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High-risk human papilloma virus infection, tumor pathophenotypes, and *BRCA1/2* and *TP53* status in juvenile breast cancer

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Abstract Juvenile breast cancer is rare and poorly known. We studied a series of five breast cancer patients diagnosed within 25 years of age that included two adolescents, 12- and 15-years-old, and 3 young women, 21-, 21-, and 25-years-old, respectively. All cases were scanned for germline mutations along the entire *BRCA1/2* coding sequences and *TP53* exons 4–10, using protein truncation

test, denaturing high performance liquid chromatography and direct sequencing. Paraffin-embedded primary tumors (available for 4/5 cases), and a distant metastasis (from the 15-years-old) were characterized for histological and molecular tumor subtype, human papilloma virus (HPV) types 16/18 *E6* sequences and tumor-associated mutations in *TP53* exons 5–8. A *BRCA2* germline mutation (p.Ile2490Thr), previously reported in breast cancer and, as compound heterozygote, in Fanconi anemia, was identified in the 21-year-old patient diagnosed after pregnancy, negative for cancer family history. The tumor was not available for study. Only germline polymorphisms in *BRCA1/2* and/or *TP53* were detected in the other cases. The tumors of the 15- and 12-years-old were, respectively, classified as glycogen-rich carcinoma with triple negative subtype and as secretory carcinoma with basal subtype. The tumors of the 25-year-old and of the other 21-year-old were, respectively, diagnosed as infiltrating ductal carcinoma with luminal A subtype and as lobular carcinoma with luminal B subtype. No somatic *TP53* mutations were found, but tumor-associated HPV 16 *E6* sequences were retrieved from the 12- and 25-year-old, while both HPV 16 and HPV 18 *E6* sequences were found in the tumor of the

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Gitana Maria Aceto, Angela Rosaria Solano equally contributed to the work. GMA, ARS, RMC, and EJP contributed to study conception and design, and acquisition, analysis, and interpretation of data; and drafted and revised the manuscript critically for important intellectual content. MIN contributed to sample preparation and genetic analyses, and participated in data analysis. SV carried out genetic analyses, data analyses, coordinated data collection and drafted the manuscript. AMo contributed to the somatic genetic analysis and data collection. SM performed the pathological examination of the samples. RDCh critically reviewed the manuscript and participated clinically in the diagnosis and follow up of patients and sample provision. CP participated clinically in the diagnosis and follow up of patients and sample provision. ML participated clinically in the diagnosis and follow up of patients and sample provision. PB contributed to genetic data analyses and manuscript preparation. AMA designed and supervised pathological analyses data and contributed to drafting the manuscript. All authors read and approved the final manuscript.

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15-year-old and in its associated metastasis. Blood from the 15- and 25-year-old, diagnosed with high-stage disease, resulted positive for HPV 16 *E6*. All the HPV-positive cases were homozygous for arginine at *TP53* codon 72, a genotype associated with HPV-related cancer risk, and the tumors showed p16(INK4A) immunostaining, a marker of HPV-associated cancers. Notably menarche at 11 years was reported for the two adolescents, while the 25-year-old was diagnosed after pregnancy and breast-feeding. Our data suggest that high-risk HPV infection is involved in a subset of histopathologically heterogeneous juvenile breast carcinomas associated with menarche or pregnancy and breast-feeding. Furthermore we implicate *BRCA2* in a juvenile breast carcinoma diagnosed at 21 years of age, 4 years after an early full-term pregnancy, in absence of cancer family history.

Keywords Juvenile breast cancer · *BRCA1* · *BRCA2* · *TP53* · Mutation · Human papilloma virus · Reproductive factors

HPV in juvenile breast cancer

BC	Breast cancer
jBC	Juvenile breast cancer
HPV	Human papilloma virus
HRDC	Laboratorio de Hormonas en la Regulacion y Diferenciacion Celular
PAS	Periodic acid-Schiff
ER	Estrogen receptor
PgR	Progesterone receptor
CK5	Cytokeratin 5
SMA	Smooth muscle actin
PTT	Protein truncation test
DHPLC	Denaturing high performance liquid chromatography
HGVS	Human Genome Variation Society
PCR	Polymerase chain reaction
BIC	Breast Cancer Information Core
pRb	Retinoblastoma protein
p16(INK4A)	Cyclin-dependent kinase-4 inhibitor
IARC	International Agency for Research on Cancer

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Introduction

Breast cancer (BC), worldwide the commonest major malignancy in women, is a sex hormone-related disease influenced by multi-factorial gene-environment interactions, most notably involving reproductive factors [1–4]. BC incidence increases with age, peaking in the post-menopausal period [5, 6], while the vast majority of the proliferative breast lesions that manifest in adolescence and young adulthood, i.e., between 12 and 24 years of age [7], are benign [8]. Juvenile BC (jBC), i.e., BC diagnosed within 25 years of age [7], is rare (estimated incidence $\leq 10/100,000$), Globocan (<http://www-dep.iarc.fr/globocan/database.htm>), but tends to be more advanced at presentation and more aggressive than BC in older women [9]. BC manifesting in children and adolescents within 14 years of age is even more rare ($\leq 1/100,000$), but its incidence seems to vary geographically with a pattern contrasting with that of BC in women Globocan (<http://www-dep.iarc.fr/globocan/database.htm>) [10]. This suggests that environmental factors, distinct from those involved in BC later in life, could be implicated in very early onset jBC.

At puberty the striking proliferative expansion of the terminal ductal and lobular epithelia of the breast, induced by sex hormones, could promote tumorigenesis from cells initiated by highly penetrant genetic and/or environmental factors [11–15]. Indeed genetic factors, most notably deleterious germline mutations in *BRCA1* (MIM no. 113705), *BRCA2* (MIM no. 600185), and *TP53* (MIM no. 191170), the major genes responsible for autosomal-dominant hereditary BC, are clearly implicated in jBC [16–20], IARC Database (<http://www.iarc.fr/en/websites/index.php>), but there is no reported evidence of germline *BRCA1/2* or *TP53* mutations in BC-affected children and adolescents [20], IARC Database (<http://www.iarc.fr/en/websites/index.php>). Due to disease rarity, the extent and nature of the biological and molecular heterogeneity of jBC and the identity of the risk factors that could be implicated in different jBC subsets remains largely unknown.

