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ORIGINAL ARTICLE

# *APOBEC3B* high expression status is associated with aggressive phenotype in Japanese breast cancers

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**Abstract**
*Background* The members of AID/APOBEC protein family possess cytidine deaminase activity that converts cytidine residue to uridine on DNA and RNA. Recent studies have shown the possible influence of *APOBEC3B* (*A3B*) as DNA mutators of breast cancer genome. However, the clinical significance of *A3B* expression in Japanese breast cancer has not been studied in detail.
*Methods* Ninety-three primary breast cancer tissues (74 estrogen-receptor (ER) positive, 3 ER and HER2 positive, 6 HER2 positive, and 10 triple negative) including 37 tumor-normal pairs were assessed for *A3B* mRNA expression using quantitative real-time RT-PCR. We analyzed the relation between *A3B* expression, mutation analysis of *TP53* and *PIK3CA* by direct sequencing, polymorphic *A3B* deletion allele and human papillomavirus (HPV) infection in tumors.
*Results* *A3B* mRNA was overexpressed in tumors compared with normal tissue. Patients with high *A3B* expression were associated with subtype and progression of lymph node metastasis and pathological nuclear grade. However, the expression was not related to any other clinicopathological factors, including mutation of *TP53* and *PIK3CA*, polymorphic *A3B* deletion allele, HPV infection and survival time.
*Conclusion* The expression of *A3B* in breast cancer was higher than in non-cancerous tissues and was related to the lymph node metastasis and nuclear grade, which are reliable aggressive phenotype markers in breast cancer. Evaluation of *A3B* expression in tumor may be a marker for breast cancer with malignant potential.

**Keywords** Breast cancer · APOBEC3B · Nuclear grade

## Introduction

Tens of thousands of genomic mutations have been observed in human cancers, and several mutations are prerequisites for carcinogenesis. These mutations promote clonal diversities among cancer cells, and clinically they also promote metastasis and resistance to therapy [1]. Base substitutions are the most frequent mode of mutations, and some of them can be induced by DNA damage from extrinsic carcinogens and erroneous DNA repair machineries [2]. For most base substitutions, however, the specific mechanism that introduces them to the cellular genome remains unclear.

Recent genome-wide analysis of the mutation pattern of breast cancers confirmed the domination of C to T

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transitions with a strong bias toward 5′-TCA-3′ context [3]. A nuclear localizing cytidine deaminase, *APOBEC3B* (*A3B*), which shows the sequence preference of TCA signature in vitro, has been identified as a possible contributor to the C to T transitions in breast cancer genome: It works as the mutational source on genomic DNA of many types of cancers including breast cancers [4].

In one cohort in the U.S., high expression of *A3B* mRNA has been observed in about half of breast cancers [4]. The authors have also showed in cultured breast cancer cells that A3B has mutational induction activity on *TP53* gene [4]. *A3B* expression has been also reported to be induced transcriptionally by the human papillomavirus (HPV) 16 infection [5]. Breast cancers with high *A3B* mRNA expression harbor more mutations in genome and show poorer post-operative disease-free survival (DFS) among ER-positive, lymph node negative breast cancers without adjuvant therapy [6]. This observation suggests that the mutational function of *A3B* may contribute to breast cancer carcinogenesis and/or have prognostic significance among breast cancers.

In some reports, breast cancers in Asia including Japan have shown some clinical differences from Western countries in terms of incidence [7] and outcomes [8]. Those clinical differences might be, at least in part, attributed to genetic differences among ethnicities [9]. A common polymorphism of *APOBEC3s* gene clustered on 22q13 deletes 29.5 kb region including the entire *A3B* coding region, leaving only 3′-UTR. The prevalence of the polymorphic deletion allele greatly differs among groups: Higher incidence (40–90 %) of deletion allele has been reported in East Asian and Pacific Islander populations than in African and European populations (0.9–6 %) [10, 11]. On the other hand, an intriguing observation on the deletion polymorphism and genome-wide mutations has reported that the deletion alleles are found to be a risk allele for incidence of breast cancer [12]. They have higher content of *APOBEC* mutation signature even without *A3B* coding regions [2, 13]. It is still under debate how the *A3B* deletion allele contributes to breast cancer mutations. However, the wide variations in prevalence of *A3B* deletion allele among various populations strongly suggests different influences of *A3B* in breast cancer carcinogenesis, development, and prognosis among the population groups.

