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Clinical Implications of APOBEC3-mediated Mutagenesis in Breast Cancer

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Abstract

Over recent years, members of the APOBEC3 family of cytosine deaminases have been implicated in increased cancer genome mutagenesis, thereby contributing to intra- and inter-tumor genomic heterogeneity and therapy resistance in, amongst others, breast cancer. Understanding the available methods for clinical detection of these enzymes, the conditions required for their (dysregulated) expression, the clinical impact they have, and the clinical implications they may offer is crucial in understanding the current impact of APOBEC3-mediated mutagenesis in breast cancer. Here, we provide a comprehensive review of recent developments in the detection of APOBEC3-mediated mutagenesis and responsible APOBEC3 enzymes, summarize the pathways that control their expression, and explore the clinical ramifications and opportunities they pose. We propose that APOBEC3-mediated mutagenesis can function as a helpful predictive biomarker in several standard-of-care breast cancer treatment plans and may be a novel target for treatment.

Keywords

APOBEC3; breast cancer; tumor evolution; therapy resistance; immunotherapy

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Introduction

The genomic landscape of breast cancer is shaped by many mutagenic processes, which promote intra- and inter-tumor genomic heterogeneity and contribute to tumor evolution and thereby treatment resistance (1, 2). These mutational processes are computationally distinguishable as signatures with different etiologic causes (1). For example, a mutational signature attributable to deamination of cytosine bases in DNA catalyzed by apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-3 (APOBEC3) enzymes is evident in a large proportion of breast cancers (1). Two of the seven APOBEC3 enzymes found in humans, APOBEC3A [A3A] and APOBEC3B [A3B], have been causally linked to the observed APOBEC mutation signature in breast cancer. This review focuses firstly on how APOBEC3-positive tumors can be diagnosed, secondly on how the proteins responsible may become dysregulated in breast cancer, and finally on the clinical impact and implications of APOBEC3-mediated mutagenesis for novel and patient-specific treatment opportunities. Of note, APOBEC3 enzymes may also have roles in cancer that are independent of mutagenesis, for example estrogen dependent gene expression (3), R-loop homeostasis (bioRxiv 2021.08.30.458235v1) and RNA editing (bioRxiv 2022.06.01.494353), which are beyond the scope of this review.

The APOBEC3 ABC's and how to detect them

Family member profiles

APOBEC3 proteins catalyze the deamination of cytosines, thereby converting them into pre-mutagenic uracils [reviewed in (4)]. Human cells can express up to seven APOBEC3 proteins, A3A, A3B, A3C, A3D, A3F, A3G, and A3H, which can be further distinguished by single amino acid variants, of which A3H is the most variable in the human population with over a dozen phylogenetically distinct haplotypes (5, 6) [Fig. 1A]. In addition, although all A3 members are structurally similar, differences in amino acid sequence and functionality allow sub-classification into different domain groupings [called Z1, Z2, and Z3, respectively color-coded green, orange and blue in Fig. 1A]. The composition of these domains is evolutionarily conserved amongst higher primates and most of these three domains are also expressed in other mammalian orders, including even- and odd-toed ungulates, bats, and afrotheres (5). In humans, the Z1 domain provides the catalytically active pocket in A3A, A3B and A3G, while the catalytically active pockets of A3C-F and A3H are encoded by Z2 and Z3 domains, respectively [Fig. 1A]. Additionally, a catalytically inactive form of Z2 is present as the N-terminal domain of A3B, A3D, A3F, and A3G, possibly serving to regulate catalytic activity, subcellular localization and the packaging into viral particles (7–9). As such, several A3 members, including A3D, A3F, A3G, and A3H, are capable of restricting HIV-1, whereas other virus types may be restricted by A3A, A3B, and A3H [(10–13), and reviewed in (4, 14)]. For instance, the DNA-based hepatitis B virus may be restricted through the editing capabilities of A3B, A3C, A3F, and A3G (15), and the large DNA herpesviruses by A3B and potentially also by A3A (16).

A3A and A3B are major contributors to cancer

Whole genome sequencing found that off-target activity of APOBEC3 to chromosomal DNA constitutes a major source of somatically acquired mutations in a variety of malignancies, including breast cancer (17–20). Mutational activity by APOBEC3 proteins can be computationally identified in sequencing data as mutations occurring at cytosines within a 5'-TCW [W = A or T] trinucleotide context (21). The mutational process within this context starts with the deamination of cytosine into uracil, which then templates for thymine during replication and base pairs with adenosine. After a round of replication, a C-to-T transition is then immortalized into the genome. Alternatively, uracil excision by DNA glycosylases and subsequent error-prone repair by translesion synthesis polymerases can generate C-to-G transversions. These two distinguishable single base substitution [SBS] mutations are included in the over 40 etiologically distinct mutation signatures found in pan-cancer datasets [referred to as SBS2 and SBS13, respectively: see (1)]. Importantly, these mutation signatures are consistent with the mutagenic characteristics of A3A, A3B, and A3H haplotype I [A3H-I]. Although previously proposed as a likely contributor, the role of A3H-I in cancer-related mutagenesis has recently been questioned (bioRxiv 2022.04.26.489523v2 (22, 23). In comparison, A3A and A3B are currently seen as major contenders to the origin of APOBEC3 deaminase activity in cancer, which is further described below.

