HIGH-RISK HUMAN PAPILLOMA VIRUS INFECTION, TUMOR PATHOPHENOTYPES, BRCA1/2 AND TP53 STATUS IN JUVENILE BREAST CANCER PATIENTS

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Abstract

Introduction. Juvenile breast cancer is rare and poorly known. We studied a series of 5 breast cancer patients diagnosed within 25 years of age, that included 2 adolescents, 12- and 15-year-old, and 3 young women, 21-, 21- and 25-year-old respectively.

Methods. 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-year-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.

Results. 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-year-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 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.

Conclusions. 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.

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 ≤ 10/100,000) [9], but tends to be more advanced at presentation and more aggressive than BC in older women [10]. BC manifesting in children and adolescents within 14 years of age is even more rare (≤ 1/100,000), but its incidence seems to vary geographically with a pattern contrasting with that of BC in women [9, 11]. 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 [12-16]. 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 [17-22], but there is no reported evidence of germline *BRCA1/2* or *TP53* mutations in BC-affected children and adolescents [21-22]. 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 favour 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 [23-25] and were found in relevant fractions of unselected BCs (range: 25-29%) [26-27]. Furthermore, in women affected with both mammary and cervical cancers, HPV 16 was retrieved from both cancer types [28]. 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 favour HPV persistence and replication in clonally-expanding and differentiating hormone-dependent epithelia, facilitating HPV-induced carcinogenesis [29-30].

To investigate the role of genetic and environmental factors in jBC, we studied a small but highly selected group of 5 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 [31], but jBC remains very rare also in this country [1,9]. 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 5 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 9x8x5 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: 4.5x4 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), two years after delivery of her only child and soon after finishing breast-feeding. 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.

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 Josè 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 Josè de San Martín, Hospital de Niños "Dr. Ricardo Gutierrez", Instituto Alexander Fleming*. BCs were classified and staged according to World Health Organization and American Joint Committee on Cancer [32,33]. Histological grading was performed using the Nottingham modification of the Bloom-Richardson system [34].

PAS-diastase 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^{TM} (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 [35]. The entire *BRCA1* and *BRCA2* coding sequences, including intron-exon boundaries, as well as *TP53* exons 4-10 [36] 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 [37-39]. DHPLC conditions were adapted based on manufacturer's software and literature [37,38]. Samples that showed PTT shifts or altered DHPLC profiles were directly sequenced using an ABI PRISM^{TM}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.fhcrc.org/sift/SIFT.html*) [40]. Predictions of sequence changes on splicing were obtained using the ESEFinder software [41].

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-µm-thick sections lightly counterstained with hematoxylin, microdissected for DNA extraction as previously reported [42] and analysed for mutations in exons 5-8 of *TP53* [43]. Amplification of a 147 bp β-actin gene fragment served as a control for DNA quality.

The presence of HPV 16/18 *E6* sequences (gene bank accession numbers K02718 and AY262282 respectively) [44] 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*) [44] 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 (1μg) 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.

Results

Table 1 summarizes the individual, clinical and pathological features of the 5 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 (Figure 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 and metastatic tumor cells resulted strongly positive for p16(INK4A) (Figure 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 [45]. The tumor of the other adolescent patient (A11-12y) was composed of a predominant invasive component with papillary, cribriform and microcystic patterns (Figure 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 (Figure 1E). A clear immunoreaction for p16(INK4A) was seen in the intraductal component and in focal invasive areas with cribriform pattern (Figure 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 [45]. 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 [45].

The tumor of case A18-21y was classified as lobular carcinoma and, being positive for ER, PR and 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.

Germ-line mutational analysis identified 9 synonymous or intronic *BRCA1* variants in the 5 tested cases and a total of 6 *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 aminoacid change affects protein function. Furthermore, the ESEFinder software [41] 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 3 polymorphisms in *BRCA1* and 4 in *BRCA2*; A18-21y for 1 polymorphism in *BRCA1* and 2 in *BRCA2* (Table 2).

No deleterious germline mutations were found in *TP53* exons 4-10, which are known to harbour 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 [21]. However, A31-21y (which was positive for a probably pathogenetic *BRCA2* missense mutation) carried a new *TP53* intronic variant (IVS10+30 A>T), not 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 [46]. The remaining case (A18-21y) was Arg/Pro heterozygous. Analysis of tumor DNA from the 4 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 [36].

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 (Figure 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 [47]. 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 [48-50]. In the 3 HPV-positive cases, the retrieval of *E6* sequences by PCR was fully consistent with the immunohistochemical evidence of p16(INK4A) expression (Figure 1). Notably in case A9-15y both the primary BC and its ovarian metastasis were *E6*-positive and showed strong p16(INK4A) immunostaining (Figure 1B-C), while no p16(INK4A) staining was observed in A18-21y, negative for HPV *E6*.

