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Breast Cancer Specificities of Patients With Anti-Ri Paraneoplastic Neurologic Syndromes

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Abstract

Background and Objectives

Breast cancers (BCs) of patients with paraneoplastic neurologic syndromes and anti-Yo antibodies (Yo-PNS) overexpress human epidermal growth factor receptor 2 (HER2) and display genetic alterations and overexpression of the Yo-onconeural antigens. They are infiltrated by an unusual proportion of B cells. We investigated whether these features were also observed in patients with PNS and anti-Ri antibodies (Ri-PNS).

Methods

Using clinicopathologic data, DNA sequencing, and whole-transcriptome analysis, 28 BCs associated with Ri-PNS were characterized regarding oncological characteristics. Genetic alteration of the onconeural antigens and differential gene expression profiles were analyzed in the 12 available tumor samples and compared with those of 5 Yo-PNS tumors.

Results

Ri-PNS BCs were mainly luminal B invasive carcinomas that did not overexpress HER2 and were a subtype of BCs different from the ones observed in Yo-PNS BCs. They had a low expression of wild-type *TP53* and deletions of 1p chromosome. Neither overexpression nor genetic alteration of the Ri onconeural antigens was found in Ri-PNS BCs. Conversely, the nature of the antitumor immune reaction in Ri-PNS BCs was similar to the one found in Yo-PNS BCs. Ri-BCs also had a high propension for early nodal regional metastasis.

Discussion

BCs associated with Ri-PNS are uncommon and the tumor subtype and their molecular and oncological characteristics are different from those of Yo-PNS and controls. Overexpression or sequence variation in the gene of the onconeural antigen is not mandatory. Conversely, Ri-PNS–associated and Yo-PNS–associated BCs display the same atypical B-cell–mediated intratumoral immune response, and tumor escape in the lymph nodes is frequent.

Introduction

Paraneoplastic neurologic syndromes (PNSs) are cancer-related autoimmune disorders mainly associated with certain type of cancers, namely lung and testicular cancers, lymphomas, thymomas, and gynecologic malignancies. These rare diseases are thus associated with very frequent cancer subtypes, which early raised the hypothesis that particularities of the tumors were needed to trigger a PNS. The selective association of each syndrome with one or few different

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Glossary

BC = breast cancer; **CNV** = copy number variation; **ER** = estrogen; **FFPE** = formalin-fixed paraffin-embedded; **GO** = Gene Ontology; **HER2** = human epidermal growth factor receptor 2; **HR** = hormone receptor; **IHC** = immunohistochemistry; **LN** = lymph node; **MCP-counter** = Microenvironment Cell Populations–counter; **NST** = no special type; **PR** = progesterone; **Ri-PNS** = PNS and anti-Ri antibodies; **Yo-PNS** = PNS and anti-Yo antibodies.

cancer subtypes suggests a specificity of the couple tumor-PNS. One could hypothesize that for each subtype of cancer, there is 1 histomolecular subtype favoring the apparition of paraneoplastic autoimmunity. In PNS-associated breast cancers (BCs), it has been shown that most of the BCs associated with PNS and anti-Yo antibodies (Yo-PNS) overexpress human epidermal growth factor receptor 2 (HER2) and do not express the hormone receptors (HRs)[1] and it could thus be expected that other PNS-associated BCs share this specificity.[2,3] Another central characteristic of Yo-PNS is the presence of genetic alterations (sequence variation and copy number variation [CNV]) and overexpression of the onconeural antigens in the associated tumors,[1,4] which was found in no other PNSs so far.[5] Whether these antigens' alterations are specific to PNSs associated with BCs remains to be determined. The antitumor immune reaction is another important feature of PNS-associated cancers and has been described to be strikingly B-cell mediated in Yo-PNS BCs. Whether this B-cell reaction is typical of PNS-associated BCs also remains in question. Hence, to clarify whether this histomolecular subtype, the antigen alterations, and the particular immune environment are common features of BC triggering PNS, we conducted a study on a series of PNS and anti-Ri antibodies (Ri-PNS) BCs. Indeed, anti-Ri autoantibodies, which are directed against the NOVA1 protein and its paralog NOVA2,[6] are the second most frequent autoantibodies associated with BCs.[3] The main aim of this study was to assess the tumor oncological characteristics (histomolecular subtype, cytogenetic profile, and oncogenic mutations), to identify putative alterations of the antigens, and to analyze the intratumor immune reaction in Ri-PNS BCs combining clinical, histologic, and transcriptomic analyzes.

