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Original Paper

Loss of Heterozygosity of *BRCA1*, *TP53* and *TCRD* Markers Analysed in Sporadic Endometrial Cancer

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Genetic alterations of tumour suppressor genes, for which loss of heterozygosity (LOH) is one mechanism of gene inactivation, are important steps in the development of endometrial cancer. To investigate the clinical relevance of LOH of *BRCA1* (17q21), *TP53* (17p13) and *TCRD* (14q11) in endometrial cancer, polymerase chain reaction (PCR)-based fluorescent DNA technology for the detection of microsatellite polymorphisms was applied. One hundred and thirteen archival endometrial cancer samples with matched normal tissues were examined. Allele loss at three loci were correlated with age, tumour size, lymph node status, metastases, stage, histological types, grade, expression of oestrogen receptor (ER) and progesterone receptor (PgR), family history of cancer, previous history of cancer or precursor lesions, and previous history of hormone replacement therapy (HRT). LOH for *BRCA1* was detected in 18.1%, of *TP53* in 26.9%, and of *TCRD* in 26.3% of informative cases. LOH of *BRCA1* correlated with medium grade, positive ER status, and family history of cancer; LOH of *TP53* correlated with younger age, high grade, positive PgR status, and with tumours from patients without HRT; LOH of *TCRD* correlated only with family history of cancer. LOH at all three loci correlated only with grade and positive family history. Allele loss of one of the three tumour suppressor loci did not correlate with disease-free survival (DFS), but LOH of *BRCA1* correlated significantly with decreased overall survival (OS). The latter, together with the correlation of LOH of *BRCA1* locus with steroid hormone receptor expression, might give a hint to the potential involvement of the co-localised 17β-hydroxysteroid dehydrogenase (HSD) gene in the development of endometrial cancer. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: *BRCA1*, *TP53*, *TCRD*, endometrial cancer, LOH
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INTRODUCTION

ENDOMETRIAL CANCER is the most common female genital cancer, with a significant increase in the cause-specific death rate over the last few years, which may reflect the aging population [1, 2]. According to the FIGO classification (1988), the staging of endometrial cancer is based upon surgical findings. The three major criteria for therapeutic decisions include myometrial invasion, tumour grading and extrauterine tumour spread [3, 4]. The search for prognostic and/or predictive factors in endometrial cancer include DNA ploidy and S-phase fraction [5, 6] and expression of the steroid hormone receptors [7–9], the epidermal growth factor receptor (EGFR), the Her-2/neu protein and the p53 protein [10–15]. In these studies, tumours with high FIGO classification showed decreased oestrogen receptor (ER) and progesterone receptor (PgR) [7, 8] and increased EGFR, Her-2/neu, and p53 expression [6, 11–15]. By univariate or multivariate analyses, none of these parameters proved to be more relevant than the three major prognostic parameters. Cytogenetic and molecular genetic analyses of endometrial cancer samples have found amplification of the *Her-2/neu* gene

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[10, 11], mutations in the *p53* gene [14–16], microsatellite instability and mutations in the c-Ki-*Ras* gene [17–19], deletions of the *DCC* gene [20], and loss of heterozygosity (LOH) at chromosomes 1p, 3p, 8p, 9p, 10q, 14q, 16q, 17q, and 18q [16, 21–23]. The main focus of these studies was mostly directed towards their relevance for tumour development and their role in the multistep carcinogenesis of endometrial cancer. They encompass a small number of cases and do not focus on prognostic value or correlations with histological or clinical parameters.

