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Emerging Roles of Pseudogene RNAs in Antitumor and Antiviral Immunity

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Abstract

Innate immunity mediates anti-tumor responses through a variety of mechanisms including stimulation of cytokine production, activation of cytotoxic immune cells, and induction of cancer cell apoptosis. Together, these anti-tumor defense mechanisms can increase the efficacy of chemo- or immunotherapies. Intriguingly, recent evidence suggests that innate immune responses are intricately regulated not only by exogenous non-self RNA but also by host-derived RNAs such as pseudogene transcripts. Indeed, although pseudogenes have long been considered as non-functional artifacts of evolution, accumulating evidence indicates that pseudogene transcripts function as important gene expression regulators or immune modulators. In this article, we highlight recent findings that unveiled novel roles for pseudogene RNAs in antiviral or antitumor immunity, with a focus on the *BRCA1* pseudogene transcripts that serve as immunoregulatory RNAs in breast cancer. Considering the importance of innate immune sensing and signaling in anti-tumor immunity, recent findings on the regulation of innate immunity by pseudogene RNAs may impact the design of next-generation antitumor therapies.

The Role of Antiviral Innate Responses in Tumor Immunity and Immunotherapy

Tumor immunity and immunotherapy have become increasingly important in treatment strategies for a variety of malignancies including advanced triple negative breast cancer [1,2]. Although immunotherapy has been shown to be effective, patient response rates vary significantly and only a small fraction of patients respond favorably to the treatment [3]. The efficacy of cancer immunotherapy appears to depend on the host immune system recognizing and eliminating cancer cells [4]. Increasing evidence demonstrates a positive correlation between the presence of host antitumor immune responses and favorable patient outcomes for many cancers [5-8]. As an example, tumors with a high density of tumor-infiltrating lymphoid cells (TILs) in the tumor microenvironment are more likely to respond to immune checkpoint inhibitors, whereas those with low or no TILs are less likely to respond to the inhibitors [9-11]. Thus, interventions that render non-responding tumors to become responding tumors and hence promote antitumor immunity bear tremendous therapeutic potential.

Antiviral innate immune responses hold intrinsic anticancer benefits by promoting antitumor immunity and thereby increasing efficacy of chemotherapy and immunotherapy [4,12-16]. They mediate essential antitumor responses through several mechanisms, including stimulation of cytokine production, activation of cytotoxic immune cells, and induction of cancer cell apoptosis. Activation of innate immunity is triggered by pattern recognition receptors (PRRs) that are expressed in many different cells types (both immune and non-immune cells) and detect invariant molecular structures shared by pathogens of various origins [17,18]. PRRs can be categorized into two subfamilies depending on their subcellular locations: the membrane-bound Toll-like receptors (TLRs) and the cytosolic nucleic acid sensors, such as RIG-I-like receptors (RLRs) and the cyclic GMP-AMP synthase (cGAS)-stimulator of interferon gene (STING) pathway [19-22].

RIG-I and MDA5 of the RLR family are important PRRs involved in the detection of RNA viruses [19,20,23]. RLRs initiate host signaling that induces type I and III interferons (IFNs) and other cytokines, leading to the transcription of hundreds of IFN-stimulated genes (ISGs) [24]. Activation of RLRs by double-stranded RNA ligands not only triggers host immune responses but can also directly induce apoptosis of cancer cells, in an IFN-dependent or independent manner [25]. Consequently, cancer cells are highly susceptible to RLR-induced cell death via intrinsic and extrinsic apoptosis and immune activation, indicating that the RLR signaling pathway is a promising molecular pathway to target in cancer immunotherapy. Thus, boosting innate immune responses may be key for establishing clinically desired antitumor immunity. Clinical trials evaluating the safety and efficacy of RLR agonists, STING agonists, TLR agonists, or poly(I:C) derivatives (synthetic analogues of double-stranded RNA) are currently completed or are ongoing [26-32]. Oncolytic viruses have also emerged as important agents in cancer treatment as they offer the attractive therapeutic combination of tumor-specific cell lysis and immune stimulation [4,22,33].