Throughout the years several studies provided evidences in favor of an involvement of oncogenic viruses in human BC. In particular the high-risk human papilloma viruses (HPVs) types 16 and 18 (HPV 16/18), worldwide the major risk factors for cancer of the uterine cervix, are capable of immortalizing human mammary epithelial cells IARC Database (<http://www.iarc.fr/en/websites/index.php>), [21–23] and were found in relevant fractions of unselected BCs (range: 25–29%) [24, 25]. Furthermore, in women affected with both mammary and cervical cancers, HPV 16 was retrieved from both cancer types [26]. These data suggest that HPV infection of mammary epithelia could be implicated in BC. At puberty, increased estrogen and

progesterone levels are predicted to induce proliferation of hormone-responsive epithelia and to down-modulate immune responses. This could favor HPV persistence and replication in clonally expanding and differentiating hormone-dependent epithelia, facilitating HPV-induced carcinogenesis [27, 28].

To investigate the role of genetic and environmental factors in jBC, we studied a small but highly selected group of five Argentinean cases diagnosed with BC within 25 years of age. This jBC series included two adolescents, with BC diagnosed after menarche, and three young women, two with BC manifesting after full-term pregnancy. These cases were characterized for histological and molecular tumor subtype and investigated for germline *BRCA1/2* and *TP53* mutations, for somatic *TP53* mutations and for molecular evidence of HPV16/18 infection. Our results suggest that HPV 16/18 are associated with jBC arising after physiological hormonal stimulation, such as menarche and pregnancy. Moreover we document an association between a germline *BRCA2* mutation and an HPV-negative 21-year-old jBC case with no reported cancer family history, diagnosed 4 years after an early full-term pregnancy.

Patients and methods

Patients

Argentina has one of the highest age-standardized BC incidence rates in the world [29], but jBC remains very rare also in this country [1], Globocan (<http://www-dep.iarc.fr/globocan/database.htm>). To identify jBC cases, we revised a selected series of 88 Argentinean BC patients referred to the HRDC for *BRCA1/2* genetic diagnosis as a public health service between 2000 and 2008. We identified five female patients, all of Caucasian ethnicity, with BC manifesting within 25 years of age. These included [code-age in years (y) at BC diagnosis]: A9-15y, A11-12y, A17-25y, A18-21y, and A31-21y.

Relevant individual and clinical characteristics available for these patients are given below and summarized in Table 1.

A9-15y, from the province of Salta, Northern Argentina, was referred to HRDC in 2003 from *Hospital de Niños “Dr. Ricardo Gutierrez”*. This young girl had been diagnosed in 2002, at 15 years of age, with stage IV BC associated with mastitis. At diagnosis total body computerized tomography scan documented a $9 \times 8 \times 5$ cm pelvic mass and multi-focal bone metastases. Oophorectomy confirmed left ovarian metastasis. Despite chemotherapy, the patient died with metastatic disease in 2005. A9-15y was born by normal delivery and underwent menarche at 11 years of age. Her reported cancer family history included ovarian cancer (mother, deceased at 30 years of age), and colorectal cancer (father, age unreported). Clinical records, pathology reports, and paraffin blocks documenting the tumors in parents could not be retrieved.

A11-12y, from the province of Buenos Aires, was referred to HRDC in 2004 from *Hospital de Niños “Dr. Ricardo Gutierrez”*. She had been diagnosed in 2004, at 12 years of age, with stage II BC metastatic to 4/13 axillary lymph nodes (primary tumor size: 4.5×4 cm). The patient was treated with chemotherapy and was disease-free at the end of 2008. A11-12y was born by normal delivery and reported menarche at 11 years of age. Her reported cancer family history included only a maternal aunt diagnosed with ovarian cancer at 35 years of age.

A17-25y, from the province of Buenos Aires, was referred to HRDC in 2004 from *Instituto Alexander Fleming*. She had been diagnosed in 1999, at 25 years of age, with stage I BC (tumor size: 2.3 cm), 2 years after delivery of her only child and soon after finishing breastfeeding. The patient was treated with local radiotherapy, chemotherapy, and tamoxifen and remained clinically disease-free until October 2003, when hepatic and skeletal metastases manifested. A17-25y reported use of oral contraceptives from 17 to 20 years of age and no family history of cancer.

Table 1 Clinicopathological and immunohistochemical features of five juvenile breast cancer cases

Cases	Cancer family history ^a	Histotype	Stage	Grade	PAS	Alcian blue	SMA	P16 (INKA4)	TP53	CK5	S100	ER	PR	Her2/neu
A9-15y ^b	Yes	GRC	IV	G2	+	+	–	+	–	–	ND	–	–	–
A11-12y ^c	Yes	SC	II	G1	+	+	–	+	–	+	+	–	–	–
A17-25y	No	IDC	IV	G2	ND	ND	ND	+	–	–	ND	+	+	+
A18-21y	No	ILC	I	G2	ND	ND	ND	–	–	–	ND	+	+	–
A31-21y	No	IDC	II	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

^a As reported by the patient; ^b mother: ovary, father: colon; ^c maternal aunt: ovary

ND Not done, NA not available, GRC glycogen-rich breast carcinoma, SC secretory carcinoma, IDC infiltrating ductal carcinoma, ILC infiltrating lobular carcinoma

A18-21y, from the province of Buenos Aires, was referred to HRDC in 2004 from *Instituto Alexander Fleming*. She had been diagnosed in 2002, at 21 years of age, with stage I BC (tumor size: 2.5 cm). The patient was treated with local radiotherapy and chemotherapy but is currently affected with stage IV disease. A18-21y was nulliparous and reported no cancer family history.