Methods

Patients

Patients with Ri-PNS and a BC diagnosed between October 2001 and March 2023 were identified retrospectively by the French Reference Center for PNS and autoimmune encephalitis (Lyon, France). Patients were included if they had (1) a PNS diagnosis according to the international guidelines[7]; (2) the presence of Ri antibodies in serum and/or CSF detected using both immunohistochemistry (IHC) on rat brain sections and dot blot using commercial tests (RAVO diagnostika and Euroimmun); and (3) a histologically proven BC. Tumor and PNS synchronism was defined by a delay of ≤12 months between the diagnosis of the cancer and that of the PNS. Tumors occurring in a delay >12 months before or after PNS diagnosis were labeled as asynchronous. Data concerning clinical features and oncological history were retrieved retrospectively from patients' medical files. Complete clinical description of this cohort has been published elsewhere.[3]

Control Specimens

Ten BCs without Ri-PNS from the biopathology department of the *Centre Léon Bérard* (BB-0033-00050, CRB *Centre Léon Bérard*, Lyon, France) were selected to match the Ri-PNS BCs regarding histopathologic type, HER2 and HR expression (namely estrogen [ER] and progesterone [PR] receptors), and the Nottingham grade.[8] Of note, all control tumors were primary BC.

Clinical data were compared with those from a literature-drawn cohort.[9]

Yo-PNS Samples

Another cohort of 5 primary BC samples with Yo-PNS previously described[1] was used for comparison with primary Ri-PNS BCs.

Tumor Pathology Study

Formalin-fixed paraffin-embedded (FFPE) tissue sections of 4-µm thick were stained with hematoxylin-phloxine-saffron (HPS). A referent pathologist (IT) assessed the subtype of BC according to the 2019 World Health Organization classification.[10]

Immunohistochemistry

Detailed chromogen IHC protocols and antibodies are described in eMethods. Classical diagnostic markers, including ER and PR receptors, were obtained using a routine automated protocol. HER2 expression was assessed using prediluted monoclonal anti-HER2 antibody 4B5 (Roche Diagnostics, Basel, Switzerland). Expressions of NOVA1 antibody PA5-18895 (ThermoFisher Scientific, Waltham, MA) and NOVA2 antibody PA5-83784 (ThermoFisher Scientific) were assessed using an automated IHC protocol. A staining intensity value from 0 (no staining) to 3 (high staining) was given by manual quantification conducted by 2 evaluators (IT and VD) blinded to the provenance (patient or control) of the sample.

RNA Sequencing

Sequencing was performed (paired end, 2 × 75 cycles) using the NextSeq 500/550 High Output V2 kit on a NextSeq 500 machine (Illumina, San Diego, CA). The mean number of reads per sample was around 80 million. Alignment and quantification were conducted using grape, a Nextflow pipeline[11] using STAR[12] 2.4.0j and RSEM 1.2.21.[13] The GRCh38 version of the human reference genome and GENCODE 41

Table 1 Clinicopathologic Description of Ri-Paraneoplastic Neurologic Syndrome Breast Cancer (BC) Cohort

	28 BCs, n (%)
Pathology	
Invasive carcinoma NST	24 (85.7)
Lobular carcinoma	1 (3.6)
Papillary carcinoma	1 (3.6)
NA	2 (7.1)
Nottingham grade[a]	
III	7 (25.0)
II	11 (39.2)
I	1 (3.6)
NA	9 (32.1)
Tumor subtype	
NA	6
HER2- hormone receptor +	18 (81.8)
HER2+ hormone receptor +	3 (13.6)
HER2+ hormone receptor -	0 (0.0)
TNBC	1 (4.5)
Receptor expression	
NA	4
ER	23 (95.8)
PR	13 (54.1)
HER2	3 (12.5)
Tumor size	
T0	6 (21.4)
Tis	1 (3.6)
T1	14 (50.0)
T2	4 (14.2)
T3	0 (0.0)
Lymph node metastasis	
N0	7 (25.0)
N+	18 (64.2)
NA	3 (10.7)
Tumor-PNS delay	
Synchronous	21 (75.0)
Asynchronous	7 (25.0)
Neurologic features	
Cerebellar syndrome	15 (53.6)
Extracerebellar features	13 (46.4)
Opsoclonus	11
Myoclonus	11
Dystonia	6
Hypertonia	12
Oculomotor palsy	12
Tremor	5
Limbic encephalitis	2
mRS score at onset	
>2	11
<2	11
NA	6
mRS score at last follow-up	
>2	18
<2	8
NA	2

Abbreviations: ER = estrogen receptor; HER2 = cErbB2 protein; mRS = modified Rankin Scale; N+ = cancer cells in at least one lymph node; N0 = no cancer cell in any lymph node; NA = not available; NST = of no special type; PR = progesterone receptor; T0 = no detectable tumor; T1 = tumor is ≤2 cm across; T2 = tumor is >2 cm but ≤5 cm across; T3 = tumor is >5 cm across; Tis = *in situ* tumor; TNBC = triple-negative breast carcinoma; TNM = tumor node metastasis staging.
[a] Nottingham grade is a composite pathological prognostic score taking into account differentiation, atypia, and mitotic activity ranging from grade I (best prognosis) to III (worse prognosis).

were used. Three samples with a number of unique reads less than 10 million (5 million paired-reads) were discarded from the analysis.

Comparative Genomic Hybridization Array

The fragmentation, labeling, and cohybridization on 4 × 180 K Agilent SurePrint G3 Human whole-genome oligonucleotide arrays (Agilent Technologies), as well as scanning and analysis, are fully described in eMethods. The accession number in National Center for Biotechnology Information's Gene Expression Omnibus is GSE96039.

