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Somatic DNA Damage Response and Homologous Repair Gene Alterations and Its Association With Tumor Variant Burden in Breast Cancer Patients With Occupational Exposure to Pesticides

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Homologous recombination is a crucial pathway that is specialized in repairing double-strand breaks; thus, alterations in genes of this pathway may lead to loss of genomic stability and cell growth suppression. Pesticide exposure potentially increases cancer risk through several mechanisms, such as the genotoxicity caused by chronic exposure, leading to gene alteration. To analyze this hypothesis, we investigated if breast cancer patients exposed to pesticides present a different mutational pattern in genes related to homologous recombination (*BRCA1*, *BRCA2*, *PALB2*, and *RAD51D*) and damage-response (*TP53*) concerning unexposed patients. We performed multiplex PCR-based assays and next-generation sequencing (NGS) of all coding regions and flanking splicing sites of *BRCA1*, *BRCA2*, *PALB2*, *TP53*, and *RAD51D* in 158 unpaired tumor samples from breast cancer patients on MiSeq (Illumina) platform. We found that exposed patients had tumors with more pathogenic and likely pathogenic variants than unexposed patients ($p = 0.017$). In general, tumors that harbored a pathogenic or likely pathogenic variant had a higher mutational burden ($p < 0.001$). We also observed that breast cancer patients exposed to pesticides had a higher mutational burden when diagnosed before 50 years old ($p = 0.00978$) and/or when carrying *BRCA1* ($p = 0.0138$), *BRCA2* ($p = 0.0366$), and/or *PALB2* ($p = 0.00058$) variants, a result not found in the unexposed group. Our results show that pesticide exposure impacts the tumor mutational landscape and could be associated with the carcinogenesis process, therapy response, and disease progression. Further studies should increase the observation period in exposed patients to better evaluate the impact of these findings.

Keywords: breast cancer, pesticides, occupational exposure, mutational burden, somatic

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INTRODUCTION

Brazil is one of the leading agricultural pesticide-consuming countries in the world (1). The extensive use of pesticides raises concerns about human health (2). Since 2008, Brazil has become the world's top pesticide importer, with more than 1,400 formulations authorized by the government legislation (3). Only 3.5% of the total pesticides authorized in Brazil are approved in other countries due to their high toxicity and their recognized carcinogenic potential. In this context, a total of 52 pesticides used in Brazil are classified as "probable" or "possible" carcinogens for humans, 16 had evidence suggestive of the carcinogenic potential for humans, and eight had insufficient information about the carcinogenic potential for humans (4). Although the use of pesticides has been widespread in Brazil since the 1960s, the exact data concerning pesticide consumption from small-scale farmers are scarce (5–7). Improper pesticide application associated with farmers' limited knowledge regarding its harmful effects and poor adherence to safety precautions, such as the correct use of personal protective equipment, represent a considerable health risk for chronically exposed populations (8, 9).

Pesticide exposure has the potential to increase cancer risk through several mechanisms, including oxidative stress generation, changes in adhesion molecules, acetylcholinesterase inhibition, endocrine disruption, and contribution to genomic instability (5), which are known hallmarks of cancer (10). Double-strand breaks can be a threat to genomic stability. Multiple DNA repair mechanisms are available to counteract its deleterious effects, as failure to repair double-strand breaks can result in chromosome aberrations, apoptosis, and oncogenesis (11). In this regard, homologous recombination is a crucial pathway specialized in repairing double-strand breaks that occur mainly during DNA replication and through cell damage (12). Specific hereditary cancer predisposition syndromes, such as hereditary breast and ovarian cancer (HBOC) and Fanconi anemia, are associated with germline mutations in *BRCA1/2* and *PALB2* genes, respectively (13). These genes are essential for homologous recombination, and alterations in genes involved in this pathway are closely associated with carcinogenic features such as high mutational burden, which is caused by the accumulation of unrepaired DNA damage (11, 13). The loss of function of tumor suppressor genes in tumors, such as *TP53* gene, has shown that DNA damage response pathways must be downregulated to guarantee cell proliferation and for cells to avoid apoptosis (14). Thus, tumoral cells often harbor alterations in genes responsible for these DNA damage response pathways, which may lead to loss of genomic stability and cell growth suppression (10, 15).

It is suggested that only 5%–10% of breast cancer cases are hereditary; the remaining ~90% are associated, to a greater or lesser extent, with environmental factors that result in the occurrence and accumulation of somatic and epigenetic alterations during a person's life (16). Thus, lifestyle and environmental conditions have a significant impact on breast cancer risk. Evaluation of mutational patterns related to DNA damage and repair processes in cancer revealed that several signatures could be associated with carcinogen exposures and defects in DNA maintenance pathways, such as specific base transversions found in smoking-associated lung cancer and caused by tobacco exposure (17). While factors such as obesity, physical activity, and consumption of tobacco and alcohol are known to be associated with breast cancer risk (18–20), the potential roles of environmental exposure to pesticides in breast cancer development are not well understood. Even so, the lack of high-quality and significant evidence, concerning the relationship between pesticide exposure and cancer risk from epidemiological studies, makes it challenging to infer causality. While most studies indicate a trend toward increased risk, only a few are statistically significant, as reviewed elsewhere (21). Although inconsistent results are found in the literature regarding pesticide exposure and breast cancer risk, it is suggested that hormone-positive breast cancer could have an increased association with pesticide exposure (22).

Considering the genotoxicity potential of pesticides, and that the homologous repair pathway may be an essential mechanism for DNA maintenance under such circumstances, we investigated if the profile of acquired genetic mutations in breast tumors is related to patients' occupational exposure. We hypothesized that it might be associated with increased genomic instability and truncating variants, especially in genes responsible for DNA damage response and known tumor suppressor genes, which could directly impair cell response against genotoxicity and favor oncogenesis and/or disease progression.

MATERIALS AND METHODS

Study Population

A total of 541 patients were enrolled in this study between January 2015 and September 2019. They were managed at Francisco Beltrão Cancer Hospital (Ceonc), which assists 27 municipalities in Paraná state, Brazil. After patients signed consent forms, tumor samples were collected consecutively with no selection bias during the diagnostic breast cancer biopsy surgery. The study was reviewed and approved by the Ethics Committees of the State University of West Paraná, under the number CAAE 35524814.4.0000.0107, and was performed following the Declaration of Helsinki.