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 age 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 analysed a series of 5 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 3 cases were respectively 21-, 21- and 25-year-old at BC diagnosis and comprised two patients, 21- and 25-year-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 [14].

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 [51]. 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 [52-54]. 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 of 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 4 cases for which paraffin blocks were available presented with 4 different histotypes, including 2 rare histotypes, *i.e.*, glycogen-rich and secretory BC, in the adolescents, and 2 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(INKA4), 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 [50, 55].

Only the two adolescent patients reported cancer family history, in both cases early onset ovarian cancer on the maternal side, in A9-15y also colorectal cancer in the father. 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 [17], 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 [56-58]. 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 [59]. 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 A18-21y, diagnosed with stage II BC four 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 [60]. 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 [60,17]. 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 [61-65].

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 [22]. However, to our knowledge, BC in children and adolescents is not documented in Li-Fraumeni and Li-Fraumeni-like families [22]. 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 C>G polymorphism at *TP53* codon 72. This polymorphism is functionally significant [66]. In fact, the Arg72 isoform appears to be more efficient at inducing apoptosis, while the Pro72 isoform may work

better at inducing G1 arrest [67]. Such functional differences may influence cancer risk following exposure to specific carcinogens [68]. In particular, Arg/Arg homozygosity was associated with increased risk of HPV-related cervical cancer [69-70].

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 [22-71]. In this study, no mutations were detected in the 4 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 [72]. Co-infection with HPV-16 and 18 commonly occurs in cervical cancer [24]. Cancer-related HPV 16 integrations up-regulate HPV *E6* and *E7*, essential for HPV-induced malignant transformation and never lost during cancer progression [73]. E7 triggers excessive cell cycling by inactivation of pRb, and E6 sequesters TP53, which promotes escape from apoptosis and accumulation of secondary mutations [73-75].

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 [26]. Notably, the HPV-positive BC cases were reported to be significantly younger than the HPV-negative cases [76], less ER-positive, more proliferative and of higher grade [76]. 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 the HPV *E6* 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 [77]. 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 [78-81].

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 [82]. 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 [82]. 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. 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.

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 5 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 3 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 4 of 5 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*.

Abbreviations

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.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

GMA contributed to study conception and design, and acquisition, analysis, and interpretation of data; and drafted and revised the manuscript critically for important intellectual content. ARS 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. RMC contributed to study conception and design, and acquisition, analysis and interpretation of data; and drafted and revised the manuscript critically for important intellectual content. EJP contributed to study conception and design, and acquisition and interpretation of data; and drafted and revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

References

1. Parkin D.M: International variation, *Oncogene* 2004, 23: 6329-6340.
2. Levine PH, Pogo BG, Klouj A, Coronel S, Woodson K, Melana SM, Mourali N, Holland JF: Increasing evidence for a human breast carcinoma virus with geographic differences. *Cancer* 2004, 101(4):721-6.
3. Tavani A, Braga C, La Vecchia C, Negri E, Russo A, Franceschi S: Attributable risks for breast cancer in Italy: education, family history and reproductive and hormonal factors. *Int J Cancer.* 1997 Jan 17; 70(2):159-63.
4. Hunter DJ, Willett WC: Diet, body size, and breast cancer. *Epidemiol Rev.* 1993; 15(1):110-32.
5. Hulka BS, Moorman P.G: Breast cancer: hormones and other risk factors. *Maturitas* 2001, 38:103-113.
6. Dumitrescu RG, Cotarla I: Understanding breast cancer risk -- where do we stand in 2005? *J Cell Mol Med.* 2005, 9(1):208-21.
7. Lagerros YT, Hsieh SF, Hsieh CC: Physical activity in adolescence and young adulthood and breast cancer risk: a quantitative review. *Eur J Cancer Prev.* 2004 Feb; 13(1):5-12. Review.
8. Dehner LP, Hill DA, Deschryver K: BC in young patients. *Semin Diagn Pathol.* 1999.
9. Globocan (http://www-dep.iarc.fr/globocan/database.htm)
10. Wilson M, Cranor ML, Rosen PP: Papillary duct hyperplasia of the breast in children and young women. *Mod Pathol.* 1993, 6(5):570-4.
11. Parkin DM, Whelan SL, Ferlay J, Teppo L, Thomas DB: Cancer incidence in 5 continents. Vol. 8 (*IARC Scientific Publication* No. 155). Lyon, France: International Agency for Research on Cancer (IARC), 2002.
12. Medina D: Mammary developmental fate and breast cancer risk. *Endocr Relat Cancer* 2005, 12(3):483-95.
13. Russo J, Balogh GA, Chen J, Fernandez SV, Fernbaugh R, Heulings R, Mailo DA, Moral R, Russo PA, Sheriff F, Vanegas JE, Wang R, Russo IH: The concept of stem cell in the mammary gland and its implication in morphogenesis, cancer and prevention. *Front Biosci.* 2006, 11:151-72.
14. Russo IH, Russo J: Role of hormones in mammary cancer initiation and progression. *J Mammary Gland Biol Neoplasia.* 1998, 3:49-61. Review.
15. Hiraku Y, Yamashita N, Nishiguchi M, Kawanishi S: Catechol estrogens induce oxidative DNA damage and estradiol enhances cell proliferation. *Int J Cancer.* 2001, May 1; 92(3):333-7.
16. Hilakivi-Clarke L, de Assis S: Fetal origins of breast cancer. *Trends Endocrinol Metab.* 2006, 17(9):340-8.
17. Brooks GA, Stopfer JE, Erlichman J, Davidson R, Nathanson KL, Domchek SM: Childhood cancer in families with and without BRCA1 or BRCA2 mutations ascertained at a high-risk breast cancer clinic. *Cancer Biol Ther.* 2006, 5(9): 1098-102.
18. Miki Y, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, Tavtigian S, Liu Q, Cochran C, Bennett LM, Ding W, et al: A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science.* 1994, 266(5182):66-71.
19. Wooster R, Bignell G, Lancaster J, Swift S, Seal S, Mangion J, Collins N, Gregory S, Gumbs C, Micklem G: Identification of the breast cancer susceptibility gene BRCA2. *Nature.* 1995, 378(6559):789-92.