The purpose of this research is to clarify the clinical significance of *A3B* expression in Japanese breast cancer patients. Therefore, we examined *A3B* mRNA expression in primary breast cancer in a Japanese cohort. We also analyzed the association between *A3B* expression, *A3B* insertion/deletion allele, mutation status of *TP53* and *PIK3CA*, HPV infection, clinicopathological characteristics and clinical outcome.

## Materials and methods

### Patient samples

Ninety-three patients who underwent surgical excision of breast cancer at Gunma University Hospital between 2007 and 2012 were involved in the study. Tumor samples were immediately frozen after surgery and stored at −80 °C until DNA and RNA isolation. The study was approved by Ethics Committee for Human Genome Research of Gunma University Graduate School of Medicine (protocol number 182) and patients provided written informed consent.

### RT-qPCR assay for *APOBEC3B* gene

Total RNA was extracted from breast cancer and adjacent normal breast tissues using NucleoSpin RNA II kit (Macherey–Nagel) with Mixer Mill MM300 (Retsch) according to the manufacturer's instructions. RNA concentration was determined by a Thermo Scientific™ NanoDrop Lite. One µg of total RNA was then used for synthesis of cDNA using a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) according to the manufacturer's recommendations. One µl of each reverse transcript reaction was used for PCR analysis. The *A3B* qPCR assay reactions were performed using KAPA PROBE FAST qPCR Kits (Kapa Biosystems) in a 20-µl reaction volume on StepOnePlus™ Systems (Life Technologies) with a VIC probe for ACTB gene (Life Technologies, 4326315E) as internal control. The primers and a probe used for the *A3B* gene were: forward, 5-CGCCAGACCTACTTGTGC TA-3 and the reverse, 5-GCCACAGAGAAGATTCTTAG CC-3 (111 bp), Universal Probe Library #39 (Roche). The cycling conditions used were: initial denaturation at 95 °C for 3 min, followed by 40 cycles at 95 °C for 3 s and 60 °C for 20 s.

### Mutation status of *TP53* and *PIK3CA* genes

PCR reactions using cDNA were performed using Kapa HiFi HotStart ReadyMix PCR Kit (Kapa Biosystems) in a 25-µl reaction volume with an initial denaturation step at 95 °C for 3 min, followed by 30 cycles of 98 °C 20 s, 60 °C 15 s, and 72 °C at 1 min. The final extension step was at 72 °C for 1 min. Mutation hotspots in exon 2–11 of *TP53* and exon 9/20 of *PIK3CA* were assessed by direct sequencing of the PCR products. Primers used were TP53fragment1-F: GACACGCTTCCCTGGATTGGC, TP53fragment1-R: GCAAAACATCTTGTTGAGGGCA, TP53fragment1-seq: CAGGGGAGTACGTGCAAGTCAC

AG, TP53fragment2-F: GTTTCCGTCTGGGCTTCTTG CA, TP53fragment2-R: GGTACAGTCAGAGCCAACCT, TP53fragment2-seq: GCCAACCTCAGGCGGCTCATA, TP53fragment3-F: TGGCCCCTCCTCAGCATCTTA, TP5 3fragment3-R: CAAGGCCTCATTCAGCTCTC, TP53 fragment3-seq: CGAGTGGAAGGAAATTTGCGT, TP53 fragment4-F: CGGCGCACAGAGGAAGAGAATC, TP53 fragment4-R: CAAGGCCTCATTCAGCTCTC, TP53 fragment4-seq: GGGGAGCCTCACCACGAGCTG, PIK 3CA_ex9-F: TGGCCAGTACCTCATGGATTAGAA, PIK3CA_exon9-R: GAGGCCAATCTTTTACCAAGCA, PIK3CA_exon9-seq: TACATCTGGGCTACTTCATCTC-TAG, PIK3CA_exon20-F: AATGCACAAAGACAAGAG AATTTGAG, PIK3CA_exon20-R: AATTCCTATGCAAT CGGTCTTTGC, PIK3CA_ex20-seq: GCAGTGTGGAAT CCAGAGTGAG.