All enrolled patients were invited to answer a questionnaire with 61 questions about their current and past occupational history (23). Among the 167 women diagnosed with breast cancer, 158 were eligible for this study. Based on their answers, the study population was categorized as occupationally exposed or not to pesticides. The study was composed of 91 exposed patients and 67 unexposed to pesticides (**Figure 1**).

DNA Extraction and Clinicopathological Data Obtention

Tumor samples were stored under refrigeration at −20°C until genomic DNA extraction with QIAamp DNeasy Blood & Tissue kit (QIAGEN, Valencia, CA, USA), following the manufacturer's recommendations. All clinicopathological variables were collected from medical records. Patients were categorized based on their age, tumor grade, hormonal receptor status (estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2)), molecular subtype, lymph nodal

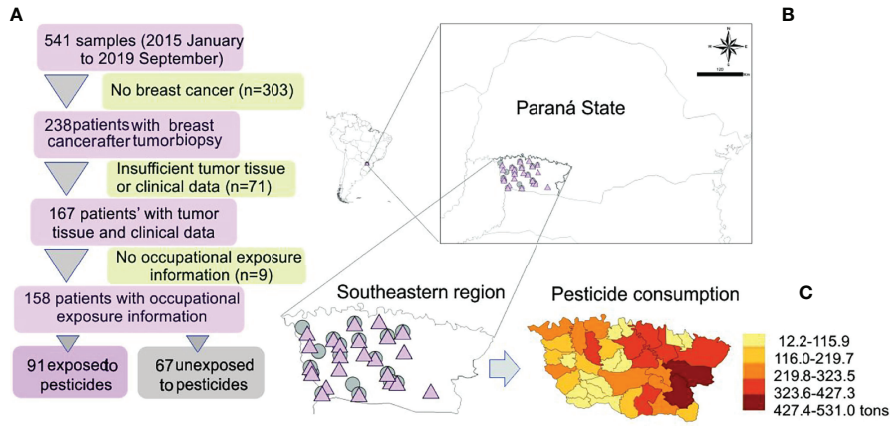

FIGURE 1 | **(A)** Study design and map showing the sample distribution according to exposure. **(B)** Amplified map of Brazilian Paraná State with symbols representing exposed (pink triangle) and unexposed (gray circle) patients' localities. **(C)** Detail of pesticide consumption in the study area; modified from Gaboardi et al. (24).

invasion, tumor size, distant metastasis, menopausal status, chemoresistance and/or tumor persistence, disease relapse, and survival status. For HER2 status, an immunohistochemistry score of 0 and 1+ was considered negative, and 2+ and 3+ were considered positive, where fluorescence *in situ* hybridization (FISH) further confirmed 2+. Early disease onset was categorized as <50 years old and late disease onset as ≥50 years old.

Sample Preparation and Next-Generation Sequencing of Unpaired Tumor

Multiplex PCR-based assays were performed on 158 unpaired tumor samples, designed to cover the entire coding regions and flanking splicing sites of *BRCA1*, *BRCA2*, *PALB2*, *TP53*, and *RAD51D* (25). Tumor DNA sequencing was prepared with Nextera XT DNA Kit (Illumina, San Diego, CA, USA) and performed in three independent runs, using paired-end methodology on MiSeq Genome Analyzer (Illumina), with up to 150 bp of reading length.

Somatic Variants Calling and Enrichment in Unpaired Tumor Samples

Base quality score recalibration, indel realignment, and variant calling were performed following the Genome Analysis Toolkit v4.1 (GATK, Broad Institute) best practice (https://software.broadinstitute.org/gatk/best-practices) (26). Somatic variants were called by Mutect2 from GATK, using default parameters for unpaired tumor samples. Somatic variants were functionally annotated using GATK Funcotator (27) and the Ensembl Variant Effect Predictor (VEP) (28). To enrich in true positives, all the following parameters were applied: i) removal of single-nucleotide polymorphisms (SNPs) observed in the 1000 Genomes Project, the Exome Aggregation Consortium (ExAC), observed in common populations with minor allele frequency >0.5%, according to Genome Aggregation Database (gnomAD); ii) support from ≥20 reads in the tumor; iii) a variant allele fraction (VAF) of ≥0.02; iv) support from reads mapped to both strands; v) synonymous variants were excluded.

Tumor Mutational Burden Analysis

To explore the mutational landscape according to pesticide exposure, somatic variant data were processed and analyzed using the R programming language (version 4.0.2) with the "maftools" package (29). Tumor mutational burden (TMB) was measured by the total number of somatic non-synonymous variants in the target region by the total size of the target region per mega-base. Breast cancer patients were then separated into low- and high-TMB groups using the median value (**Supplementary Table I**). To analyze the correlations with clinicopathological features of patients with breast cancer, the mutational burden data were merged with corresponding clinical information. The Wilcoxon rank-sum test (30) and Fisher's exact test (31) were used for comparisons between two groups of clinical variables, with $p < 0.05$ considered significant.

Classification of Somatic Variants

All intronic, 5′Flank, 3′UTR, and silent variants were excluded. The pathogenicity of variants was determined predominantly based on the clinical data reported in ClinVar (http://www.ncbi.nlm.nih.gov/clinvar/), including benign, likely benign, variant of uncertain significance (VUS), likely pathogenic, and pathogenic. All nonsense and frameshift mutations not registered in the ClinVar, Catalogue of Somatic Mutations in Cancer (COSMIC), or International Agency for Research on Cancer (IARC) database were determined as pathogenic, according to the American College of Medical Genetics and Genomics (ACMG) standard terminology (32). Novel variants in a splice site that were not yet described were determined as likely pathogenic if there were known pathogenic variants in the same splice site already registered in ClinVar. Variants registered in ClinVar as "conflicting interpretations of pathogenicity" were classified as benign if "benign" or "likely benign" reports were predominant and were classified as likely pathogenic if "pathogenic" or "likely pathogenic" reports were predominant. If there was no meaningful information on the pathogenicity of variants in

ClinVar, the COSMIC (http://cancer.sanger.ac.uk/cosmic) database was referred to. For *TP53* variants, a functional classification based on its translational activity in the IARC *TP53* Database (http://p53.iarc.fr/) was also referred to. All variants classified as "benign" and "likely benign" were lumped together into benign.