A31-21y, from the province of Buenos Aires, was referred to HRDC in 2004 from *Hospital de Clínicas José de San Martín*, University of Buenos Aires. She had been diagnosed at 21 years of age with stage II BC. A31-21y reported no family history of cancer. She delivered her only child after full term pregnancy at age 17 years. No information is available regarding breast-feeding.

Fifty blood donors from Buenos Aires were used as controls. Cases and controls were anonymized. Informed written consent to genetic studies was obtained from the patients themselves or from direct relatives acting as legal representatives. All study procedures were approved by the Institutional Ethical Committees of *G. d'Annunzio* University and of the Argentinean Society of Clinical Investigation.

Histopathology and immunohistochemistry

Archival formalin-fixed paraffin-embedded tumor blocks were retrieved from the pathology files of *Hospital de Clínicas Gral José de San Martín*, *Hospital de Niños "Dr. Ricardo Gutiérrez"*, *Instituto Alexander Fleming*. BCs were classified and staged according to World Health Organization and American Joint Committee on Cancer [30, 31]. Histological grading was performed using the Nottingham modification of the Bloom-Richardson system [32].

PAS-diastrase and alcian blue special stains were performed for diagnostic purposes. Consecutive sections were immunostained with antibodies to estrogen receptor (ER), diluted 1/200; progesterone receptor (PgR), diluted 1/200; cytokeratin 5 (CK5), diluted 1/100; TP53, diluted 1/250; smooth muscle actin (SMA), diluted 1/50 (all from Novocastra Laboratories Ltd., Newcastle upon Tyne, UK); S100, diluted 1/1000, and Her2/neu HerceptTest™ (Dako, Glostrup, DK); P16INK4A, diluted 1:200 (MTM Laboratories, Heidelberg, Germany). Heat-induced epitope retrieval was performed using water bath (Tris/EDTA buffer) and microwave (citrate buffer). Slides were immunostained using Dako Autostainer Plus (Dako, Glostrup, DK). Detection was accomplished with mouse non-avidin-biotin EnVision + polymer (Dako). Immunostained slides were evaluated by two of the authors (A.M., S.M). Results were recorded as percentages of immunoreactive cells. Only nuclear reactivity was taken into account for ER, PgR, P16INK4A, and TP53. For CK5, S100, and SMA cytoplasmic staining was considered. The

immunohistochemical staining was classified as negative (immunoreactivity in 0–10% of the neoplastic cells), or positive (immunoreactivity in >10% of the neoplastic cells). Only moderate to intense and complete membrane staining in >10% of the tumor cells was taken as evidence of Her2/neu overexpression (2+ and 3+ scores). Archival positive cases for each marker were chosen from the Pathological Anatomy files of the *SS. Annunziata* Hospital in Chieti and used as positive external controls, whereas normal ductal or lobular epithelium in the tumor blocks served as internal positive (ER, PR, CK5, SMA) or negative (TP53, Her2/neu, P16INK4A, S100) controls.

Germline mutation analysis

Genomic DNA was isolated from blood using either the salting-out method (case A9-15y) or the QIAamp DNA Blood Mini Kit (QIAGEN Inc, Chatsworth, CA). Protein truncation test (PTT), denaturing high performance liquid chromatography (DHPLC), and direct sequencing were used for mutation detection. *BRCA1* exon 11 and *BRCA2* exons 10–11 were screened by PTT as described [33]. The entire *BRCA1* and *BRCA2* coding sequences, including intron–exon boundaries, as well as *TP53* exons 4–10 [34] were analyzed by DHPLC using the Wave® Nucleic Acid Fragment Analysis System (Transgenomic Inc., San Jose, CA). Primers for exons 2–24 of *BRCA1*, 2–27 of *BRCA2*, and 4–10 of *TP53* were as reported in the literature [35–37]. DHPLC conditions were adapted based on manufacturer's software and literature [35, 36]. Samples that showed PTT shifts or altered DHPLC profiles were directly sequenced using an ABI PRISM™310 genetic analyzer (Applied Biosystems, Foster City, CA). Variants nomenclature follows the guidelines of the Human Genome Variation Society (HGVS). Variants were confirmed on replicate blood aliquots. Virtual analyses of functional compatibility for aminoacid changes were performed using SIFT version 2 (<http://blocks.fhcr.org/sift/SIFT.html>) [38]. Predictions of sequence changes on splicing were obtained using the ESEFinder software [39].

Analysis of somatic *TP53* mutations and HPV 16/18 sequences

Formalin-fixed, paraffin-embedded BC blocks were retrievable for 4/5 cases. For case A9-15y, blocks from both primary and metastatic BC were retrieved. Tumor areas were identified on deparaffinized 10- μ m-thick sections lightly counterstained with hematoxylin, microdissected for DNA extraction as previously reported [40], and analyzed for mutations in exons 5–8 of *TP53* [41]. Amplification of a 147 bp β -actin gene fragment served as a control for DNA amplificability.

The presence of HPV 16/18 *E6* sequences (gene bank accession numbers K02718 and AY262282, respectively) [42] was evaluated by nested polymerase chain reaction (PCR) using two sets of highly specific primers designed on BLAST database alignments (<http://www.ncbi.nlm.nih.gov/BLAST>) [42] and confirmed by sequencing. Primers and PCR conditions are reported in Table S1, provided as supplementary online material. The same primer sets were used to search for HPV 16/18 *E6* sequences in the peripheral blood of cases and controls. Diluted DNAs extracted from HPV 16-positive cervical ThinPreps (PAP TEST Preserv Cyt Solution) were used as positive controls for HPV 16. DNA (1 µg) from HeLa cells, which contain 10–50 integrated HPV 18 copies, was diluted 1:100 and used as positive control for HPV 18. To avoid contaminations, positive control amplifications and analyses of all amplified products were strictly performed in a laboratory located in a building distinct from that used for the manipulation of test samples. All the primer sets used for the analysis of somatic *TP53* mutations and HPV 16/18