Evidence for the involvement of a tumour suppressor gene (TSG) in tumour development is based on allelic loss at the TSG locus, the second step of Knudson's model of TSG inactivation. Prognostic use of the assessment of allelic loss of TSGs has been limited by the position and frequency of heterozygosity when classical restriction length polymorphisms (RFLP) are used. Because of their abundance, polymorphic nature and amenability to amplification by polymerase chain reaction (PCR), short tandem repeats (STR) are much better markers for genomic mapping and genetic linkage analysis. STRs provide a source of highly informative loci for use in the identification of individual allele patterns. The ability to resolve PCR products differing in size by just 1 base on polyacrylamide gels allows precise allele determination, even though enzyme slippage during amplification may result in artificial stutter bands. Still, the ability to amplify multiple loci by PCR using different fluorescent primers in a single reaction combined with direct detection of the fluorescent-labelled PCR products on polyacrylamide gels makes STR DNA profiling amenable to automated fluorescent DNA technology.

For endometrial cancer, the clinical assessment of allelic loss of three genes seems to be of interest: (1) the *BRCA1* gene located on chromosome 17q21 [24], a region which also includes the 17β-hydroxysteroid dehydrogenase 2 gene (*EDH17B2*), one of the key enzymes in oestrogen metabolism [25, 26]; (2) the *TP53* gene located on chromosome 17p13, for which mutations and protein overexpression have already been described [13–15]; and (3) the T-cell antigen receptor (*TCRD*) gene located on chromosome 14q11, a locus which had shown the highest loss rate in an allelotyping study of sporadic endometrial cancers [21]. To get insight into the putative biological role and the potential prognostic or predictive value of *BRCA1*, *TP53* and *TCRD* in sporadic endometrial cancer, LOH of these three genes was analysed using a technique based on PCR amplification of STR and fluorescent DNA technology.

MATERIALS AND METHODS

Patients' characteristics, surgical procedures, and adjuvant therapy

The study included 113 patients, who were treated for primary endometrial cancer in the Department of Gynaecology and Obstetrics, Heinrich-Heine-Universität, Düsseldorf, Germany between January 1980 and December 1994 and from whom complete follow-up data were available. An abdominal hysterectomy and bilateral oophorectomy was performed in all patients. In 75 patients, lymphadenectomy was performed by removing the lymphatic tissue along the external and internal iliac veins and arteries and in the obturator fossae. In 38 patients, iliacal lymph nodes were palpable negative (N0) and, therefore, not removed. Paraaortic lymphadenectomy was only performed in cases of positive palpation. Patients with deep myometrial invasion (> 50%) or undifferentiated tumours (G3) received postoperative brachytherapy to the vaginal vault. 8 patients received external beam radiation, 11 patients adjuvant hormonal therapy (tamoxifen, medroxy-progesterone acetate), none chemotherapy. Patients were followed clinically for up to 186 months (median 98 months). Recurrences were confirmed by histological examination. Primary staging and histological grade was performed according to the FIGO classification (1988). TNM status was given according to the UICC classification (1987). Anamnestic information included age, positive family history (at least one case of breast or ovarian cancer below the age of 60 years, and at least one cancer of other type), previous history of cancer or precursor lesions, history of hormonal therapy (oral contraceptives (OC), hormone replacement therapy (HRT)) and death due to primary disease. In cases of recurrence, disease-specific treatment options (surgery, chemotherapy, hormonal therapy, radiation) were applied.

Histology and DNA extraction

Formalin-fixed, paraffin-embedded blocks of tumour specimens and additional non-tumour tissue from 113 patients were used. Ninety-three per cent of the tumours encompassed endometrioid adenocarcinoma of various types (papillary, secretory, ciliated cell, adenoacanthoma, adenosquamous); 7% were clear cell, serous or mucinous carcinoma. In addition, five different myometrium samples randomly chosen from each year (1980–1994; total $n = 90$) served as controls to test the influence of fixatives, fixation time, etc. on DNA quality. For tumour samples, haematoxylin–eosin staining (HE) was used for a repeated pathological evaluation (TNM status, myometrial invasion, grading, histological typing, lymph node involvement). ER and PgR expression were determined and scored on newly prepared 5 μm sections by immunohistochemistry [27]. The number of tumour cells in sections of endometrial carcinomas was estimated by visual examination of 5 μm HE-stained sections. Adjacent 40 μm toluidine-stained tumour sections with a high content of tumour cells (> 70%) were used for microdissection [28]. DNA was extracted from the tumour sections as previously described [29]. As a source of normal DNA for LOH analysis, sections of myometrium or lymph nodes without tumour infiltration (evaluation of serial sections under light microscopy by a pathologist) were used.