### DNA extraction and PCR with HPV 16-specific primers

Among 93 breast cancer samples in which *A3B* mRNA levels were measured, HPV16 infection was examined in 88 genomic DNA samples from the available frozen breast cancer tissue. DNA extraction was performed using NucleoSpin Tissue kit (Takara bio, Japan) according to the manufacturer's protocol. PCR reaction using DNA were performed using Kapa HiFi HotStart ReadyMix PCR Kit (Kapa Biosystems) in a 25-µl reaction volume with an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of 98 °C 20 s, 60 °C 15 s, and 72 °C at 1 min, and a final extension step at 72 °C for 1 min. Primers used were HPV16-F; AGGGCGTAACCGAAATCGGT: HPV16-R: CTGAGCTGTCATTTAATTGCTCA. The final PCR product of 10 µl was loaded onto 1 % agarose gel, stained with ethidium bromide, and visualized under UV illumination.

### DNA extraction and PCR genotyping assay of *A3B* deletion

Among 93 breast cancer samples in which *A3B* mRNA levels were measured, *A3B* deletion allele was examined in 32 genomic DNA samples from available adjacent normal breast tissue. DNA extraction was performed using NucleoSpin Tissue kit (Takara bio, Japan) according to the manufacturer's protocol. We performed PCR using oligonucleotide sequences as described previously [10, 14]: Deletion_F; TAGGTGCCACCCCGAT: Deletion_R; TTGAGCATAATCTTACTCTTGTAC (700 bp): Insertion1_F; TGTCCCTTTTCAGAGTTTGAGTA: Insertion1_R; TGGAGCCAATTAATCACTTCAT (705 bp) [10]: Insertion2_F; GAGTGGAAGCGCCTCCTC: Insertion2_R; CTCCTGGCCAGCCTAGC (811 bp) [14].

Insertion and deletion PCR assays were performed separately. The final PCR product of 10 µl was loaded onto 1 % agarose gel, stained with ethidium bromide, and visualized under UV illumination.

PCR reactions using DNA (50 ng) were performed using Phusion High-Fidelity DNA Polymerase (New England Biolabs Japan) in a 50-µl reaction volume with an initial denaturation step at 98 °C for 5 min, followed by 30

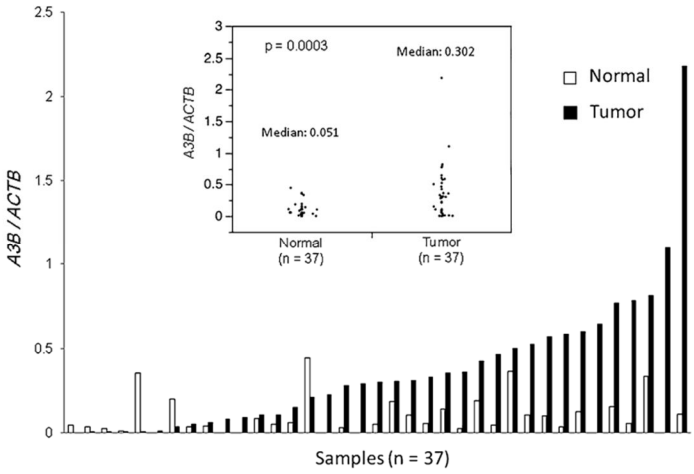


**Fig. 1** *A3B* expression in breast tumors and adjacent normal breast tissues. *A3B* mRNA expression relative to *ACTB* in 37 breast cancer tissue samples and paired with adjacent normal breast tissues. *A3B* expression level is significantly higher in tumor tissue compared with normal tissue (Mann–Whitney test, $p = 0.003$)