DNA Sequencing

NOVA1 and *NOVA2* sequences were assessed along with a panel of breast oncogenesis genes (namely *NOTCH1*, *NOTCH2*, *ATM*, *TP53*, *ESR1*, *PIK3CA*, *BRCA1*, and *BRCA2*) using the Miseq next-generation sequencing platform (Illumina) according to the manufacturer's instructions. The sequence data generated were aligned using Next-GENe (Softgenetics, State College, PA) on human reference

sequence GRCh38. The Catalogue of Somatic Mutations in Cancer database[14] was used as control data.

Statistical and Bioinformatics Analyses

Statistical analyses were performed using R 4.0.3.[15] Comparisons were made using the χ^2 test, Fisher exact test or Wilcoxon rank-sum test according to the preanalytical conditions. The p values were adjusted using the Benjamini and Hochberg method.

Bioinformatics analyses are detailed in eMethods. In brief, differential gene expression analysis was performed using the DESeq2 1.32.0[16] R package by comparing gene expression between Ri-PNS and control samples. Hierarchical clustering was performed with the pheatmap 1.0.12 R package using the Euclidean distance and the Ward clustering method. Gene Ontology (GO) enrichment was performed using the clusterProfiler 4.10.1[17] R package on overexpressed and underexpressed Ri-PNS genes separately. Immune cell populations were analyzed using the Microenvironment Cell Populations–counter (MCP-counter) method[18] with the MCPCounter 1.1 R package.

Ethical Considerations

This study is part of the project Gene PNS (NCT03963700) and was approved by the ethics and scientific committee of the *Hospices Civils de Lyon*. Tumors and other biological samples were collected after patients gave informed and written consent.

Data Availability

Anonymized data used for this study are available on request.

Results

Clinical and Pathologic Cohorts

Among the 47 patients with anti-Ri PNSs diagnosed in the French Reference Center between November 2001 and October 2022, we identified 28 patients with an associated BC and collected their histopathologic data (Table 1). Twelve patients had available FFPE tissue samples: 3 axillary lymph nodes (LNs) and 9 primary BCs. Histologic data on each available tumor sample are provided for each patient in Table 2, and the study flowchart summarizing the methods and analyses applied to characterize Ri-PNS BCs compared with controls and Yo-PNS BCs is presented in Figure 1. Regarding clinical presentations, 15 patients (53.6%) had a cerebellar syndrome at onset while 13 (46.4%) presented only with extracerebellar features. None of the patients had both cerebellar and extracerebellar impairment in this cohort. Tumor and PNS were synchronous in 75.0% of the cases (Table 1). Clinical characteristics were not significantly different between the synchronous and asynchronous groups.

Ri-PNS BCs Are Luminal B BCs but With an Uncommon Genetic Background

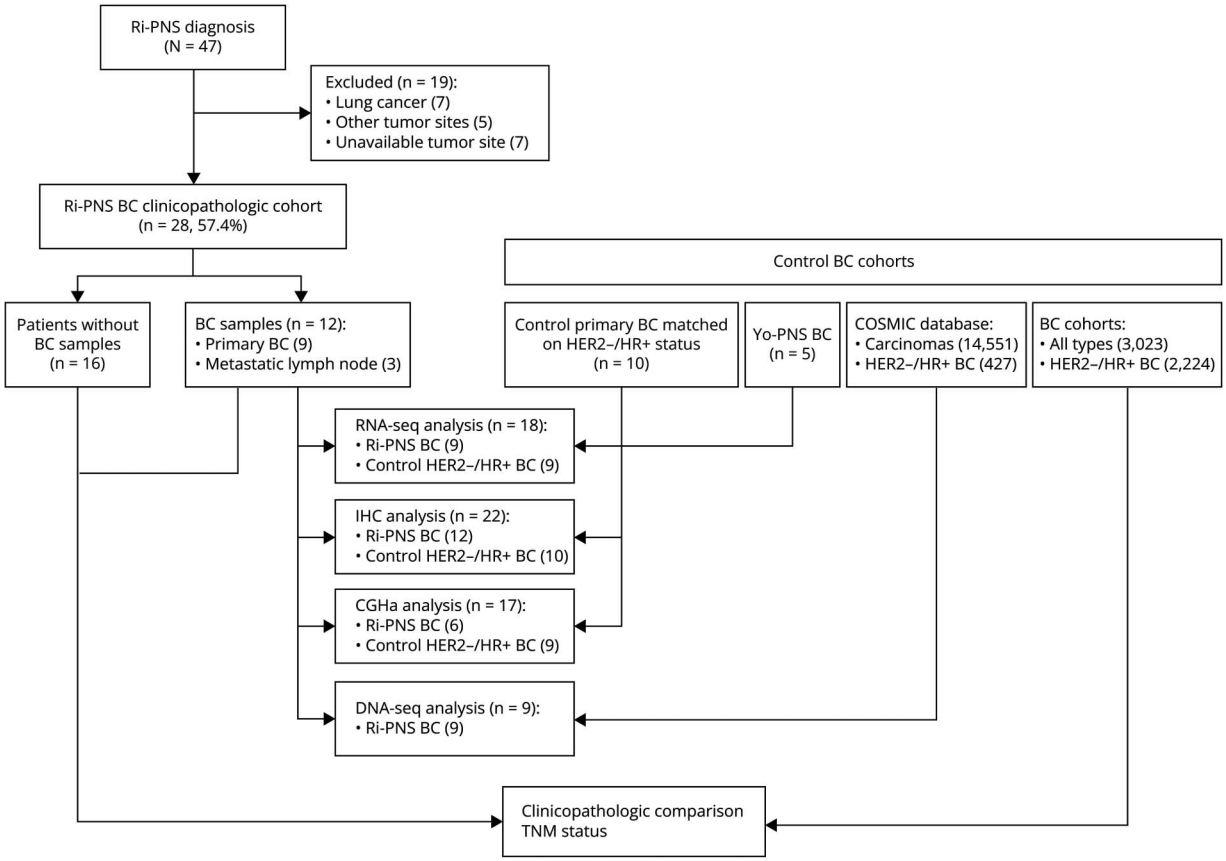
Twenty-four of the 28 Ri-PNS BCs (85.7%) were invasive carcinomas of no special type (NST). The Nottingham grade[8] was available for 19 samples: 18 BCs were grade 2 or 3 (94.8%) while only 1 was grade 1 (5.2%). IHC found that HER2 was overexpressed in only 3 Ri-PNS BCs (12.5%). Concerning HR, ER was positive in 23 of 24 (95.8%) and PR in 13 of 24 (54.1%) Ri-PNS BCs.

