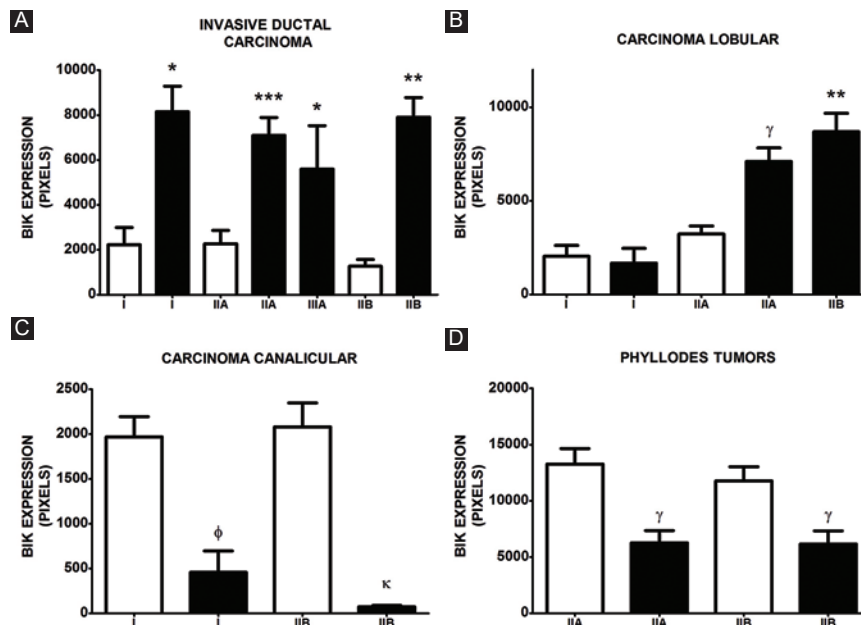


Figure 1. A-D: BIK expression in breast cancer microarrays. Both IDC and lobular carcinoma (LC) showed increased BIK expression levels. By contrast, BIK was down-expressed in canalicular carcinoma and phyllodes tumors. Data represent the mean \pm SEM in triplicated. * ($p < 0.04$; Student's t-test vs. control [I]); ** ($p < 0.007$; Student's t-test vs. control [IIB]); *** ($p < 0.0007$; Student's t-test vs. control [IIA]); γ ($p < 0.01$; Student's t-test vs. control [I]); φ ($p < 0.017$; Student's t-test vs. control [IIB]); κ ($p < 0.02$; Student's t-test vs. control [IIA]).

using an ECL Western blot detection kit (Millipore). The blots were subjected to a densitometry analysis and data were analyzed using the GraphPad Prism 5 software (San Diego, CA). The intensity values for BIK were normalized against the GAPDH loading control. Data were the mean \pm SEM from three independent experiments ($p < 0.05$; Student's t-test).

Results

BIK expression

To establish the expression of BIK on different types and grades of BC tissues, IHC studies were performed on TMA. Invasive ductal carcinoma (IDC) showed an overexpression of BIK in Grade I ($142 \pm 34\%$; $P < 0.04$; Student's t-test vs. control [I]) and Grades IIA ($226 \pm 37\%$; $p < 0.0007$; Student's t-test vs. control [IIA]) and IIB ($521 \pm 68\%$; $p < 0.007$; Student's t-test vs. control [IIB]) relative to adjacent tissues (Fig. 1A). Similarly, lobular carcinoma (LC) Grades IIA and IIB had a considerable BIK signal increase by $293 \pm 53\%$ ($p < 0.02$; Student's t-test vs. control [IIA]) and $347 \pm 86\%$ ($p < 0.007$; Student's t-test vs. control [IIB]) when compared to the adjacent tissue (Fig. 1B).