**Table 1** Patient characteristics and *A3B* mRNA expression level

| Characteristics | *A3B* relative expression | | *p* value |
|---|---|---|---|
| | Low expression $n = 63$ | High expression $n = 30$ | |
| Age (years) | 57.9 ± 13.1 | 54.9 ± 11.6 | 0.29 |
| Menopausal status | | | 0.52 |
| Premenopausal | 25 | 14 | |
| Postmenopausal | 38 | 16 | |
| Subtype | | | 0.034* |
| ER (+) | 54 | 20 | |
| ER (+)/HER2 (+) | 0 | 3 | |
| HER2 (+) | 4 | 2 | |
| Triple negative | 5 | 5 | |
| PgR | | | 0.64 |
| − | 18 | 10 | |
| + | 45 | 20 | |
| Deletion allele (*n*=32) | | | 0.0019* |
| Insertion/insertion | 1 | 10 | |
| Deletion/insertion | 14 | 7 | |
| Stage | | | 0.66 |
| 0 | 1 | 0 | |
| I | 21 | 7 | |
| II | 35 | 20 | |
| III | 6 | 3 | |
| Lymph node metastasis | | | 0.033* |
| − | 40 | 12 | |
| + | 23 | 18 | |
| Lymphatic invasion | | | 0.079** |
| − | 19 | 4 | |
| + | 44 | 26 | |
| Venous invasion | | | 0.066** |
| − | 51 | 19 | |
| + | 12 | 11 | |
| Nuclear grade | | | 0.009* |
| NG1 | 17 | 2 | |
| NG2 | 24 | 8 | |
| NG3 | 22 | 20 | |

* $p < 0.05$; ** $p < 0.1$

cycles of 98 °C 10 s, 50 °C 30 s, and 72 °C at 10 s, and a final extension step at 72 °C for 3 min.

### Statistical analysis

Statistical significance was estimated using Student's *t* test, Mann–Whitney test, ANOVA, and Chi-square test. Kaplan–Meier curves were generated for overall survival (OS) and DFS, and statistical significance was determined using the log-rank test. A probability value of <0.05 was considered significant. All statistical analyses were performed using JMP software (SAS Institute Inc.).

## Results

### *A3B mRNA is expressed in tumor tissues of Japanese breast cancer patients*

First, to determine whether *A3B* was highly expressed in Japanese breast cancers, we quantified *A3B* mRNA expression by RT-qPCR from fresh frozen tumor samples. Thirty-seven paired adjacent normal breast tissue samples were also available for *A3B* quantification (Supplementary Table 1). *A3B/ACTB* mRNA levels (median 0.302) in the breast cancer tissues were significantly higher than in the

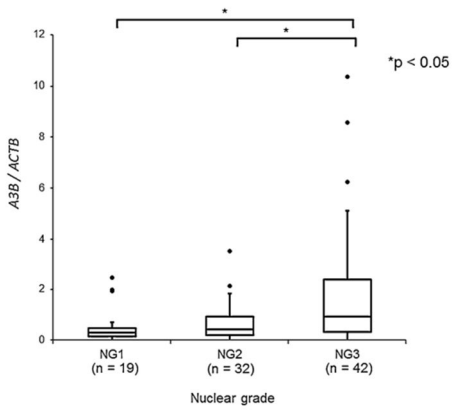


**Fig. 2** *A3B* mRNA expression level correlates with pathological nuclear glade. Relative expression levels of *A3B* mRNA are plotted in each pathological nuclear grade group. *Box plot* shows ±1SD. *Asterisk* denotes $p < 0.05$

adjacent normal breast tissues (median 0.051) ($p = 0.0003$) (Fig. 1). Approximately half of the tumor tissues showed higher *A3B* mRNA expression than did the adjacent normal tissues.