Table 2 Histologic and Molecular Characteristics of Ri-PNS BCs

Patient number	Sample type	Nottingham grade	HER2 IHC expression	ER	PR	Tumor-PNS delay[a], mo	Neurologic features	Genetic alteration on *NOVA1* or *NOVA2* gene	*NOVA 1* CNV	*NOVA 2* CNV
1	PBT	III	−	+	−	4.5	Extracerebellar	p.A155P (*NOVA2*)	None	None
2	PBT	II	−	+	+	−0.2	Cerebellar	None	None	None
3	MLN	II	−	+	+	−3.7	Cerebellar	None	None	None
4	PBT	II	−	+	−	−19.8	Extracerebellar	None	None	None
5	PBT	II	−	+	+	13.9	Cerebellar	None	None	None
6	PBT	II	−	+	−	−8.5	Cerebellar	None	Gain	Gain
7	PBT	III	+++	+	−	7.6	Cerebellar	None	NA	NA
8	PBT	III	+++	+	+	0	Cerebellar	None	NA	NA
9	PBT	II	+++	+	+	13	Extracerebellar	NA	NA	NA
10	MLN	NA	−	+	−	0.4	Cerebellar	NA	NA	NA
11	MLN	NA	−	+	−	0.2	Extracerebellar	NA	NA	NA
12	PBT	II	−	+	+	−12.8	Extracerebellar	NA	NA	NA

Abbreviations: BC = breast cancer; CNV = copy number variation; ER = estrogen receptor; HER2 = cErbB2 protein; IHC = immunohistochemistry; MLN = metastatic lymph node; NA = not available; PBT = primary breast tumor; PNS = paraneoplastic neurologic syndrome; PR = progesterone receptor.
[a] The delay between the tumor and the PNS was calculated using tumor diagnosis as Day 0; negative interval thus corresponds to a patient for whom PNS was diagnosed before the tumor.

Figure 1 Flowchart of the Study



Control cohorts are either from (1) in-house control luminal B BCs for the RNA-seq and IHC analyses, (2) the COSMIC database[14] for the DNA sequencing of *NOVA1* and *NOVA2*, or (3) a literature-drawn cohort[9] for the clinicopathologic comparison. BC = breast cancer; CGHa = Comparative Genomic Hybridization array; HER2- = no overexpression of human epidermal growth factor receptor 2; COSMIC = Catalogue of Somatic Mutations in Cancer; HR+ = overexpression of hormone receptor; IHC = immunohistochemistry; PNS = paraneoplastic neurologic syndrome; TNM = tumor node metastasis staging.

Overall, most of the Ri-PNS BCs (81.8%) were HR-positive/HER2-negative high-grade invasive carcinomas NST, also called luminal B BCs (Table 1). Amongst the 12 samples of our pathologic cohort, 2 presented with limited comedonecrosis, 2 displayed intravascular tumor emboli, and none showed signs of neurotropism. DNA-seq analysis focused on breast tumor oncogenes (*NOTCH1*, *NOTCH2*, *ATM*, *TP53*, *ESR1*, *PIK3CA*, *BRCA1*, *BRCA2*) found none of the mutations expected in regular luminal B BCs; especially, all tumors had a wild-type *TP53* profile. Comparative genomic hybridization analysis found few significant differences between genomic profiles of Ri-PNS BCs and control luminal B BCs (eFigure 1): when altered, the chromosome 1p was more frequently lost in Ri-PNS BCs (3/6, 50%) while gained in control luminal B BCs (3/10, 30%) while the chromosome 12p was frequently gained only in control luminal B BCs (4/10, 40%). None of these alterations were found in Yo-PNS BCs.[1] There was no significant link between the oncological characteristics of the Ri-PNS BC (subtype, grade, and oncogenic background) and clinical features (cerebellar and extracerebellar).