Carcinoma canalicular Grades I and IIB expressed lower levels of BIK than their adjacent tissues. The decrease observed was of $71 \pm 12\%$ ($p < 0.01$;

Student's t-test vs. control [I]) for Grade I and by $97 \pm 0.53\%$ ($p < 0.017$; Student's t-test vs. control [IIB]) for Grade IIB (Fig. 1C). Similarly, rare breast tumor phyllodes Grades IIA and IIB expressed lower BIK levels $-61 \pm 12\%$ ($p < 0.02$; Student's t-test vs. control [IIA]) and $-59 \pm 14\%$ ($p < 0.02$; Student's t-test vs. control [IIB]) than their adjacent tissues, respectively (Fig. 1D).

p-BIK expression

Similar to BIK expression levels, the phosphorylated form of BIK "p-BIK" (Thr-33) gradually increased in Grades I ($72 \pm 22\%$; $p < 0.001$; Student's t-test vs. control [I]), IIA ($168 \pm 24\%$; $p < 0.0001$; Student's t-test vs. control [IIA]), and IIB ($256 \pm 26\%$; $p < 0.0001$; Student's t-test vs. control [IIB]) of IDC, relative to adjacent tissues (Fig. 2A). By contrast, p-BIK expression did not differ from adjacent tissues and tumor samples and between distinct tumor grades of LC (Fig. 2B). In the same way, p-BIK did not exhibit expression differences in intraductal carcinoma and mixed ductal and LC (Fig. 2C and D).

BIK expression in BC cell lines

The expression of BIK was tested at mRNA and protein levels in the HMEC, MCF10A, MCF-7, and

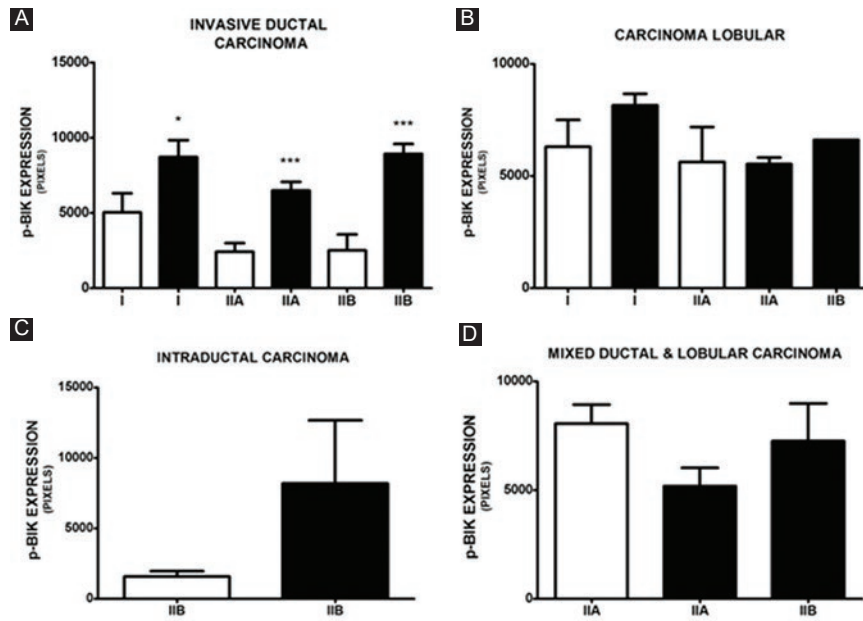


Figure 2. A-D: p-BIK expression in breast cancer microarrays. The phosphorylated form of BIK showed expression changes in invasive ductal carcinoma, but it was not statistically different in the other tumor subtypes. Values are the mean \pm SEM performed in triplicated. *($p < 0.001$; Student's t-test vs. control [I]); ***($p < 0.0001$; Student's t-test vs. control).