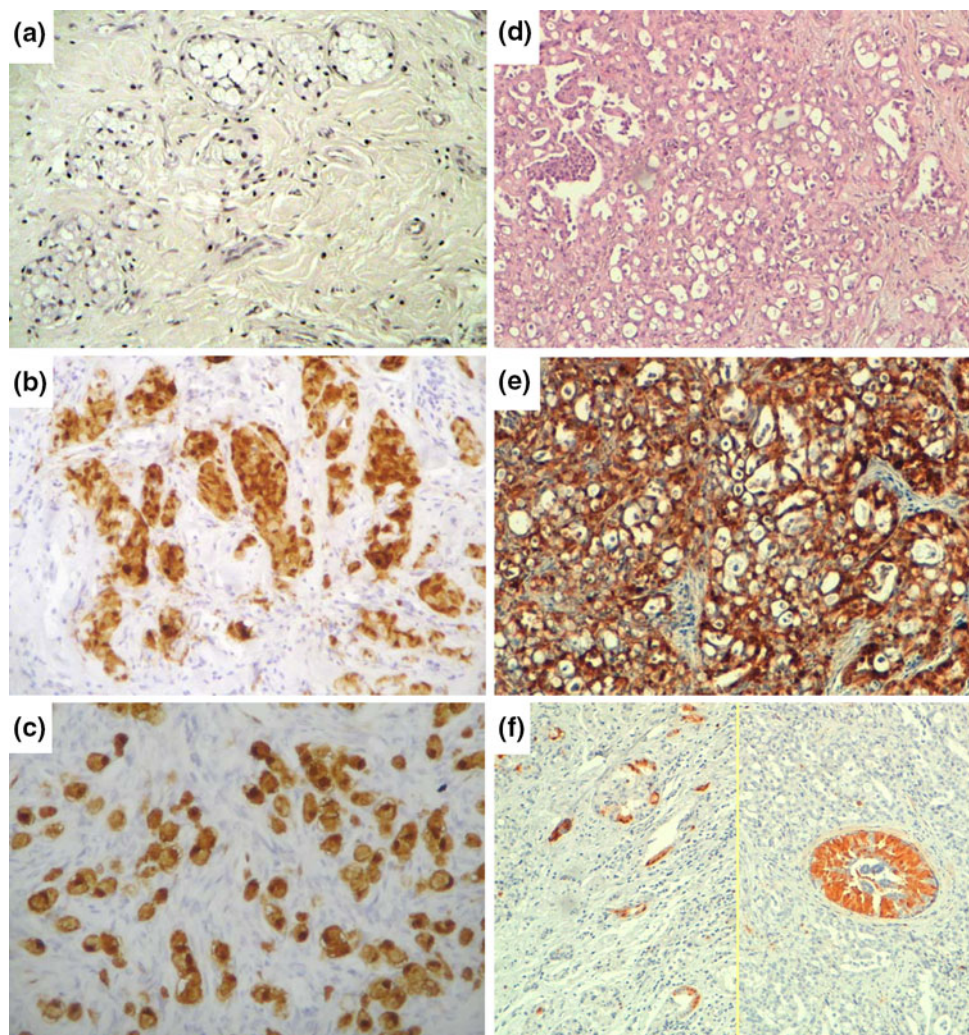
sequences were specifically designed to amplify targets within 200 bp, due to the low amplicon sizes obtainable from the DNA extracted from the available paraffin-embedded sections.

Results

Table 1 summarizes the individual, clinical, and pathological features of the five jBC cases.

The four cases for which paraffin-embedded tumor blocks could be retrieved were reviewed pathologically and characterized for molecular subtype by immunohistochemistry. For case A9-15y the tumor consisted of nests of neoplastic cells, sometimes with signet-ring appearance, which showed rather pleomorphic nucleus and clear cytoplasm (Fig. 1a), reacting positive for PAS and, focally, for alcian blue. The tumor was classified as glycogen-rich breast carcinoma. The same histotype was observed in the ovarian metastasis. Immunohistochemically both primary

Fig. 1 Histological and immunohistochemical characteristics of the breast tumors from the two adolescent patients. The primary tumor of case A9-15y, classified as glycogen-rich breast carcinoma with triple negative subtype on the basis of morphology, special stains, and immunohistochemistry, consisted of nests of roundish or polygonal neoplastic cells with large clear cytoplasm, sometimes showing a signet ring-like morphology (Panels a, b). Strong and diffuse p16(INK4A) immunoreactivity was observed in both the primary tumor (Panel b) and the associated ovarian metastasis, which showed the same histotype (Panel c). The tumor of A11-12y, classified as secretory breast carcinoma with basal subtype on the basis of morphology, special stains, and immunohistochemistry, showed a predominantly invasive component with papillary, cribriform, and microcystic patterns (Panel d), and was strongly and diffusely positive for S100 (Panel e) and CK5 (not shown). The intraductal component and focal invasive areas strongly immunoreacted for p16(INK4A) (Panel f)



and metastatic tumor cells resulted strongly positive for p16(INK4A) (Fig. 1b, c), but negative for CK5, S100, ER, PR, Her2/neu, TP53, and SMA. Based on these results, the molecular subtype of the A9-15y tumor was classified as triple negative and p16(INK4A)-positive [43]. The tumor of the other adolescent patient (A11-12y) was composed of a predominant invasive component with papillary, cribriform, and microcystic patterns (Fig. 1d). Glands and microcystic spaces contained abundant pink secretion with vacuolated appearance, positive with PAS and alcian blue. The tumor was classified as secretory breast carcinoma. The immunohistochemical staining showed strong and diffuse reactions for CK5 and S100 (Fig. 1e). A clear immunoreaction for p16(INK4A) was seen in the intra-ductal component and in focal invasive areas with cribriform pattern (Fig. 1f). The tumor resulted negative for ER, PR, Her2/neu, TP53, and SMA. Accordingly, the molecular subtype of the A11-12y tumor was classified as basal and p16(INK4A)-positive [43]. The other 2 cases, A17-25y and A18-21y, occurred in young women aged 25 and 21 years at diagnosis. The tumor of case A17-25y was classified as ductal infiltrating breast carcinoma and was positive for p16, ER, PR, and Her2/neu(2+). Accordingly, this tumor was classified as luminal B, p16(INK4A)-positive, with regard to molecular subtype [43].