Statistical Analysis

Statistical models included subjects with complete data on the specific environmental variable of interest and the adjustment variables. All statistical analyses and the visualizations were performed in the R programming language (version 4.0.2). The Shapiro–Wilk test (33) was applied to determine the normality of the data. Fisher's exact test was used to compare frequencies between groups. Fisher's exact test for co-occurrence analysis between mutated genes and clinical features was performed. For continuous variables and group comparisons, the Mann–Whitney U test (34) was performed. p-Values <0.05 were considered statistically significant.

RESULTS

Clinical and demographic characteristics of 158 women included in this study were observed (**Table 1**). The median age of diagnosis was 56.8 years (31–86 years). Statistically significant data showed that exposed patients presented a higher frequency of ER-negative (p = 0.0161) and PR-negative (p = 0.0014) tumors than did unexposed patients. Tumor samples were sequenced with a high depth, with a mean coverage of samples of 520× (102× to 1,068×). After processing and filtering steps for somatic variant identification, of 158 samples, 120 harbored 258 variants that were classified as missense, frameshift, nonsense, or splice-site variants and were distributed in *BRCA1*, *BRCA2*, *PALB2*, *TP53*, and *RAD51D* genes. We found 78 samples with variants in *BRCA2* (65%), 47 in *PALB2* (39.1%), 36 in *BRCA1* (30%), 27 in *TP53* (22.5%), and 13 in *RAD51D* (10.8%). The proportion of missense variants was the highest among other mutation types (~88.4%), and all variants were found in heterozygosity.

Variant Landscape of Breast Cancer Samples and Grouped According to Patients' Pesticide Exposure

The distribution of variants in all samples showed *BRCA2* as the most affected gene (**Figure 2A**). The most frequent variant type was SNPs/single-nucleotide variants (SNVs), and the most frequent nucleotide substitution was T>C, followed by T>G (**Figures 2B, C**). Approximately 75% of all variants identified by our study were classified as benign, 3.5% as likely pathogenic, 13.5% as pathogenic, 5% as VUS, and 3.5% have no classification

TABLE 1 | Comparison of selected characteristics of the 158 breast cancer patients exposed and unexposed to pesticides.

Variable		Exposed (n = 68)	Unexposed (n = 52)	p-Value
Age at diagnosis (years, mean ± SD)		57.66 ± 14.5	55.63 ± 11.7	
Tumor grade	I/II	44 (64.7%)	40 (76.9%)	0.06
	III	24 (35.3%)	12 (23.1%)	
ER status	Positive	40 (58.8%)	39 (75%)	**0.0161**
	Negative	28 (41.2%)	13 (25%)	
PR status	Positive	26 (38.2%)	31 (59.6%)	**0.0014**
	Negative	42 (61.8%)	20 (38.4%)	
	Not informed	–	1 (1%)	
HER2 status	Positive	9 (13.2%)	7 (13.5%)	>0.9999
	Negative	59 (86.8%)	45 (86.5%)	
Molecular subtype	Luminal A	19 (28%)	19 (36.5%)	0.2899
	Luminal B	21 (30.9%)	17 (32.7%)	
	HER2+	9 (13.1%)	7 (13.5%)	
	Triple negative	19 (28%)	9 (17.3%)	
Lymph node metastasis	Positive	30 (44.1%)	23 (44.2%)	>0.9999
	Negative	38 (55.9%)	29 (55.8%)	
Tumor size	<2 cm	22 (32.3%)	20 (38.5%)	0.5885
	2–5 cm	35 (51.5%)	23 (44.2%)	
	>5 cm	11 (16.2%)	9 (17.3%)	
Distant metastasis	Yes	10 (14.7%)	8 (15.4%)	>0.9999
	No	58 (85.3%)	44 (84.6%)	
Menopausal status	Premenopause	19 (28%)	16 (30.8%)	0.6418
	Postmenopause	49 (72%)	36 (69.2%)	
Chemoresistance	Positive	22 (32.3%)	14 (27%)	0.4382
	Negative	46 (67.7%)	38 (73%)	
Disease relapse	Yes	8 (11.3%)	9 (17.3%)	0.3153
	No	60 (88.2%)	43 (82.7%)	
Survival status	Alive	60 (88.2%)	46 (88.4%)	>0.9999
	Deceased	8 (11.3%)	6 (11.6%)	

The bold values represent significant statistical differences (Fisher's exact test, p < 0.05).

ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

in any database. Afterward, we explored whether variants were mutually exclusive or likely to co-occur (**Figure 2C**), and we observed the co-occurrence of variants in *BRCA1* and *TP53* in a significant manner ($p < 0.05$). In general, missense variants were the most frequent type (~88.5%), followed by frameshift (~7%), nonsense (3.5%), and splicing variants (1%) (**Figure 2D**).

Among breast cancer samples with variants detected, 56.6% (n = 68) belonged to patients with occupational exposure to pesticides whereas 43.3% (n = 52) to patients with no exposure. The most mutated genes in the exposed group were *BRCA2* and *PALB2* (**Figure 3A**) and in the unexposed group were *BRCA2* and *BRCA1* (**Figure 3B**). Concerning the co-interaction analysis, we observed a different correlation result among the exposed and unexposed groups (**Figures 3C, D**). Variants in *TP53* co-occur with *BRCA1* mutations in the exposed group ($p < 0.05$), while *PALB2* variants were found to occur in a mutually exclusive manner concerning *BRCA2* in the unexposed group ($p < 0.05$). Transversions T>G were higher in the exposed group than in the unexposed group, with more T>C transitions (**Figures 3E, F**).