Ri-PNS BCs Metastasize Early in Regional LNs

The study of the oncological characteristics of the patients showed that 21 of 28 Ri-PNS BCs (75.0%) were T1 or lower (in situ or no primary tumor) and 18 of 28 BCs (64.2%) had ipsilateral axillary LN metastasis at diagnosis (Table 1). Six of the 28 BCs (21.4%) had a definitive pathologic diagnosis after LN biopsy but no detectable primary breast tumor after appropriate radiologic and clinical assessment (including ad hoc nuclear imaging), also called occult BC with axillary LN metastasis. No patient had distant metastasis. No significant differences in the tumor size, LN, or distant metastasis were found when comparing patients with cerebellar impairment with those with extracerebellar features.

Onconeural Antigens Are Neither Mutated nor Overexpressed in Ri-PNS BCs

DNA-seq analysis of *NOVA1* and *NOVA2* sequences was performed on 9 Ri-PNS BCs. No sequence variation was found in Ri-PNS BCs nor in controls. Information on *NOVA1* and *NOVA2* CNV was available for 6 samples and 9 controls (eFigure 1). Only one Ri-PNS patient (patient no. 6) displayed a significant but moderate gain in copy numbers on

both onconeural gene *loci* (4 copies of *NOVA1* and 5 copies of *NOVA2*). Overall, we found no genetic alteration of the 2 onconeural antigens.

Concerning expression levels, bulk RNA-seq analysis on Ri-PNS BCs (n = 9) compared with control luminal B BCs without PNS (n = 9) found no differential expression of *NOVA1* or *NOVA2* mRNA (Figure 2, A and C). It is important to note that *NOVA1* expression was significantly higher in all luminal B BCs (Ri-PNS and matched controls) compared with the expression level in the other histologic subtypes of BCs (HER2-driven and triple-negative BCs, eFigure 2). IHC analysis on Ri-PNS BCs (n = 12) compared with controls (n = 10) confirmed the absence of significant difference in the expression of NOVA1 and NOVA2 proteins between Ri-PNS BCs and controls (Figure 2, B and D).

Ri-PNS BCs Are Characterized by a Strong Antitumor Immune Reaction

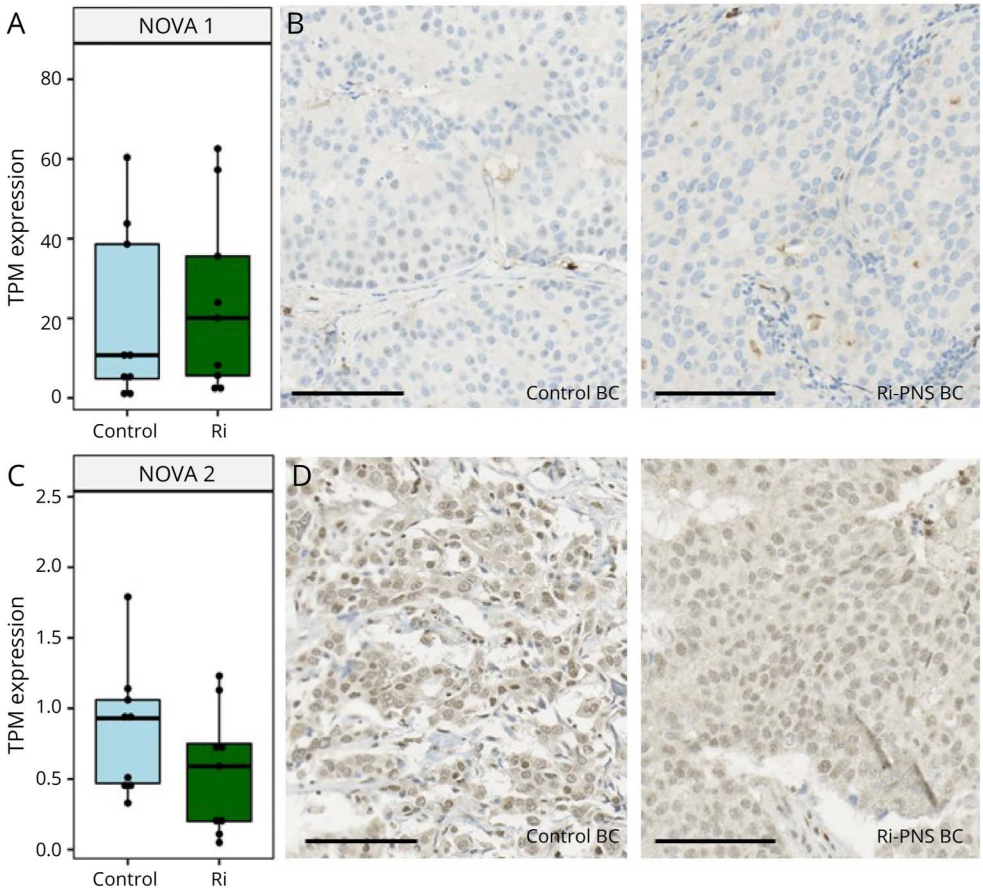
RNA-seq analysis found 81 differentially expressed genes (36 downregulated and 45 upregulated) in Ri-PNS luminal B BCs (n = 9) compared with luminal B BCs without PNS (n = 9; Figure 3). The exhaustive list of differentially expressed genes is presented in eTable 1. None of the significantly differentially expressed genes in Ri-PNS was held by the chromosome 1p concerned by the loss of heterozygosity. The hierarchical clustering based on differentially expressed RNA clearly separated Ri-PNS from their controls. Enrichment analysis on the genes overexpressed in Ri-PNS was mainly related to immune activation. Detailed GO enrichment analysis is provided in eTables 2 and 3.