Regulation of Innate Antiviral Immunity by Host-derived RNAs

Interestingly, recent evidence suggests that antiviral innate immunity is regulated not only by exogenous non-self RNA but also by host-derived RNAs such as pseudogene transcripts. Pseudogenes have been considered non-functional artifacts of evolutionary processes due to degenerative features such as the accumulation of disruptive mutations or their lack of regulatory elements [34,35]. However, a growing body of evidence indicates a biological role for pseudogenes as gene expression regulators or immune modulators. According to the estimate of GENCODE [36], the human genome expresses 14,112 pseudogenes, a figure comparable to the number of protein-coding genes. The human genome also contains pseudogenes of tumor suppressors and oncogenes, including *PTEN* [37], *KRAS* [38], *BRAF* [39], *p53* [40], and *BRCA1* [41-43]. Pseudogenes of *PTEN*, *KRAS*, and *BRAF* were reported to regulate expression of their parent genes by sequestering microRNAs [44], or to function as competitive endogenous RNAs [45,46]. Other evidence indicates that pseudogenes may regulate gene expression by generating siRNAs [47,48] or by modulating RNA stability [44,49].

Pseudogene transcripts also serve as immune modulators. 5S ribosomal RNA pseudogene transcripts (in particular *RNA5SP141*) were shown to bind to RIG-I and induce the expression of antiviral or proinflammatory cytokines during infection with herpes simplex virus type 1 or the related herpesvirus Epstein-Barr virus [50]. During the course of infection with these viruses, specific *RNA5SP141*-binding proteins are downregulated, which leads to 'unmasking' of these pseudogene transcripts and thereby activation of RIG-I. A ribosomal protein S15a pseudogene transcript (*RPS15AP4, Lethe*) is selectively induced by proinflammatory cytokines or glucocorticoid receptor agonists, and serves as a functional regulator of inflammatory signaling through an interaction with NF-κB [51]. Many long noncoding RNAs (lncRNAs) are also known to function as positive or negative regulators of the innate antiviral response (as reviewed in detail elsewhere [52-54]). *NEAT1* (nuclear paraspeckle assembly transcript 1) binds splicing factor proline and glutamine rich (SFPQ), which is required for the proper expression of several innate immune-related genes, and thereby increases antiviral gene induction (e.g. IL8, RIG-I and MDA5) [55]. *THRIL* (TNF-α and hnRNAPL related immunoregulatory lncRNA) interacts with hnRNPL at the *TNF* promoter and induces TNF-α transcription [56]. These data indicate that host-derived pseudogene RNAs and lncRNAs play pivotal roles in regulating antiviral defense and inflammatory signaling pathways.

The Presence of *BRCA1* and *BRCA1* Pseudogene (*BRCA1P1*) on Chromosome 17 in Breast Cancer

The chromosome 17q21 region that contains the *BRCA1* gene has a partially duplicated pseudogene, *BRCA1P1* (Figure 1A). It is of note that somatic abnormalities in chromosome 17 are common in breast tumors, including whole chromosome and gene-copy-number anomalies, allelic losses, and structural rearrangements [57-60]. These chromosome abnormalities have been linked to mechanisms of breast-cancer pathophysiology [61] associated with different clinicopathological features and gene-expression subtypes of breast cancer [62]. Abnormalities of specific loci on chromosome 17 including *BRCA1* loss, *P53* loss, *ERBB* amplification, and *TOP2A* amplification or deletion are known to have important roles in breast-cancer pathophysiology [63].

The *BRCA1P1* pseudogene contains only three of the 24 exons of *BRCA1* [42,43,64]. It also includes an insertion of the acidic ribosomal phosphoprotein P1 pseudogene (*RPLP1P4*) in exon 1a, and displays unique features of a chimeric pseudogene derived from two parent genes, *BRCA1* and *RPLP1*. The presence of *BRCA1P1* on the same chromosome close to *BRCA1* appears to create a hotspot for homologous recombination, leading to genomic rearrangements between *BRCA1P1* and *BRCA1* in members of families with a high risk of breast cancer [65]. While the roles of BRCA1 in regulating homologous recombination and DNA damage repair have been studied extensively [66,67], the biological relevance of the *BRCA1P1* pseudogene in breast cancer has not been

