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Original article

## APOBEC3B expression in drug resistant MCF-7 breast cancer cell lines



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### ABSTRACT

APOBEC3B belongs to a protein family of cytidine deaminases that can insert mutations in DNA and RNA as a result of their ability to deaminate cytidine to uridine. It has been shown that APOBEC3B-catalysed deamination provides a chronic source of DNA damage in breast cancers. We investigated APOBEC3B expression in four drug resistant breast cancer cell lines (Doxorubicin, Etoposide, Paclitaxel and Docetaxel resistant MCF-7 cell lines) using a novel RNA in situ hybridization technology (RNAscope) and compared expression levels with drug sensitive MCF-7 cell line. After RNAscope staining, slides were scanned and saved as digital images using Aperio scanner and software. Quantitative scoring utilizing the number of punctate dots present within each cell boundary was performed for the parameters including positive cell percentage and signal intensity per positive cell. In Doxorubicin and Etoposide resistant MCF-7 cell lines, APOBEC3B expression was approximately five-fold increased (23% and 24% respectively) with higher signal intensity (1.92 and 1.44 signal/cell, respectively) compared to drug sensitive MCF-7 cell line (5%, 1.00 signal/cell) with statistical significance. The increase of APOBEC3B expression in Docetaxel resistant and Paclitaxel resistant MCF-7 cell lines was not very high. In conclusion, APOBEC3B expression was increased in some population of tumor cells of drug resistant cell lines. At least for some drugs, APOBEC3B expression may be related to drug resistance, subjecting to some tumor cells to frequent mutation.

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### 1. Introduction

The chemical biology of cytosine is interesting and deamination, methylation and oxidation of cytosine can be the modulators of genomic potential [1]. The human APOBEC3 (A3) family consisted of seven genes encoding six functional cytidine deaminases (A3A, A3B, A3C, A3F, A3G and A3H) which are host cell mutators of viral DNA [2–4]. They represent a somewhat unusual protein family that can insert mutations in DNA and RNA as a result of their ability to deaminate cytidine to uridine [5]. They are closely related to activation-induced deaminase (AID), which is responsible for immunoglobulin class switch recombination and somatic hypermutation or rearranged VDJ genes [6]. APOBEC3 proteins have different subcellular localizations: cell-wide, cytoplasmic or nuclear during interphase. Lackey et al.

showed that several APOBEC3 family members have access to the nuclear compartment and can impede the cell most likely through DNA deamination.

The family of AID/APOBEC proteins may play a role in the carcinogenesis of several tumors including lymphomas, carcinomas of bladder, cervix, lung, breast, ovary, head and neck [7–11]. The aberrant expression of AID causes B cell leukemias and lymphomas, including Burkitt's lymphoma caused by c-myc/IgH translocation [10]. Shinohara et al. found that APOBEC3B is highly expressed in several lymphoma cells and transfection of its gene into lymphoma cells induces base substitutions in cMYC gene. They suggested that aberrant expression of APOBEC3B can evoke genomic instability by inducing base substitutions into human genome, which might lead to tumorigenesis in human cells [7]. Sasaki et al. [8] have shown that APOBEC3B mRNA expression was significantly higher in non-small-cell lung cancers than in adjacent normal lung. Burns et al. [9] analyzed gene expression data and mutation patterns, distributions and loads for 19 different cancer types. They found that notably, APOBEC3B is upregulated, and its

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preferred target sequence is frequently mutated and clustered in at least six distinct cancers: bladder, cervix, lung (adenocarcinoma and squamous cell carcinoma), head and neck, and breast. In their another study with breast cancers, they also showed that APOBEC3B-catalysed deamination provides a chronic source of DNA damage in breast cancers that could select TP53 inactivation and explain how some tumors evolve rapidly and manifest heterogeneity [12]. Because of these findings supporting association of APOBEC3B with carcinogenesis causing high mutation burden, it might also be related with drug resistance in breast cancers. In this study we investigated APOBEC3B expression in the drug resistant breast cancer cell lines using RNAscope method and compared expression levels with drug sensitive MCF-7 cell line.