Deconvolution using the MCP-counter method found a significant enrichment in B lineage, cytotoxic lymphocytes, and NK cells in Ri-PNS BCs compared with control luminal B BCs (Figure 4A, complete MCP results in eFigure 3). Conversely, no statistically significant difference was found between the 2 clinical groups (cerebellar vs extracerebellar features). Comparison of Ri-PNS BCs and Yo-PNS BCs showed similar quantity and repartition of immune cells between the 2 groups, besides an overrepresentation of neutrophils in Yo-PNS tumors (Figure 4B, complete MCP results in eFigure 4).

Discussion

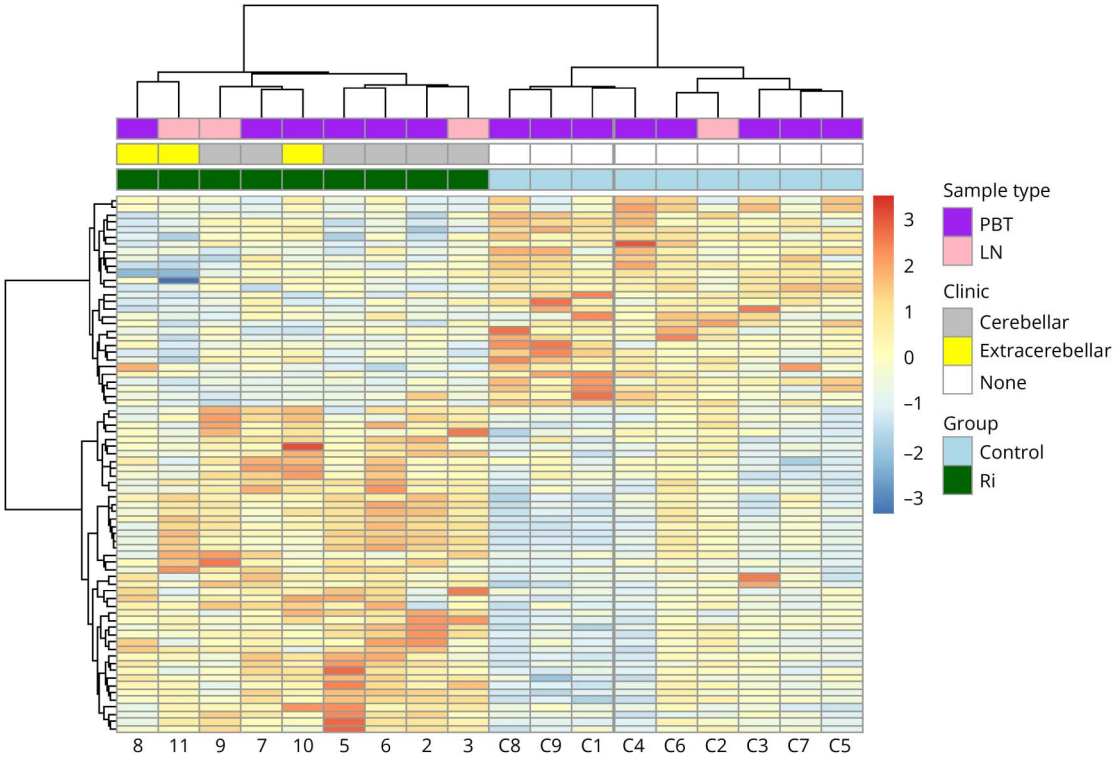
This study shows that Ri-PNS BCs are luminal B BCs that overexpress HR and do not express HER2, contrary to Yo-

Figure 2 Expression of the Onconeural Antigens in Ri-PNS BCs Compared With Controls



(A and C) Median boxplots of TPM expression of *NOVA1* (A) and *NOVA2* (C) in control luminal B BCs (left, light blue) vs Ri-PNS tumors (right, green). (B and D) Expression of the NOVA1 and NOVA2 protein in 10x magnification. Representative NOVA1 IHC staining in Ri-PNS BCs (top right corner) vs control tumors (top left corner). Representative NOVA2 IHC staining in Ri-PNS BCs (bottom right corner) vs control tumors (bottom left corner). Scale bars: 200 µm. BC = breast cancer; IHC = immunohistochemistry; PNS = paraneoplastic neurologic syndrome; TPM = transcripts per million.