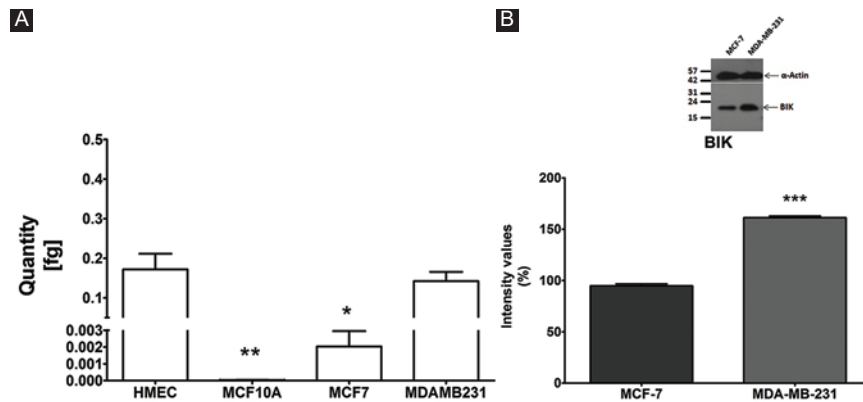


Figure 3. A: BIK expression in BC cell lines. BIK mRNA levels were determined by quantitative polymerase chain reaction (qPCR) – using a standard curve (RNA, 18S ribosomal gene) – and **B:** Western blot. qPCR values are the mean \pm SEM of three experiments performed in triplicated. *($p < 0.02$; Student's t-test vs. human mammary epithelial cells [HMEC] or MDA-MB-231); **($p < 0.0002$; Student's t-test vs. HMEC or MDA-MB-231). Western blot data represent the mean \pm SEM of three independent experiments. ***($p < 0.0001$; Student's t-test vs. MCF-7).

MDA-MB-231 cell lines. At mRNA level, HMEC (0.17 ± 0.04 fg) and MDA-MB-231 (0.14 ± 0.0023 fg) cells showed higher mRNA expression levels than those observed for MCF-10A ($1.9 \times 10^{-5} \pm 6.3 \times 10^{-8}$ fg) and MCF-7 (0.002 ± 0.00092 fg) cell lines (Fig. 3A). In a similar manner, Western blot analysis showed that BIK expression was higher in MDA-MB-231 ($67 \pm 4\%$) than in MCF-7 cells (Fig. 3B).

Discussion

BIK protein levels were shown to be different among the BC subtypes studied: BIK was overexpressed in

IDC and LC but underexpressed in canalicular and phyllodes subtypes. This contrasting expression change of BIK might be related to the control of specific cellular processes in each BC subtype.

Different lines of evidence have shown that BIK expression is either decreased or increased in BC. The processes that lead to a decrease in the expression of BIK are diverse and involve genomic deletions at BIK locus⁵, selective silencing of the BIK gene expression^{5,11,12}, and BIK post-translational downregulation by proteasomal degradation¹². Importantly, BIK levels significantly increased in response to proteasome inhibition and to estrogen receptor antagonism in

the BC cell lines 293T and MCF-7¹² and MCF-7¹³, respectively. This increase in BIK levels resulted from the apoptosis induction and the cell cycle arrest. Similarly, the apoptosis of tumor-initiating cells (TICs) was induced by the transfection of a constitutively active form of BIK, which resulted in a decrease in the TIC number and tumor growth¹⁴. Despite the fact that mechanisms reducing BIK expression were shown to be different, BIK acts in all cases by functioning as pro-apoptotic protein. If BIK levels decrease, BAX continues to interact with BCL2 and BAX does not translocate to the mitochondria; consequently, apoptosis is not induced.

On the other hand, BIK overexpression was not related to the control of apoptosis in BC⁶ and non-small cell lung cancer (NSLCC)⁷. In NSLCC, BIK overexpression was accompanied by increased levels of pro-inflammatory genes, such as BCL-2, which suggests that an inflammatory response is participating in the promotion of tumorigenesis⁷. Furthermore, the involvement of BIK in the control of autophagy of MDA-MB-231 cells was recently demonstrated, predicting its participation in the MDA-MB-231 cell survival and proliferation⁸. Interestingly, Maycotte et al.¹⁵ showed that MDA-MB-231 cells – among other BC cells – were dependent on autophagy for survival, which strongly suggest the participation of BIK in tumorigenesis. In fact, very recent data indicated the association of BIK overexpression with poor outcomes of BC in an autophagy-dependent manner⁶.

Together, evidence strongly suggest that decreased expression levels of BIK – observed in canalicular carcinoma and phyllodes tumors – could be related to apoptosis evasion; however, other mechanisms cannot be excluded. As mentioned earlier, BIK overexpression was shown to be associated with malignancy of IDC in an autophagy-dependent manner, which correlates with BIK overexpression we found. Meanwhile, the impact of BIK overexpression in LC remains to be elucidated. It is important to mention that not only the changes on BIK expression seem to have an impact on BC but it also was recently demonstrated its interaction with the MDA-MB-231 cell genome¹⁶.

Conclusions

BIK expression changes pointed out the involvement of BIK in the control of different cellular pathways in specific BC subtypes.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors have obtained the written informed consent of the patients or subjects mentioned in the article. The corresponding author is in possession of this document.

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