REGULATION OF FOXK2 TRANSCRIPTION FACTOR BY AKT ONCOGENIC KINASE: ROLE IN BREAST CANCER DRUG RESISTANCE

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INTRODUCTION

Breast cancer is a tumor type with high mortality rates. Despite therapeutic advances, drug resistance is still the main cause of treatment failure. Recently, we demonstrated that the FOXK2 transcription factor modulates drug sensitivity in breast cancer cells through the induction of FOXO3a expression. However, we found constitutively high levels of FOXK2 in drug-resistant cells and poor outcome patients, suggesting that FOXK2 deregulation possibly contributes to the development of drug resistance and poor prognosis. We then hypothesized that FOXK2 is regulated post-translationally, which leads to impairment of its function, contributing to a resistant and aggressive phenotype of breast tumors. Thereby, we performed an in silico analysis and found the oncogenic serine-threonine kinase AKT (isoform 1) as a putative regulator of FOXK2. Thus, the aim of this study was to evaluate whether AKT can regulate the expression and function of FOXK2 and investigate how this regulation affects drug resistance in breast cancer **(Figure 1)**.

METHODS AND RESULTS

Our results show that increased expression of FOXK2 is associated with higher levels of AKT phosphorylation and with a resistant phenotype in breast cancer cells **(Figure 2)**, as assessed by Western blot and MTT assay. Following AKT1 gene silencing by small interfering RNA **(Figures 3a and 3b)**, MDA-MB-231 cells exhibited decreased short-term viability and colony formation capacity **(Figures 3c and 3d)**, as evaluated by MTT and clonogenic assays, respectively. Moreover, AKT1-inhibited cells showed greater sensitivity to doxorubicin treatment **(Figures 3e and 3f)**, suggesting that AKT1 is required for survival and promotes doxorubicin resistance. We also observed that the pattern of FOXK2 expression is altered following AKT1 knockdown **(Figures 4a and 4b)**. By silencing AKT1, there was no modulation in the expression of AKT2 and AKT3 isoforms **(Figure 4b)**, suggesting that the effects found on FOXK2 expression are AKT1-specific. Nevertheless, the induction of FOXK2 overexpression did not affect the growth and the sensitivity to doxorubicin in AKT1-inhibited cells **(Figures 4c, 4d and 4e)**. Corroborating these data, the induction of AKT overexpression by transient transfection with the pBABE-(myr)Akt:ER vector **(Figure 5a)** resulted in increased FOXK2 expression **(Figure 5b)**, further confirming that FOXK2 is regulated by AKT.

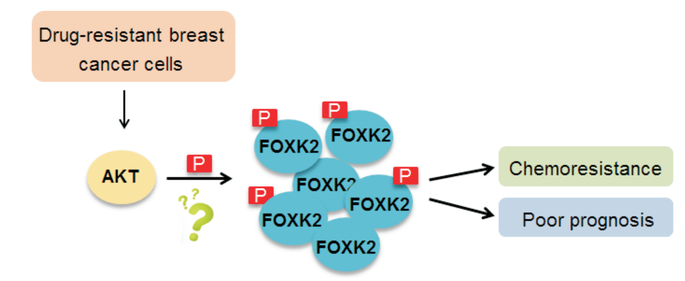


Figure 1 – Study hypothesis. The present study is based on the hypothesis that AKT promotes the phosphorylation of FOXK2, inactivating its tumor suppression function and contributing to a drug resistance phenotype and poor prognosis in breast cancer.