Frequency of Pathogenic, Likely Pathogenic, and Uncertain Significance Variants

We identified 28 pathogenic, 10 likely pathogenic, and 12 VUS variants (**Table 2**; **Supplementary Figure 1**). All variants were identified in 47 tumor samples from different patients. Several variants were detected in more than one sample: a) the pathogenic variants p.M296fs* in *PALB2* (6 samples), p.C61G in *BRCA1* (2 samples), and p.E198* in *TP53* (2 samples); b) the likely pathogenic variant p.H193R in *TP53* (2 samples); c) the VUS p.S46C in *RAD51D* (2 samples). Concerning *TP53*, we found 26 variants classified as pathogenic and likely pathogenic on ClinVar, being 21 of them also predicted as pathogenic on COSMIC. Six *BRCA1* variants are classified as pathogenic on ClinVar, 21 as pathogenic on the COSMIC database, and only one ranked as pathogenic in both databases (*BRCA1*

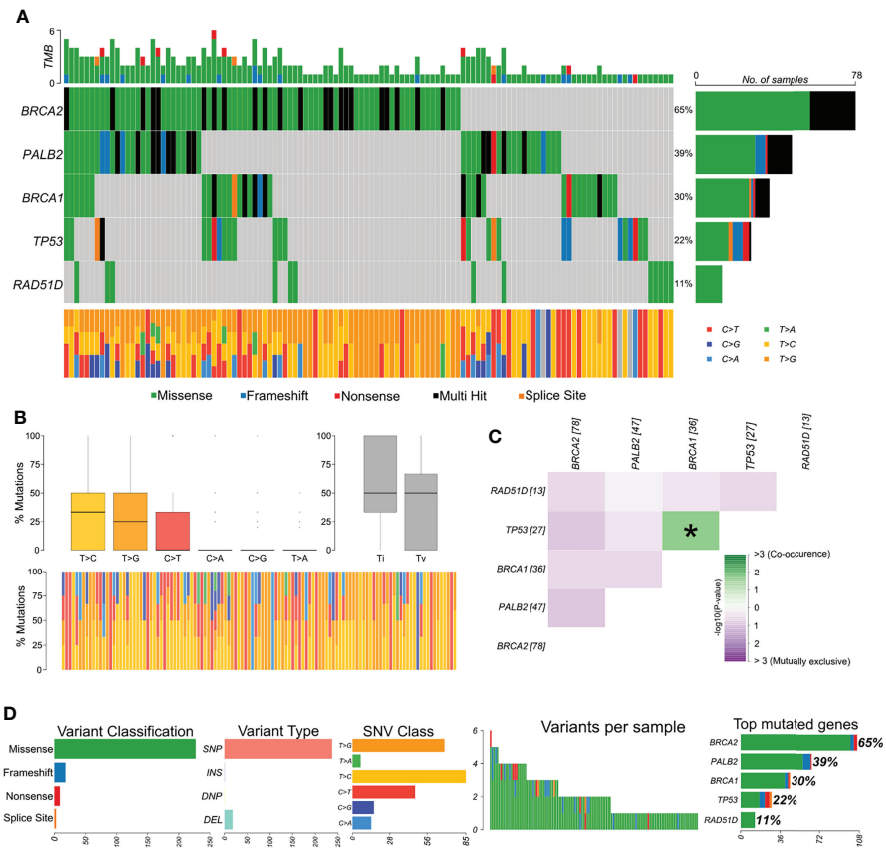

FIGURE 2 | Genomic landscape and potential associations between mutations identified in tumor breast cancer samples. **(A)** Oncoplot showing the mutational profile of each gene in all tumor samples ordered by variant frequency. **(B)** Distribution of transitions and transversions in all tumor samples showing boxplot with overall summary of single-nucleotide variants (SNV) classified into six substitution classes, boxplot with distribution of SNVs classified into transitions (Ti) and transversions (Tv), and proportion of SNVs per sample classified into six substitution classes. **(C)** Co-occurrence or exclusive variant associations of the evaluated genes. **(D)** Panels with variant classification, type (SNP, single-nucleotide polymorphism; INS, insertion; DNP, double nucleotide polymorphism; DEL, deletion), SNV class, number of variants per sample, variant classification summary, and genes ordered by total number of variants. Color legend of panel A is the same for panels **(B, D)**.

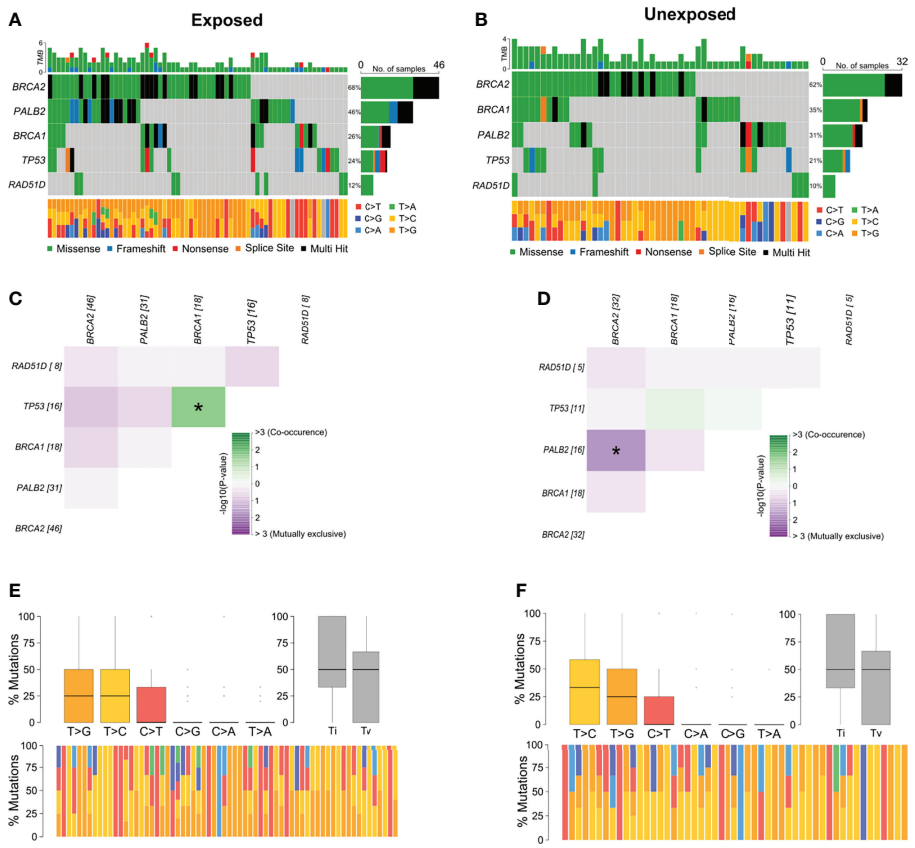

FIGURE 3 | Genomic landscape and potential associations between mutations in tumor breast cancer samples grouped according to pesticide occupational exposure. **(A, B)** Oncoplots showing the mutational profile of each gene in groups exposed and unexposed to pesticides. **(C, D)** Mutational co-occurrence or exclusive associations between evaluated genes in **(C)** exposed and **(D)** unexposed groups. **(E, F)** Distribution of transitions and transversions in **(E)** exposed and **(F)** unexposed groups showing boxplot with overall summary of SNVs classified into six substitution classes, boxplot with distribution of SNVs classified into transitions (Ti) and transversions (Tv), and proportion of SNVs per sample classified into six substitution classes. Color legend of panels **(A, B)** is the same for panels **(E, F)**, respectively. The symbol * means statistical significance ($p < 0.05$).

c.181T>G). Four *BRCA2* variants are pathogenic on ClinVar and six on COSMIC, without concordance among both databases. Eight *PALB2* variants were classified as pathogenic on ClinVar, 12 on COSMIC, and only one classified as pathogenic in both databases (*PALB2* c.1240C>T). We found two *RAD51D* classified as VUS on ClinVar, and one of them was predicted as pathogenic in COSMIC (*RAD51D* c.137C>G).