The tumor of case A18-21y was classified as lobular carcinoma and, being positive for ER, PR, and Her2/neu negative, was classified as luminal A. Case A31-21y, for which paraffin embedded blocks were not available, was reported as an infiltrating ductal carcinoma in the medical records.

Germline mutational analysis identified nine synonymous or intronic *BRCA1* variants in the five tested cases and a total

of six *BRCA2* variants in 4/5 cases (Table 2). All detected *BRCA1* variants are listed in the Breast Cancer Information Core (BIC) database (<http://research.nhgri.nih.gov/bic/>) as polymorphisms. Importantly, one of the *BRCA2* variants, found in A31-21y, notably with no reported cancer family history, was a missense mutation listed in BIC as unclassified (p.Ile2490Thr). This mutation was not detected in 100 chromosomes from 50 Argentinean controls. Virtual analysis of functional compatibility by SIFT version 2 (<http://blocks.fhcrc.org/sift/SIFT.html>) predicts that the amino acid change affects protein function. Furthermore, the ESEFinder software [39] predicts that the sequence change affects splicing by increasing affinity for the SF2/ASF and SRp40 factors, required for efficient splice site usage. Lack of fresh blood for RNA analysis did not allow verification of these ESEFinder predictions. The other *BRCA2* variants (Table 2) were of no pathological relevance. Search for large rearrangements within *BRCA1/2* could not be performed due to lack of sufficient high-quality genomic DNA and unavailability of additional blood samples, particularly for case A9-15y, who died shortly after blood sampling. However A9-15y was heterozygous for one *BRCA1* polymorphism; A11-12y for four polymorphisms in *BRCA1* and three in *BRCA2*; A18-21y for one polymorphism in *BRCA1* and two in *BRCA2* (Table 2).

No deleterious germline mutations were found in *TP53* exons 4–10, which are known to harbor germline mutations responsible for the Li-Fraumeni and Li-Fraumeni-like syndromes, rare genetic disorders that greatly increase the risk of developing various cancers, most notably BC, at a young age [20]. However, A31-21y (which was positive for a probably pathogenetic *BRCA2* missense mutation) carried a new *TP53* intronic variant (IVS10 + 30 A > T), not

Table 2 Germline *BRCA1* and *BRCA2* variants identified in five juvenile breast cancer cases

Cases	BRCA1				BRCA2			
	Mutation	Effect	Type	Status	Mutation	Effect	Type	Status
A9-y15	c.1067A > G	p.Gln356Arg	M	P	–	–	–	–
A11-y12	c.442-34C	–	IVS7	P	c.426-89T > C	–	IVS4	P
	c.4308C > T	p.Ser1436Ser	Syn	P	c.426 + 67A > C	–	IVS4	Novel
	c.548-58delT	–	IVS8	P	c.7435 + 53C > T	–	IVS14	P
	c.5152 + 66G > A	–	IVS18	P	–	–	–	–
A17-y25	c.4308C > T	p.Ser1436Ser	Syn	P	–	–	–	–
	c.548-58delT	–	IVS8	P	–	–	–	–
	c.5152 + 66G > A	–	IVS18	P	–	–	–	–
A18-y21	c.442-34T > C	–	IVS7	P	c.681 + 56C > T	–	IVS8	P
	–	–	–	–	c.7242A > G	p.Ser2414Ser	Syn	P
A31-y21	–	–	–	–	c.7469T > C	p.Ile2490Thr	M	UV

P Polymorphism, Syn synonymous, IVS intron variant sequence, M missense, UV unverified variant

predicted by ESEfider to affect splicing. This *TP53* variant was not detected in 50 Argentinean controls. Furthermore 4/5 cases, including the two adolescents, resulted Arg/Arg homozygous at codon 72 in the proline-rich domain of TP53, a polymorphism that could influence BC risk [44]. The remaining case (A18-21y) was Arg/Pro heterozygous. Analysis of tumor DNA from the four cases with available BC tissue blocks did not reveal tumor-associated *TP53* mutations in exons 5–8, which encode the TP53-DNA-binding domain, where most of such mutations occur [34].

Most interestingly, the HPV 16-specific *E6* sequence was amplified from the primary BC of 3/5 cases, including A9-15y, A11-12y, and A17-25y, the latter with BC diagnosed after pregnancy and lactation. Moreover HPV 18-specific *E6* was also amplified from the A9-15y primary tumor. Notably, the A9-15y ovarian metastasis resulted positive for HPV 16/18 *E6*. Furthermore the relevant HPV *E6* sequences were retrieved from peripheral blood of the two cases with stage IV disease, i.e., A9-15y and A17-25y (Fig. 2). Blood samples from A11-12y, with HPV-positive stage II BC, and from A18-21y, with HPV-negative stage I

BC, did not yield HPV *E6* sequences. HPV 16/18 *E6* sequences were not retrieved from 50 control blood samples.

The high-risk HPV oncoproteins E6 and E7 promote carcinogenesis by interfering with two key tumor suppressor proteins [22]. While E6 binds TP53, promoting its degradation, E7 sequesters pRb, leading to compensatory overexpression of the cyclin-dependent kinase-4 inhibitor p16(INK4A). Thus, p16(INK4A) is strongly expressed in HPV-associated cancers of the uterine cervix [45–47]. In the three HPV-positive cases, the retrieval of *E6* sequences by PCR was fully consistent with the immunohistochemical evidence of p16(INK4A) expression (Fig. 1). Notably in case A9-15y both the primary BC and its ovarian metastasis were *E6*-positive and showed strong p16(INK4A) immunostaining (Fig. 1b, c), while no p16(INK4A) staining was observed in A18-21y, negative for HPV *E6*. The lack of RNA for the search of integrant-derived transcripts, the low amplicon sizes obtainable from the available paraffin-embedded sections, and the weak *E6* signals from peripheral blood impeded the analysis of HPV integration status by PCR-based methods [48, 49].