## 2. Materials and method

### 2.1. Cell lines

The Doxorubicin-resistant MCF-7 cell line (MCF-7/Dox), Etoposide resistant MCF-7 cell line (MCF-7/Eto), Paclitaxel-resistant MCF-7 cell line (MCF-7/Pac) and Docetaxel-resistant MCF-7 cell line (MCF-7/Doc) previously established in Ufuk Gunduz Lab by stepwise selection of cells after prolonged treatment to increasing concentrations of drugs within the medium. [13–16] MCF-7/Dox and MCF-7/Eto are resistant to 1000 nM etoposide, MCF-7/Pac to 400 nM paclitaxel and MCF-7/Doc to 120 nM doxorubicin, respectively. Parental MCF-7 sensitive (MCF-7/S), MCF-7/Eto, MCF-7/Pac and MCF-7/Doc and MCF-7/Dox cell lines are maintained in 1640 RPMI medium (Biochrom AG, Germany) with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Biochrom AG, Germany) and 1% (v/v) Gentamycin Sulfate Solution (Biological Industries, Israel) in tissue culture treated filter cap flasks. These cell cultures are incubated in a Heraeus incubator (Hanau, Germany) at 37 °C in a 95% (v/v) humidified atmosphere with 5% (v/v) CO<sub>2</sub>.

### 2.2. Cell pelleting procedure

We made cell pellets for RNAscope assay according to procedure as follows. Culture the cells in the large flask, remove cells with trypsin or versene as normal for cell type, pellet the cells gently as typical for cell type, wash the cells by gently re-suspending in cold PBS followed by gentle spin, remove supernatant and repeat two more times, gently re-suspend pellet in 1 mL of 10% neutral buffered formalin, mix well and transfer to a 1.5 mL microfuge tube, spin at 40 °C for 10 min at low speed, pour the fixative out, add another 1 mL of 10% NBF, mix well, and spin at 40 °C for 30 min at fastest speed (>12,000g), remove supernatant, gently add 1 mL of 10% neutral buffered formalin and store at 40 °C overnight, the pellet is ready for processing and if not processing immediately, remove neutral buffered formalin and replace with 70% ethanol and store at 4 °C.

### 2.3. RNAscope assay

RNAscope is a novel RNA in situ hybridization technology, based on Advanced Cell Diagnostics (ADC)'s patented probe design and signal amplification methodology. This platform has the sensitivity to detect every gene in the human transcriptome in situ, and to simultaneously quantify multiple mRNA transcripts at a single cell level. We applied this assay using RNAscope 2.0HD detection kit brown (Catalog No. 320497, ADC, Hayward, CA) and APOBEC3B probe (Catalog No. 561751, NM\_001270411.1, probe region 223–1454, ADC, Hayward, CA). Cell pellets were mounted on SuperFrost Plus slides. Then slides were air dried and stained with the RNAscope 2.0 FFPE assay skipping the deparaffinization step. This assay briefly has following steps: pretreatment, target probe

hybridization, signal amplification, detection (colorimetric reaction), counterstain, dehydration and mounting. DapB negative control probe (Catalog No. 310043 ADC, Hayward, CA) and PPIB positive control probe (Catalog No. 313901, ADC, Hayward, CA) were used for the control slides.

### 2.4. RNAscope APOBEC3B evaluation for cell lines

RNAscope can enhance the value of in situ hybridization results by enabling a quantitative scoring guideline utilizing the number of punctate dots present within each cell boundary. All the slides stained with RNAscope method were scanned with Aperio at ×40 magnification. For every cell line in the experiment, at least 500 cells with preserved morphology were evaluated for RNAscope staining intensity. Only intranuclear brown dots were taken into consideration for evaluation. After evaluation, for every slide that is representative for a cell line, following parameters were calculated: total signal count, positive cell count, total cell count, percentage of positive cells, signal per total cell, signal per positive cell.

