Modern Pathology (2017) 30, 15–25

Molecular determination of the clonal relationships between multiple tumors in *BRCA1/2*-associated breast and/or ovarian cancer patients is clinically relevant

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Female *BRCA1/2* mutation carriers affected with breast and/or ovarian cancer may develop new tumor deposits over time. It is of utmost importance to know the clonal relationships between multiple tumor localizations, enabling differentiation between multiple primaries or metastatic disease with consequences for therapy and prognosis. We evaluated the value of targeted next generation sequencing in the diagnostic workup of *BRCA1/2* mutation carriers with ≥2 tumor localizations and uncertain tumor origins. Forty-two female *BRCA1/2* mutation carriers with ≥2 tumor localizations were selected. Patients with inconclusive tumor origin after histopathological revision were 'cases'; patients with certain tumor origin of ≥3 tumors served as 'controls'. Tumors of cases and controls were analyzed by targeted next generation sequencing using a panel including *CDKN2A*, *PTEN* and *TP53*, hotspot mutation sites for 27 different genes and 143 single nucleotide polymorphisms for detection of loss of heterozygosity. Based on prevalence of identical or different mutations and/or loss of heterozygosity patterns, tumors were classified as 'multiple primaries' or 'one entity'. Conventional histopathology yielded a conclusive result in 38/42 (90%) of patients. Four cases and 10 controls were analyzed by next generation sequencing. In 44 tumor samples, 48 mutations were found; 39 (81%) concerned *TP53* mutations. In all 4 cases, the intra-patient clonal relationships between the tumor localizations could be unequivocally identified by molecular analysis. In all controls, molecular outcomes matched the conventional histopathological results. In most *BRCA1/2* mutation carriers with multiple tumors routine pathology work-up is sufficient to determine tumor origins and relatedness. In case of inconclusive conventional pathology results, molecular analyses using next generation sequencing can reliably determine clonal relationships between tumors, enabling optimal treatment of individual patients.

Modern Pathology (2017) **30,** 15–25; doi:10.1038/modpathol.2016.145; published online 9 September 2016

Female *BRCA1/2* mutation carriers have a cumulative lifetime risk of developing breast cancer of 55–85% by the age of 70.[1–4] The cumulative lifetime risk of developing ovarian cancer varies between 15–60% for *BRCA1* and 10–35% for *BRCA2* mutation carriers.[1–4] Moreover, susceptibility for other cancers also seems to be increased in *BRCA1/2* mutation carriers.[5,6]

It has been reported that *BRCA1*-associated breast cancers more frequently develop visceral metastasis and fewer bone metastases[7,8] and *BRCA2*-associated breast cancers tend to develop more lymph node metastases compared with sporadic breast cancer.[8] Metastatic sites of sporadic ovarian cancer mostly confine to the intraperitoneal cavity,[9,10] whereas it

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Received 20 March 2016; revised 1 July 2016; accepted 4 July 2016; published online 9 September 2016

has been described that *BRCA1/2*-associated ovarian cancer patients frequently (74%) present with visceral metastases to liver, lung and spleen.[11] Although this can be of some help, the non-specific metastatic patterns in *BRCA1/2*-associated breast and ovarian cancer patients impede careful differentiation between breast cancer, ovarian cancer and other tumor origins when multiple cancer localizations occur in one patient. It is of clinical importance, however, to make this distinction, as it guides surgical and chemotherapeutic treatment and determines prognosis.[12,13]

A potentially helpful tool in determining clonal relationships between multiple tumors is DNA next Generation Sequencing.[14] With next generation sequencing, selected genes known to be frequently mutated in specific tumor types can be analyzed. Additionally, single nucleotide polymorphisms can be analyzed to detect any DNA copy number changes present in the tumor cells. Identical molecular aberrations of different tumor localizations indicate a common tumor origin (eg, metastatic disease), whereas different mutations and/or copy number changes in different tumor samples indicate two primary malignancies.

The aim of the current study was to evaluate the value of next generation sequencing in the diagnostic workup of *BRCA1/2*-associated breast and ovarian cancer patients with multiple tumor localizations.

Materials and methods

Patient Selection: Cases and Controls

Patients at increased risk of breast and/or ovarian cancer visiting the Family Cancer Clinic of the Erasmus Medical Center Cancer Institute for counseling and surveillance programs are registered in an institutional ongoing database. All women provide written informed consent for registration of their clinical data and storage of genetic material (if relevant) for research purposes. From this database, we selected all female germline *BRCA1* or *BRCA2* mutation carriers with ≥ 2 synchronous or metachronous tumor localizations of which tumor material had been obtained by fine needle aspiration (FNA), biopsy or surgical excision. Tumor localizations of which no suitable material was available for histopathological or molecular analysis were excluded. Included were *BRCA1/2* mutation carriers with multiple tumors of which at least one was located in the breast or ovary. Inclusion and exclusion criteria are depicted in Table 1.

If possible, the origin of the tumor localizations was identified based on H&E staining. If tumor histology did not provide a conclusive diagnosis, immunohistochemical staining was applied. Patients for whom the origin of one or more tumor localizations remained uncertain after histological and immunohistochemical evaluation were selected for next generation sequencing molecular analysis ('cases'). Patients with ≥ 3 tumor localizations of conclusive origin, based on histology and immunohistochemistry, served as 'controls'. Controls were selected for next generation sequencing, as well, to validate the versatility of the next generation sequencing approach for tumor clonality determinations.