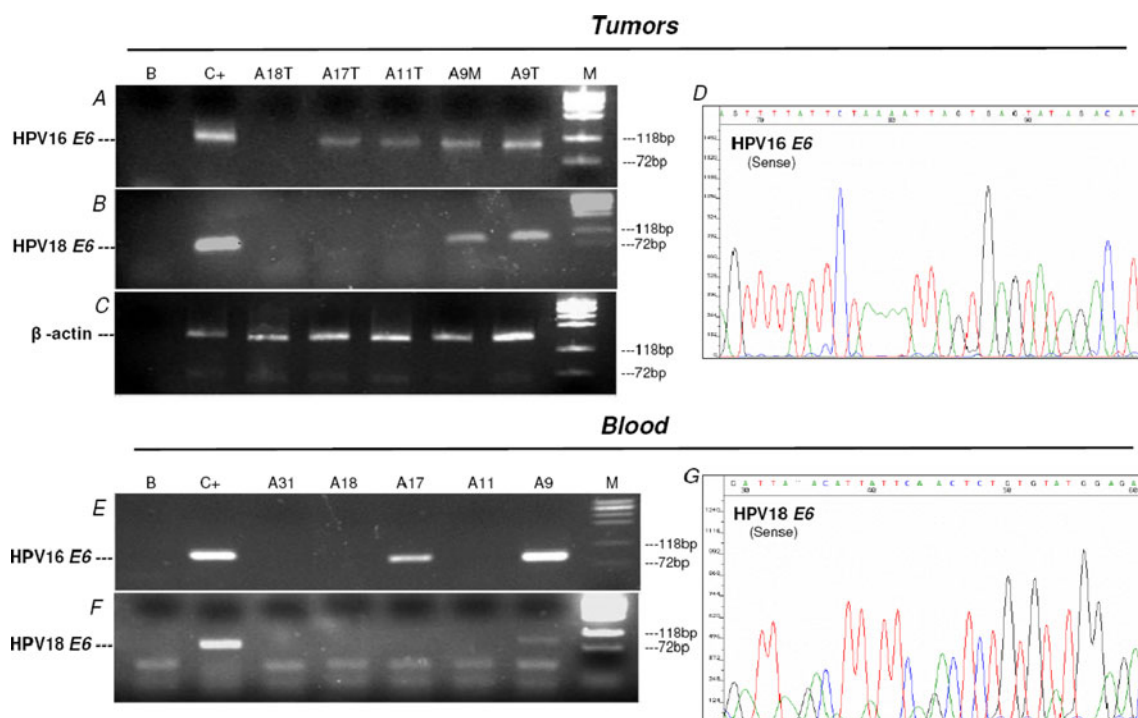


Fig. 2 Electrophoretical and sequence analyses of HPV *E6* PCR products. Panels *a* and *b* show HPV 16 (*a*) and 18 (*b*) *E6* sequences amplified from the primary tumors (A18T, A17T, A11T, A9T) and from the unique ovarian metastasis (A9M), where the bands corresponding to the expected *E6* fragments were obtained from both the primary tumor and the ovarian metastasis of A9-15y (for both HPV 16 and 18), and from the tumors of A11-12y and A17-25y (for HPV 16 only), while A18-21y resulted HPV *E6* negative. Panel *c* shows β -actin amplification, used as a control for DNA extracted

from paraffin-embedded tumor samples. Panel *d* exemplifies the HPV 16 *E6* sequence obtained from the primary tumor of A9-15y. Panels *e* and *f* show results obtained for HPV 16 (*e*) and 18 (*f*) *E6* sequences amplified from peripheral blood, where only cases A9-15y (HPV 16 and 18) and A17-25y (HPV 16), both at advanced stage, resulted positive. Cases A18-21y and A31-21y resulted negative for HPV 16 *E6* in blood (note that the tumor from A31-21y was not available for study). Panel *g* exemplifies the HPV 18 *E6* sequence obtained from the peripheral blood of A9-15y

In summary, evidence of an association between high-risk HPV infection and jBC was obtained for two ER/PR/Her2/neu-negative and p16(INK4A)-positive jBC cases in post-menarchal adolescents, of which one classified as a glycogen-rich breast carcinoma, the other as a secretory carcinoma, and for an ER/PR/Her2/neu/p16(INK4A)-positive ductal carcinoma which manifested after pregnancy and prolonged lactation at the age of 25 years. A germline missense mutation in *BRCA2*, most probably of pathogenetic relevance, was associated with a jBC that manifested in a 21-year-old patient, 4 years after an early full-term pregnancy. In this latter case the tumor was reported as infiltrating ductal carcinoma, but pathophenotype and tumor-associated genetic alterations could not be verified (due to lack of paraffin-embedded blocks).

Discussion

We analyzed a series of five jBC cases selected, based on diagnosis within 25 years of age, among the pre-menopausal BC patients referred for *BRCA1/2* genetic analysis as a public health service in the city of Buenos Aires between 2000 and 2008. Notably this series included the only two BC-affected adolescent girls (respectively 12- and 15-year-old at diagnosis) in the records of *Hospital de Niños “Dr. Ricardo Gutierrez”*, one of the major pediatric hospitals in Argentina. Both adolescents reported menarche at 11 years of age. The other three cases were, respectively, 21-, 21-, and 25-years-old at BC diagnosis and comprised two patients, 21- and 25-years-old, diagnosed with BC 4 and 2 years after a full-term pregnancy. Thus, four out of these five jBC cases became clinically manifest after menarche or pregnancy, suggesting a promoting role of sex hormones [13].