Pathogenic or likely pathogenic variants represented 18% of all variants detected (**Figure 4A**) and included missense, frameshift deletions, and nonsense variants, with a high prevalence in *TP53* and *PALB2* (**Figure 4B**). The proportion of pathogenic and/or likely pathogenic variants was higher in the tumor sample of exposed patients than in unexposed patients (**Figure 4C**). When analyzing variants predicted as pathogenic, likely pathogenic, or VUS, exposed patients also presented a significantly higher number than individuals unexposed to pesticides ($p = 0.017$, **Figure 4D**). We also found a significant difference in the frequency of variant types (missense, splice site, frameshift, and nonsense) in the group of patients exposed or not to pesticides ($p = 0.043$, **Figure 4E**).

The Correlation Between Tumor Mutational Burden and Breast Cancer Clinicopathological Parameters and Pesticide Exposure

We analyzed the mutational burden and its association with different clinicopathological variables and pesticide exposure. In general, samples that presented a pathogenic or likely pathogenic variant had a higher mutational burden (**Figure 5A**). We observed that breast cancer patients exposed to pesticides had no difference regarding mutational burden in the presence or absence of pathogenic variants in the tumor. However, in the unexposed group, tumors harboring any deleterious variant had a higher mutational burden than those with variants of no clinical and/or functional impact ($p < 0.02$, **Figure 5B**). We also found that only the exposed group of patients diagnosed with breast cancer before 50 years old ($p = 0.00978$, **Figure 5C**) and patients carrying tumors with *BRCA1* ($p = 0.0138$, **Figure 5D**), *BRCA2* ($p = 0.0366$, **Figure 5E**), and/or *PALB2* ($p = 0.00058$, **Figure 5F**) variants had a higher mutational burden; the same was not observed in the unexposed group (**Supplementary Figure 2**). We found mutational burden increased in tumors that

TABLE 2 | List of variants classified as pathogenic, likely pathogenic, and VUS.

Gene	cDNA change	Protein change	Type	Mutated samples	Previously reported	Pesticide exposure
BRCA1	c.181T>G	p.C61G	SNP	2	Yes	Exposed/unexposed
BRCA1	c.3329delA	p.K1110fs	DEL	1	Yes	Exposed
BRCA1	c.3790_3797delAAGAATAG	p.K1264fs	DEL	1	No	Exposed
BRCA1	c.1687C>T	p.Q563*	SNP	1	Yes	Exposed
BRCA1	c.3765_3786delCACAGAGGAGAATTTATTATCA	p.T1256fs	DEL	1	No	Exposed
BRCA1	c.5129G>A	p.G1710E	SNP	1	Yes	Exposed
BRCA1	c.546G>T	p.L182F	SNP	1	Yes	Unexposed
BRCA1	c.1996C>G	p.L666V	SNP	1	Yes	Unexposed
BRCA2	c.2806_2809delAAAC	p.A938fs	DEL	1	Yes	Unexposed
BRCA2	c.7879A>T	p.I2627F	SNP	1	Yes	Exposed
BRCA2	c.5067delA	p.K1691fs	DEL	1	Yes	Exposed
BRCA2	c.1314delT	p.T441fs	DEL	1	Yes	Exposed
BRCA2	c.5687C>T	p.A1896V	SNP	1	Yes	Exposed
BRCA2	c.5096A>G	p.D1699G	SNP	1	Yes	Unexposed
BRCA2	c.670G>A	p.D224N	SNP	1	Yes	Unexposed
BRCA2	c.3562A>G	p.I1188V	SNP	1	Yes	Exposed
PALB2	c.1314delA	p.F440fs	DEL	1	Yes	Exposed
PALB2	c.886delA	p.M296fs	DEL	6	Yes	Exposed
PALB2	c.1240C>T	p.R414*	DEL	1	Yes	Unexposed
PALB2	c.2453T>C	p.F818S	SNP	1	Yes	Unexposed
PALB2	c.2201C>A	p.T734N	SNP	1	Yes	Unexposed
PALB2	c.2608G>A	p.V870I	SNP	1	Yes	Exposed
TP53	c.481delG	p.A161fs	DEL	1	No	Exposed
TP53	c.592G>T	p.E198*	SNP	2	Yes	Exposed
TP53	c.856G>A	p.E286K	SNP	1	Yes	Exposed
TP53	c.730G>A	p.G244S	SNP	1	Yes	Exposed
TP53	c.734G>A	p.G245D	SNP	1	Yes	Unexposed
TP53	c.578A>C	p.H193P	SNP	1	Yes	Unexposed
TP53	c.617delT	p.L206fs	DEL	1	No	Unexposed
TP53	c.736A>G	p.M246V	SNP	1	Yes	Exposed
TP53	c.454_466delCCGCCCGGCACCC	p.P152fs	DEL	1	Yes	Exposed
TP53	c.586C>T	p.R196*	SNP	1	Yes	Exposed
TP53	c.626_627delGA	p.R209fs	DEL	1	Yes	Exposed
TP53	c.637C>T	p.R213*	SNP	1	Yes	Exposed
TP53	c.742C>T	p.R248W	SNP	1	Yes	Unexposed
TP53	c.818G>A	p.R273H	SNP	1	Yes	Exposed
TP53	c.447delC	p.T150fs	DEL	1	No	Unexposed
TP53	c.517G>A	p.V173M	SNP	1	Yes	Exposed
TP53	c.796G>A	p.G266R	SNP	1	Yes	Exposed
TP53	c.535C>A	p.H179N	SNP	1	Yes	Unexposed
TP53	c.536A>G	p.H179R	SNP	1	Yes	Unexposed
TP53	c.578A>G	p.H193R	SNP	2	Yes	Exposed/unexposed
TP53	c.711G>T	p.M237I	SNP	1	Yes	Unexposed
TP53	c.832C>T	p.P278S	SNP	1	Yes	Exposed
TP53	c.614A>G	p.Y205C	SNP	1	Yes	Exposed
TP53	c.613T>C	p.Y205H	SNP	1	Yes	Unexposed
TP53	c.96+2T>CTGGT	Splice site	INS	1	No	Unexposed
TP53	c.559+1G>C	Splice site	SNP	1	No	Exposed
RAD51D	c.56T>C	p.L19P	SNP	1	Yes	Unexposed
RAD51D	c.137C>G	p.S46C	SNP	2	Yes	Exposed/unexposed