Table 1 Inclusion and exclusion criteria

Inclusion
Women with a proven *BRCA1* and/or *BRCA2* mutation
With ≥ 2 synchronous or metachronous tumor localizations[a]
Tumor material available for next generation sequencing analysis (obtained by fine needle aspiration, histological biopsy or surgical excision)
One of the 4 clinical scenarios:
1. Breast cancer and ovarian cancer
2. Breast cancer and second other tumor
3. Ovarian cancer and second other tumor
4. Breast cancer, ovarian cancer, and third or additional other tumor localizations
Exclusion criteria for 'other tumor localization'
Hematological malignancies
Dermatological malignancies (ie. melanomas, basal cell carcinomas)
Ipsilateral lymph node metastases in the presence of breast cancer
Premalignant lesions, such as ductal carcinoma in situ
Contralateral breast cancer or second ipsilateral breast cancer, except in the presence of a third tumor localization
Peritoneal tumor localization in the presence of ovarian cancer
If reported that the ovarian cancer was growing per continuum into the peritoneal cavity
Confining to the ipsilateral adnexa

[a]Isolated site of invasive cancer as diagnosed by radiological examination, intra-operatively or during pathological examination.

Conventional Diagnostics

Tumor histology. Formalin-fixed paraffin-embedded tumor tissues were collected from the Department of Pathology of the Erasmus Medical Center Cancer Institute and from regional hospitals. Two pathologists specialized in breast and gynecological cancer (C.v.D., P.v.D.) independently reviewed haematoxylin and eosin (H&E) stained tissue sections of the tumor localizations for histology, with a subsequent consensus discussion.

Immunohistochemistry. Immunohistochemical tissue markers were chosen according to the institutional protocol and depended on clinical and histological differential diagnosis of the origin of the various tumor localizations. Estrogen receptor (ER) was used as a breast cancer marker. Immunohistochemical markers used for differentiation of ovarian cancer were cancer antigen 125 (CA125), Wilms' tumor 1 (WT1) and PAX-8, all known to be frequently expressed in ovarian cancer.[15–17] To differentiate with primary lung carcinoma, TTF-1 was used.[18]

Molecular Analysis

For cases and controls, p53 immunohistochemistry was performed on all tumor tissues if formalin-fixed paraffin-embedded tissue blocks were available. Nuclear expression of p53 in tumor cells was scored as either heterogeneous (no indication for *TP53* mutation), strong in all tumor cells (indication for missense *TP53* mutation) or absent in all tumor cells (indication for frameshift, nonsense, or splice site *TP53* mutation). For next generation sequencing analysis, normal and tumor tissues were manually microdissected from haematoxylin-stained tissue sections of formalin-fixed paraffin-embedded tissue blocks or if unavailable, from original routine H&E, immunohistochemical stained sections or cytological preparations. DNA was extracted using proteinase K and 5% Chelex resin, as previously described;[19] DNA concentrations were measured with the Qubit 2.0 Fluorometer. To assess the quality of DNA amplification a multiplex control PCR was performed as previously described;[20] PCR products were analyzed on an agarose gel. All DNA samples were screened with the Ion Torrent Personal Genome Machine, with supplier's materials and protocols (Life Technologies, Carlsbad, CA, USA). A custom made primer panel was used, designed using Ion AmpliSeq Designer 2.2.1, for diagnostic use in clonality determinations of various tumor types including breast and ovarian cancer. Because this panel was designed for analysis of a broad range of tumor types, it includes genes frequently mutated in breast and ovarian cancer, as well as genes rarely mutated in these tumors. The panel targets almost the entire open reading frame of *CDKN2A*, *PTEN* and *TP53* (coverage 95–99%), multiple hotspot mutation sites for 27 different genes and 143 single nucleotide polymorphisms at 15 different loci for the detection of loss of heterozygosity (see Supplementary Table 1 for primer details). In total, the panel consisted of 254 amplicons with a mean amplicon size of 160 base pairs. With this panel, libraries were created using the Ion AmpliSeq 2.0 Library Kit. Template was prepared using the Ion OneTouch 2 with the Ion OneTouch 200 Template Kit v2 DL or using the IonChef with the Ion Personal Genome Machine Hi-Q Chef Kit. Sequencing was performed on an Ion 318v2 chip with the Ion Personal Genome Machine sequencing 200 kit v2 or the Ion Personal Genome Machine Hi-Q sequencing kit. Data was analyzed with Variant Caller v4.0 or v.4.4.2.1. Annotation of the variants was previously described.[21] For mutation detection, all exonic and splice variants with a variant percentage $\geq 20\%$ were reported, excluding synonymous single-nucleotide variants and variants present in patient-matched normal tissue. Variants with a total coverage of <100 reads, reference coverage <10 reads, and/or a variant coverage of <5 reads for either the forward or reverse strand were excluded. For loss of heterozygosity analysis, single nucleotide polymorphisms with a total coverage of <100 reads or a strand bias (ratio forward:reverse reads not between 1:10 and 10:1 for reference and/or variant reads) were excluded. If a mutation was detected in one or more tumor samples of a patient, the specific locus was manually checked using the integrative genomics viewer (IGV) in normal DNA as well as all tumor samples of that patient. Furthermore, *TP53* was manually checked for mutations if no mutation was detected and immunohistochemistry showed aberrant staining or was unavailable.

Samples for which the control PCR showed no signal for amplicons larger than 100 base pairs and for which next generation sequencing analysis showed $<70\%$ of reads on target and/or $<70\%$ of amplicons with at least 100 reads were defined low quality samples. For low quality samples with more than 3 variants, we focused on variants present in other tumors of the patient, or if not present, on *TP53* variants. For all low quality samples, mutations were confirmed by Sanger sequencing or by a second next generation sequencing run. For Sanger sequencing, primers from the AmpliSeq design were extended with M13 tails. PCR protocol was previously described,[22] data was analyzed using Mutation Surveyor v.4.0 software (SoftGenetics).