### 2.5. RNAscope Apobec3B evaluation for negative and positive control slides

We examined slides under a standard bright field microscope at 20–40× magnification and first assessed cell morphology. The cell morphologies were preserved in all the cell lines that evaluated. Positive control signals were visible as punctuate dots within cell nuclei at 20–40× magnification. We also counted positive control signal intensity. Then we assessed negative control background. One dot to every 10 cells displaying background DAB staining per 20× microscope field is acceptable for RNAscope evaluation. In the negative control slides from all cell lines in our study, no background signal was observed.

### 2.6. Immunohistochemistry

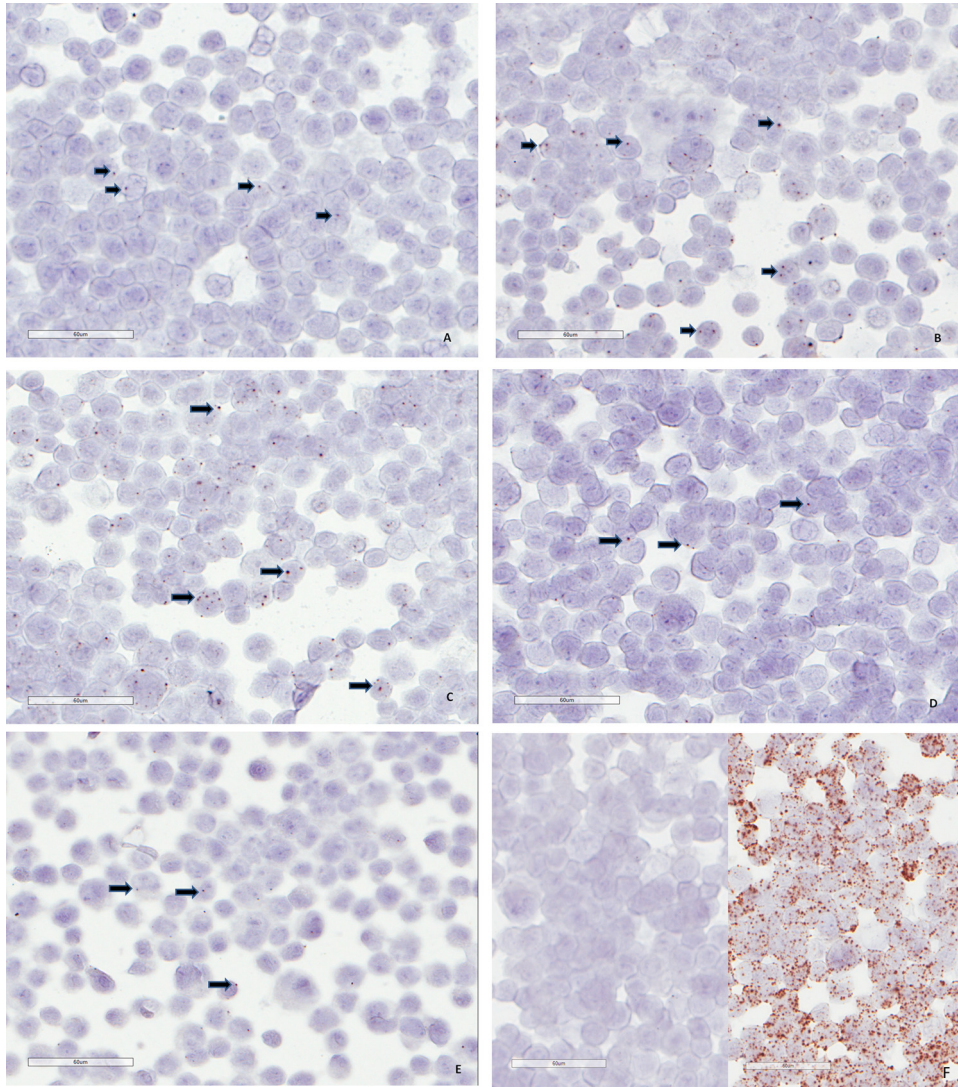
Rabbit polyclonal APOBEC3B (bs-12494, Bioss Inc, Massachusetts, USA) IgG primary antibody was used for immunohistochemical staining. Bond Polymer Refine Detection System (Catalog No: DS9800, Leica Biosystems Newcastle Ltd, UK) was applied for the immunohistochemical detection. This is a biotin-free, polymeric horseradish peroxidase-linker antibody conjugate system for the detection of tissue-bound mouse and rabbit IgG and some mouse IgM primary antibodies. Immunohistochemical staining has been performed automatically using Leica Biosystems Bond™ (Leica Microsystems Inc., Buffalo Grove, IL) according to the procedure below. Antibody against APOBEC3B (Bioss, bs-12494R) were tested at 1:100 and 1:200 dilutions. Heat-induced epitope retrieval was done for 20 min with ER2 solution (Leica Microsystems AR9640) or ER1 solution (Leica Microsystems AR9961).