20. Malkin D, Li FP, Strong LC, Fraumeni JF Jr, Nelson CE, Kim DH, Kassel J, Gryka MA, Bischoff FZ, Tainsky MA, et al: Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. *Science*. 1990, 250(4985): 1233-8.
21. Lalloo F, Varley J, Moran A, Ellis D, O'Dair L, Pharoah P, Antoniou A, Hartley R, Shenton A, Seal S, Bulman B, Howell A, Gareth D, Evans R: BRCA1, BRCA2 and TP53 mutations in very early-onset breast cancer with associated risks to relatives. *European Journal of Cancer*. 2006, 42:1143–1150.
22. IARC Database (http://www.iarc.fr/en/websites/index.php).
23. Liu Y, Klimberg VS, Andrews NR, Hicks CR, Peng H, Chiriva-Internati M, Henry-Tillman: Human papillomavirus DNA is present in a subset of unselected breast cancers. *J Hum Virol*. 2001, 4(6):329-34.
24. Pagano JS, Blaser M, Buendia M, Da mania B, Khalili, K, Raab-Traub N, Roizman B: Infectious agents and cancer: criteria for a causal relation. *Seminars in Cancer Biology*. 2004, 14:453–471.
25. Band V, Zajchowski D, Kulesa V, Sager R: Human papilloma virus DNAs immortalize normal human mammary epithelial cells and reduce their growth factor requirements. *Proc Natl Acad Sci* U S A. 1990, 87(1):463-7.
26. Damin AP, Karam R, Zettler CG, Caleffi M, Alexandre CO: Evidence for an association of human papillomavirus and breast carcinomas. *Breast Cancer Res Treat*. 2004, 84(2):131-7.
27. Di Lonardo A, Venuti A, Marcante ML: Human papillomavirus in breast cancer. *Breast Cancer Res Treat*. 1992, 21(2):95-100.
28. Hennig EM, Suo Z, Thoresen S, Holm R, Kvinnsland S, Nesland JM: Human papillomavirus 16 in breast cancer of women treated for high grade cervical intraepithelial neoplasia (CIN III). *Breast Cancer Res Treat*. 1999, 53(2):121-35.
29. Muñoz N, Franceschi S, Bosetti C, Moreno V, Herrero R, Smith JS, Shah KV, Meijer CJ, Bosch FX: Role of parity and human papillomavirus in cervical cancer: the IARC multicentric case-control study. International Agency for Research on Cancer. Multicentric Cervical Cancer Study Group. *Lancet*. 2002, 359(9312):1093-101.
30. Sethi S, Müller M, Schneider A, Blettner M, Smith E, Turek L, Wahrendorf J, Gissmann L, Chang-Claude J: Serologic response to the E4, E6, and E7 proteins of human papillomavirus type 16 in pregnant women. *Am J Obstet Gynecol*. 1998, 178(2):360-4.
31. Schwartsmann G. Breast cancer in South America: challenges to improve early detection and medical management of a public health problem. *J Clin Oncol*. 2001, 19(18 Suppl):118S-124S.
32. Ellis IO, Schnitt SJ, Sastre-Garau X, Bussolati G, Tavassoli FA, Eusebi V, et al: Invasive breast carcinoma. in pathology and genetics of the breast and female genital organs. Tavassoli FA, Devilee P (eds). *IARC Press*: Lyon, 2003, 13-59.
33. Greene FL, Page DL, Fleming ID, et al: AJCC Cancer Staging Manual. 6th ed. New York: Springer; 2002.
34. Frierson HF Jr, Wolber RA, Berean KW, Franquemont DW, Gaffey MJ, Boyd JC et al: Interobserver reproducibility of the Nottingham modification of the Bloom and Richardson histologic grading scheme for infiltrating ductal carcinoma. *Am J Clin Pathol*. 1995, 103:195-8.
35. Ottini L, Masala G, D'Amico C, Mancini B, Saieva C, Aceto G, Gestri D, Vezzosi V, Falchetti M, De Marco M, Paglierani M, Cama A, Bianchi S, Mariani-Costantini R, Palli D: BRCA1 and BRCA2 mutation status and tumor characteristics in male breast cancer: a population-based study in Italy. *Cancer Res*. 2003, 63(2):342-7.