Primers, PCR reaction, fragment analysis, and assessment of allelic loss

Primer sequences of *BRCA1* marker (D17S855), *TP53* (AFM051), *TCRD*, *ESR*, *D11S35*, and of *D16S511* used for the amplification of STRs were available from GenomeDataBase (Johns Hopkins Welch Library, Johns Hopkins University, Baltimore, Maryland, U.S.A.). The primers were purchased from Pharmacia Biotech (Freiburg, Germany). Primer sequences were: *D17S855* (5′-F-GGATGGCCTTTTAGAAAGTGG, 5′-ACACAGACTTGTCCTACTGCC), *TP53* (5′-F-TACAGGGATAGGTAGCCCGAG, 5′-GGATTTGGGCTCTTTTGTAA), *TCRD* (5′-F-GCTGAGACTAAACCTACCAC, 5′-GTTAGTGGAAGAGCAGAGCA), *ESR* (5′-F-GACGCATGATATACTTCACC-3′, 5′-GCAGAATCAAATATCCAGATG), *D11S35* (5′-F-ACAATTGGATTACTACTAGC-3′, 5′-TGTATTTGTATCGATTAACC-3′) and *D16S511* (5′-F-CCCCGGAGCAAGTTCA-3′, 5′-CAGCCCAAAGCCAGATTA-3′). PCR

reactions, fragment analysis, and assessment of allelic loss were performed as described previously [28]. LOH results were correlated with the clinical and histological parameters mentioned above.

Statistical methods

Associations of LOH of the different markers with other clinicopathological factors were calculated using the chi-square test. In cases where the total number was less than 40 or the theoretical percentage was less than 5% exact Fisher's test was used. Probabilities of disease-free survival (DFS) and overall survival (OS) were calculated using the method of Kaplan–Meier, statistical differences were evaluated using the log-rank test. Relative risk estimations were performed by means of Cox proportional hazard models. Statistical analyses were performed using the SPSS statistical package.

RESULTS

In DNA extracted from sections of a total of 113 primary endometrial cancers with reference tissue from the same patient, and from 90 benign, randomly chosen myometrium samples, LOH of three different markers (*BRCA1*, *TP53*, *TCRD*) was analysed. All analyses were performed at least twice, and the mean signal reduction was calculated. LOH has been defined as a signal reduction of at least 40% [21, 28, 30]. Incomplete losses are commonly observed and may reflect either normal cell contamination or tumour heterogeneity. To define the cut-off to discriminate between specific allele loss and cases with both alleles retained, the range of allele ratios was determined for all informative cases and markers (data not shown). The graph showed a bimodal distribution of allele ratios with ratios ranging from 0.8 to 1.0 (both alleles present) and a broader range of allele ratios between 0.0 and 0.6 (indicating allele losses) separated by a few cases with borderline values between 0.6 and 0.8 (less than 6% of all tumours analysed).

To establish conditions for the detection of LOH of the three markers, fluorescent PCR (fPCR) using primers flanking STR regions was applied. PCR was optimised in terms of amplimers, enzyme concentration and cycle number. To determine exact allele sizes, an allelic ladder was constructed by combining PCRs derived from DNAs of different patients having different heterozygous allelic patterns. The allelic ladder encompassed all known alleles. An exact assignment of both alleles of each patient or of each tumour sample was feasible. During each electrophoretic separation, one lane was reserved for an aliquot of this allele mixture (Figure 1) and together with the internal size marker in the sample buffer, lane to lane variations were excluded.