Results

Patients

Fifty-six *BRCA1/2* mutation carriers with multiple tumor localizations were selected. Fourteen were excluded due to missing or unsuitable tumor material, leaving 42 women (39 *BRCA1*, 3 *BRCA2*) for analyses. Clinical classification of tumor origins was 'breast cancer+ovarian cancer' in 31 patients, 'breast cancer+other' in nine, 'ovarian cancer+other' in one, and 'breast cancer+ovarian cancer+other' in one woman (data not shown). Median number of tumor localizations was 2 (range 2–5), and median time from first to last cancer diagnosis was 5 years (range 0–23).

Conventional Diagnostics

For 21/42 women (50%) the origin of the tumor localizations was conclusive based on histology only. In an additional 17 (40%) a conclusive diagnosis was reached after immunohistochemistry for relevant markers. Ten of 38 women with conclusive outcomes based on histology and/or immunohistochemistry had ≥ 3 tumor localizations (controls; 8 *BRCA1* and 2 *BRCA2* mutation carriers).

In four women (10%) one or more tumor localizations remained of uncertain origin after histological and immunohistochemical evaluation (cases; all *BRCA1* mutation carriers).

Case no. 1 presented with tumors in the right and the left breast, and a tumor in the lung seven years

Table 2 Cases and controls: patient characteristics and outcomes of tumor histology, immunohistochemistry and molecular analysis

BRCA1/2+ Age	*Tumor sites analyzed, timeline (years)*			*Histology (H&E) with immunohistochemistry (IHC), if applicable*				*Molecular analysis*				*Agreement molecular analysis with histopathology*
				Conclusive[a]								
				H&E	*IHC*	*Diagnosis*[b]	*Tumor origin*	*p53 IHC*[c]	*Variants by next generation sequencing per tumor*	*Entity*[d]	*Agreement with loss of heterozygosity analysis*	
Case 1 *BRCA1+* 41 y	0	Right breast[e]	T1	+		Invasive ductal carcinoma	Breast	+	TP53 c.646G>A; p.V216M	**1**	+	NA
	0	Left breast	T2	+		Invasive ductal carcinoma	Breast	–	TP53 c.637C>T; p.R213*	**2**		
	+7	Lung[e]	T3	–	–	Non-small cell carcinoma	Unknown	+	TP53 c.646G>A; p.V216M	**1**		
Case 2 *BRCA1+* 55 y	0	Ovary	T1	+		Serous carcinoma	Ovary	–	TP53 c.158G>A; p.W53*	**1**	+/–	NA
	+6	Breast[e]	T2	–	ND[f]	Adenocarcinoma	Unknown	ND[f]	TP53 c.158G>A; p.W53*	**1**		
Case 3 *BRCA1+* 33 y	0	Breast[e]	T1	+		Invasive ductal carcinoma	Breast	ND[f]	TP53 c.686_687del; p.C229fs*10	**1**	NE	NA
	+10	Ascites[e]	T2	–	–	Adenocarcinoma	Unknown	ND[f]	TP53 c.527G>A; p.C176Y	**2**		
Case 4 *BRCA1+* 38 y	0	Breast	T1	+		Invasive ductal carcinoma	Breast	+	TP53 c.318_326delinsAAA; p.S106_F109delinsRN	**1**	+	NA
	+3	Retroperitoneal lymph node	T2	–	ND[f]	Large cell carcinoma	Unknown	ND[f]	TP53 c.514G>T; p.V172F	**2**		
	+3	Ovary & uterus	T3	+		Serous carcinoma	Ovary	+	TP53 c.514G>T; p.V172F	**2**		
Control 1 *BRCA1+* 38 y	0	Right breast[e]	T1	+		Invasive ductal carcinoma	Breast	–	No mutations	**1**	+	+
	+13	Right breast	T2	+		Invasive ductal carcinoma	Breast	+	TP53 c.722C>T; p.S241F PTEN c.176C>G; p.S59*	**2**		
	+19	Ovary	T3	–	+	Serous carcinoma	Ovary	+	TP53 c.400T>G; p.F134V	**3**		
Control 2 *BRCA1+* 49 y	0	Right breast[e]	T1	+		Invasive ductal carcinoma	Breast	+	No mutations	**1**	NE	+
	+2	Adnexa	T2	–	+	Serous carcinoma	Ovary	+	TP53 c.645T>G; p.S215R	**2**		
	+23	Left breast	T3	+		Invasive ductal carcinoma	Breast	+/–	PIK3CA c.3140A>G; p.H1047R STK11 c.484G>A; p.D162N	**3**		
Control 3 *BRCA1+* 37 y	0	Breast	T1	+		Invasive ductal carcinoma	Breast	+	TP53 c.817C>T; p.R273C	**1**	+/–	+
	+2	Ovary	T2	–	+	Serous carcinoma	Ovary	–	TP53 c.406C>T; p.Q136*	**2**		
	+5	Ovary & uterus (+inguinal & cervical lymph nodes)	T3	–	+	Serous carcinoma	Ovary	–	TP53 c.406C>T; p.Q136*	**2**		
Control 4 *BRCA2+* 60 y	0	Larynx[e]	T1	+		Squamous cell carcinoma	Larynx	–	TP53 c.375_375+1delinsTT	**1**	+/–	+
	+2	Lung	T2	–	+	Adenocarcinoma	Lung	+	TP53 c.814G>T; p.V272L BRAF c.1405_1406delinsTT; p.G469L	**2**		
	+5	Uterus & omentum	T3	+		Serous carcinoma	Ovary	–	TP53 c.528C>A; p.C176*	**3**		
	+9	Breast	T4	+		Invasive ductal carcinoma	Breast	+/–	PIK3CA c.3140A>G; p.H1047R	**4**		
Control 5 *BRCA1+* 37 y	0	Breast	T1	+		Invasive ductal carcinoma	Breast	+	TP53 c.743G>A; p.R248Q	**1**	+/–	+
	+6	Ovary	T2	+		Serous carcinoma	Fallopian tube	–	TP53 c.395del; p.K132fs*38	**2**		
	+7	Omentum	T3	+		Serous carcinoma	Fallopian tube	–	TP53 c.395del; p.K132fs*38	**2**		