The primary tumor and the matched metastasis of A9-15y were classified as glycogen-rich breast carcinoma, a rare histological BC variant, not previously associated with juvenile onset, and usually reported to have poor prognosis [50]. This histotype may manifest with different molecular subtypes, in this case triple negative and p16(INK4A)-positive. The primary tumor of A11-12y was classified as secretory breast carcinoma. This is also a rare BC subtype, frequently found in young females and previously reported in a 12-year-old girl, in association with juvenile papillomatosis of the breast, a condition related to jBC risk, not documented in A11-12y [51–53]. This tumor was assigned to the basal molecular subtype, and was also p16(INK4A)-positive. The tumor of A17-25y was classified as infiltrating ductal carcinoma of luminal B subtype, with p16(INK4A) reactivity. The tumor of A18-21y manifested in a nulliparous young woman and was classified as lobular carcinoma, a histotype more frequently seen in

postmenopausal patients. This tumor resulted in luminal A subtype, and was p16(INK4A)-negative. As stated before, the primary tumor of case A31-21y, which manifested after pregnancy, was originally diagnosed as infiltrating ductal carcinoma, but unavailability of paraffin-embedded blocks did not allow further study. Overall, these data highlight the broad pathologic spectrum and the biological heterogeneity of jBC. In fact, the four cases for which paraffin blocks were available presented with four different histotypes, including two rare histotypes, i.e., glycogen-rich and secretory BC, in the adolescents, and two common histotypes, i.e., infiltrating ductal and infiltrating lobular carcinoma, in the young women. The molecular subtypes were also heterogeneous, including triple negative, basal, luminal B, and luminal A. However, 3/4 cases for which paraffin-embedded tumor blocks were available resulted positive for p16(INK4A), a cyclin-dependent kinase-4 inhibitor expressed in a limited range of normal tissues and tumors, regarded as a surrogate marker of the presence of high-risk HPVs [47, 54].

Only the two adolescent patients reported cancer family history, in both cases early onset ovarian cancer on the maternal side, also colorectal cancer in the father were observed. This could suggest a role of germline mutations in cancer-predisposing genes, particularly *BRCA1/2*. However, there are no data in the literature on *BRCA1/2* germline mutations associated with BC in childhood and adolescence, although other tumor types were reported [16], and no mutations were identified in these cases after scanning the entire *BRCA1/2* coding sequences. Such negative results should be interpreted with caution, as the screening techniques used, although highly sensitive for point mutations and small insertions/deletions, do not detect genomic rearrangements, mutations in regulatory sequences, and epigenetic alterations, known to be responsible for a fraction of *BRCA1/2*-related tumors [55–57]. Lack of evidence for *BRCA1/2* mutations was consistent with the fact that glycogen-rich and secretory carcinomas were not reported in *BRCA1/2* mutation carriers, although it is known that *BRCA1*-associated BCs are frequently of triple negative or basal subtype [58]. The other three patients did not report cancer family history and did not result positive for pathogenic germline mutations in *BRCA1*. However, we identified a missense germline *BRCA2* mutation, p.Ile2490Thr, in A31-21y, diagnosed with stage II BC 4 years after an early full-term pregnancy. *BRCA2* p.Ile2490Thr, predicted to affect protein function, is listed as unclassified in BIC, and was reported in more than 70 individuals of Latin American ancestry, affected and unaffected with BC, and, as compound heterozygote, in a Fanconi anemia case of Latin American origin diagnosed with medulloblastoma at 2.5 years of age [59]. It is important to stress that *BRCA2* is known to be implicated

in jBC, as *BRCA2* 6174delT, a mutation frequent in Ashkenazi Jews, was reported in a 20-year-old BC patient [16, 59]. Here we report another extraordinarily early manifestation of *BRCA2*-associated BC, particularly interesting in view of its relationship with an early full-term pregnancy. In this regard, the role of parity and age at first full-term pregnancy as possible BC risk factors in *BRCA1/2* mutation carriers is currently debated [60–64].

BC is one of the cancers most often associated with the Li-Fraumeni and Li-Fraumeni-like syndromes, rare genetic disorders due to germline *TP53* mutations, which greatly increase the risk of developing multiple cancers beginning in childhood [20]. However, to our knowledge, BC in children and adolescents is not documented in Li-Fraumeni and Li-Fraumeni-like families [20]. Furthermore none of the cases in the presently described jBC series reported a family history consistent with these syndromes. This was in agreement with the fact that no pathogenetic mutations were identified in *TP53* exons 4–10, where Li-Fraumeni syndrome-associated mutations are generally detected. The new intronic *TP53* variant IVS10 + 30A > T, not predicted to affect mRNA splicing, is unlikely to be of pathogenic relevance, and was detected in the *BRCA2* carrier. Nonetheless, quite remarkably, 4/5 cases, including A9-15y, A11-12y, and A17-25y, resulted Arg/Arg homozygous at the common *TP53* codon 72 C > G polymorphism, while A31-21y, who resulted as a carrier of a *BRCA2* mutation, was heterozygous (Arg/Pro). The *TP53* codon 72 polymorphism is functionally significant [65]. In fact, the Arg72 isoform appears to be more efficient at inducing apoptosis, while the Pro72 isoform may work better at inducing G1 arrest [66]. Such functional differences may influence cancer risk following exposure to specific carcinogens [67]. In particular, Arg/Arg homozygosity was associated with increased risk of HPV-related cervical cancer [68, 69].

Somatic tumor-associated mutations in *TP53* exons 5–8, encoding the DNA-binding region of the protein, are found in about 21% of all BC cases [20, 70]. In this study, no mutations were detected in the four jBC cases for which tumor sections were available. This was consistent with lack of *TP53* protein overexpression and nuclear accumulation, as assessed by immunohistochemistry.