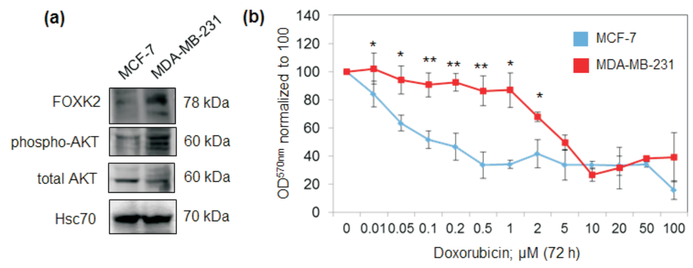
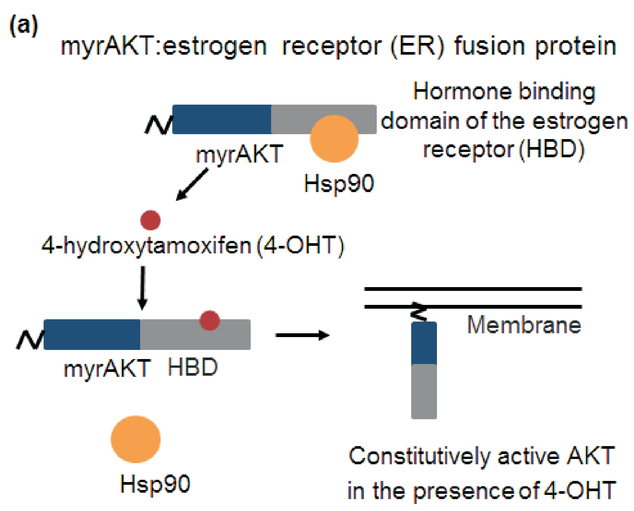


Figure 2 – Expression profile of FOXK2 and AKT in breast cancer cell lines exhibiting distinct drug resistance phenotypes. (**a**) MCF-7 and MDA-MB-231 were harvested and had proteins extracted and subjected to Western blot analysis, where FOXK2, phospho-AKT, total AKT and Hsc70 levels were determined. Hsc70 expression was used as a constitutive control. (**b**) MCF-7 and MDA-MB-231 were seeded in 96-well plates and treated with increasing concentrations of doxorubicin for 72 h. Cell viability was measured by the MTT assay. Bars represent average ± s.d. of three experiments. Statistical significance was determined by Student's t-test. (two-sided; (* $p \leq 0,05$; ** $p \leq 0,01$; significant).



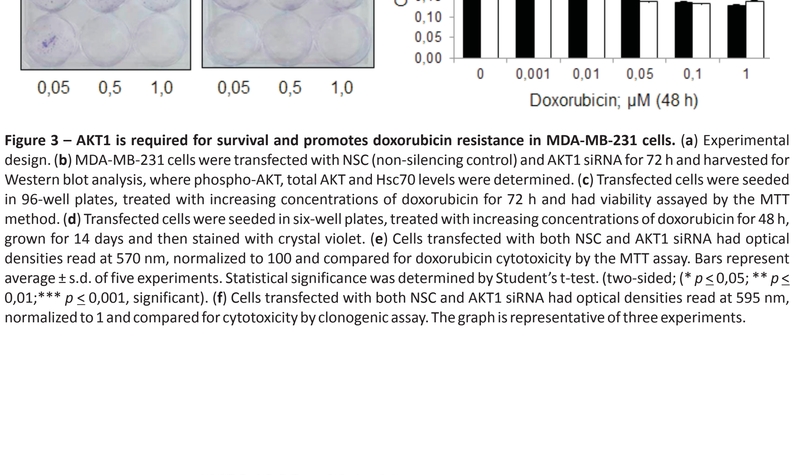


Figure 3 – AKT1 is required for survival and promotes doxorubicin resistance in MDA-MB-231 cells. (**a**) Experimental design. (**b**) MDA-MB-231 cells were transfected with NSC (non-silencing control) and AKT1 siRNA for 72 h and harvested for Western blot analysis, where phospho-AKT, total AKT and Hsc70 levels were determined. (**c**) Transfected cells were seeded in 96-well plates, treated with increasing concentrations of doxorubicin for 72 h and had viability assayed by the MTT method. (**d**) Transfected cells were seeded in six-well plates, treated with increasing concentrations of doxorubicin for 48 h, grown for 14 days and then stained with crystal violet. (**e**) Cells transfected with both NSC and AKT1 siRNA had optical densities read at 570 nm, normalized to 100 and compared for doxorubicin cytotoxicity by the MTT assay. Bars represent average ± s.d. of five experiments. Statistical significance was determined by Student's t-test. (two-sided; (* $p \leq 0,05$; ** $p \leq 0,01$;*** $p \leq 0,001$, significant). (**f**) Cells transfected with both NSC and AKT1 siRNA had optical densities read at 595 nm, normalized to 1 and compared for cytotoxicity by clonogenic assay. The graph is representative of three experiments.