Table 2 (Continued)

BRCA1/2+ Age		*Tumor sites analyzed, timeline (years)*		*Histology (H&E) with immunohistochemistry (IHC), if applicable*				*Molecular analysis*			*Agreement molecular analysis with histopathology*
				Conclusive[a]							
				H&E	*IHC*	*Diagnosis*[b]	*Tumor origin*	*p53 IHC*[c]	*Variants by next generation sequencing per tumor*	*Entity*[d]	*Agreement with loss of heterozygosity analysis*
Control 6 *BRCA1+* 41 y	0	Breast	T1	+		Invasive ductal carcinoma	Breast	+	TP53 c.743G>A; p.R248Q	**1**	+	+
	0	Cervix	T2	–	+	Adenocarcinoma	Genital tract	–	TP53 c.721del; p.S241fs*6 CAPZB c.491C>A; p.T164N	**2**		
	+1	Uterus	T3	–	+	Serous carcinoma	Genital tract	–	TP53 c.721del; p.S241fs*6 CAPZB c.491C>A; p.T164N	**2**		
	+1	Omentum	T4	–	+	Serous carcinoma	Genital tract	–	TP53 c.721del; p.S241fs*6	**2**		
Control 7 *BRCA1+* 50 y	0	Ovary	T1	+		Serous carcinoma	Ovary	+	TP53 c.722C>A; p.S241Y	**1**	+	+
	+4	Breast	T2	+		Invasive ductal carcinoma	Breast	+/–	No mutations	**2**		
	+4	Rectosigmoid	T3	+		Serous carcinoma	Ovary	+	TP53 c.722C>A; p.S241Y	**1**		
Control 8 *BRCA1+* 42 y	0	Ovary	T1	+		Serous carcinoma	Ovary	+	TP53 c.818G>T; p.R273L	**1**	+	+
	0	Omentum	T2	+		Serous carcinoma	Ovary	+	TP53 c.818G>T; p.R273L	**1**		
	0	Breast	T3	–	+	Invasive ductal carcinoma	Breast	ND[f]	TP53 c.524G>A; p.R175H	**2**		
	+1	Abdominal wall (scar)[e]	T4	+		Serous carcinoma	Ovary	ND[f]	TP53 c.818G>T; p.R273L	**1**		
	+1	Pleural effusion	T5	–	+	Serous carcinoma	Ovary	+	TP53 c.818G>T; p.R273L	**1**		
Control 9 *BRCA2+* 59 y	0	Breast	T1	+		Invasive ductal carcinoma	Breast	–	TP53 c.327_328dup; p.R110fs*14	**1**	+	+
	+2	Ovary[e]	T2	–	+	Serous carcinoma	Ovary	–	TP53 c.112del; p.Q38fs*6 FBXW7 c.1347G>C; p.E449D	**2**		
	+2	Uterus	T3	–	+	Serous carcinoma	Ovary	–	TP53 c.112del; p.Q38fs*6 FBXW7 c.1347G>C; p.E449D	**2**		
Control 10 *BRCA1+* 45 y	0	Breast	T1	+		Metaplastic carcinoma	Breast	+	TP53 c.488A>G; p.Y163C	**1**	+	+
	+6	Adnexa	T2	+		Serous carcinoma	Ovary	+	TP53 c.524G>A; p.R175H	**2**		
	+6	Rectouterine pouch	T3	+		Serous carcinoma	Ovary	+	TP53 c.524G>A; p.R175H	**2**		

Abbreviations: H&E: haematoxylin and eosin stained slides; IHC: immunohistochemical analysis; NA: not applicable; ND: not done; NE: not evaluable.
[a]Conclusive diagnosis, based on tumor histology (H&E) and immunohistochemistry, if applicable: yes (+) or no (–).
[b]Based on tumor histology (H&E) and immunohistochemistry if applicable.
[c]Nuclear expression of p53 in tumor cells was scored as either heterogeneous (+–), strong in all tumor cells (+) or absent in all tumor cells (–).
[d]Entity: tumor or tumors most probably of the same origin (clonally identical). 1 is one independent entity, 2 is a second independent entity, etc. Various tumors that form one entity may represent advanced disease, cancer relapse, or distant metastases.
[e]Low quality sample.
[f]No formalin-fixed paraffin-embedded tissue block available or no tissue left in the formalin-fixed paraffin-embedded tissue block.