**Table 1**

RNAscope expression parameters of APOBEC3B for MCF-7/S, MCF-7/Doc, MCF-7/Dox, MCF-7/Eto, MCF-7/Pac cell lines.

Cell Lines	Percentage of positive cells, median (min-max)	Signal per positive cell median (min-max)
MCF-7/S	5% (1–8%)	1.00 (1.00–1.17)
MCF-7/Doc	7% (5–11%)	1.05 (1.00–1.10)
MCF-7/Dox	23% (16–24%)	1.92 (1.90–2.29)
MCF-7/Eto	24% (16–32%)	1.44 (1.11–1.86)
MCF-7/Pac	8% (6–15%)	1.05 (1.00–1.33)

Doxorubicin-resistant MCF-7 cell line (MCF-7/Dox), Etoposide resistant MCF-7 cell line (MCF-7/Eto), Paclitaxel-resistant MCF-7 cell line (MCF-7/Pac) Docetaxel-resistant MCF-7 cell line (MCF-7/Doc) and MCF-7 sensitive (MCF-7/S).



**Fig. 1.** APOBEC3B expression in breast cancer cell lines stained with RNAscope method. MCF-7 sensitive cell line (1A), Doxorubicin-resistant (1B), Etoposide resistant (1C), Docetaxel-resistant (1D), and Paclitaxel-resistant (1E) MCF-7 cell lines were shown with negative (1F left) and PPIB positive control probes (1F right). Cells with brown dots (arrows) show APOBEC3B expressions. Doxorubicin-resistant and Etoposide resistant MCF-7 cell lines demonstrate higher APOBEC3B expression compared to MCF-7 sensitive cell line.

### 2.7. Western blot

Lysates were prepared from MCF-10A, SK-BR-3, MCF-7, T-47D or MDA-MB-231 cells. Cells were lysed in a buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 70% Tergitol, 0.1% SDS, 1 mM MgCl<sub>2</sub> and protease inhibitor cocktail (Sigma, MO). Lysates were centrifuged at 12,000g for 15 min at 4 °C. Proteins were solubilized in sample buffer (Life Technologies, NY) and subjected to SDS-PAGE. Proteins

were electroblotted onto PVDF. Membranes were immunoblotted with APOBEC3B (Bioss, MA) and Vinculin (Sigma, MO) antibodies. After washing, membranes were incubated with HRP-conjugated secondary antibody (BIO-RAD, CA). Bands were visualized by using the enhanced chemiluminescence (ECL) Western blotting detection system. Based on the data of Burns et al. [12], we selected BT474 and MDA-MB231 as positive control and SK-BR-3 as negative control for both immunohistochemistry and western blot analysis.