Of all APOBEC3 proteins possibly involved in breast cancer mutagenesis, A3B is the only deaminase overexpressed and steadily present in the nuclear compartment (7, 17, 24, 25). Various studies have directly and indirectly connected A3B-activity with APOBEC3-mediated mutagenesis in several cancer types, including breast cancer [Fig. 1A] (bioRxiv 2022.04.26.489523v2, (17, 26). However, APOBEC3-mediated mutagenesis can still be detected in breast cancers of patients carrying loss of A3B. Loss of A3B presents as a chimeric allele, where the *A3A* coding region is fused to the *A3B* 3'UTR, which is rare in European and African populations, but present in ~37%, ~58%, and ~93% of East Asians, native American, and Oceanic populations, respectively (27, 28). This *A3A-B* fusion allele indicates that additional APOBEC3 members, such as A3A, also contribute to the overall level of APOBEC signature SBS mutations in breast cancer.

Like A3B, the potent deamination activity of A3A has also been causally linked to increased levels of APOBEC3-mediated mutagenesis [Fig. 1A] (bioRxiv 2022.04.26.489523v2, (23, 29–32). In fact, A3A has been proposed as the dominant deaminase over A3B in breast cancer, predominantly based on the reported computationally distinguishable mutational signatures of A3A and A3B as established in yeast, cell lines and subsequently tumors (30, 33, 34). However, although potentially useful in the detection of A3A-mediated mutagenesis specifically, more recent analyses in HAP1 cells have shown that this approach may not provide the necessary resolution between these two deaminases and highlights the appreciable contribution of A3A as well as A3B (bioRxiv 2022.04.26.489523v2). Furthermore, while A3A was recently proposed as a major contributor to SBS2 and SBS13 in breast cancer cells, A3B still has an appreciable contribution to APOBEC3-mediated mutagenesis (23). Therefore, since both A3A and A3B are directly implicated with the accumulation of APOBEC signatures, and a detailed correlation of the most relevant

deaminase in relation to breast cancer development is yet to be fully established (and they may also contribute combinatorially), APOBEC3-mediated mutagenesis in this review will not specifically be attributed to either enzyme Fig. 1A]. Reproducible and clinically implementable detection methods, discussed below, can further consolidate the clinical relevance of APOBEC3 proteins and their mutagenic activity.

Options for clinical detection of APOBEC3-mediated mutagenesis

As part of the initial histopathological assessment of malignancy, expression of APOBEC3 proteins can be detected through immunohistochemistry [IHC] in, for instance, diagnostic core needle biopsies [Fig. 1B]. A rabbit monoclonal antibody has recently proven to be suitable for the specific detection of A3A protein using immunofluorescence (bioRxiv 2022.04.26.489523v2). For detection of A3B, the most frequently used antibody is 5210-87-13, a rabbit monoclonal that detects A3A, A3B, and A3G due to a shared epitope (35). As the only APOBEC3 protein with dominant nuclear localization, A3B expressed by tumor cells can be clearly distinguished from other APOBEC3 proteins, including A3A, which are present as cell-wide or cytoplasmic proteins (36). The immunohistochemical detection of A3B has been demonstrated in tumor tissue from several cancer types, including head/neck and ovarian cancer (37–39). Given the low expression of APOBEC3 proteins in most healthy cells, staining of A3A and A3B in tumor cells can be readily detected. It is currently unknown which molecular breast cancer subtype is most likely to score positive for the immunohistochemical detection of A3A and A3B protein. However, protein expression can be seen at early stages of tumor development (40) and, therefore, immunohistochemical detection of A3A and A3B may conveniently be included in histopathologic analysis and stained in parallel to molecular markers such as estrogen receptor [ER] and human epidermal growth factor receptor 2 [HER2]. We therefore recommend all samples be subjected to the A3A/B immunohistochemical analysis. Furthermore, to establish mutational contributions, the resected primary tumor may also be analyzed by DNA sequencing [Fig. 1B]. To the best of our knowledge, targeted sequencing approaches have yet to be optimized to detect APOBEC SBS signatures. Therefore, whole exome sequencing [WES] or whole genome sequencing [WGS] is necessary to gain a comprehensive overview of APOBEC3-mediated mutagenesis. Based on available sequencing data, HER2-amplified breast cancers are most likely to display pan-genomic APOBEC SBS signatures, and ER+ disease may additionally contain APOBEC-induced mutations with clinical relevance (41, 42). These samples may be prioritized in sequencing efforts. Moreover, because tumors with homologous recombination [HR] deficiencies such as loss of BRCA1 or BRCA2 rarely show APOBEC SBS signatures (43, 44), testing labs may be prudent to focus APOBEC diagnostic efforts on HR-proficient tumors.

Importantly, by combining genome sequencing with IHC, historical APOBEC3 activity [*i.e.*, presence of APOBEC SBS signatures, but absence of A3A and A3B protein], may be distinguished from ongoing APOBEC3 activity [*i.e.*, presence of both APOBEC SBS signatures and IHC-positivity]. Other techniques that can consolidate the expression and/or activity of A3A and A3B include quantification by RNA-based methods [*i.e.*, RT-qPCR, RNA sequencing, and/or RNA scope], immunoblotting, A3A dependent RNA editing (45), and DNA deaminase assays [Fig. 1B] (1, 12, 17, 39, 46–50). In order to understand the

biological context surrounding APOBEC3-mediated mutagenesis it is important to know how these enzymes can be expressed in breast cancer [discussed below].

(Dys)regulation of APOBEC3 enzymes in breast cancer

Due to their prominent role in the innate immune system, much of the initial data on APOBEC3 regulation stems from virology research. These observations have proven insightful in the identification of mechanisms underlying APOBEC3 (dys)regulation, even though viruses are unlikely to be directly involved in breast cancer [reviewed in (51)].