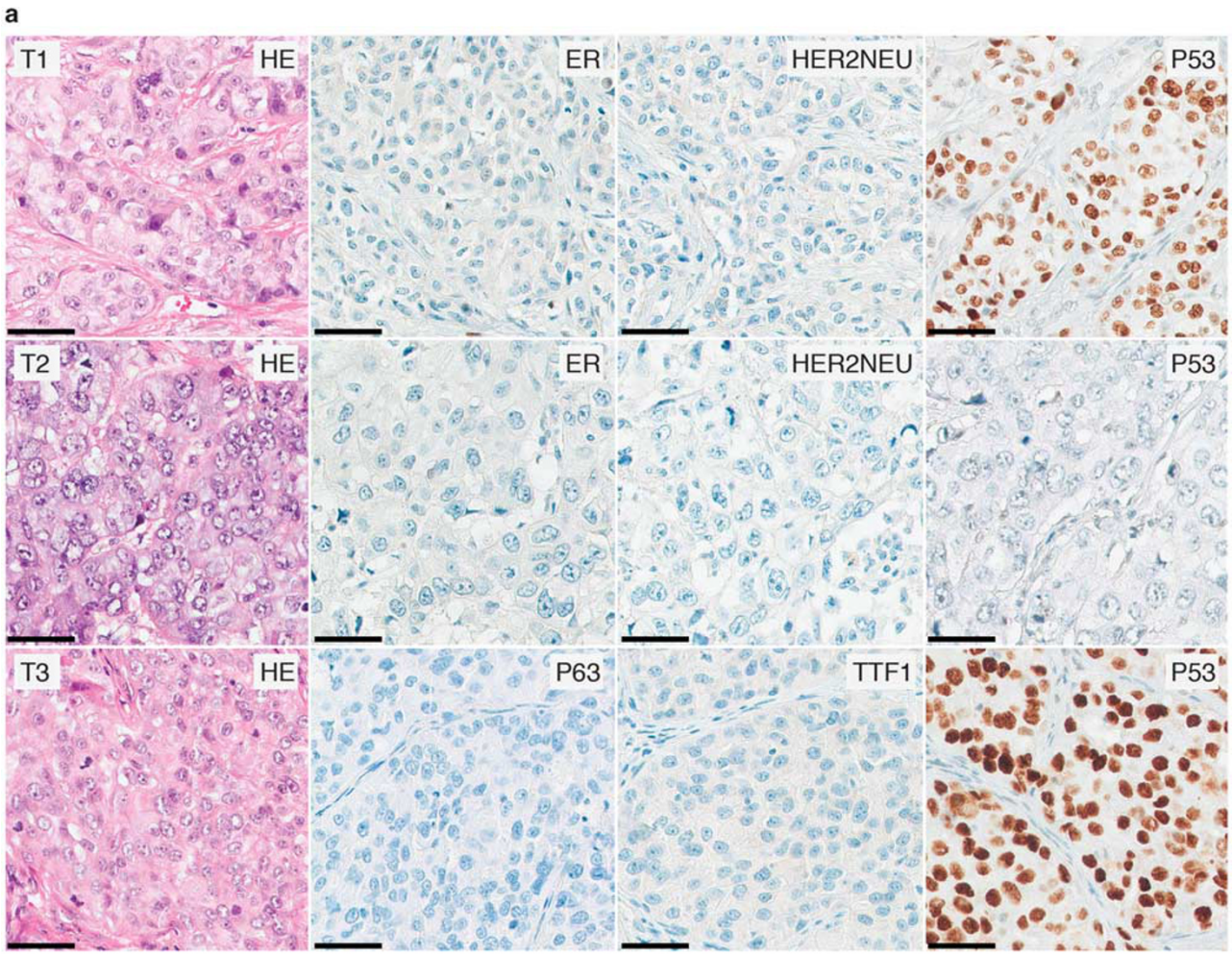

Figure 1 Conventional diagnostics and molecular analysis results for case no. 1. Case no. 1 presented with tumors of the right (T1) and the left (T2) breast, and a tumor in the lung 7 years later (T3). (**a**). Both tumors in the breast (T1 and T2) could be conclusively diagnosed invasive ductal carcinoma of the breast based on haematoxylin and eosin (H&E) stainings only. Additionally, ER and HER2NEU stainings are shown, which were negative in both tumors. Conclusive diagnosis regarding the origin of the non-small cell carcinoma in the lung (T3) based on HE stainings and immunohistochemistry (P63 and TTF1 both negative) was not possible. As part of the molecular analysis p53 immunohistochemistry was performed, showing strong nuclear expression in the tumor cells of T1 and T3, and absent expression in the tumor cells of T2. Scale bars represent 50 μm. (**b**). Targeted next generation sequencing results of *TP53* exon 6 for DNA isolated from normal and tumor tissues of the patient. Each grey line represents an individual read; only aberrations from the wildtype sequence are indicated. Sequencing results are shown in reverse complement, which means that TCG is actually CGA. T1 and T3 show an identical *PT53* missense mutation (c.646G>A; p.V216M), whereas T2 shows a different *TP53* nonsense mutation (c.637C>T; p.R213*). (**c**). Loss of heterozygosity was analyzed using single nucleotide polymorphisms, the variant allele frequencies of 17 single nucleotide polymorphisms at 5 different loci (chromosome 8p, *PTEN*, *BRCA2*, *BRCA1* and *SMAD4*) are shown for the three tumor samples. Loss of the reference allele is indicated in red and loss of the variant allele in green; a more intense color (either red or green) indicates a higher tumor percentage. As expected for a *BRCA1* germline mutation carrier, all tumor samples show loss of the same *BRCA1* allele. For all other loci shown, T1 and T3 show corresponding loss of heterozygosity patterns (both tumors show either red or green), whereas T2 shows a different loss of heterozygosity pattern. Chr: Chromosome.

later. Both breast tumors were diagnosed IDC of the breast based on HE staining. The lung tumor was diagnosed non-small cell carcinoma, however, conclusive diagnosis regarding the origin of the tumor was not possible based on HE and immunohistochemistry (see Figure 1a for details).

Case no. 2 presented with a tumor in the ovary and a tumor in the breast six years later. The tumor of the ovary was diagnosed serous carcinoma of the ovary based on HE staining. The breast tumor was diagnosed adenocarcinoma based on cytological preparations; however, no tissue was available for immunohistochemistry. Therefore, tumor origin could not be determined.