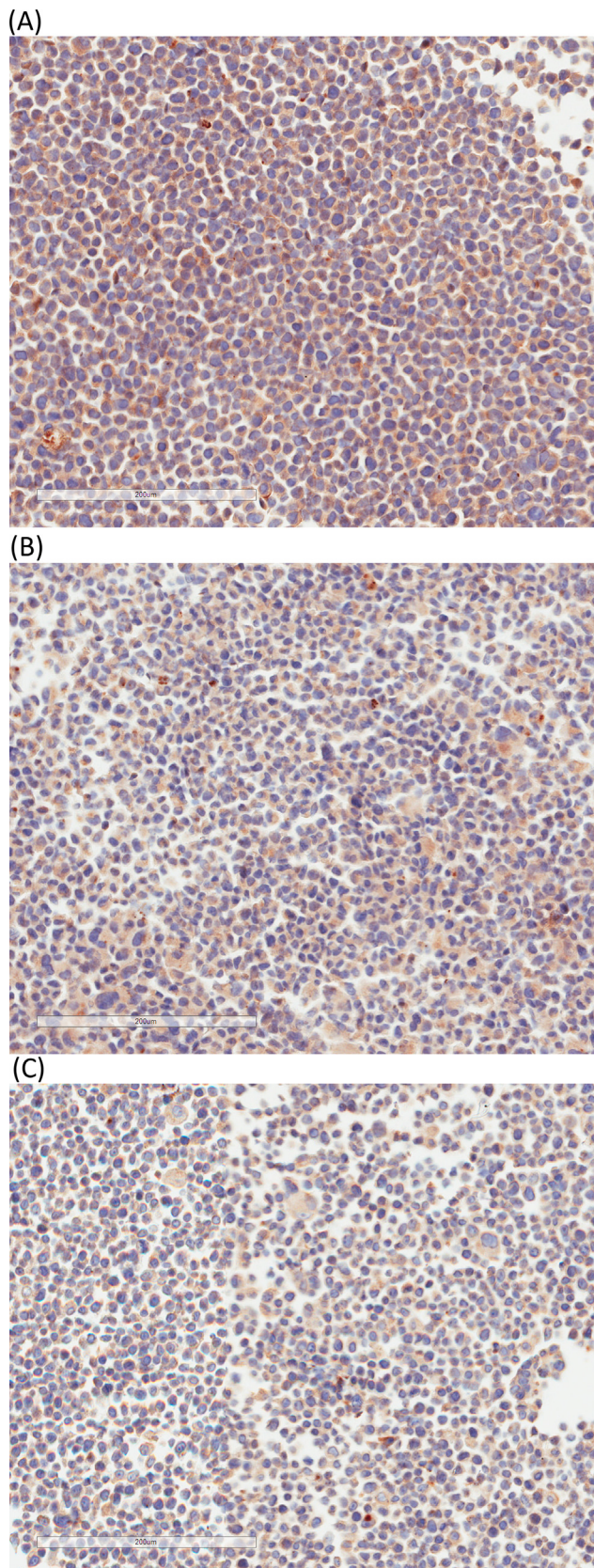
**Table 2**

Statistical comparison of resistant cell lines with MCF-7/S for APOBEC3B expression.

	Percentage of positive cells		Signal per positive cells (p)	
	Z <sup>a</sup> score	(p)	Z <sup>a</sup> score	(p)
MCF-7/S vs MCF-7/Doc	2.197	0.028	1.630	0.103
MCF-7/S vs MCF-7/Dox	2.612	0.009	3.093	0.002
MCF-7/S vs MCF-7/Eto	4.165	<0.001	4.255	<0.001
MCF-7/S vs MCF-7/Pac	2.554	0.011	1.541	0.123

Doxorubicin-resistant MCF-7 cell line (MCF-7/Dox), Etoposide resistant MCF-7 cell line (MCF-7/Eto), Paclitaxel-resistant MCF-7 cell line (MCF-7/Pac) Docetaxel-resistant MCF-7 cell line (MCF-7/Doc) and MCF-7 sensitive (MCF-7/S).

<sup>a</sup> Mann-Whitney U test.