Regulation of A3A expression

One of the most prominent factors that induce A3A expression are interferons [IFNs]. The pleotropic group of IFNs, most commonly type-I and type-II IFN, are produced as first-responder inflammatory cytokines by, amongst others, tumor-resident immune cells. Type-I IFNs potently induce A3A, while type-II IFNs only activate A3A marginally (52). In breast epithelial cells *A3A* transcriptional activation through type-I IFNs requires the transcription factor complex STAT2 and its upstream regulator JAK [Fig. 2A] (53). Type-I IFN also induces *A3A* in tumor cell lines, including those from lung, bladder, and breast cancer (45, 46, 54–56).

A3A transcription can also be activated by the canonical PKC/ncNF-κB pathway, which itself is activated by a myriad of inflammatory and genotoxic stresses [Fig. 2A and discussed below] (53, 57). However, the activation of *A3A* upon genotoxic insults is closely guarded by the protein Ataxia Telangiectasia and Rad3-related [ATR], which generally serves as a protective protein during DNA replication stress [Fig. 2A](58). Interestingly, ATR also prevents direct induction of A3A through commonly used cancer treatments, particularly those that cause replication stress (53). However, whether anti-cancer treatments can stimulate inflammatory pathways and subsequently induce A3A expression, and whether this impacts disease trajectory, remains unclear. Moreover, the current body of knowledge on the transcriptional regulation of A3A strongly indicates that chemical inhibition of the IFN and/or PKC/ncNF-κB pathways may be a useful approach to limit A3A expression. Currently, the best-defined inhibitor that has been directly investigated within this context targets JAK2 and effectively prevents A3A induction by IFN (53) [Fig. 2].

Regulation of A3B expression

The PKC/ncNF-κB signaling pathway and its associated proteins PI3K and AKT are at the center of *A3B* transcriptional activation (39, 59, 60). PKC/ncNF-κB and related AP-1 complexes are recruited to sites within the *A3B* promoter, intronic regions, and a distant enhancer, thus activating *A3B* transcription [Fig. 2B] (61). Various upstream stimuli converge to activate the PKC/ncNF-κB signaling pathway, thereby eliciting an increase in A3B expression in breast cancer. For example, tumor necrosis factor alpha [TNFα], a pro-inflammatory cytokine, activates the PKC/ncNF-κB signaling pathway and stimulates *A3B* expression [Fig. 2B] (61). Furthermore, the pro-inflammatory cytokine IL-6, produced by both leukocytes and several solid tumor cell lines, can activate PKC/ncNF-κB [reviewed in (62)] and thereby A3B [Fig. 2B] (61, 63).

Induction of *A3B* by type I and type II IFNs by PKC/ncNF-κB cross-activation is also observed in (oropharangeal and lung) cancer cell lines (54, 56). However, relative to *A3A*, this induction of *A3B* by IFNs is less consistent between different tissue types, indicating the presence of currently unknown regulatory mechanisms [Fig. 2B]. Finally, DNA double strand breaks, which commonly occur in response to ionizing radiation, various chemotherapeutic drugs, or advanced genomic instability [reviewed in (64)] can increase A3B expression in, amongst others, breast cancer cell lines (55, 60, 61, 65–67). Induction of A3B through the PKC/ncNF-κB pathway is also dependent on several main responders to DNA double strand breaks, specifically DNA-PKcs and ATM [ataxia telangiectasia mutated] [Fig. 2B] (60, 61). Interestingly, and further emphasizing the central role of the PKC/ncNF-κB pathway in A3B induction, several pre-clinical and clinically approved PKC inhibitors have been shown to effectively, and dose-responsively, inhibit expression of A3B in various [breast] cancer cell lines (39, 61) [Fig. 2]. Future studies could further explore the usefulness of these compounds in restricting the mutagenic activities of A3B.

Additionally, several viral oncoproteins, including human papillomavirus [HPV] E6, E7, and polyomavirus T-antigen, are strongly implicated with *A3B* transcriptional dysregulation and the accumulation of APOBEC SBS signatures in several virally induced cancers [Fig. 2B] (52, 68–73). Specifically, HPV-E6 may drive expression of *A3B* through recruitment of the transcription factor TEAD to two distinct binding sites at the *A3B* promoter (68). Additionally, both HPV-E7 and polyomavirus T-antigen target the transcriptional repressor DREAM which, as an integral component of the RB/E2F pathway, facilitates the timely expression of cell cycle-associated genes during proliferation [Fig. 2B]. *A3B* is repressed by the DREAM and the PRC1.6 complex, which are recruited to an E2F binding site within the *A3B* promoter in normal-like breast epithelial cells [Fig. 2B] (26, 72). Importantly, disruption of the RB/E2F pathway is common in breast cancer and associates with increased APOBEC SBS signatures (26, 74). Moreover, given the functional implication of the RB/E2F pathway with proliferation, it is likely that *A3B* expression is regulated in a fashion similar to many cell cycle genes. Multiple lines of evidence have indeed classified *A3B* as a gene that associates strongly with cell cycle progression and proliferation in cancer cells (26, 37, 75).

Thus, in contrast to A3A, A3B is readily induced by therapeutic agents. In fact, the occurrence of treatment-induced mutations is relatively well documented and predominantly showcases the direct induction of mutations by cancer drugs, including cis-platin (44), and induction of APOBEC-mutagenesis has been documented comparing gliomas before and after irradiation (76). However, it would be insightful, although challenging to control, to investigate the contribution of treatment-induced A3B on the total APOBEC signature load of breast cancer patients. A minor contribution of treatment-induced A3B-activity is to be expected and might, on a background level, amplify the impact of A3B on disease progression [discussed below].

APOBEC3-mediated mutagenesis and disease trajectory

Recent tumor sequencing efforts have revealed the genetically heterogeneous and evolving nature of tumor cells, changing their genetic makeup during different cancer stages or when

facing anti-cancer treatments (1, 77, 78). Specifically, APOBEC3-mediated mutagenesis has been shown to, in varying degrees, influence the mutational landscape of pre-malignant breast lesions, primary disease, and metastatic breast cancer.