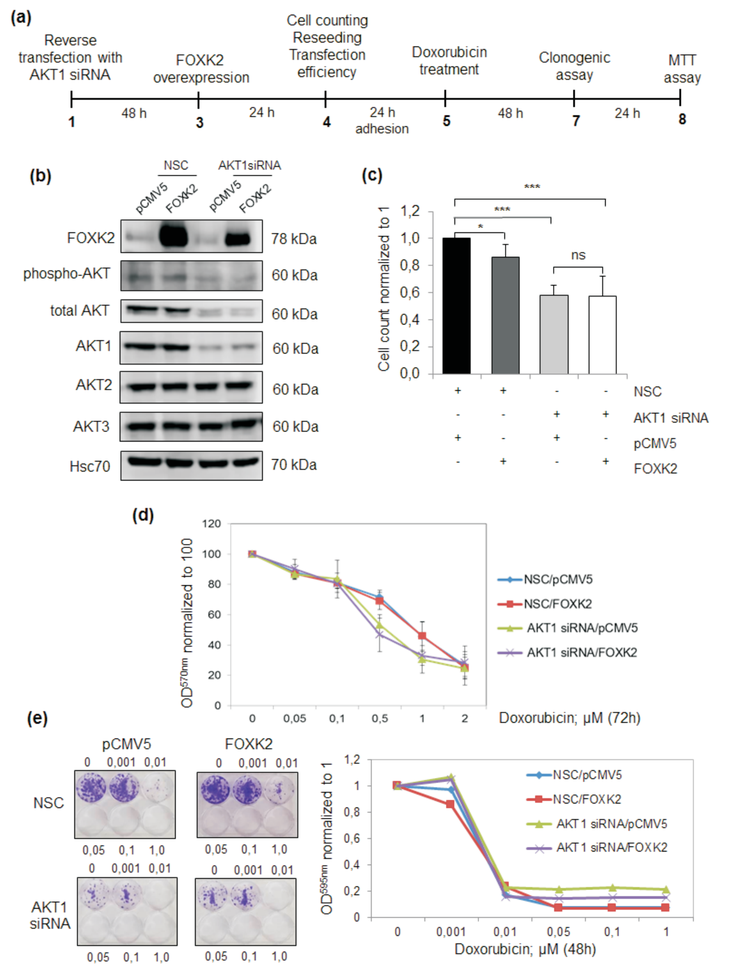


Figure 4 – FOXK2 expression pattern is modified following AKT1 knockdown, without interfering with cell growth and sensitivity to doxorubicin in AKT1-inhibited cells. (**a**) Experimental design. (**b**) MDA-MB-231 cells were transfected with NSC (non-silencing control) and AKT1 siRNA. After 48 h, cells were transfected with the empty vector (pCMV5) and the wild-type FOXK2 vector and harvested for Western blot analysis, where FOXK2, phospho-AKT, total AKT, AKT1, AKT2, AKT3 and Hsc70 levels were determined. (**c**) After 24 h of transfection, cells were counted by trypan blue exclusion. Bars represent average ± s.d. of seven experiments. (**d**) Transfected cells were seeded in 96-well plates, treated with increasing concentrations of doxorubicin for 72 h and had optical densities read at 570 nm, normalized to 100 and compared for doxorubicin cytotoxicity by the MTT assay. Bars represent average ± s.d. of five experiments. Statistical significance was determined by Student's t-test. (two-sided; (* $p \leq 0,05$; ** $p \leq 0,01$;*** $p \leq 0,001$, significant). (**e**) Transfected cells were seeded in six-well plates and treated with increasing concentrations of doxorubicin for 48 h. Cells were then grown for 14 days and stained with crystal violet. Optical densities were obtained at 595 nm, normalized to 1 and compared for cytotoxicity by the clonogenic assay. The graph is representative of three experiments.

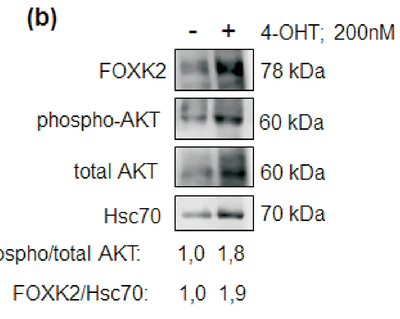


Figure 5 –FOXK2 expression is induced following transient myrAKT overexpression. (**a**) pBABE-(myr)Akt:ER vector operating scheme. (**b**) MDA-MB-231 cells were transfected with pBABE-(myr)Akt:ER vector for 24 h, after which they were treated with 200 nM 4-hydroxytamoxifen or ethanol vehicle control for 24 h. Cells were then harvested for Western blot analysis, where FOXK2, phospho-AKT, total AKT and Hsc70 levels were determined. The relative expression levels of phospho-AKT and FOXK2 were determined based on the expression levels of the target gene product versus the reference, total AKT and Hsc70, respectively. The values are shown under the respective Western blot bands. The intensities of the unsaturated Western blot bands were determined using the Image Studio Digits software. 4-OHT: 4-hydroxytamoxifen.