### *The clinical significance of A3B expression in breast cancer patients*

We divided 93 breast cancer patients into two groups according to the levels of *A3B* expression in tumor tissues. The cut off point was the mean expression level of *A3B* in tumor (high expression group, $n = 30$; low expression group, $n = 63$). Clinicopathological factors were significantly different in the *A3B* high expression group. *A3B* high expression correlated with the breast cancer subtype ($p = 0.034$) and progression of lymph node metastasis ($p = 0.033$), lymphatic invasion ($p = 0.079$), venous invasion ($p = 0.066$), and pathological nuclear grade ($p = 0.009$) (Table 1; Fig. 2), compared with the *A3B* low expression group. Moreover, *A3B* expression in cases with *A3B* deletion allele was significantly lower than that of cases with *A3B* insertion allele ($p = 0.0019$) (Table 1). No significant differences were observed regarding age, menopausal status, PgR status, or clinical stage (Table 1). The status of polymorphic *A3B* deletion allele in 32 breast cancer patients was not significantly associated with clinicopathological factors (Table 2; Supplementary Fig. 1).

### *A3B mRNA expression levels were not predictors of clinical outcome among Japanese breast cancer patients*

To determine whether *A3B* mRNA expression was associated with clinical outcome in our Japanese cohort, we compared DFS and OS in the high and low *A3B* expression groups. As a result, we did not find significant differences in DFS and OS among any patients or in the ER-positive group (Fig. 3). Among patients with no lymph node metastasis, patients with high *A3B* expression were not associated with DFS or OS (Supplementary Fig. 2).

### *TP53/PIK3CA driver mutations and HPV infection were not associated with A3B expression in breast cancers in this study*

We performed PCR-direct sequence of *TP53* and *PIK3CA* mutational hotspots from cDNA to determine whether the level of *A3B* mRNA expression was associated with *TP53* and *PIK3CA* driver gene mutation status. *TP53* mutation at exon 2–11 and *PIK3CA* mutations at exon 9 and exon 20 hotspots were found in 16.7 and 15.7 %, respectively in of the samples. No correlation was found between *A3B* expression and *TP53/PIK3CA* mutation status in our cohort (Fig. 4a, b). HPV16 infection, known as an *A3B*-inducer, was detected in 27.3 % (24/88) of patients in our cohort, but the infection was not significantly associated with *A3B* expression (Fig. 4c).

## Discussion

In the present study, we found that expression levels of *A3B* mRNA in breast cancer tissue were significantly higher than in adjacent normal breast tissue of Japanese patients. Moreover, breast cancer patients with high *A3B* expression were associated with breast cancer subtype, progression of lymph node metastasis and pathological nuclear grade. However, the expression was not related to the other clinicopathological factors, i.e. mutation of *TP53* and *PIK3CA*, status of polymorphic *A3B* deletion allele, HPV16 infection and survival time.

The *A3B* was previously reported overexpressed in breast tumoral tissue compared to the normal counterpart [3]. In Western populations, prognostic significance of *A3B* expression in ER-positive lymph node-negative patients without adjuvant therapy had previously been reported [5]. The Japanese cohort in this study showed no difference in terms of DFS and OS, even though the overexpression of *A3B* observed was the same as in Western countries. This difference in clinical outcomes suggests a different mode

**Table 2** Relationship of *A3B* mRNA expression and patients characteristics in 32 breast cancer patients with or without *A3B* deletion allele

| Characteristics | *A3B* allele | | *p* value |
|---|---|---|---|
| | Insertion/insertion $n = 11$ | Deletion/insertion $n = 21$ | |
| Age (years) | 56.6 ± 14.7 | 51.6 ± 12.2 | 0.34 |
| Menopausal status | | | 0.91 |
| Premenopausal | 6 | 11 | |
| Postmenopausal | 5 | 10 | |
| Subtype | | | |
| ER (+) | 2 | 18 | 0.06** |
| ER (+)/HER2 (+) | 2 | 0 | |
| HER2 (+) | 0 | 3 | |
| Triple negative | 1 | 0 | |
| PgR | | | 0.95 |
| − | 2 | 4 | |
| + | 9 | 17 | |
| Stage | | | 0.86 |
| I | 4 | 6 | |
| II | 6 | 12 | |
| III | 1 | 3 | |
| Lymph node metastasis | | | 0.17 |
| − | 3 | 11 | |
| + | 2 | 10 | |
| Lymphatic invasion | | | 0.21 |
| − | 1 | 6 | |
| + | 10 | 15 | |
| Venous invasion | | | 0.65 |
| − | 7 | 15 | |
| + | 4 | 6 | |
| Nuclear grade | | | 0.57 |
| NG1 | 0 | 2 | |
| NG2 | 3 | 5 | |
| NG3 | 8 | 14 | |