APOBEC3 activity during pre-malignancy

Although samples sizes remain limited, APOBEC3 expression and/or -mediated mutagenesis is found in about 8% of ductal carcinoma *in situ* [DCIS] samples and is detectable in approximately 16% of specimens when DCIS is associated with invasive disease (50, 79–82). However, although APOBEC3-mediated mutagenesis is appreciable in DCIS, no clear evidence of APOBEC3-mediated mutagenesis towards driver genes has been found (80, 82). This indicates that although APOBEC3-mediated mutagenesis can influence the cellular genome at the precursor-stage, this frequently takes place before clonal selection overtakes the overall genomic makeup [Fig. 3]. Therefore, the overall impact on tumor evolution is yet to be fully determined.

APOBEC3 activity throughout malignant disease

APOBEC3-mediated deamination is actively involved in tumor evolution in early and advanced breast cancer (2, 44, 56, 78, 81, 83, 84), adding novel “branches” to the cancer evolutionary “tree” that may unfavorably impact disease trajectory. For instance, in hypermutated breast tumors APOBEC3 activity can account for almost two-thirds of the total mutational burden (44, 85). Indeed, mutations in ~25% of cancer driver genes occur within the preferred 5’-TCW APOBEC context [see Fig. 3 and Table 1 for examples].

As driver mutations occur predominantly in the early stages of tumor evolution, this indicates that APOBEC3-mediated mutagenesis provides mutagenic fuel during the early stages of breast cancer (78, 83). However, APOBEC3-mediated alterations of driver genes can also occur as late events (44, 81, 83) [Fig. 3]. Notable APOBEC3-associated driver mutations are the E542K and E545K hot spot mutations in *PIK3CA*, the second most frequently altered breast cancer driver gene (2). These mutations account for ≥ 1/3rd of the *PIK3CA* single-point mutations in breast cancer and are thought to predominantly occur as early events (29, 42, 83). Tumors can also carry multiple APOBEC-associated *PIK3CA* mutations which are enriched in metastatic breast cancer as compared to primary breast cancer (83). This *cis-PIK3CA* mutational genotype provides enhanced downstream signaling, associates with lower progression-free survival, and has been recognized as a contributing factor to acquired treatment resistance (42, 86–88).

Other genes affected by APOBEC3-mediated mutagenesis include, amongst others, *KMT2C* and *ARID1A* (78), which exhibit widespread, non-hotspot truncating S>X and Q>X mutations in an APOBEC context (83). Importantly, loss of *KMT2C* is associated with resistance to endocrine therapy, while *ARID1A* mutations may confer resistance to both endocrine therapy and chemotherapy (89–91). Moreover, the emergence of truncating *NF1* mutations bearing APOBEC SBS signatures may also occur during endocrine therapy (92). Loss of *NF1* has been shown to confer resistance to endocrine treatment, possibly by enabling cell cycle progression overdrive (93). An APOBEC-associated mutation in the tumor suppressor *CDH1*, unique to the metastatic tumor, grew to dominance during

chemotherapy (81). Pathogenic mutations in *CDH1* have been associated with cellular decohesion and hyperplasia and contribute to lobular breast cancer [reviewed in (94)]. Finally, post-mortem sequencing of metastases of endocrine-resistant ER^+ breast cancer revealed novel acquired APOBEC-associated mutations in *SPEN* and *ESR1* (95), genes that have been associated with acquired resistance to endocrine treatment (96, 97). Importantly, APOBEC-associated mutations in almost all the aforementioned driver genes [*PIK3CA*, *KMT2C*, *ARID1A*, *NF1*, and *CDH1*] have recently been shown to be enriched in metastatic breast cancer, strongly emphasizing their relevance in cancer development (83). Other genes carrying APOBEC-associated mutations in these studies were, amongst others, the tumor suppressors *MAP3K1*, *TP53*, and *ZFHX3*.

Leveraging APOBEC3 activity for clinical benefit

APOBEC3 as a prognostic biomarker

Considering the active contribution of APOBEC3-mediated mutagenesis to disease trajectory it can be expected that APOBEC3 expression and/or APOBEC SBS signatures can serve as prognostic biomarkers in breast cancer. Indeed, in ER^+ breast cancer high expression of *A3B* correlates with unfavorable clinical parameters, including disease-free survival, metastasis-free survival, and overall survival (48). Although prognostic studies are rare as these have to be performed in the absence of systemic treatment to distinguish them from predictive biomarkers, the independent nature of these findings emphasizes the suitability of *A3B* as a prognostic marker in ER^+ breast cancer.

APOBEC3 as a predictive biomarker

The role of APOBEC3 expression and/or APOBEC SBS signatures as predictive biomarkers has become increasingly established over recent years. Increased *A3B* expression in breast cancer has been strongly associated with treatment failure of adjuvant endocrine drugs (49), implying that commonly used endocrine drugs [such as tamoxifen] are less suitable for APOBEC3-positive tumors [Fig. 4]. Conversely, in breast cancer as well as ovarian carcinoma and bladder cancer, APOBEC3-mediated mutagenesis was found to predict beneficial treatment response (38, 55, 98, 99). Although diverse treatment regimens were used in these studies, all applied DNA intercalating agents. These observations suggest that APOBEC3-mediated mutagenesis and DNA intercalators combine to exert synergistic levels of DNA damage during breast cancer treatment [Fig. 4]. Overall, APOBEC3-mediated mutagenesis is a predictive biomarker for response to both endocrine- and chemotherapy. Of note, recent work in lung cancer suggests A3A and A3B may also contribute to resistance to targeted therapies (bioRxiv 2021.01.20.426852v1; bioRxiv 2020.12.18.423280v2).