36. Soussi T: The p53 tumor suppressor gene: from molecular biology to clinical investigation. *Ann N Y Acad Sci*. 2000, 910:121-37.
37. Gross E, Arnold N, Pfeifer K, Bandick K, Kiechle M: Identification of specific BRCA1 and BRCA2 variants by DHPLC. *Hum Mutat*. 2000, 16(4):345-53.
38. Wagner T, Stoppa-Lyonnet D, Fleischmann E, Muhr D, Pagès S, Sandberg T, Caux V, Moeslinger R, Langbauer G, Borg A, Oefner P: Denaturing high-performance liquid chromatography detects reliably BRCA1 and BRCA2 mutations. *Genomics*. 1999, 15;62(3):369-76.
39. Yamanoshita O, Kubota T, Hou J et al: DHPLC is superior to SSCP in screening p53 mutations in esophageal cancer tissues. *Int J Cancer*. 2005, 114(1): 74–79.
40. Ng PC, Henikoff S: Accounting for human polymorphisms predicted to affect protein function. *Genome Res* 2002, 12:436–446.
41. Cartegni L, Wang J, Zhu Z, Zhang MQ, Krainer AR: ESEfinder: A web resource to identify exonic splicing enhancers. *Nucleic Acids Res*. 2003, 31(13):3568-71.
42. Ottini L, Palli D, Falchetti M, D'Amico C, Amorosi A, Saieva C, Calzolari A, Cimoli F, Tatarelli C, De Marchis L, Masala G, Mariani-Costantini R, Cama A: Microsatellite instability in gastric cancer is associated with tumor location and family history in a high-risk population from Tuscany. *Cancer Res*. 1997, 57(20):4523-9.
43. Mahdavinia M, Bishehsari F, Verginelli F, Cumashi A, Lattanzio R, Sotoudeh M, Ansari R, Semeraro D, Hormazdi M, Fakheri H, Rakhshani N, De Lellis L, Curia MC, Cama A, Piantelli M, Malekzadeh R, Iacobelli S, Mariani-Costantini R: P53 mutations in colorectal cancer from northern Iran: Relationships with site of tumor origin, microsatellite instability and K-ras mutations. *J Cell Physiol*. 2008, 216(2):543-50.
44 Morris BJ: Cervical human papillomavirus screening by PCR: advantages of targeting the E6/E7 region. *Clin Chem Lab Med*. 2005, 43:1171–7.
45. Sørlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, Deng_S, Johnsen H, Pesich R, Geisler S, Demeter J, Perou CM., Lønning PE,. Brown PO, Børresen-Dale A L, and Botstein D: Repeated observation of breast tumor subtypes in independent gene expression data sets. *PNAS*. 2003, 100: 8418–8423.
46. Aoki MN, da Silva do Amaral Herrera AC, Amarante MK, do Val Carneiro JL, Fungaro MH, Watanabe MA: CCR5 and p53 codon 72 gene polymorphisms: implications in breast cancer development. *Int J Mol Med*. 2009, 23(3):429-35.
47. Pagano JS, Blaser M, Buendia MA, Damania B, Khalili K, Raab-Traub N, Roizman B: Infectious agents and cancer: criteria for a causal relation. *Semin Cancer Biol*. 2004, 14(6):453-71.
48. Scheurer ME, Tortolero-Luna G, Adler-Storthz K: Human papillomavirus infection: biology, epidemiology, and prevention. *Int J Gynecol Cancer*. 2005, 15(5):727-46.
49. Stoler MH: Human papillomaviruses and cervical neoplasia: a model for carcinogenesis. *Int J Gynecol Pathol*. 2000, 19(1):16-28.
50. Klaes R, Friedrich T, Spitkovsky D, Ridder R, Rudy W, Petry U, Dallenbach-Hellweg G, Schmidt D, von Knebel Doeberitz M: Overexpression of p16(INK4A) as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri. *Int J Cancer*. 2001, 92(2):276-84.
51. Kuroda H, Sakamoto G, Ohnisi K, Itoyama S: Clinical and pathological features of glycogen-rich clear cell carcinoma of the breast. *Breast Cancer*. 2005, 12(3):189-95.
52. Nonomura A, Kimura A, Mizukami Y, Nakamura S, Ohmura K, Watanabe Y, Tanimoto K, Ikegaki S: Secretory carcinoma of the breast associated with juvenile papillomatosis in a 12-year-old girl. A case report. *Acta Cytol*. 1995 May-Jun;39(3):569-76.