** $p < 0.1$

of action by *A3B* according to the etiology of breast cancer among different ethnic/geographical populations [10, 11]. In our study, *A3B* expression in breast cancer patients with *A3B* deletion was significantly lower than those with *A3B* insertion. Despite the limited number of samples, A3B expression levels correlated to the genotype and malignant potential of the tumor in breast cancer patients (Table 1). Our data show that *A3B* expression is possibly a marker of tumor progression (lymph node metastasis, $p = 0.033$ and nuclear grade, $p = 0.009$) (Table 1). The *A3B* expression related better to pathological findings than *A3B* genotyping (Table 2). However, this might be caused by lower patient numbers in the genotyping data. More patients will need to be involved in future studies in order to fully elucidate clinical potential of A3B genomic analysis.

*APOBEC3* family is expressed in virally infected cells where it plays a significant role in host defense against virus. Therefore, it is possible that viral infection, including HPV [15] and Epstein–Barr virus [16], may play a role in the regulation of *A3B* gene expression, in at least some breast cancers. In this study, the HPV16 infection rate was not associated with the *A3B* expression (Table 1). As for the relation between viral infection and carcinogenesis, there has been a long-standing debate about the contribution of tumor-inducing viruses [17, 18]. Future elucidation of a mechanism of *A3B* regulation may become a lead in prevention and/or treatment of viral associated-breast cancers.

*A3B* expression levels were reported to positively correlate with levels of genomic uracil contents,