In 113 tumours analysed, the loss rate for the *BRCA1* marker (*D17S855*) was 18.1% ($n_{LOH}/n_{inf} = 13/72$), for the *TP53* (*AFM051*) marker 26.9% ($n_{LOH}/n_{inf} = 18/67$), and for

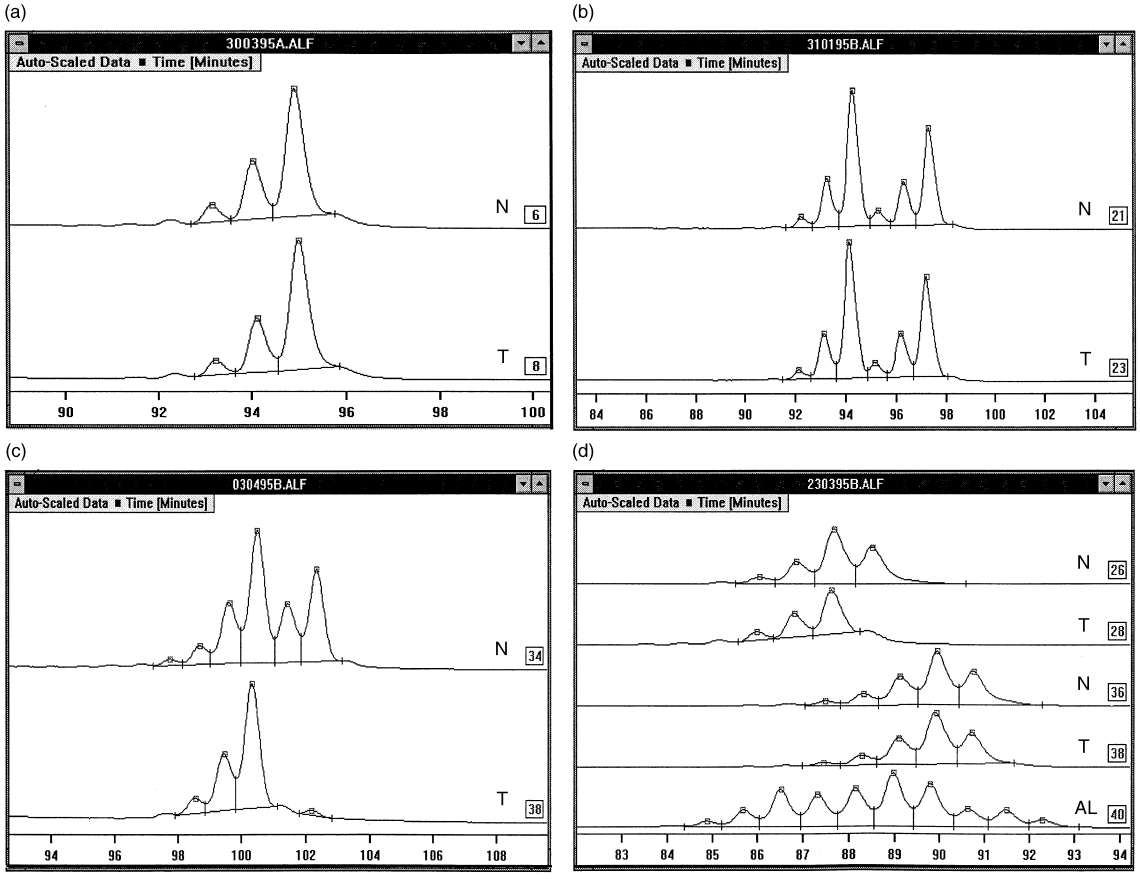

Figure 1. Printouts from the automated DNA sequencer of various DNA probes analysed with the internal *BRCA1* marker *D17S855*. The *x*-axes show electrophoresis running time in minutes. In each run an allele mixture (AL) encompassing 10 alleles (dinucleotide repeats) from 138 to 156 bp (shown in lane 40 (d)) was used as a size marker. (a), (b), (c) Results of matched DNA samples extracted from normal tissue (upper curve) and tumour tissue (lower curve), homozygous (a), heterozygous (b) and loss of heterozygosity showing 95% signal reduction of one allele (c).