53. Rice HE, Acosta A, Brown RL, Gutierrez C, Alashari M, Mintequi D, Rodriguez A, Chavarrfa O, Azizkhan RG: Juvenile papillomatosis of the breast in male infants: two case reports. *Pediatr Surg Int.* 2000,16(1-2):104-6.
54. Paul Peter Rosen: Rosen's Breast Pathology. 1997, p441-447 Lippincot-Raven Publishers.
55. Sano T, Oyama T, Kashiwabara K, Fukuda T, Nakajima T: Expression status of p16 protein is associated with human papillomavirus oncogenic potential in cervical and genital lesions. *Am J Pathol.* 1998, 153(6):1741-8.
56. Ford D, Easton DF, Stratton M, Narod S, Goldgar D, Devilee P, Bishop DT, Weber B, Lenoir G, Chang-Claude J, Sobol H, Teare MD, Struewing J, Arason A, Scherneck S, Peto J, Rebbeck TR, Tonin P, Neuhausen S, Barkardottir R, Eyfjord J, Lynch H, Ponder BA, Gayther SA, Zelada-Hedman M, et al: Genetic heterogeneity and penetrance analysis of the BRCA1 and BRCA2 genes in breast cancer families. The Breast Cancer Linkage Consortium. *Am J Hum Genet.* 1998 Mar, 62(3):676-89.
57. McClain MR, Nathanson KL, Palomaki GE, Haddow JE: An evaluation of BRCA1 and BRCA2 founder mutations penetrance estimates for breast cancer among Ashkenazi Jewish women. *Genet Med.* 2005, 7(1):34-9.
58. Veschi S, Aceto G, Scioletti AP, Gatta V, Palka G, Cama A, Mariani-Costantini R, Battista P, Calò V, Barbera F, Bazan V, Russo A, Stuppia L: High prevalence of BRCA1 deletions in BRCAPRO-positive patients with high carrier probability. *Ann Oncol.* 2007, 18 Suppl 6:vi86-92.
59. Palacios J, Robles-Frías MJ, Castilla MA, López-García MA, Benítez J: The molecular pathology of hereditary breast cancer. *Pathobiology.* 2008, 75(2):85-94.
60. Offit K, Levran O, Mullaney B, Mah K, Nafa K, Batish SD, Diotti R, Schneider H, Deffenbaugh A, Scholl T, Proud VK, Robson M, Norton L, Ellis N, Hanenberg H, Auerbach AD: Shared genetic susceptibility to breast cancer, brain tumors, and Fanconi anemia. *J Natl Cancer Inst.* 2003, 95(20):1548-51.
61. Narod SA: Modifiers of risk of hereditary breast cancer. *Oncogene.* 2006, 25(43):5832-6.
62. Lee E, Ma H, McKean-Cowdin R, Van Den Berg D, Bernstein L, Henderson BE, Ursin G: Effect of reproductive factors and oral contraceptives on breast cancer risk in BRCA1/2 mutation carriers and noncarriers: results from a population-based study. *Cancer Epidemiol Biomarkers Prev.* 2008, 17(11):3170-8.
63. Andrieu N, Goldgar DE, Easton DF, Rookus M, Brohet R, Antoniou AC, et al: Pregnancies, breast-feeding, and breast cancer risk in the International BRCA1/2 Carrier Cohort Study (IBCCS). *J Natl Cancer Inst.* 2006, 98:535–44.
64. Keinan-Boker L, Lerner-Geva L, Kaufman B, Meirow D: Pregnancy-associated breast cancer. *Isr Med Assoc J.* 2008, 10(10):722-7.
65. Awadelkarim KD, Aceto G, Veschi S, Elhaj A, Morgano A, Mohamedani AA, Eltayeb EA, Abuidris D, Di Gioacchino M, Battista P, Verginelli F, Cama A, Elwali NE, Mariani-Costantini R. BRCA1 and BRCA2 status in a Central Sudanese series of breast cancer patients: interactions with genetic, ethnic and reproductive factors. *Breast Cancer Res Treat.* 2007,102(2):189-99.
66. Matlashewski GJ, Tuck S, Pim D, Lamb P, Schneider J, Crawford LV: Primary structure polymorphism at amino acid residue 72 of human p53. *Mol Cell Biol.* 1987, 7(2):961-3.
67. Pim D, Banks L: p53 polymorphic variants at codon 72 exert different effects on cell cycle progression. *Int J Cancer.* 2004, 108(2):196-9.