Case no. 3 presented with a tumor in the breast and peritonitis carcinomatosa 10 years later. The tumor in the breast was diagnosed IDC of the breast based on HE staining. The tumor cells found in the ascites were diagnosed adenocarcinoma based on cytological preparations. CA-125 and WT-1 immunohistochemistry performed on de-stained cytological preparations was not conclusive, therefore,

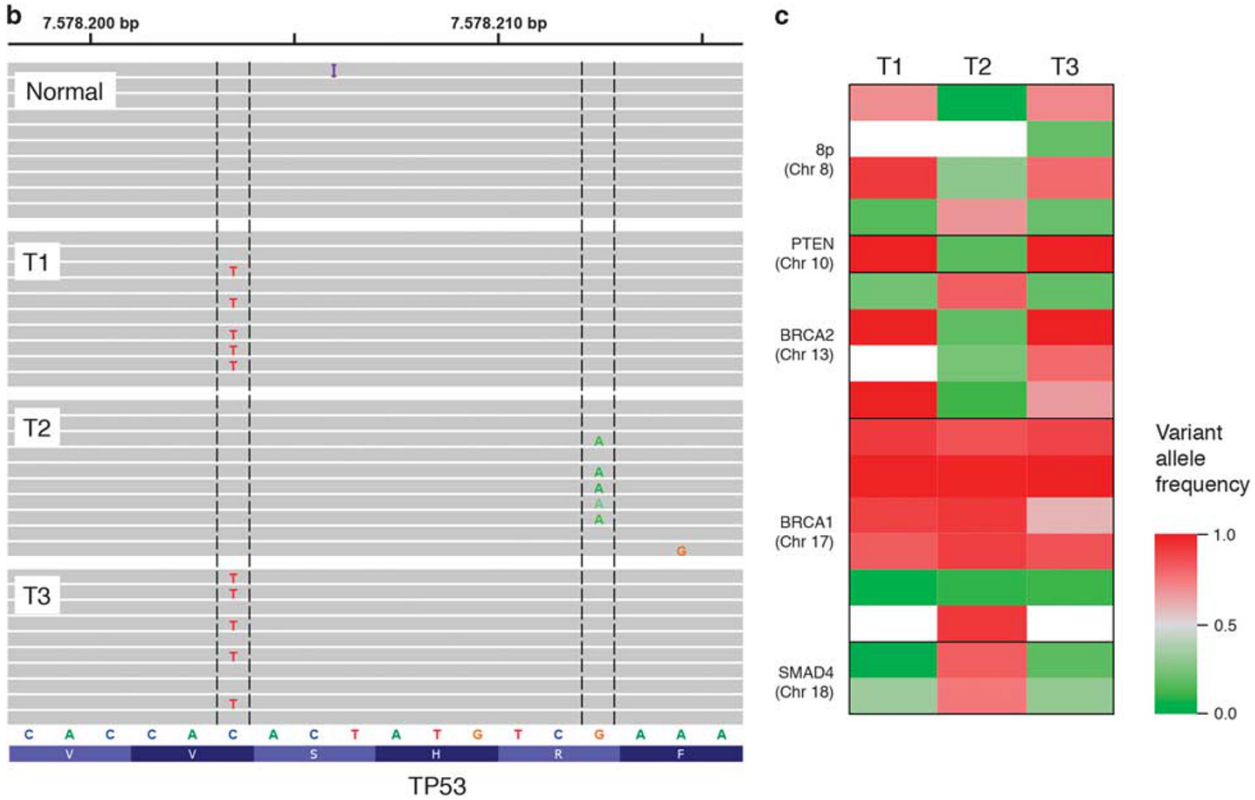

Figure 1 (Continued).

determining the site of the origin of this tumor was not possible.

Case no. 4 presented with a tumor in the breast and tumors in the retroperitoneal lymph nodes as well as in the ovary and uterus three years later. The tumor in the breast was diagnosed IDC of the breast and the tumor in the ovary and uterus serous carcinoma of the ovary, both based on HE staining. The tumor in the retroperitoneal lymph nodes was classified as a large cell carcinoma based on the HE staining. However, only a small biopsy was available, from which no tissue was left in the formalin-fixed paraffin-embedded tissue block for additional analyses.

Characteristics and outcomes of tumor histology and immunohistochemistry of cases and controls are outlined in Table 2. Median age at first cancer diagnosis was 41.5 years (range 33–59). Median year of first cancer diagnosis was 1997 (range 1983–2012). Clinical classification of tumor origins was breast cancer+ovarian cancer in 11, breast cancer+other in two, and breast cancer+ovarian cancer+other in one woman.

Molecular Analysis

Outcomes of molecular analysis are depicted in Table 2. The formalin-fixed paraffin-embedded tissues used for DNA isolation were relatively old, ranging from 2 to 32 years old at time of isolation. Six out of 38 (16%) DNA samples isolated from formalin-fixed paraffin-embedded tumor tissue were of low quality (see Supplementary table 2 for quality parameters). For 6 tumors no formalin-fixed paraffin-embedded tissue was available and DNA was isolated from original routine HE and/or immunohistochemical sections or from cytology preparations. Four out of 6 (67%) DNA samples isolated from original sections were of low quality.