APOBEC3 as a predictor for immune checkpoint inhibition response

Cancer growth relies on a disturbance in the balance between detection and subsequent elimination of cancer cells by immune cells and, conversely, the escape of cancer cells from immune cells. At the core of this interaction are antigen-presenting dendritic cells [DCs] and $CD4^+$ or $CD8^+$ T cells. DCs are innate immune cells specialized in recognizing neoantigens, which are proteins released by tumor cells that contain non-autologous antigens as a result from somatic mutations (100). Upon recognition, these neoantigens are used to prime naïve

$CD4^+$, and $CD8^+$ T cells, which infiltrate tumor tissue and eliminate tumor cells displaying these neoantigens [reviewed in (101)]. Due to the highly plastic nature of tumors, cancer cells need to tip the scale in their favor in order to avoid cytotoxic elimination. Proteins expressed by tumor cells, such as PD-1, can interact with inhibitory ligands expressed by T cells, such as PD-L1, initiating a shift towards immune tolerance. This interaction, called an immune checkpoint, forms the basis for immune checkpoint inhibition [ICI], which seeks to enhance immunogenic tumor cell killing by using antibodies against key immune checkpoint proteins [Fig. 4, and reviewed in (102, 103)].

Given the involvement of APOBEC activity with shaping the tumor genome, and thereby the antigen repertoire, its suitability to predict ICI response in breast cancer has become subject of investigation. In a recent study (104), murine breast cancer cells that normally do not possess the *A3B* gene, were engineered to express A3B and orthotopically injected. Interestingly, expression of A3B alone already significantly slowed tumor growth as compared to cells devoid of A3B. This partial inhibition of tumor growth was dependent on $CD4^+$ and $CD8^+$ immune cells, suggesting that at least some cytotoxic tumor cell killing was achieved. A3B expression also promoted tumor infiltration by T cells that were likely primed with APOBEC3-induced neoantigens. Strikingly, when combined with ICI, potent and sustained growth inhibition was achieved in A3B-expressing cells, but not control cells.

Increased APOBEC3-mediated mutagenesis has also been associated with immunoactivation in human breast cancer. For instance, firstly, indicators of APOBEC3-mediated mutagenesis such as increased APOBEC SBS signatures and expression of *A3A* or *A3B* often correlate positively with infiltration of tumor tissue by immune cells, including DCs and $CD8^+$ T cells (105–107). Secondly, the same indicators of APOBEC3-mediated mutagenesis correlate positively with expression of PD-1 and PD-L1 (108). Thirdly, in several studies -although with a limited number of breast cancer patients- the presence of APOBEC SBS signatures significantly improved the chance of ICI response (85, 108–110). Combined, these findings suggest that APOBEC3 activity can function as a powerful predictor of ICI responsiveness [Fig. 4], which merits further investigation with larger cohorts of breast cancer patients.

Synthetically lethal interactions with APOBEC3-mediated mutagenesis

In addition to somatic C-to-T and C-to-G mutations the activity of APOBEC3 enzymes can also induce DNA double-strand breaks and replication stress (17, 41, 111–113). In order to counteract these pressures, and to ensure survival, cancer cells that display APOBEC3 activity increasingly rely on DNA damage repair (111–113). This vulnerability has led to a number of synthetic lethality approaches that target DNA damage repair in A3A or A3B expressing cancer cells [Fig. 4]. Cells with high APOBEC3 activity are exceptionally vulnerable to inhibition of ATR, an important DNA damage checkpoint (29, 113). There are currently multiple ATR inhibitors being evaluated for clinical use (114). Furthermore, inhibition of at least three major repair factors involved in the resolution or neutralization of deaminated lesions, including UNG, HMCES, and APE1, show similar synthetic lethal phenotypes (111, 115–117). Altogether, these synthetic lethality models represent a rational design to systematically attack the vulnerabilities of cancers that show ongoing APOBEC3-mutagenesis and warrant further research into their suitability in breast cancer.

Dampening APOBEC3 activity using inhibitors

Efforts to inhibit A3A and A3B enzymes have chiefly relied on the design of chemical inhibitors and, so far, revolve around two molecular classes [Fig. 5]. One such approach exploits the trinucleotide preference of the Z1 domains of A3A and A3B, and features a chemically modified cytosine, called dZ, in an oligo-based substrate. These efforts yielded promising substrate-like inhibitors within the low micromolar range in *in vitro* assays (118–121). Additionally, another recent approach identified several candidate small molecules [as opposed to substrate-like inhibitors], with comparable *in vitro* effectiveness within the low micromolar range (122). After further characterization *in vitro*, APOBEC3 inhibitors can subsequently be investigated in clinically relevant pre-clinical platforms, as relevant genetically engineered mouse models for A3A and A3B have become available recently (31, 104). Future studies should aim to determine possible systemic toxicity of candidate inhibitors and their ability to lower the accumulation of APOBEC3 SBS signatures in murine cancers. Moreover, considering the established role of both these enzymes in the development of [breast] cancer, further research into APOBEC3 inhibition should stay focused on the dual-inhibition of both A3A and A3B. Ultimately, APOBEC3 inhibitors should be investigated as as synergistic treatments in conjunction with existing anti-breast cancer therapies, including surgery and targeted treatments based on genetic markers [Fig. 4].