Figure 3 Heatmap of Differentially Expressed Genes Between Control and Ri-PNS Breast Cancers

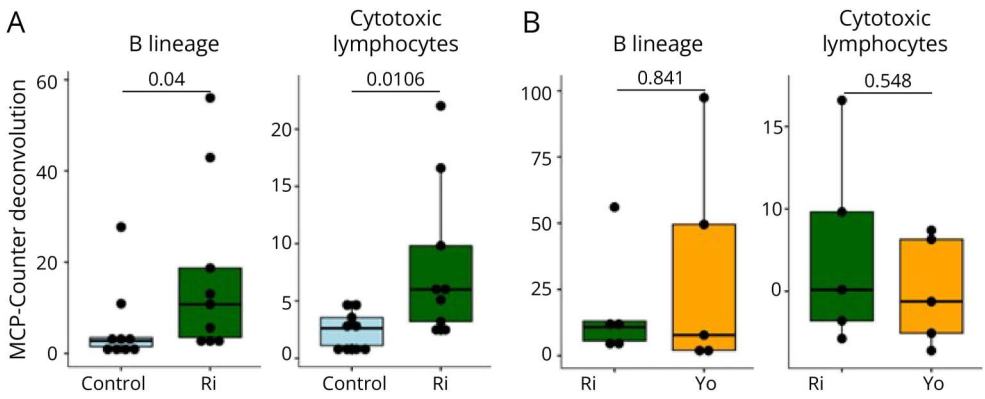


Heatmap of differentially expressed genes (in rows) between Ri-PNS BC (green) and control luminal B BC (light blue) samples (in columns). Tumor sites are separated in primary BCs (PBT, purple) and metastatic lymph nodes (LN, pink). Clinical features of Ri-PNS are categorized into cerebellar (gray) or extracerebellar (yellow). TPM expression values were first transformed into $\log_{10}$(TPM + 0.01) and then transformed into a Z-score per gene. BC = breast cancer; PBT = primary breast tumor; PNS = paraneoplastic neurologic syndrome; TPM = transcripts per million.

PNS BCs that are HER2-driven tumors (HER2+ HR-), a rare subtype of BCs.[1] However, Ri-PNS BCs are not classical luminal B BCs. They have an uncommon genetic background singularizing them from regular luminal B BCs, the most frequent molecular subtype, representing around 15% of all BCs[19]: they all have a wild-type *TP53* (0% of *TP53* mutation in Ri-PNS BC samples while such mutations are found in 41% of luminal B BCs)[14,20] and significant loss of 1p chromosomal arms, for which the signification remains unknown. These types of tumor specificities are likewise found in other PNS-associated tumors: loss of chromosome 10 in Hu-PNS small cell lung cancer associated with loss of tumor suppressor genes, gain or amplification of 17q chromosome holding *ERBB2*, and the Yo antigen *CDR2L* in Yo-PNS BCs and ovarian tumors. These kinds of genetic alterations seem recurrent in PNS-associated tumors and probably participate in PNS pathophysiology. This overall suggests that, for each PNS, there is 1 specific type of tumor with its oncogenic drivers, mutations, and genetic alterations that lay ground for each specific syndrome. On a clinical perspective, refining the profiling of tumors associated with each PNS could allow us to better predict the risk of autoimmunity associated with each patient's tumor and thus to improve the personalized decision-making strategies in the field of cancer immune therapy.

Concerning the antigens, we found no genetic abnormalities nor overexpression of the onconeural antigens *NOVA1* and *NOVA2*, contrary to what was described for the Yo-PNS antigens. These antigen alterations are thus not a hallmark of PNS BCs but maybe rather a particularity of Yo-PNS. Nevertheless, a high expression of NOVA1 and NOVA2 was found in Ri-PNS, but at the same level than in other luminal B BCs,[21] which mirrors what is known of the expression of the antigen ELAVL4 in anti-Hu lung cancers.[5] Hence, antigen expression by the tumor may well be a *sine qua non* condition for paraneoplastic autoimmunity, whereas overexpression seems to be restricted to certain PNSs only.[1,4,22] A link between antigen overexpression and cytogenetic abnormalities has been shown in Yo-PNS (gain or amplification in 17q locus and overexpression of CDR2L) and in GabaBR-PNS (gain of 5q and overexpression of KCTD16, one of the main intracellular interactors of GabaBR; KCTD16 autoantibodies are observed only in paraneoplastic GabaBR PNS[23,24]). In these latter PNSs, the trigger of this specific autoimmunity seems well correlated with tumor abnormalities. The absence of overexpression and cytogenetic alterations of the NOVA protein in Ri-PNS questions 2 possibilities: alternative antigenic targets that could present the same antigenic alterations, as in Yo-PNS with CDR2 and CDR2L, or

Figure 4 Comparative MCP-Counter Deconvolution Between Ri-PNS, Yo-PNS, and Control BCs