the *TCRD* marker 26.3% (n_{LOH}/n_{inf} = 20/76). To validate the significance of LOH rates, the background incidence of allele loss was determined. In 30 tumours, three other loci on chromosomes 6q25.1 (*ESR*), 11q22-q23 (*D11S35*), and 16q24 (*D16S511*) were analysed. The LOH rate for the *ESR* marker was 4.0% (n_{LOH}/n_{inf} = 1/25), for the *D11S35* marker 7.7% (n_{LOH}/n_{inf} = 2/26), and the *D16S511* marker 5.3% (n_{LOH}/n_{inf} = 1/19). LOH of *D17S855* (Table 1) correlated with high grade (P = 0.012), positive ER status (P = 0.032), and family history of cancer (P = 0.008). LOH of *AFM051* correlated with younger age (P = 0.04), high grade (overall P = 0.015), positive PgR status (P = 0.047), and no use of HRT (P = 0.04). LOH of *TCRD* correlated only with positive family history of cancer (P = 0.01), but with none of the other ten parameters analysed. For information about genetic instability, LOH data for the three markers were combined. The only consistent correlation was seen for tumour grade with P overall = 0.055 for one LOH, P overall = 0.067 for two LOHs, and P overall = 0.077 for LOH at all three loci.

In the overall study group, routine parameters (high tumour size, high grade, presence of metastases, low PgR status) correlated significantly (P < 0.05) with decreased DFS and OS. None of the other anamnestic parameters, stage or ER status, correlated with DFS or OS. Allele loss of the *BRCA1* region correlated significantly with a decreased OS (Table 2 and Figure 2), but not with DFS. LOH of the two other loci, *TP53* and *TCRD*, did not correlate significantly with OS or DFS.

Table 1. *Loss of heterozygosity in the three marker regions* (BRCA1, TP53, TCRD) *in endometrial cancer* (n = 113) *correlated with patient's age, histopathological findings, stage, and steroid hormone receptor expression by chi-square test (significance at* P = 0.05*)*

		Chromosome 17q21 *BRCA1* (*D17S855*)	Chromosome 17p13 *TP53* (*AFM051*)	Chromosome 14q11 *TCRD*
Criteria (n = 113)	n	P value	P value	P value
Age (years)				
< 50	8			
≥ 50	105	1	0.04	0.84
Tumour				
T1	71			
T2	20	0.17	0.68	0.27
T3/4	22			
Nodes				
N0	98			
N1/2	15	0.97	0.56	0.31
Metastasis				
M0	99			
M1	14	0.33	0.62	0.66
Grade				
I	52			
II	30	0.012	0.015	0.28
III	21			
Stage				
I	69			
II	18	0.42	0.77	0.34
III	15			
VI	11			
Family history				
neg	97	0.008	0.12	0.01
pos	16			
HRT				
neg	96	0.28	0.04	0.35
pos	17			
ER				
neg	52			
pos	51	0.032	0.067	0.082
nr	10			
PgR				
neg	52			
pos	51	0.38	0.047	0.47
nr	10			

nr, no result; neg, negative; pos, positive; HRT, hormone replacement therapy; ER, oestrogen receptor; PgR, progesterone receptor.