68. Dumont P, Leu JI, Della Pietra AC 3rd, George DL, Murphy M: The codon 72 polymorphic variants of p53 have markedly different apoptotic potential. *Nat Genet*. 2003, 33(3):357-65.

69. Katiyar S, Thelma BK, Murthy NS, Hedau S, Jain N, Gopalkrishna V, Husain SA, Das BC: Polymorphism of the p53 codon 72 Arg/Pro and the risk of HPV type 16/18-associated cervical and oral cancer in India. *Mol Cell Biochem*. 2003, 252(1-2):117-24.

70. Siddique MM, Balram C, Fiszer-Maliszewska L, Aggarwal A, Tan A, Tan P, Soo KC, Sabapathy K: Evidence for selective expression of the p53 codon 72 polymorphs: implications in cancer development. *Cancer Epidemiol Biomarkers Prev*. 2005, 14(9):2245-52.

71. Olivier M, Langerød A, Carrieri P, Bergh J, Klaar S, Eyfjord J, Theillet C, Rodriguez C, Lidereau R, Bièche I, Varley J, Bignon Y, Uhrhammer N, Winqvist R, Jukkola-Vuorinen A, Niederacher D, Kato S, Ishioka C, Hainaut P, Børresen-Dale AL: The clinical value of somatic TP53 gene mutations in 1,794 patients with breast cancer. *Clin Cancer Res*. 2006, 12(4):1157-67.

72. Muñoz N, Castellsague X, de Gonzalez AB, Gissman L: HPV in the etiology of human cancer. *Vaccine*. 2006, 24:S1-S10.

73. Morris BJ: Cervical human papillomavirus screening by PCR: advantages of targeting the E6/E7 region. *Clin Chem Lab Med*. 2005, 43:1171-7.

74. International Agency for Research on Cancer (IARC). IARC monographs on the evaluation of carcinogenic risks to humans.. *Human papillomaviruses*. IARC, Lyon (France). 1995, Vol. 64.

75. Xavier Castellsague´, Nubia Muñoz: Journal of the National Cancer Institute Monographs. No. 31, 2003.

76. Kroupis C, Markou A, Vourlidis N, Dionyssiou-Asteriou A, Lianidou ES: Presence of high-risk human papillomavirus sequences in breast cancer tissues and association with histopathological characteristics. *Clin Biochem*. 2006, 39(7):727-31. Epub 2006, Jun 15.

77. Pornthanakasem W, Shotelersuk K, Termrungruanglert W, Voravud N, Niruthisard S, Mutirangura A: Human papillomavirus DNA in plasma of patients with cervical cancer. *BMC Cancer*. 2001, 1:2. Epub 2001 Mar 5.

78. Samama B, Lipsker D, Boehm N: p16 expression in relation to human papillomavirus in anogenital lesions. *Human Pathology*. 2006, 37:513-519.

79. Nindl I, Meyer T, Schmook T, Ulrich C, Ridder R, Audring H, Sterry W, Stockfleth E: Human papillomavirus and overexpression of P16INK4a in nonmelanoma skin cancer. *Dermatol Surg*. 2004, 30:409-14.

80. Hafkamp HC, Speel EJ, Haesevoets A, Bot FJ, Dinjens WN, Ramaekers FC, Hopman AH, Manni JJ: A subset of head and neck squamous cell carcinomas exhibits integration of HPV 16/18 DNA and overexpression of p16INK4A and p53 in the absence of mutations in p53 exons 5-8. *Int J Cancer*. 2003, 107(3):394-400.

81. Klussmann JP, Gültekin E, Weissenborn SJ, Wieland U, Dries V, Dienes HP, Eckel HE, Pfister HJ, Fuchs PG: Expression of p16 protein identifies a distinct entity of tonsillar carcinomas associated with human papillomavirus. *Am J Pathol*. 2003, 162(3):747-53.

82. Shai A, Pitot HC, Lambert PF: p53 Loss synergizes with estrogen and papillomaviral oncogenes to induce cervical and breast cancers. *Cancer Res*. 2008, 68(8):2622-31.

Figure Legends

Figure 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).

Figure 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.

Case	Cancer family history*	Histotype	Stage	Grade	PAS	Alcian Blue	SMA	P16 (INKA4)	TP53	CK5	S100	ER	PR	Her2/ neu
A9-15y[1]	Yes	GRC	IV	G2	+	+	-	+	-	-	ND	-	-	-
A11-12y[2]	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

Table 1. Clinicopathological and immunohistochemical features of 5 juvenile breast cancer cases
*As reported by the patient; [1] mother: ovary, father: colon; [2] 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).