Boxplots of the median value and interquartile range (IQR) for the concerned variable of control luminal B BC (on the left, in blue) and Ri-PNS BC (on the right, in green) samples (A) or Ri-PNS BCs (on the left, in green) and Yo-PNS BCs (on the right, in orange) (B); the upper whisker extends from the hinge to the largest value no further than 1.5 × IQR, and the lower whisker extends to the smallest value to a maximum of 1.5 × IQR from the hinge; each dot represents the value of a sample. *p* values of comparisons between groups using the Wilcoxon rank-sum test are adjusted with the Benjamini and Hochberg method (details in eMethods) and shown on the top of each couple of boxplots. Ri-PNS and Yo-PNS samples from metastatic lymph nodes were removed. (A) Comparison of selected signatures between Ri-PNS and control luminal B BCs. (B) Comparison of selected signatures between Yo-PNS and Ri-PNS BCs. BC = breast cancer; MCP = Microenvironment Cell Populations; Ri-PNS = paraneoplastic neurologic syndrome and anti-Ri antibodies; Yo-PNS = paraneoplastic neurologic syndrome and anti-Yo antibodies.

another original mechanism of immune tolerance breakdown that is still to fathom.

This study highlights the fact that the oncological factors implicated in the paraneoplastic immunogenesis are not ubiquitous, even in the same type of cancer. Nevertheless, it is noteworthy that although these 2 PNSs have proven themselves to be very different, they also share commonalities. First, Ri-PNS BCs seem to display the same potential for LN metastasis, as described with Yo-PNS BCs.[1] Indeed, we observed a high proportion of LN metastasis, at the time of diagnosis, in our cohort (64.2%, higher than the expected rate of 36%–43.8% in luminal B BCs).[9,25,26] PNS-associated cancer propensity for metastasis, already hinted by case reports and radiologic studies,[27–30] is confirmed in PNS-associated BCs, whatever the associated syndrome and underlying subtype of BC. This could indicate a particular aggressiveness of PNS-associated BCs, irrespective of the nature of the associated syndrome and the tumor histomolecular subtype. The extremely high proportion of occult BCs in Ri-PNS (21.4%) and Yo-PNS (20.5%)[1] compared with the general population of BCs (0.1%)[31] is also a striking similarity. These data add credit to the already current practice of promptly proposing PET-FDG scanners to patients with Ri-PNS and Yo-PNS without detectable tumor after the first screening following ENFS recommendations (mammogram and MRI).[32] On a pathophysiologic perspective, this suggests discrepancies in antitumor immunity effectiveness between the primary and secondary sites in both PNSs. At the primary tumor site, we describe an unusually intense immune infiltration of the tumors by B cells that explains the major part of the differentially expressed genes between Ri-PNS BCs and their controls. Again, this resembles what was described in Yo-PNS[1,4]: a surprisingly important implication of humoral immunity at the tumor site. The intensity of the antitumor immune response, comparable between Yo-PNS BCs and Ri-PNS BCs, is significantly higher than in controls. The only difference between Yo-PNS BCs and Ri-PNS BCs is the overrepresentation of neutrophils in Yo-PNS BCs, which is probably reflecting the difference of the predominant tumor subtype between the 2 cohorts. Indeed, tumor-associated neutrophils have been associated with the negativity of HR in BCs,[33] which is the case of nearly all samples in the Yo-PNS cohort and virtually none in Ri-PNS. Overall, there are similar B-cell–mediated immune responses in both PNSs, which seem to contain tumor growth in situ but fail to prevent LN metastasis. The determinants for this remote tumor immune escape in PNS are still to be understood.

This study is naturally limited by the small size of the cohort because of the rarity of this syndrome. Moreover, the extended timespan required to conduct the collection of these rare samples probably led to tumor tissue alterations, rendering RNA extraction difficult in some of the oldest samples and further reducing the number of samples available for analysis. Further studies, ideally on larger cohorts, would be needed to confirm the present conclusions and deepen the molecular and mechanistic analyses to understand the link between the salient genetic and cytogenetic characteristics, the histomolecular subtype, the antigenic targets, and the

autoimmunity. Eventually, this could help physicians and patients in decision making when confronted with the question of whether to start an immunotherapy, given the risk of neurologic side effect. Personalizing the strategy based on the study of patient's tumor would be a substantial step forward in this challenging field.

This work allows us to conclude that BCs associated with Ri-PNS are uncommon. Their molecular and oncological particularities are different between Yo-PNS and Ri-PNS, and the autoimmunity is thus not determined only by the nature of the target. Rather, BC-associated PNSs need the combination of the tumoral expression of the onconeural target and the proper type of underlying BC to occur. This suggests a strong link between oncogenesis and autoimmunity that remains to be explored. However, what stands as a commonality between all PNS-associated BCs is the atypical B-mediated intratumoral immune response that seems to fail in fully controlling the cancer. Indeed, as in Yo-PNS BCs, the rate of LN metastasis at the time of diagnosis is unexpectedly high in Ri-PNS BCs, the reason of which is still unfathomed.

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Author Contributions

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