**Fig. 2.** Immunohistochemical APOBEC3B expression in BT-474 (2A), MDA-MB-231 (2B), and MCF-7 (2C) breast cancer cell lines.

## 2.8. Statistical analysis

All analyses were performed using SPSS for Windows version 17.0 statistical software package (SPSS, Chicago, IL, USA). Descriptive statistics were presented as median (min-max). Mann-Whitney *U* test was used to measure the significance of parameters. *p*-value of <0.05 was considered statistically significant.

## 3. Results

Totally 9142 cells from MCF-7/S, MCF-7/Doc, MCF-7/Dox, MCF-7/Eto, MCF-7/Pac cell lines were evaluated for APOBEC3B expression. Of all, 1174 cells (12.27%) were positive for APOBEC3B expression and mean APOBEC3B signal per positive cell was 1.22.

The level of APOBEC3B expression for MCF-7/S, MCF-7/Doc, MCF-7/Dox, MCF-7/Eto, MCF-7/Pac cell lines were presented in Table 1 (Fig. 1A–E). The percentage of APOBEC3B positive cells was higher in MCF-7/Dox (median 23%, min-max 16–24%) and MCF-7/Eto (median 24%, min-max 16–32%) cell lines than the others with significantly higher APOBEC3B signal per cell. In MCF-7/Dox and MCF-7/Eto cell lines, the cells with positive APOBEC3B signal showed relatively high median signal intensity (1.92 and 1.44, respectively) compared to MCF-7 (1.00), MCF-7/Doc (1.05) and MCF-7/Pac (1.05) cell lines.

Statistically we compared the APOBEC3B Rnascopie parameters (percentage of positive cells, signal per cell and signal per positive cell) of MCF-7 with drug resistant cell lines (Table 2). Statistically significant differences for APOBEC3B expression including all three parameters have been found for MCF-7/Dox and MCF-7/Eto cell lines. Although statistically difference found for MCF-7/Doc and MCF-7/Pac cell lines, considerable overlap were observed for the parameters including percentage of positive cells, signal per cell and signal per positive cell.

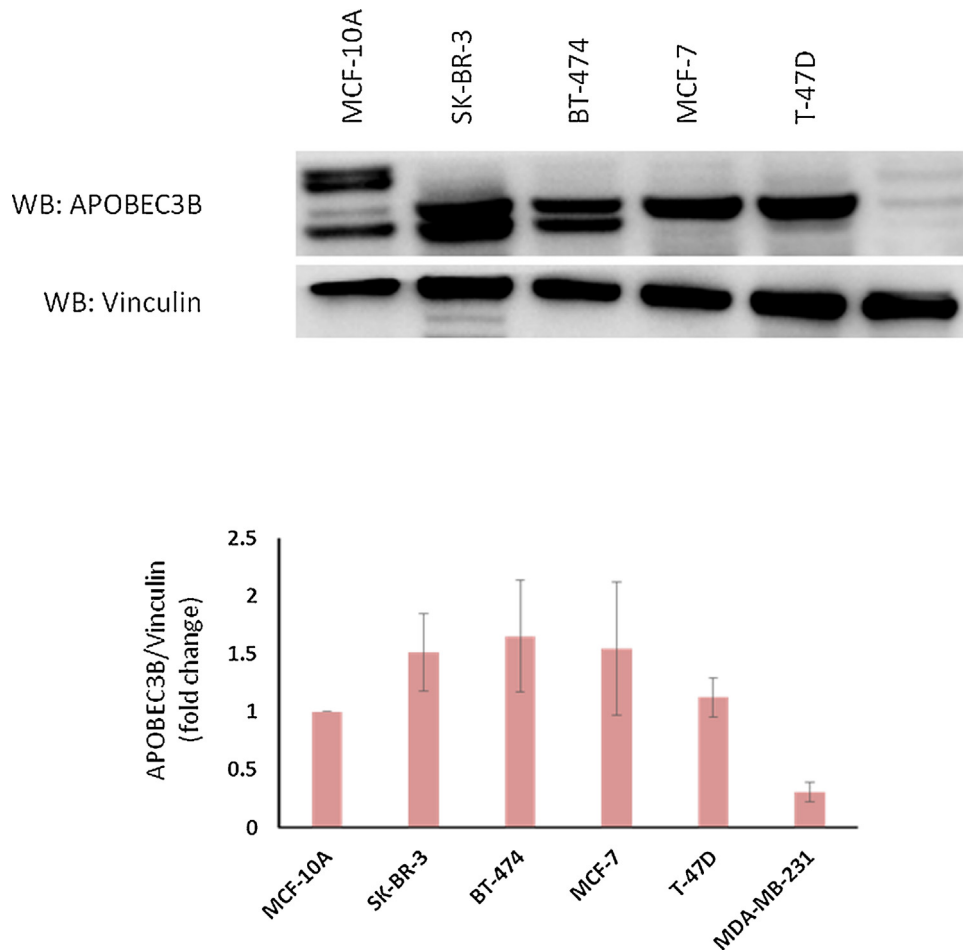
**APOBEC3B Evaluation for Negative and Positive Control Slides:** As a positive control, totally 1115 cells from MCF-7 sensitive, MCF-7/Doc, MCF-7/Dox, MCF-7/Eto, MCF-7/Pac cell lines were evaluated for PPIB expression. Of all, 1100 cells (98.8%) were positive for PPIB expression and mean PPIB signal per positive cell was 9.8 (Fig. 1F right). The range of percentage of positive cell for PPIB was 98–100% and the range of signal per cell was between 9.59 and 9.85 (Table 2). In our negative control slides, background staining was not present and all of them were evaluated as completely negative (Fig. 1F left).