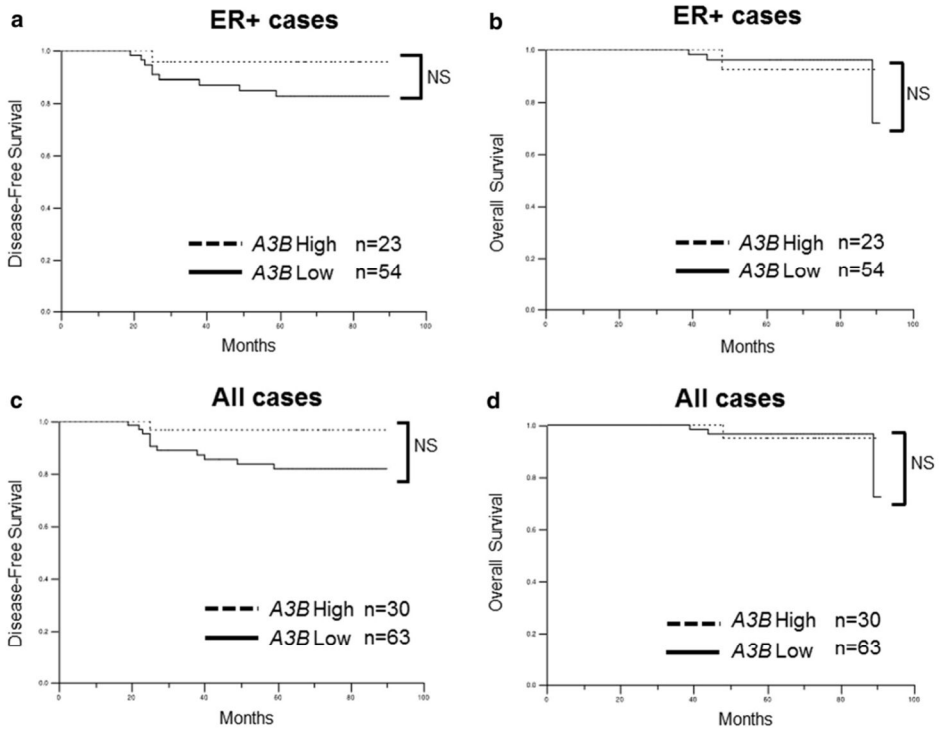


**Fig. 3** Survival curves of breast cancer patients according to the level of A3B expression. Kaplan–Meier survival analysis of *A3B* expression for high and low groups in DFS (**a**) and OS (**b**) among ER-positive cases ($n = 77$) and DFS (**c**) and OS (**d**) among all cases of this cohort ($n = 93$). Cutoff value was defined as mean of *A3B* expression. *NS* not significant

frequencies of overall base substitutions, and C-to-T transitions [4]. Since *A3B* can mutate to oncogenic driver gene, deamination activity of *A3B* might contribute to breast cancer carcinogenesis. *TP53* and *PIK3CA* are the most frequently mutated genes in breast cancers [19], and tumors with *TP53* mutations are reported to have higher levels of *A3B* mRNA in TCGA and other containing Western patients databases [4]. However, *TP53* and *PIK3CA* did not show any correlation between their mutation status and *A3B* expression in our study. It has been reported that *TP53* mutation rates in triple negative breast cancer patients are higher than that in hormone receptor-positive patients [20]. Our study included only 10.8 % (10/93) triple negative breast cancer patients because of selection for patients without preoperative chemotherapies. This sample size is smaller than the one in previous report from Western countries [3]. Therefore, our mutation data might not accord with the previous studies. Although pathological nuclear grade and lymph node metastasis correlated with clinical outcomes [21], we found no correlation between *A3B* expression status and clinical stage, breast cancer subtype, prognosis or driver mutation status in our cohort. The limited number and clinical follow-up of patients in this study might have contributed to less detection power.

In conclusion, *A3B* expression could be a useful marker of aggressive breast cancer. With respect to developing new molecular cancer therapies and cancer prevention methods based on *A3B*, further studies including IHC, whole genome sequencing and larger sample numbers will be required to fully clarify the significance of *A3B* expression in Japanese breast cancer patients.

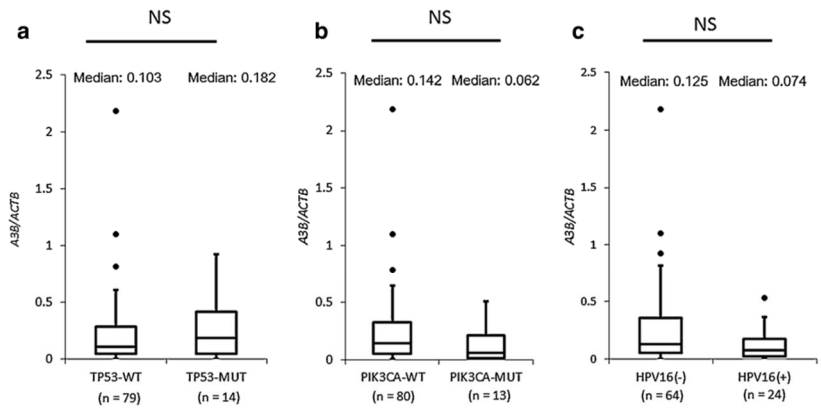


**Fig. 4** *TP53/PIK3CA* mutation status, HPV 16 infection and *A3B* expression. *A3B* mRNA expression level was compared between *TP53* (**a**) and *PIK3CA* (**b**) wild-type (WT) tumors and mutated tumor (MUT) ($n = 93$). (**c**) The relation of *A3B* expression and HPV16 infection was evaluated. *NS* not significant

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**Compliance with ethical standards**

**Conflict of interest** Masahiko Nishiyama received a research grant from Yakult Honsha Co. Ltd. The other authors declare that they have no conflict of interest.

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