Conclusions

Over the past decade the perspective on APOBEC3 enzymes, specifically A3A and A3B, has drastically shifted from beneficial members of the innate system to direct influencers of cancer development and disease trajectory most notably in breast cancer. A collection of cellular pathways, including the PKC/ncNF-κB, the RB/E2F pathway, and IFN signaling relay proliferative and inflammatory signals that stimulate expression of A3A and/or A3B. The mutagenic activities of APOBEC3 proteins can now be traced using high-throughput sequencing approaches, implicating them with genomic alterations that stand in direct association with treatment response. They also provide prognostic and predictive value and reveal potential cancer weak spots. Furthermore, the promising characteristics of potential APOBEC3 inhibitors merit further investigation and may be instrumental in restricting the mutagenic arsenal of cancer cells.

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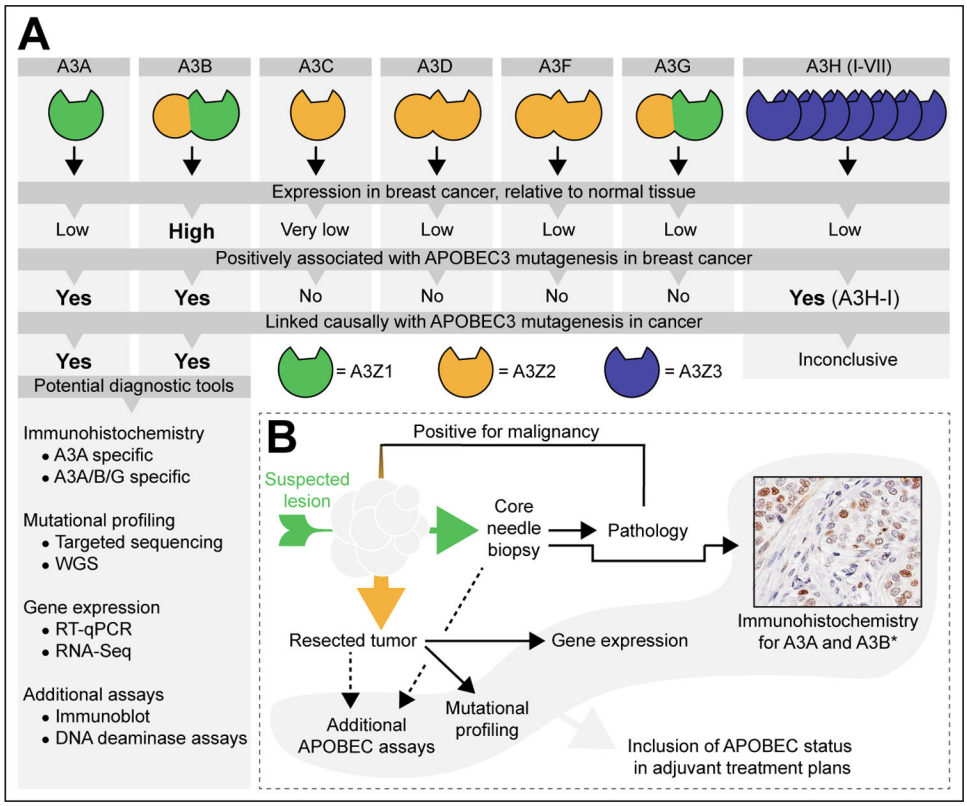


Figure 1. The APOBEC3 enzymes, their association with breast cancer, and the diagnostic methods available.

A Break-down of individual APOBEC3 family members, their conserved domain composition (5, 6), expression levels in cancer (17, 22, 30), and their causal involvement in the observed APOBEC mutagenesis pattern observed in cancer (13, 17, 22, 33). The list of potential diagnostic tools denotes published methods suitable for the detection of APOBEC3 enzymes, their deaminase activity, or the APOBEC single base substitutions [SBS] signatures (bioRxiv 2022.04.26.489523v2, (12, 17, 35, 39, 45–50).

B Proposed flow chart for the inclusion of the APOBEC status in the consideration of suitable adjuvant treatment plans. An initial core needle biopsy is taken from the suspected lesion [green arrow] and immunohistochemistry for A3A and/or A3B is performed in parallel to conventional clinical pathology. *The example shown here is considered A3B specific because of its nuclear localization. If malignant and operable, the freshly resected tumor [orange arrow] is subjected to additional assays, including mutational profiling and gene expression analyses. The resultant APOBEC status may then be included in the adjuvant treatment plans.