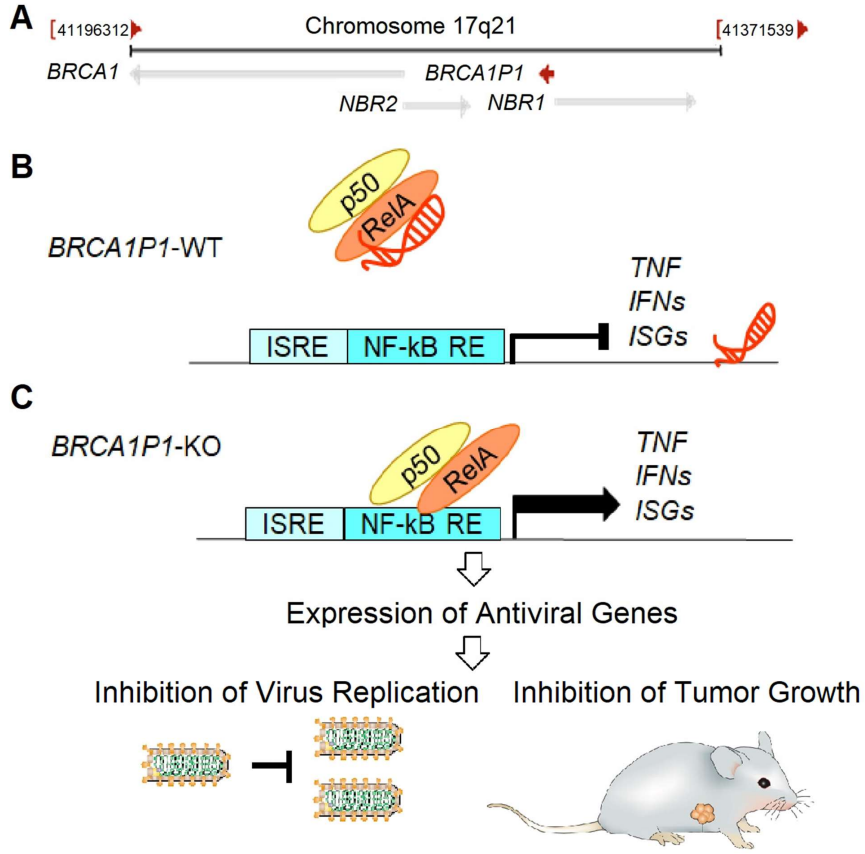

Figure 1: Regulation of Antiviral and Antitumor Immunity by the *BRCA1P1* Pseudogene. A. Schematic representation of the genomic organization of chromosome 17q21 (GRCh37/hg19), as shown in the NCBI genomic context. **B and C.** Working model for the regulation of antiviral gene expression by *BRCA1P1*-lncRNA. ISRE and NF-κB RE represent response elements for IFN-stimulated genes (ISGs) and NF-κB, respectively. In *BRCA1P1* wild type cells (*BRCA1P1*-WT), *BRCA1P1*-lncRNA binds to RelA, inhibits the activity of RelA at its target sites (NF-κB RE), and negatively regulates transcription of antiviral genes (e.g. *TNF*, *IFN*, and *ISG* genes) (**B**). In *BRCA1P1* knockout cells (*BRCA1P1*-KO), the RelA-p50 complex binds to the target promoters and positively regulates expression of antiviral genes, which suppresses virus replication in breast cancer cells and tumor growth in a xenograft mouse model of breast cancer (**C**).

elucidated. Recently, we discovered an important role for *BRCA1P1* in regulating antiviral program-like responses in breast cancer cells [68]. In this article, we highlight recent findings unveiling a novel role for *BRCA1P1* in antitumor immunity in breast cancer, and also discuss its potential impact for the design of next-generation anti-tumor therapies.

Regulation of Antiviral and Antitumor Immunity by the *BRCA1P1* Pseudogene

We recently discovered a crucial role for the *BRCA1P1* pseudogene in regulating antiviral and antitumor immunity in breast cancer [68]. *BRCA1P1* expresses a lncRNA in the nuclei of breast cancer cells through divergent transcription using the bidirectional promoter between *NBR1* and *BRCA1P1* (Figure 1A). In the nuclei of *BRCA1P1* wild type cells (*BRCA1P1*-WT), *BRCA1P1*-lncRNA binds to the NF-κB subunit RelA, inhibits the activity of RelA at its target promoters, and thereby negatively regulates transcription of antiviral genes (Figure 1B). In contrast, knockout of *BRCA1P1* (*BRCA1P1*-KO) allows RelA to bind to its target promoters and to positively regulate transcription of antiviral genes, which increases antiviral-like host innate immune responses and suppresses viral replication in breast cancer cells (Figure 1C). In a xenograft mouse model of breast cancer, depletion of *BRCA1P1* stimulates local immunity and suppresses tumor growth.