cDNA and protein change, variant type, and number of tumors harboring each variant in patients exposed and unexposed to pesticides.
VUS, variant of uncertain significance; SNP, single-nucleotide polymorphism; INS, insertion; DEL, deletion.

harbor a *TP53* pathogenic or likely pathogenic variant only in unexposed patients (**Supplementary Figure 3**).

DISCUSSION

Breast cancer tumors and other neoplasias tend to present *TP53* as the most mutated gene (35, 36). However, we found *BRCA2* (~41.5%) with the highest mutational frequency in our study. We highlight that somatic variants of clinical relevance or interest, i.e., classified as pathogenic, likely pathogenic, or VUS, do not usually occur in *BRCA* genes, and we found a slightly higher frequency of *BRCA2* and *BRCA1* (3.1% and 3.5%) variants in comparison to other studies (37–39). Our study also showed the co-occurrence of *BRCA1* and *TP53* mutations in exposed patients, a condition already observed in germline *BRCA1* mutation carriers (40, 41) and found in colorectal cancer tumors, in which co-occurrence of *BRCA1* and *TP53* mutations resulted in poorer

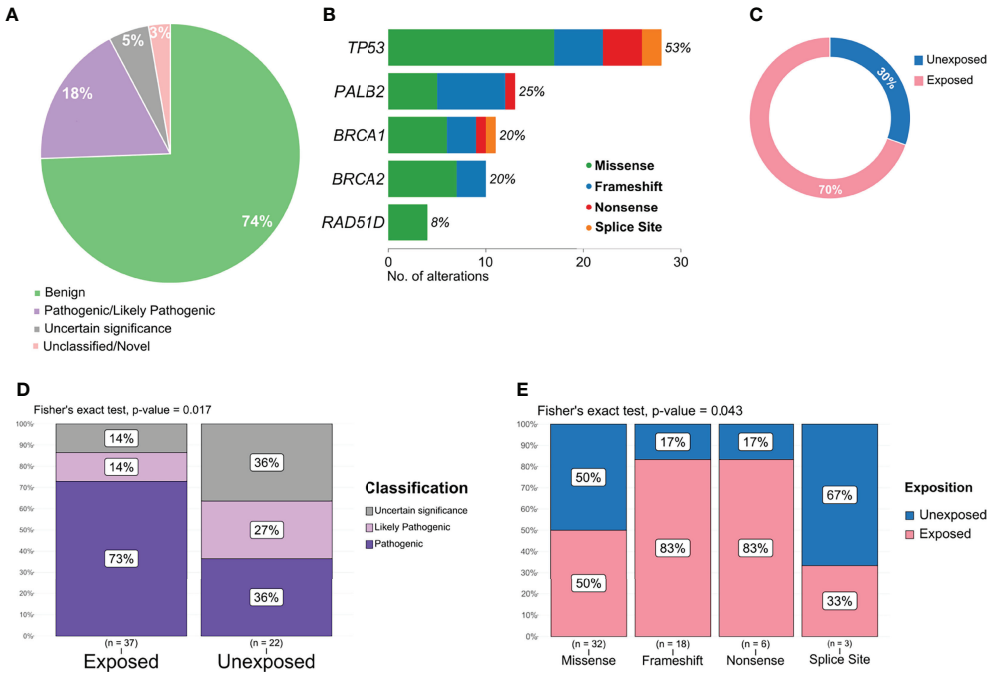

FIGURE 4 | Distribution and frequency of pathogenic, likely pathogenic, and variant of uncertain significance (VUS) variants. **(A)** Graphical representation of variant classification detected in all samples. **(B)** Frequency and type of pathogenic, likely pathogenic, and VUS variant identified in each gene. **(C)** Proportion of pathogenic and likely pathogenic variants detected according to patient exposure status. **(D)** Frequency of variants classified as pathogenic, likely pathogenic, or VUS in exposed and unexposed groups. **(E)** Frequency of variants classified as missense, frameshift, nonsense, and splice site according to exposed and unexposed samples.

prognosis (42). It must be noticed that there are discordances between variant annotations in ClinVar, a large public archive composed mainly of germline variants and the phenotype consequence (43), and COSMIC, the largest public resource of somatic mutations in human cancer (44).

Increased *BRCA1/2* variants are frequent in triple-negative tumors, a tumor type associated with the presence of germline *BRCA1* variants (45). We also detected *BRCA1/2* variants in HER2+, Luminal A, and Luminal B tumors, which indicates an underlying mechanism behind these mutations, but also a publication bias regarding germline variants of triple-negative tumors. It has been demonstrated that environmental exposure to certain toxins can induce haploinsufficiency, a mechanism proposed to contribute to breast cancer development, especially in *BRCA2* cells bearing heterozygous mutations (40, 46). This haploinsufficiency causes the reduction of *BRCA2* function, which sensitizes cells to DNA damage and compromises DNA repair, and under prolonged exposure to these toxins, it can even promote BRCA2 protein depletion in wild-type cells (40). We speculate that pesticide exposure could cause the same effect, as it tends to occur during a long lifetime, not only for tumors bearing deleterious mutations in DNA damage response genes but also in tumors with both functional alleles.

Moreover, there are several studies suggesting the endocrine-disrupting potential of certain pesticides, as reviewed elsewhere (41, 42). This capability enables these substances to mimic and/or antagonize the hormone function. Although current evidence is not fully conclusive of their mechanism of action, exposure to these compounds has been associated with an increased risk of breast cancer (47). We found that exposed patients present an increased frequency of hormone (ER/PR) negative tumors. This result could also be due to the increased presence of *BRCA1/2* mutations and may be indicative of higher genome instability and a decrease in DNA repair, which is in agreement with other studies that show a higher frequency of somatic mutations, mainly in *BRCA2*, in triple-negative tumors (45). Interestingly, it has also been shown that farmers under pesticide exposure that presented chromosomal abnormalities also had lower expression of *BRCA2* (48). This further reinforces that *BRCA2* is essential for DNA stability.