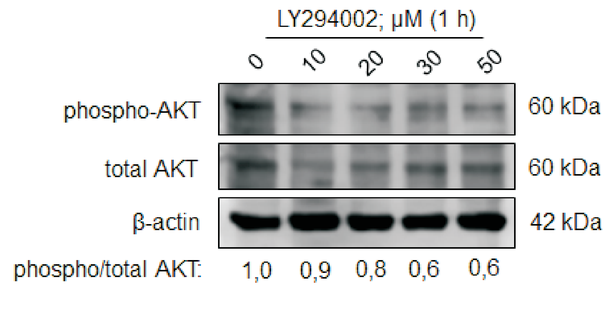


Figure 6 – Pharmacological inhibition of AKT phosphorylation with the LY294002 PI3K inhibitor . MDA-MB-231 cells were seeded in six-well plates, treated with increasing concentrations of LY294002 for one hour and harvested for Western blot analysis, where phospho-AKT, total AKT and β-actin levels were determined. The relative expression levels of phospho-AKT was determined based on the expression levels of the target gene product versus the reference, total AKT. The values are shown below the blots. The intensities of the unsaturated Western blot bands were determined using the Image Studio Digits software.

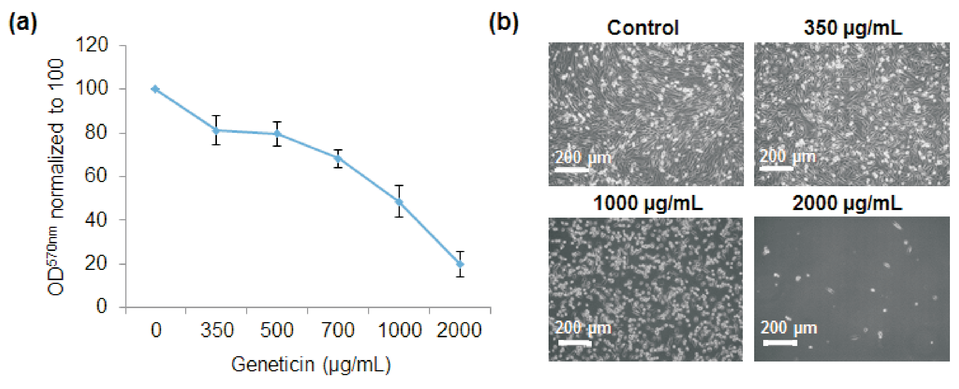


Figure 7 – Selection of clones stably expressing constitutively active AKT. (**a**) MDA-MB-231 cells were seeded in 96-well plates, treated with geneticin every 2 or 3 days up to one week and assayed by the MTT assay. Bars represent average ± s.d. of four experiments. (**b**) MDA-MB-231 cells were seeded in six-well plates for 24 h and transfected with the pBABE-(myr)Akt:ER vector. Cells that incorporated the plasmid were selected by treatment with geneticin. The images show cells untransfected (control) and transfected cells treated with 350, 1000 and 2000 μg/mL geneticin. Images were obtained by inverted optical microscope (Axio Observer.Z1, Zeiss) with magnification of 10x.

CONCLUSION

In summary, our findings suggest that AKT1 regulates FOXK2 expression in doxorubicin-resistant breast cancer cells. Experiments involving the pharmacological inhibition of AKT **(Figure 6),** the generation of MDA-MB-231 cells stably expressing constitutively active AKT **(Figure 7)** and treatment with phosphatases are in progress and will enable a better understanding on the regulation of FOXK2 expression by AKT and its impact on chemoresistance.

Keywords: Breast cancer; Drug Resistance; FOXK2 transcription factor; AKT oncogenic kinase; AKT isoforms.

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