This discovery is of high significance in several aspects. First, this is the first study that demonstrates an important role for the *BRCA1P1* pseudogene in antitumor responses

through regulation of antiviral innate immunity and tumor growth. Second, while other pseudogene RNAs were shown to regulate antiviral or anti-inflammatory responses [51,69], our data derived from breast cancer cell lines and a breast cancer xenograft mouse model demonstrate a role for *BRCA1P1* in both antiviral and antitumor immunity. Third, our results revealed that the effects of *BRCA1P1* depletion are specific to cancer cells, with no induction of apoptosis in primary human mammary epithelial cells (HMEC). This suggests that normal cells may not experience toxicity from *BRCAP1* inhibition, which induces cancer cell death specifically and immune cell activation through cytokine production. Fourth, as *BRCA1P1*-depleted cells were more sensitive to genotoxic drugs with increased apoptosis after doxorubicin and camptothecin treatment, our data suggest that *BRCA1P1* depletion could be applied to increase chemotherapy sensitivity. Finally, as boosting innate immune responses is essential for effective anti-tumor immunotherapies, and since inhibition of *BRCA1P1* robustly triggers the host innate immune system, there is therapeutic potential for *BRCA1P1* depletion to increase sensitivity of immunotherapy, which might convert non-responding tumors into responding tumors and hence promote the antitumor activity of immune checkpoint inhibitors.

Unique Features of BRCA1P1 and Future Studies

Although our study opens new possibilities to utilize antitumor immunity driven by *BRCA1P1* depletion for cancer therapeutics, there are still limitations in our study. As we used athymic nude mice that lack T-cells, we were unable to evaluate the effects of *BRCA1P1*-deficiency on T-cells or other immune cells. Therefore, future studies using humanized mice will be needed to fully understand the relevance of *BRCA1P1*-lncRNA in modulating local immunity and the tumor microenvironment. Mechanistically, *BRCA1P1*-lncRNA binds to the NF-κB subunit RelA and inhibits the activity of RelA at its target promoters. Further investigation will be required to determine the mechanistic details of how *BRCA1P1* regulates RelA activity, such as defining the RNA structural regions of *BRCA1P1* that interact with RelA, by using chemical modifications and x-ray crystallography. Defining the RelA-interacting RNA motif may contribute to the design of therapeutic methods that specifically inhibit the interaction of *BRCA1P1* with RelA.

It is of particular note that *BRCA1P1* displays unique features of a chimeric pseudogene derived from two parent genes, *BRCA1* and *RPLP1*. It contains processed sequences of *RPLP1* from exon 1 to 3 (out of four exons of *RPLP1*) inserted in exon 1a of the *BRCA1* gene. Compared to limited sequences originated from *BRCA1* (three exons out of 24 exons of *BRCA1*), it contains the majority of the *RPLP1* sequences, suggesting its role as a pseudogene transcript of ribosomal proteins. Given that *RNA5SP141* and *RPS15AP4* (*Lethe*) are also pseudogene transcripts originating from ribosomal RNA and ribosomal protein respectively [50,51], there might be a common role for ribosomal pseudogene transcripts in the regulation of innate immune pathways, which warrants further investigation. In terms of the genomic location of *BRCA1P1*, it is located in the chromosome 17q21 region close to *BRCA1,* which is frequently subject to somatic abnormalities in breast tumors [57-60]. Therefore, it is conceivable that *BRCA1P1*, along with *BRCA1*, may undergo genomic alterations during tumor evolution, which we will study further in the near future. Furthermore, it is worth noting that the *BRCA1* and *BRCA1P1* promoters have 85.7% identical sequences and are oriented in the same direction. As the two promoters have common cis-regulatory elements, they are likely regulated by similar transcription factors and upstream signals. Further studies will be required to identify the upstream signals that regulate *BRCA1P1* expression, which would increase our understanding of the regulatory circuits in *BRCA1P1*-mediated immune responses.

Future Perspectives: Potential Relevance of Pseudogene RNAs and lncRNAs in Antiviral and Antitumor Therapy Development

Recent data from our and other groups demonstrated the importance of host-derived RNAs in the regulation of antiviral innate defense mechanisms. These studies suggested that the infected host cell has evolved to express cellular pseudogene RNAs or lncRNAs that counteract viral infection by stimulating antiviral gene induction. On the other hand, viruses may transcriptionally induce pseudogene RNAs or lncRNAs that dampen innate immune responses in order to facilitate virus replication. It is therefore tempting to speculate that the *BRCA1P* transcript may be one of the immune-dampening RNAs employed by viruses, which warrants future investigation. On the other hand, blocking the synthesis or function of host RNAs that negatively regulate the innate immune response may boost antiviral defense mechanisms, thereby facilitating virus clearance. Inhibition of these host-derived RNAs also may have therapeutic potential in cancer due to their abilities to stimulate antitumor immune responses and to increase sensitivity to chemotherapy and immunotherapy. Therefore, a molecular understanding of the regulation of innate immunity by host immunoregulatory RNAs may lead to the development of new clinical immunotherapies, which is an exciting area of future research.

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