In total, 167 tumor-specific variants were detected in the 44 analyzed tumors (Supplementary table 3). Up to 27 variants were detected in the low quality tumor samples, compared to only 1 or 2 variants for good quality tumor samples. Additionally, some multinucleotide changes were incorrectly reported as 2 or 3 separate variants. Finally, 48 mutations were either detected in good quality samples or confirmed in low quality samples. In the majority of tumors ($n = 34$, 77%), one mutation was found; 7 tumors harbored two mutations. Thirty-nine (81%) of all 48 variants concerned a mutation in the *TP53* gene; in 39 of the 44 tumors (89%) a *TP53* mutation was found. Other variants included *PTEN*, *PIK3CA* and *STK11* mutations in tumors located in the breast; a *CAPZB* mutation in tumors in the uterus and cervix; a *FBXW7* mutation in tumors in the ovary and uterus; and a *BRAF* mutation in a lung lesion (Table 2). Parallel to molecular analysis, p53 immunohistochemistry was conducted and showed results consistent with molecular outcomes (Table 2). In two low quality samples with aberrant p53 staining, no

Figure 2 Loss of heterozygosity analysis. Variant allele frequencies for single nucleotide polymorphisms at 15 different loci on 11 different chromosomes (indicated on the y-axis) for the tumors samples of all patients analyzed are shown. The variant allele frequencies for the different single nucleotide polymorphisms are indicated by different colors. The example (bottom right) shows an A/T single nucleotide polymorphism, A representing the reference allele and T the variant allele. For any informative single nucleotide polymorphism without loss of heterozygosity, a variant allele frequency of 0.5 is expected (grey). If there is loss of the reference allele, a variant allele frequency >0.5 is expected (red). Alternatively, loss of the variant allele would result in a variant allele frequency <0.5 (green). A more intense color, either red or green, represents a variant allele frequency deviating further from 0.5, indicating a higher tumor percentage. Regardless of the actual nucleotides, green represents the reference allele and red the variant allele for all single nucleotide polymorphisms. Non-informative single nucleotide polymorphisms or single nucleotide polymorphisms with a strand bias or coverage <100 reads are not shown. If multiple tumors of a patient show largely concordant loss of heterozygosity patterns (all tumors show either red or green), this indicates that these tumors are most likely clonally related. Alternatively, differences in the loss of heterozygosity patterns between multiple tumors of one patient indicate multiple primary tumors. Twelve patients (all patients except control no. 4 and 9) are *BRCA1* mutation carriers. 10/12 patients show a concordant loss of heterozygosity pattern for the *BRCA1* locus in their multiple tumors. Control no. 2 shows an equivocal loss of heterozygosity pattern, which is probably due to the low quality of the data. For case no. 3 only 1 informative marker is available which does not show clear loss of heterozygosity for T1 (variant allele frequency of 0.41). Control no. 4 and 9 are *BRCA2* mutations carriers. Control no. 9 shows a concordant loss of heterozygosity pattern for the *BRCA2* locus for the three analyzed tumor samples. Control no. 4 shows a concordant loss of heterozygosity pattern for samples T2 and T3, a different loss of heterozygosity pattern for sample T1 and no loss of heterozygosity for T4. Chr: Chromosome; LOH: loss of heterozygosity.

TP53 mutation was detected, probably due to insufficient coverage of *TP53* (<100 reads for 8/19 amplicons for control no. 2, T1) or the type of *TP53* mutation (possible intronic mutation or homozygous deletion for control no. 1, T1).

As further shown in Table 2, based on the molecular analysis, all tumor localizations analyzed could be classified into one or more entities concerning their origins. Additional loss of heterozygosity analyses of the 143 single nucleotide polymorphisms at 15 different loci were confirmative of the classifications made in 8/14 patients (Figure 2;+ Supplementary Table 4 showing all single nucleotide polymorphism data). In the group of cases, where conventional histology and immunohistochemistry were not conclusive, molecular outcomes were decisive for all tumors (see Figure 1 for an example). In the group of controls, all molecular outcomes matched the diagnosis given by conventional histopathological diagnostics.

Discussion

For 38/42 (90%) *BRCA1/2* mutation carriers with multiple tumor localizations, conventional histopathological analyses (histology, immunohistochemistry) were sufficient to determine tumor origins. Results obtained by next generation sequencing provided decisive information in all four cases with inconclusive results from conventional diagnostics, enabling accurate differentiation between a second

primary or metastatic cancer. Next generation sequencing conducted on 10 control cases with ≥ 3 tumor localizations, unequivocally showed the same results as obtained by conventional histopathology, and indicate that next generation sequencing analysis of multiple tumors within one patient is a versatile procedure to determine clonal relationships between the lesions. Next generation sequencing analysis can be useful in case of ambiguous histopathology results, or if no formalin-fixed paraffin-embedded tissue block is available for immunohistochemistry.

As an illustration, the results of two patients are discussed below. First, case no. 2 comprises ovarian cancer followed by thoracic wall and axillary lymph node metastases three years later. There were no signs of breast cancer, suggesting that the ovarian cancer had metastasized to the thoracic wall and the axilla. After another three years, synchronously with progressive metastatic disease, a small breast cancer was detected. After extensive diagnostic work-up it was concluded that thoracic wall and axillary lesions actually were metastases of this formerly subclinical primary breast cancer and the patient was treated accordingly. However, retrospectively, our findings of identical *TP53* variants in the ovarian cancer and breast cancer strongly suggest that the breast cancer was actually metastatic ovarian cancer. Unfortunately, no suitable material of the thoracic wall and axillary lesions was left for molecular analysis in this study. Since the primary tumor origin determines the therapy of choice for metastatic disease, it is essential to have no doubt about the origin of the metastases. The above-mentioned case is an example of how next generation sequencing can be decisive.