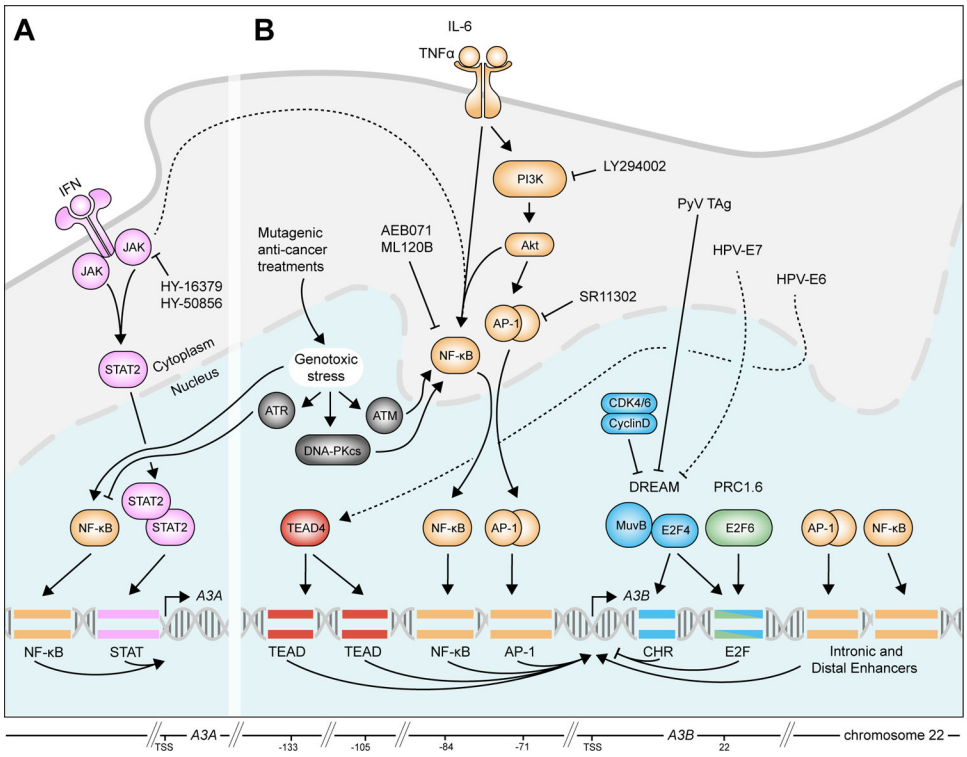


Figure 2. Transcriptional regulation of *A3A* and *A3B*

A Activation of IFN signaling facilitates the recruitment of STAT2 to the promoter of *A3A*, while genotoxic stress promotes the recruitment of the NF-κB transcription factor complex. Transcriptional activation of *A3A* through genotoxic stress is inhibited by ATR.

B The PKC/ncNF-κB pathway dominates *A3B* transcription and recruits transcription factor complexes to the *A3B* promoter, intronic, and distal enhancers. In turn, the PKC/ncNF-κB pathway is activated by genotoxic stress, TNFα, IL-6, and possibly IFN signaling. Transcriptional repression is facilitated by the DREAM complex [and the upstream RB/E2F pathway] and the PRC1.6 complex. Viral oncogenes, including HPV-E6, HPV-E7, and PyV Tag can also activate *A3B*, although this is unlikely to contribute to *A3B* expression in breast cancer.

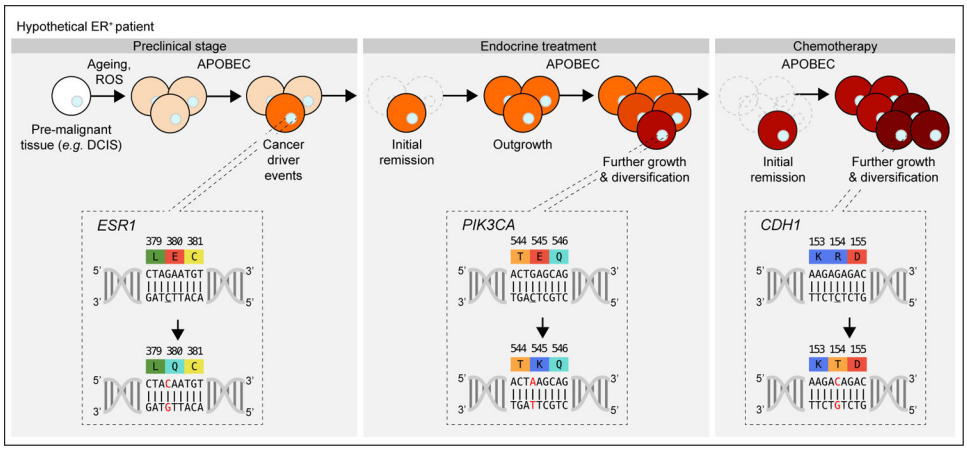


Figure 3. APOBEC3-mediated mutagenesis and disease trajectory

Clinical progression of a hypothetical ER$^+$ patient from preclinical stage [initial outgrowth and initial cancer driver events], to first- and second-line anti-cancer treatments. In this example, APOBEC3-mediated mutation of *ESR1* is the first driver mutation. Mutations in *PIK3CA* provide the tumor with resistance to adjuvant endocrine treatment. Although remission is obtained, further growth and APOBEC3-mediated diversification occurs. Second-line chemotherapeutics provided temporary remission, butfurther APOBEC3-mediated mutagenesis affected genes involved in metastatic behavior, here exemplified with *CDH1*, leading to treatment failure. The cytosine targeted by APOBEC3 proteins is underlined.