### 3.1. Immunohistochemistry and western blot

Immunohistochemically stained slides of MCF-7 sensitive, BT474 and MDA-MB-231 with APOBEC3B primary antibody were evaluated with light microscope (Fig. 2A–C). Almost all of the neoplastic cells were positive for APOBEC3B. APOBEC3B expression was mainly cytoplasmic. In rare neoplastic cells, strong dot like staining was observed. While staining intensity was mild in MCF-7 sensitive neoplastic cells, it was moderate in BT474 and MDA-MB-231 neoplastic cells. In the evaluation of WB analysis, a single band for MCF-7 and T-47D cell lines, two bands for SK-BR-3 and BT-474, and four different bands in MCF-10A were observed (Fig. 3).

## 4. Discussion

Genome sequencing studies have shown that breast cancers have somatic mutations dominated by C-to-T transitions. Burns et al. [12] demonstrated that APOBEC3B mRNA is upregulated in most primary breast tumors and breast cancer cell lines. In their knockdown experiments, increased levels of genomic uracil, increased mutation frequencies, and C-to-T transitions correlated with APOBEC3B



**Fig. 3.** APOBEC3 is expressed in breast cell lines. APOBEC3 expression in immortalized MCF-10A mammary epithelial cells and breast cancer cell lines (SK-BR-3, BT-474, MCF-7, T-47D and MDA-MB-231) as determined by Western blot. Vinculin was used as loading control. Similar results were observed in three independent experiments.

expression. They concluded that APOBEC3B could be a source of mutations. Recently, Caval et al. [17] investigated chimaeric A3A–A3B deletion allele ( $\Delta$ A3B) which is APOBEC3A hybrid allele bearing APOBEC3B 3'UTR and related to developing breast, ovarian and liver cancer. Breast cancer genomes from  $\Delta$ A3B<sup>-/-</sup> patients showed a higher overall mutation burden. They demonstrated that germline A3B can hypermutate nuclear DNA, albeit less efficiently than A3A. Chimaeric A3A mRNA resulting from  $\Delta$ A3B was more stable, resulting in higher intracellular A3A levels and greater DNA damage. In a study with 1491 primary breast cancers, Sieuwerts et al. [18] investigated the relation of APOBEC3B with breast cancer clinical outcomes. They concluded that APOBEC3B is a marker of poor prognosis and poor outcomes for estrogen positive breast cancers strongly suggesting that genetic aberrations induced by APOBEC3B contribute to breast cancer progression. In their in vivo and in vitro studies Ohba et al. [19] suggested a possible involvement of human papilloma virus (HPV) infection in the early stage of breast carcinogenesis via APOBEC3B induction in breast cancer (BC) patients with high-risk HPV in Singapore. In this study we investigated APOBEC3B expression in the drug resistant breast cancer cell lines using RNAscope method comparing expression levels. RNAscope method also let us to observe the morphologic distribution of APOBEC3B expression.