Second, control no. 1 comprises two ipsilateral breast cancers with a 13-year interval, both classified as invasive ductal carcinoma by histopathology, and ovarian cancer 6 years later. Histopathological analysis is not always able to differentiate between local recurrent and second primary breast cancer. The location of the breast cancer may help, but in this case, the first breast cancer was located in the medial upper quadrant while the second breast cancer was located centrally, leaving both options open. Some data suggest that *BRCA1/2* mutation carriers, especially when young (<40 years), show longer intervals to local recurrent breast cancer.[23,24] However, since the prognosis of a second ipsilateral breast cancer occurring <5 years is worse than after >5 years, late-recurring breast cancer are probably more often second primary tumors[25,26] and it is justifiable that they are treated accordingly. It is likely that the recurrent breast cancer after 13 years in this case was a second primary breast cancer. Molecular analysis confirmed that these tumors were two different entities.

Loss of heterozygosity -patterns were supportive of the results obtained by variant analysis in more than half of cases and controls (Figure 2). Almost all cases and controls showed corresponding loss of heterozygosity of *BRCA1* or *BRCA2* in all tumors, representing the 'second hit' of the functioning *BRCA* wild-type allele. For *BRCA1* mutation carriers, exceptions were case no. 3 with no clear loss of heterozygosity of *BRCA1* for the breast cancer and control no. 2 with no evaluable loss of heterozygosity results. For *BRCA2* mutations carriers, an exception was control no. 4 with four primary tumors showing loss of one allele of *BRCA2* in the larynx tumor, loss of the other allele in both the lung tumor and the uterus/omentum tumor, and no loss of *BRCA2* in the breast tumor. So far, *BRCA2* mutation carriers are not associated with elevated risk of lung cancer and an increased risk of laryngeal carcinoma seems improbable.[27–30] Additional Sanger sequencing showed loss of the mutated *BRCA2* allele for the tumor located in the larynx and loss of the wild-type allele for the lung lesion and the uterus/omentum tumor localizations (data not shown). The laryngeal carcinoma therefore is most likely a sporadic tumor. Loss of the wild-type *BRCA2* allele in the lung tumor may indicate either sporadic or *BRCA2*-related carcinogenesis. Furthermore, it has been described that loss of heterozygosity causes the second hit in only 80% of *BRCA1*-associated and in 60–70% of *BRCA2*-associated breast cancer,[31,32,33] fitting with the fact that we did not find (clear) loss of heterozygosity in two breast tumors. Possible alternative 'second hit' mechanisms include mutations and deletions of the wild-type allele. Epigenetic silencing as a second hit, to our knowledge, is rare in germline *BRCA1/2* mutation carriers and therefore not a plausible explanation.[31]

The diagnostic panel used in this study covered the exonic regions of the genes *CDKN2A*, *PTEN* and *TP53* almost completely, multiple hotspot mutation sites for 27 genes, and single nucleotide polymorphisms (Supplementary Table 1). In the majority of cases and controls a conclusive diagnosis concerning tumor site clonality could be made based on different or similar *TP53* variants. A *PTEN* mutation was only found once and none of the tumors harbored *CDKN2A* mutations. Up to 97% of all high grade serous ovarian cancer, typically occurring in *BRCA1/2* germline mutation carriers, harbor somatic *TP53* mutations.[12,34] *TP53* is affected in 16 to 84% of *BRCA1/2*-associated breast cancer, and in up to 97% of *BRCA1*-associated basal-like breast cancer.[35,36] Our finding of *TP53* mutations in 93% of all tumors (39/44 confirmed and 2/44 based on p53 immunohistochemistry) is in line with the high percentages found in the literature. It suggests that molecular diagnostic workup may simply consist of *TP53* analysis, rather than next generation sequencing of an entire panel. However, in two tumors without *TP53* mutations, we found mutations in other genes (*PIK3CA* and *STK11*), providing also a conclusive diagnosis for these tumor localizations. Additionally, loss of heterozygosity analysis was not only confirmative of the classifications made for most of the patients, but was also helpful if 'hotspot' *TP53* mutations were found. An example is control no. 10, for which both T2 and T3 harbor a *TP53 R175H*

mutation. Since according to somatic mutation databases this is a common *TP53* mutation these tumors potentially could still be different primary tumors. However, because loss of heterozygosity patterns were identical, we were able to reliably classify these tumors as one entity.

Immunohistochemical tissue markers were chosen according to institutional protocol depending on clinical and histological differential diagnosis of the tumor origin. Various different immunohistochemical markers of breast cancer have been investigated, such as GATA3, GCDFP, mammaglobin and SOX10. Although of potential value for differentiating breast cancer, as yet, their applicability seems limited or has not been validated well enough in triple negative breast cancer.[37–39]

A limitation of our study was that 10/44 tumor samples analyzed with next generation sequencing were of low quality, mostly due to fixation artefacts or a low amount of starting material, resulting in less reliable variant calling. Variants in low quality samples were therefore confirmed by Sanger sequencing or by a second next generation sequencing run. Furthermore, loss of heterozygosity analysis of these samples was difficult, resulting in non-evaluable loss of heterozygosity data in two patients with one or more tumor samples of low quality. Nevertheless, using a combined approach of multiple molecular analyses resulted in reliable classification of the tumors into one or more entities for all patients. Another limitation was that, due to the specific selection criteria, the study sample size was small.

In conclusion, during diagnostic workup of *BRCA1/2*-associated breast cancer and ovarian cancer patients with multiple tumor localizations, analysis of tumor histology and immunohistochemistry by a specialized pathologist may be sufficiently conclusive in most cases. However when routine pathology is inconclusive, molecular analysis using next generation sequencing can reliably determine the relationships between the tumor localizations and as such guide the most appropriate treatment for each individual patient.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on *Modern Pathology* website (http://www.nature.com/modpathol)