The T>G nucleotide transversion was the most frequent substitution in the exposed patients, whereas unexposed patients presented more T>C substitutions. An Egyptian study found a correlation between T>G and T>C substitutions regarding PIK3CA variants in breast cancer patients and suggested that the oxidative damage accumulation due to age could induce these specific substitutions (49). A high frequency of T>G was found in the presence of oxidized guanine nucleotides (8-oxo-dGTP), an established biomarker of oxidative stress (50). Another study observed that oxidation of the 5-methyl group of thymine generates 5-formyluracil (5-fU), which could induce T>G transversions, has a mutagenic potential, as it could pair wrongly with several bases (51). Another indication that T>G transversions could be associated with oxidative damage and

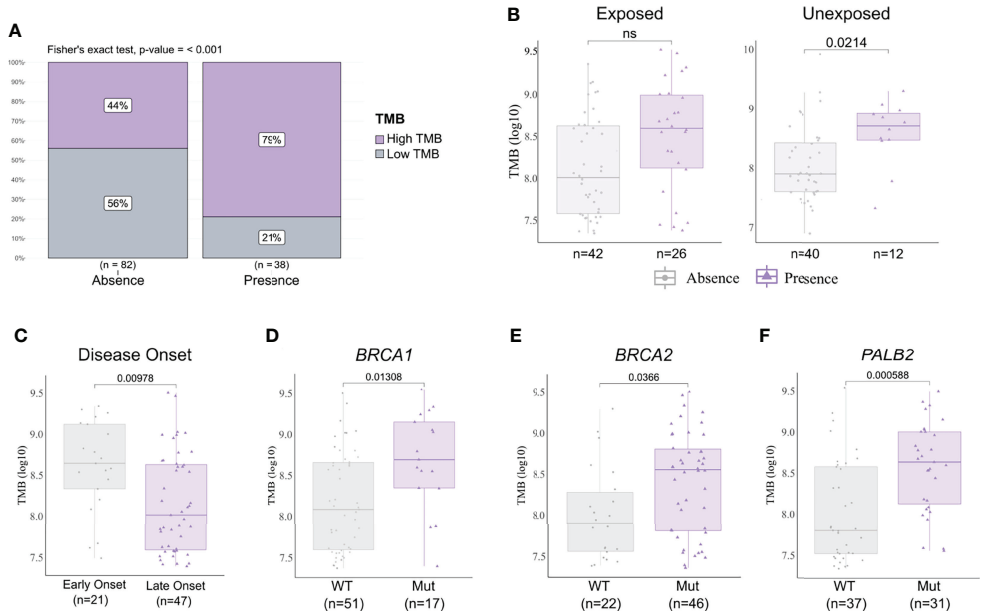

FIGURE 5 | Tumor mutational burden (TMB) and clinicopathological variables according to pesticide exposure. **(A)** Frequency of high and low mutational burden levels in tumor samples with a pathogenic and/or likely pathogenic variant detected or not. Tumors harboring any deleterious variant present increase in mutational burden ($p < 0.001$). **(B)** TMB levels in samples according to pesticide exposure status and grouped by presence or absence of any predicted pathogenic variant. Tumors from unexposed patients present increased TMB when harboring any deleterious variant ($p = 0.0214$); this result is not observed in tumors from exposed patients. **(C)** TMB levels in exposed patient samples grouped according to disease onset. We found early-onset tumors (patients with <50 years old) with significantly higher TMB ($p = 0.00978$) in comparison to late-onset tumors (patients with ≥50 years old). **(D–F)** TMB levels in exposed patient samples grouped according to *BRCA1*, *BRCA2*, and *PALB2* statuses, respectively. Tumors harboring a mutation in *BRCA1* ($p = 0.01308$), *BRCA2* ($p = 0.0366$), and/or *PALB2* ($p = 0.000588$) presented higher TMB than wild-type tumors. The expanded form of "NS" means "No significance".

exposure to exogenous substances is the high proportion found in whole-genome sequencing of arsenic exposed lung cancer patients (52). As pesticides increase oxidative stress biomarkers (53) and cause direct DNA damage (54), it is plausible to state that the higher frequency of T>G substitution might be related to the pesticide exposure in this group, as we observe a lower frequency in unexposed patients. As we did not perform whole-exome sequencing (WES), it is impossible to associate our results with any mutational signature for now. However, if we speculate that WES T>G substitutions are predominant in the tumors herein analyzed, it is not a signature associated with any mutational process yet (55, 56).

We found *TP53* variants in 17% of tumors, similar to other breast cancer studies, including one with a Brazilian cohort (36, 57). We identified higher *TP53* missense variants frequency (67.9%, 17/28) than frameshift/nonsense (32.1%, 9/28), with the latter mostly identified in pesticide-exposed patients (77.7%). Two novel splicing variants were identified in canonical splice sites, one in intron 3 (c.96+2T>CTGGT) from an unexposed patient and another in intron 5 (c.559+1G>C) from an exposed patient. Regarding the latter, a variant in this position, but with a different nucleotide change (G>A), was already predicted as pathogenic (58, 59). The missense *TP53* c.578A>G (p.H193R) variant found in two patients (one exposed and one unexposed) in our study was already documented in lung cancer tumors (60). Missense mutations in *TP53* usually produce a stable protein with a significant loss of activity, but frameshift and/or nonsense mutations cause loss of function (61). The nonsense variant c.592G>T (p.E198*) found in two exposed patients causes the premature truncation of the protein and is predicted to result in a loss of the protein function. In summary, all variants were clustered within exons 5–8, the evolutionary conserved DNA-binding region of *TP53* protein, which is considered a hotspot area by the IARC database (62). This indicates that patients under pesticide exposure are more prone to DNA damage in comparison to unexposed ones.