Case	*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

Table 2. Germline *BRCA1* and *BRCA2* variants identified in 5 juvenile breast cancer cases.
(P, polymorphism; Syn, synonymous; IVS, intron variant sequence; M, missense; UV, unverified variant).

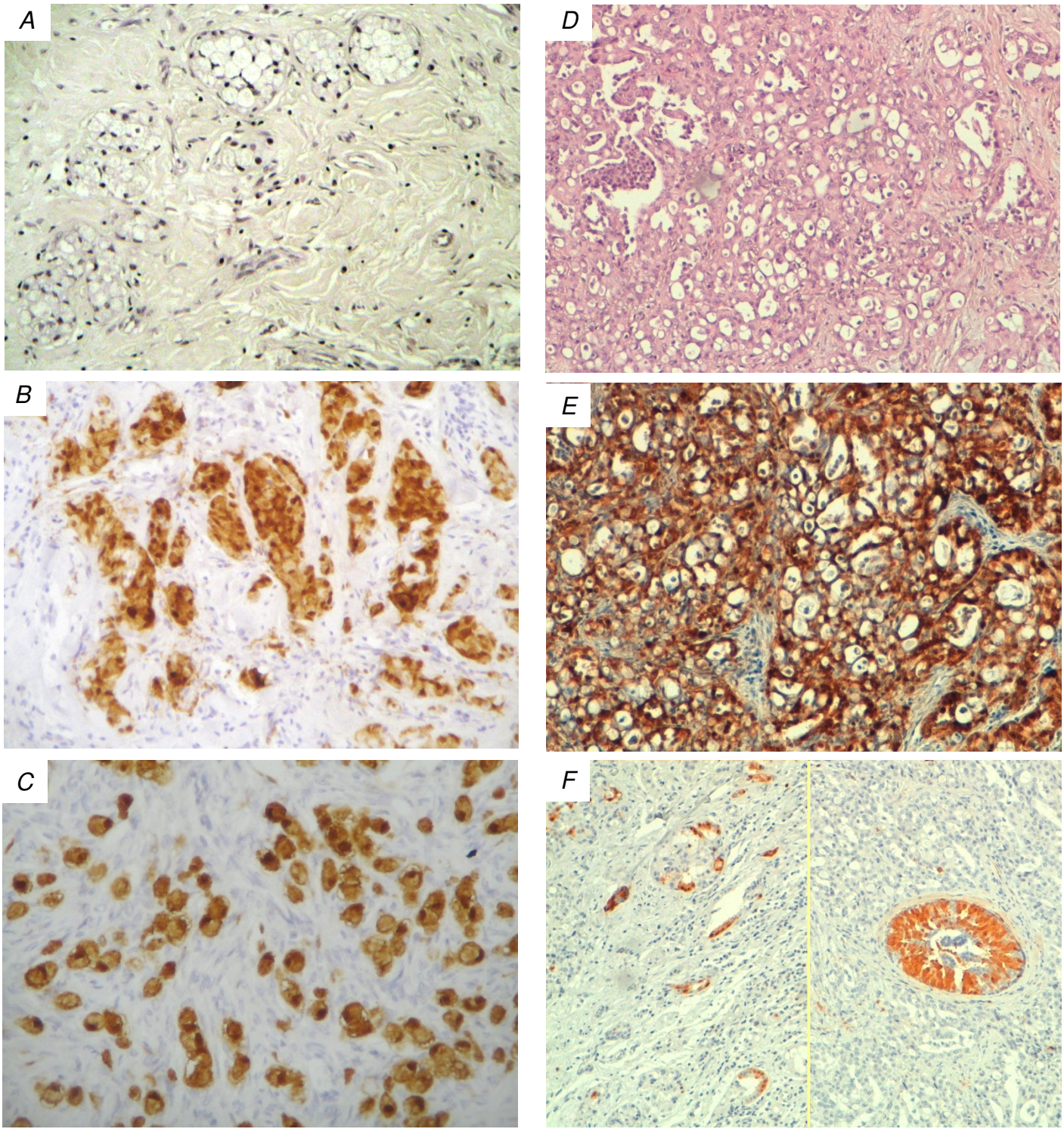
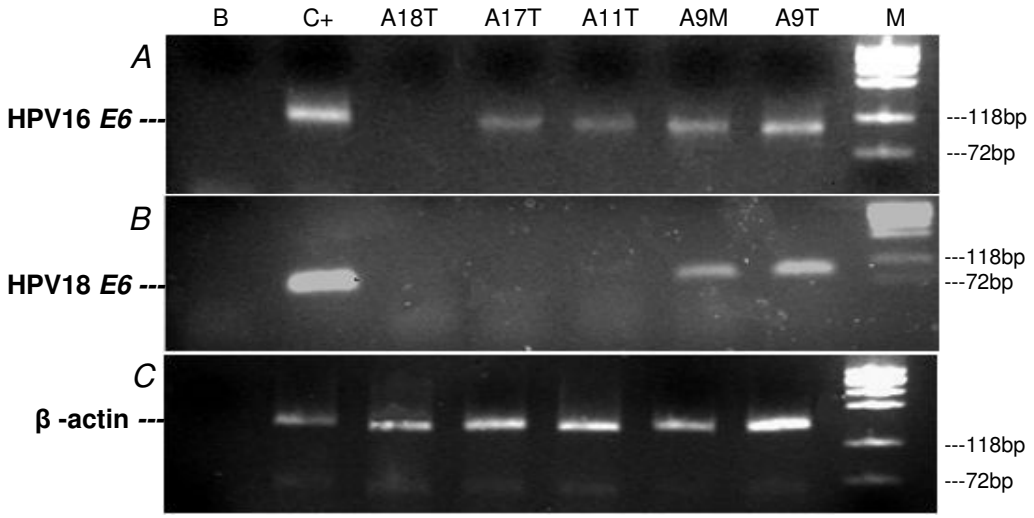
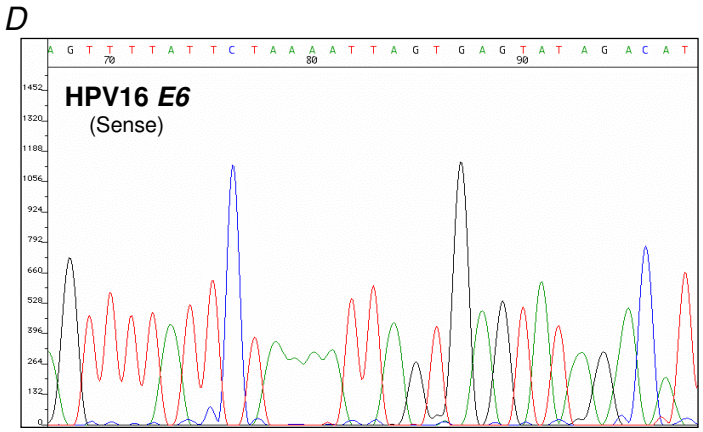