Of about 200 HPV genotypes identified to date, approximately 40 are known to be associated with genital infections, and about 30, among which most notably HPV 16 and HPV 18, are implicated in the etiology of genital cancers, most notably cervical carcinoma, where high-risk HPVs were retrieved in more than 99% of the cases studied [71]. Co-infection with HPV-16 and 18 commonly occurs in cervical cancer [22]. In infected cells high-risk HPVs

can persist in episomal forms, in integrated forms, or in both. Viral integration into the host-cell genome downstream of *E6* and *E7* seems to be an important step in the pathogenesis of HPV-associated cancers [72, 73]. Cancer-related HPV integrations up-regulate *E6* and *E7*, essential for HPV-induced malignant transformation and never lost during cancer progression [42]. *E7* triggers excessive cell cycling by inactivation of pRb, and *E6* sequesters TP53, which promotes escape from apoptosis and accumulation of secondary mutations [42, 74, 75]. In our study, lack of RNA and poor DNA quality impeded the verification of DNA integration status in the three HPV-positive jBC cases. At present data on HPV integration sites, that appear to be randomly distributed with a predilection for fragile sites, are available mainly for cervical cancer cell lines, primary cervical lesions, and some HPV-associated head and neck cancers [42, 49, 76].

High-risk HPV sequences were detected in significant fractions of age-unselected BCs from different areas of the world. In South America, HPV 16/18 DNA was detected in about 25% of the cases in a Brazilian BC series [24]. Notably, the HPV-positive BC cases were reported to be significantly younger than the HPV-negative cases [77], less ER-positive, more proliferative, and of higher grade [77]. We retrieved tumor-associated *E6* sequences of HPVs 16 in 3/4 jBC cases whose tumors could be tested, including the glycogen-rich and the secretory carcinomas of A9-15y and A11-12y, diagnosed after menarche, and the infiltrating ductal carcinoma of A17-25y, diagnosed after pregnancy and lactation. Together with HPV 16 *E6*, also HPV 18 *E6* was retrieved from both the primary and the metastatic tumor of A9-15y. This study provides the first evidence of associations between high-risk HPVs and very rare BC histotypes in adolescents. Notably, in the HPV-positive patients with stage IV disease, i.e., A9-15y and A17-25y, HPV *E6* sequences were also amplified from peripheral blood. The possibility that these sequences derived from latent genital infections is remote, since HPV DNA is found only in the blood of patients affected with high stage cervical carcinoma [78]. In the present study no HPV-associated genital lesions were documented. Furthermore, HPV positivity was consistent with the expression of p16(INK4A) in the primary tumors of A9-15y, A11-12y, and A17-25y, and in the HPV-positive metastasis of A9-15y. Expression of p16(INK4A) could be interpreted as an attempt to compensate for pRb loss, as in other HPV-associated cancers [79–82].

Data on a murine model show the importance of the synergy between estrogen and HPV *E6*-induced TP53 insufficiency for carcinogenesis in hormone-responsive mammary and uterine epithelia [83]. Interestingly, in this

model, the E6-induced mammary tumors were of mixed cell types, suggestive of origin from multipotent progenitors. In this respect the histological and molecular heterogeneity of jBC, confirmed in the present study, particularly for the 3 HPV-associated cases, would be in agreement with the murine model in suggesting origins from transformed mammary stem/progenitor cells [83]. The long latent phase of HPV infection poses difficulties for the identification of the possible modalities of mammary infection. Sexual transmission is well established, but infection through other routes could be frequent early in life [84]. Non-sexual post-natal infection with HPVs of genital origin is possible. Vertical transmission is quite rare, despite a relatively high positivity rate in the maternal cervix, but contamination of the nipple area could occur during vaginal delivery. This could result in early infection of mammary epithelia.

The rarity of jBC, combined with its pathological and molecular heterogeneity, suggests that each case should be treated taking into account its specific pathological and molecular characteristics. The possible prognostic associations of HPV-positive status in jBC, and in BC in general, and its relevance for BC treatment are as yet undetermined. Nonetheless, it has been observed that biologically aggressive basal and basal-like BCs share morphologic features with poorly differentiated HPV-related oropharyngeal and genital squamous cell carcinomas, including functional loss of RB1 and p16(INK4a) expression [85, 86]. Importantly, pRB loss and p16(INK4a) positivity are associated with p53-mediated sensitivity to chemotherapeutic agents and predicts response to adjuvant chemotherapy [87, 88]. Furthermore, it has been shown that HPV-positive status and, particularly, expression of p16(INK4A), has a major positive impact on response to radiotherapy in HPV-related head and neck cancers [89, 90].

Specific modalities of prevention and treatment could be applicable to HPV-related cancers. Treatment with interferons is useful in the management of benign HPV-related anogenital diseases harboring HPV in the episomic state, but its relevance to the prevention of other HPV-associated cancers, including jBC, is dubious and of difficult assessment, given the difficult identification of HPV-infected precursor lesions and the frequent cancer-associated impairment of interferon signaling [91, 92]. In perspective, however, chemotherapeutic agents specifically targeting HPV protein functions and/or therapeutic vaccines against unique HPV antigens could contribute to the treatment of HPV-related cancers [93–95]. Finally, the evidences for a possible involvement of high-risk HPVs in BC [96] suggest that the currently available prophylactic vaccines could have an added value in terms of BC prevention, although, given the timing of HPV vaccination, early infections, that could be linked to jBC, might not be affected [84, 97].

Conclusions

JBC is rare and the identities and roles of the hereditary and environmental factors that contribute to its etiology are still debated. We examined a small but highly selected series of five jBC cases, which most notably included two adolescents. The tumors showed remarkable histological and molecular heterogeneity, but 3/5 cases expressed p16(INK4A), a marker of HPV infection. These three cases, which included the two adolescents and a 25-year-old with BC diagnosed after pregnancy and lactation, resulted positive for HPV 16 *E6* sequences and, in one of the two adolescents, also for HPV 18 *E6*. No germline mutations in the *BRCA1* and *TP53* genes were detected, but four of five cases were Arg/Arg homozygous at *TP53* codon 72, a polymorphism which may influence HPV-related cancer risk. A germline mutation in *BRCA2* was found in a 21-year-old with HPV-negative jBC diagnosed after pregnancy. These findings suggest that puberty- and pregnancy-related hormonal changes concur in promoting extraordinarily early BC onset in mammary epithelia latently infected with high-risk HPVs and/or in carriers of germline mutations in *BRCA2*.

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