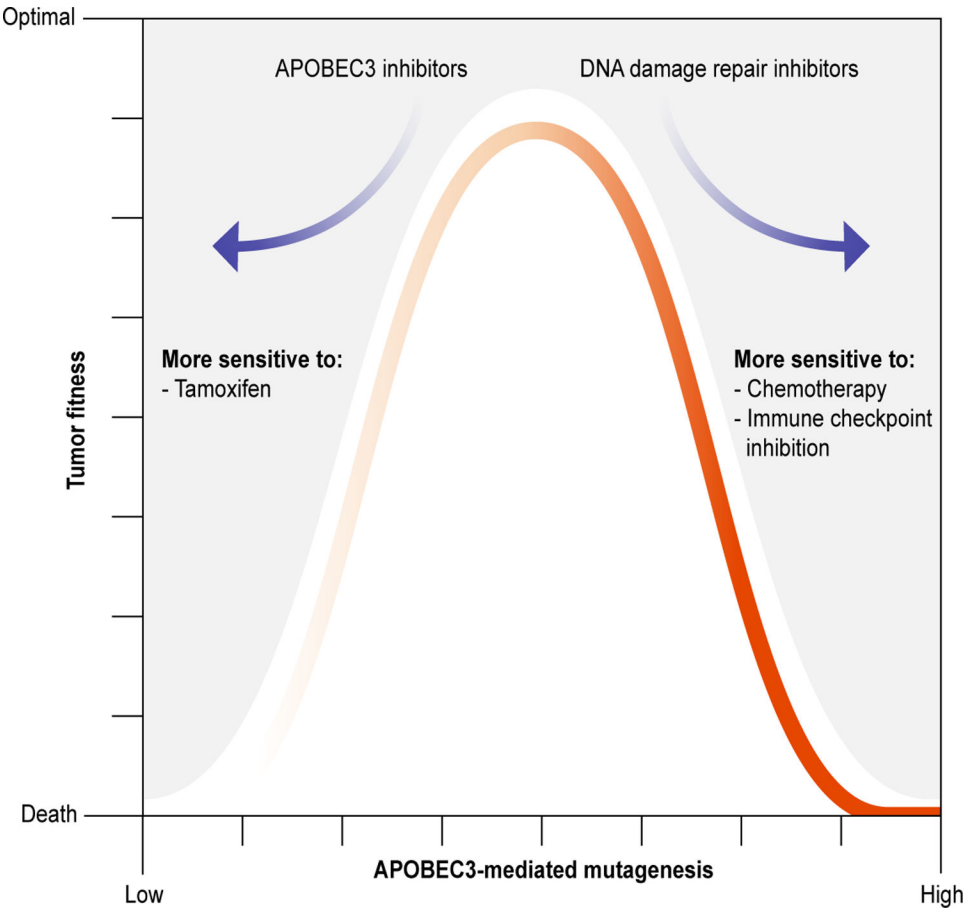


Figure 4. Clinical implications of APOBEC3-mediated mutagenesis

Overview depicting the relationship between APOBEC3-mediated mutagenesis [X-axis] and tumor fitness [Y-axis] as a function of suitable anti-cancer treatments. Standard-of-care therapies, such as tamoxifen and radiotherapy, are suitable when APOBEC3 activity is low, driving down tumor fitness. On the other hand, immune checkpoint inhibition, synergistic drugs [such as DNA intercalating agents], and synthetically lethal combinations [such as ATRi] can exploit the weaknesses brought about by APOBEC3-mediated mutagenesis. Inhibitors of A3A and/or A3B, currently in development, may also be used to limit APOBEC3-mediated genomic diversification and sensitize to other treatments.

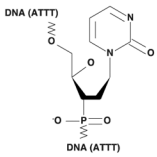
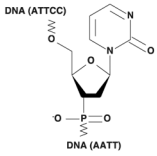
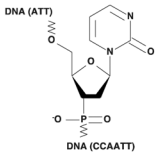
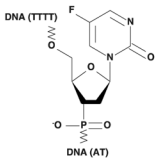
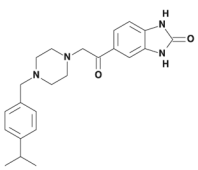
Abbreviation	Inhibitor class	Molecular structure	Binding, inhibitory and dissociative properties	References
TdZA-Oligo	Oligo-based substrate		vs. A3A K_i = 7.5 ± 1.7 μM, as per NMR spectroscopy vs. A3B K_i = 11.4 ± 2.6 μM, as per NMR spectroscopy vs. A3B K_d = 5.5 ± 0.6 μM, as per ITC assay	(119)
CCdZ-Oligo	Oligo-based substrate		vs. A3A K_i = 12.3 ± 1.8 μM, as per NMR spectroscopy vs. A3A K_d = 0.97 ± 0.15 μM, as per ITC assay vs. A3B, not an inhibitor	(118, 119)
dZCC-Oligo	Oligo-based substrate		vs. A3A, not an inhibitor vs. A3B K_i = 18.5 ± 5.5 μM, as per NMR spectroscopy	(118)
5FdZ-Oligo	Oligo-based substrate		vs. A3A IC_{50} = 0.16 ± 0.01 μM, as per oligo cleavage assay	(120)
C8.5	Small molecule inhibitor		vs. A3A IC_{50} = 9 μM, as per oligo cleavage assay vs. A3B IC_{50} = 40 μM, as per oligo cleavage assay	(122)

Figure 5. APOBEC3A and APOBEC3B inhibitors

Published inhibitors of APOBEC3A and APOBEC3B, their molecular structures and pharmacological properties.

Gene	Mutations	Trinucleotide context	Pathogenicity score (FATHMM)	Hotspot (Yes/No)	Clinical associations	References
PIK3CA	E453K	TCT > TTT	0.99	No	Loss of normal *PIK3CA* function has been recognized as a contributing factor to the acquired resistance to chemotherapy and endocrine therapy. Multi-hit mutations in *PIK3CA* are also associated with APOBEC activity.	(78, 86, 87, 95, 123)
	E542K	TCA > TTA	0.97	Yes		
	E545K	TCA > TTA	0.97	Yes		
	E545Q	TCA > TGA	0.98	Yes		
	E726K	TCA > TTA	0.99	No		
	E970K	TCT > TTT	0.99	No		
SPEN	E2151K	TCT > TTT	N/A	No	Associated with acquired resistance to endocrine treatment	(95, 96)
ESR1	E380Q	TCT > TGT	1.00	Yes	Associated with acquired resistance to endocrine treatment	(95, 97)
NF1	Q554*	TCA > TTA	0.99	No	Confers resistance to endocrine treatment, possibly by enabling cell cycle progression overdrive	(92, 124)
	Q1218*	TCA > TTA	0.98	No		
	Q1399*	TCA > TTA	0.98	No		
	Q2234*	TCA > TTA	1.00	No		
CDH1	R154T	TCT > TGT	0.98	No	Associated with cellular discohesion and hyperplasia	(81, 94)
ARID1A KMT2C	SNNN* QNNN*	not reported	various	No	Associated with acquired resistance to endocrine treatment and lowered PFS	(83)

Abbreviations: FATHMM, Functional Analysis Through Hidden Markov Models (125)