Fig.1

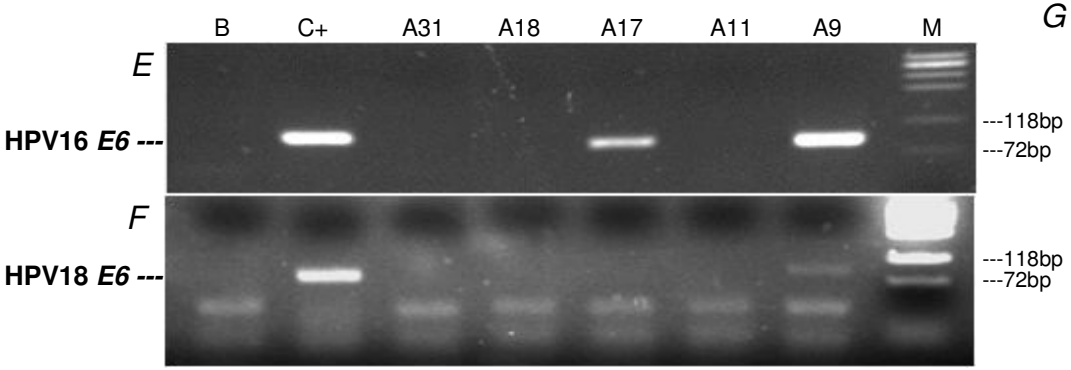
Figure 1

Tumors





Blood



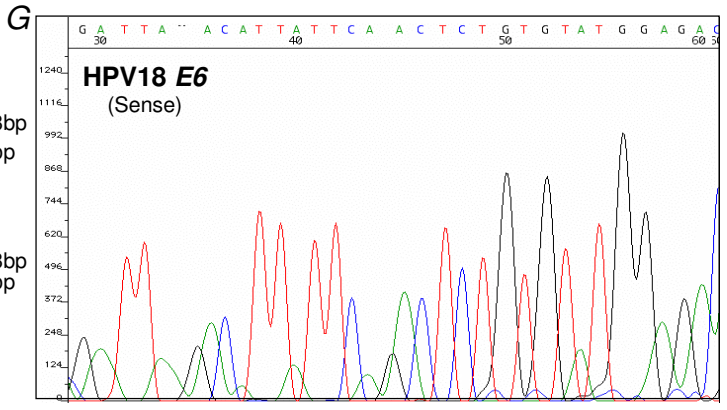


Figure 2

Additional files provided with this submission:

Additional file 1: supplement.pdf, 26K
http://breast-cancer-research.com/imedia/9437750472932872/supp1.pdf