In this study, APOBEC3B expression was observed in a small number of tumor cell population for MCF-7 cell lines. The mean percentage of APOBEC3B expressing cells for MCF-7/S cell line was

5%. However, it was sometimes expressed as low as 1% of tumor cells. In those APOBEC3B expressing tumor cells of MCF-7/S cell line, mostly only one signal was observed. In the study of Burns et al. [12], they investigated 38 independent breast cancer lines. They found APOBEC3B upregulation in 28 cell lines with expression levels exceeding 10-fold relative to controls in some of them. In their study MCF-7 cell line, APOBEC3B expression was also mild to moderately increased compared to other breast cancer cell lines. Our study may support their finding of APOBEC3B expression in MCF-7/S cell lines and contribute that its expression is confined to rare scattered cells in tumor cells with very low level of expression. Recently, in a study of Gwak et al. [20], they investigated the immunohistochemical expression of APOBEC3B in breast, gastric, colorectal and prostate cancers. They detected strong (++) immunohistochemical Apobec 3 expression in 100% of normal breast and breast cancers. As they have already expressed in their article, a polyclonal immunohistochemical antibody which might have affinity to the Apobec3 proteins other than APOBEC3B is used in their study. In our study we have also done an immunohistochemical analysis with a commercial available immunohistochemical antibody. However, immunohistochemical staining was observed extensively in the cytoplasm of almost all of breast cancer cells. Our western blot analysis of this commercial antibody showed positive bands with several proteins having different molecular weights. Similar to the study of Gwak et al. [20], antibody might have an affinity to the other members of

ApoBc3 protein. This raised the possibility of multitarget staining which must be a frequent problem for immunohistochemical analysis based on the data already published in the literature.

In the analysis of drug resistant MCF-7 breast cancer cell lines, APOBEC3B expression was detected in 23% to 24% of tumor population in Doxorubicin- and MCF-7/Eto cell lines, respectively ( $p=0.009$  and  $p<0.001$ ). The population of tumor cells with APOBEC3B expression was increased approximately five-fold compared to MCF-7/S cell line. However, the average APOBEC3B signal intensity was not that high compared to the positive cell population (1.00 signal for MCF-7/S vs 1.44 for Etoposide resistant and 1.92 signal for Doxorubicin resistant). This finding may be interpreted as that the number of tumor cells with APOBEC3B expression increased more than the signal intensity.

In the Docataxel resistant- and MCF-7/Pac cell lines, the population of tumor cells with APOBEC3B expression was also mildly increased (5% vs 7–8%) with statistically significant difference ( $p=0.028$  and  $p=0.011$ ). However, significant overlap was present for both percentage of APOBEC3B positive cells and signal intensity.

In conclusion, APOBEC3B in MCF-7/S breast cancer cell lines have been expressed in a minor population (5%) of tumor cells with a very low signal intensity. In Doxorubicin- and MCF-7/Eto cell lines, the expression of APOBEC3B was five-fold increased with more pronounced signal intensity compared to MCF-7/S cell line. Although its expression was increased, APOBEC3B was not expressed in all of the tumor cells. On the other hand, the increase of APOBEC3B expression in Docataxel resistant- and MCF-7/Pac cell lines was not very high. At least for some drugs, APOBEC3B expression may be related to drug resistance, subjecting to some tumor cells to frequent mutation.

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