PALB2 presented the second-highest proportion of pathogenic or likely pathogenic variants in our study. *PALB2* has an important role in cancer development and progression as even heterozygous mutations appear to contribute to early events of oncogenesis (63). We identified two frameshift variants in *PALB2* in seven patients of the exposed group and one nonsense variant, c.1240C>T (p.R414*), in an unexposed patient, also reported in several individuals with HBOC syndrome. The nonsense variant c.1240C>T (p.R414*) and the frameshift c.1314delA (p.F440fs) found in the same sample are localized at the evolutionary conserved chromatin-associated motif (ChAM) domain, which is responsible for *PALB2* chromatin association and DNA repair function (64). It is postulated that variants with strong evidence for pathogenicity in *PALB2* are commonly located in the coiled-coil (CC) motif or the WD40 domain (65). The recurrent frameshift variant found in six patients

exposed to pesticides occurs in the *PALB2* c.886delA (p.M296fs) and is localized at the CC motif on the amino-terminal region that mediates *PALB2* interaction with *BRCA1* and *RAD51D*.

All *BRCA1* pathogenic variants were found in exposed patients, except for the c.181T>G (p.C61G), observed in two samples, one from an unexposed patient (**Table 2**). This variant is described as a founder mutation in the Polish population and has already been reported as both germline and somatic origin (66, 67), thus called a "shared variant" (68). This missense mutation occurs in the *BRCA1* RING domain, decreasing BRCA1 availability at DNA damage sites and hindering DNA repair. It has also been demonstrated that $BRCA1^{C61G}$ mammary tumors develop cisplatin (platinum therapy) resistance, a drug that induces oxidative damage in cells (69). As for *BRCA2* pathogenic variants, three of them were in exposed patients, and only one in an unexposed patient, c.2806_2809delAAAC (p.A938fs), which was already detected in breast, ovary, and lung cancer patients by another study (70).

The increased frequency of pathogenic alterations observed for *TP53*, *PALB2*, and *BRCA1/2* in patients under pesticide exposure shown by our results suggests that exposure may have a role in oncogenesis. Mutations in these genes hinder DNA repair, leaving cells more vulnerable to DNA damage and prone to therapy-induced lethality (71) and are also related to therapy resistance mechanisms (69, 72). Somatic mutations may change over time due to selective pressure derived from therapy and genetic instability (72), and the genotoxicity effect of pesticide exposure may also impact this mutational landscape, as several toxic substance exposures produce a characteristic mutational pattern that impaired DNA repair capacity (73–75).

Deleterious variants in DNA damage response genes, mainly in lung cancer, are associated with a higher mutational burden (76), as observed in breast carcinomas with DNA damage repair gene variants (77) and in our tumor samples with *BRCA1*, *BRCA2*, and *PALB2* variants. We found a high mutational burden in patients carrying a pathogenic, likely pathogenic, or VUS variant in both groups analyzed, with the highest frequency of truncating and likely deleterious variants in exposed in comparison to unexposed patients. We found a higher mutational burden in patients exposed to pesticides with early onset of breast cancer (<50 years old) in comparison to patients with late onset (≥50 years old); usually, this finding is expected in older patients (78) due to life accumulation. Evidence suggests that the variation of high mutational burden in cancer types could also be related to chronic mutagenic exposure, i.e., lung cancer patients exposed to tobacco (79). Indeed, individuals exposed to several pesticides have increased DNA damage, including DNA strand breaks, a consequence of the direct exposure and of the oxidative stress generated from it (80–83), which could be the mechanism behind our findings in the exposed group. Interestingly, tumors from unexposed patients harboring TP53 mutations presented significantly higher TMB than those with wild-type tumors; however, this result was not observed in the exposed group. Usually, TP53 mutations are found in tumors with a high mutational burden. For example, in lung cancer, high TMB and TP53 mutations are frequently observed in tumors with the SBS4 signature, which is associated with tobacco smoking (84). This indicates that TP53 mutations are related, at least to some extent, to carcinogen exposure. Still, it has been shown, in a population chronically exposed to pesticides, that there is accumulation of DNA lesions due to low DNA repair activity even at low doses of exposure (85). We found mutations in BRCA1/2 and PALB2 genes to be significantly associated with TMB in exposed patients. Therefore, we presume that these tumors present increased mutational burden due to DNA repair deficiency and thus accumulation of DNA damage without even needing to have impairment of TP53 function.

Regarding an epidemiological overview, a study analyzed the genotoxicity of pesticides approved in the United Kingdom in workers exposed during manufacturing, formulation, or use. The authors reported that, although possible confounding variables were not considered—such as age—and the difficulty to infer causality, there is evidence of an increase in genotoxic biomarkers in pesticide-exposed workers (84). A very recent meta-analysis highlights a significant impact of DNA damage for the pesticide-exposed farmers, regardless of gender, age, pesticide type, or use of personal protective equipment (85). It is important to state that 1/3 of the products recently registered in Brazil contain active substances not approved, or even banned, by the European Commission and that the maximum residue level (i.e., pesticide concentration) considered acceptable in Brazil is higher than that allowed in the United States, Canada, European Union, and other BRICS countries (86). Several Brazilian reports, including one in the same region of our study (87), have described high DNA damage in pesticide-exposed patients. Their results were assessed mainly by cytogenetic analysis and indicate that these individuals are more prone to genetic damage and increased mutation rate (80, 88–90). Moreover, in a population from another Brazilian southern state, global DNA methylation was found to increase in exposed patients in comparison to the unexposed group, a result associated with the inactivation of DNA damage repair genes (91).

CONCLUSION

This is the first time a study has shown that occupational exposure to pesticides increases the mutational burden and the mutational status of DNA damage response genes in human breast cancer. Our results reinforce the literature that pesticide exposure causes direct DNA damage. We observed increased mutational burden and deleterious variants in the exposed group, which could be associated primarily with oncogenesis, therapy response, and disease progression. In future studies, an increased observation period should be done in these exposed patients to gather information regarding disease progression and therapeutic response, as our group has not completed a 10-year follow-up yet.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committees of State University of West Paraná. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CRB, CP, and TS conceived the overall study and supervised the research. DR, TS, FMA, and HJ contributed to sample obtention and clinical data collection. RG designed all multiplex PCR assays. CF and TS designed all next-generation sequencing (NGS) experiments. TS and SV performed the experiments. CE, NS, and EA conducted all NGS data processing and somatic variant annotation. MB supervised all the bioinformatics analyses. CE and TS built the figures and performed the statistical analysis, with additional input from all authors. TS, SF, CP, and CRB contributed to the literature search, data analysis, and data interpretation of the results. TS and CRB took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.904813/full#supplementary-material

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