DISCUSSION

In this study, three markers were analysed with routine application of PCR and fluorescent DNA technology for the detection of LOH. Microsatellite polymorphisms detecting differences in STR sequences were amplified by fluorescent PCR and analysed in an automated DNA sequencer [28, 31]. The combination of both methods offers several advantages

Table 2. *Age, histopathological findings, staging, steroid hormone receptor expression, and loss of heterozygosity of three loci in sporadic endometrial cancer* (n = 113) *correlated with disease-free survival (DFS) and (OS) overall survival (significance* P < 0.05*)*

	P values		Hazard rate
Criteria	DFS	OS	(95% CI)
Age			
< 50 versus > 50 years	0.16	0.14	1.2 (1.0–2.8)
Tumour			
T1 versus T2	< 0.01	< 0.01	1.8 (1.0–2.5)
T1 versus T3/4	< 0.01	< 0.01	1.6 (1.1–2.4)
T2 versus T3/4	0.014	0.025	2.3 (1.3–3.7)
Nodes			
N0 versus N1	0.14	0.296	0.3 (0.1–1.0)
Metastases	0.048	0.039	1.4 (0.5–3.4)
Grading			
G1 versus G2	0.32	0.721	0.9 (0.8–3.1)
G1 versus G3	< 0.01	< 0.01	1.7 (0.9–2.0)
G2 versus G3	0.01	0.015	1.1 (1.3–1.9)
Staging	0.13	0.36	0.7 (0.5–1.1)
Family history			
Negative versus positive	0.48	0.52	0.6 (0.4–2.8)
HRT			
Negative versus positive	0.78	0.63	0.5 (0.3–3.4)
ER			
Negative versus positive	0.64	0.30	1.2 (0.7–2.2)
PgR			
Negative versus positive	0.03	0.016	1.7 (0.9–2.1)
Locus 17q21			
BRCA1	0.089	0.022	1.5 (1.1–2.4)
Locus 17q13			
TP53	0.168	0.08	1.1 (0.9–1.7)
Locus 14q11			
TCRD	0.07	0.265	0.8 (0.6–1.2)

HRT, hormone replacement therapy; ER, oestrogen receptor; PgR, progesterone receptor; CI, confidence interval.

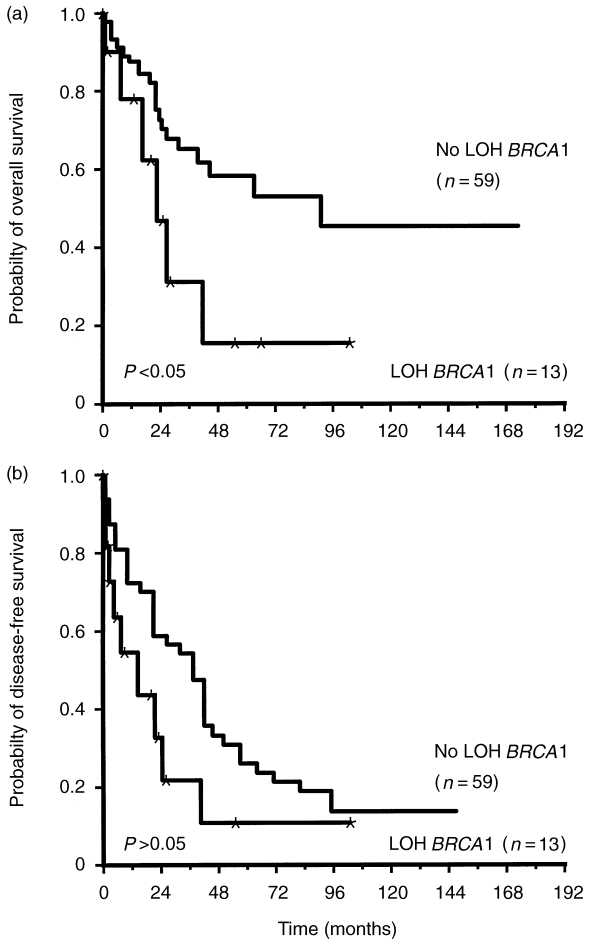

Figure 2. Kaplan–Meier curves of informative cases of patients comparing patients with informative tumours (*n*=72) with or without loss of heterozygosity of chromosome 17q21. (a) overall survival (*P*<0.05); (b) disease-free survival.

compared with other staining methods and autoradiography: (1) It is much faster than Southern blot or radioactive PCR. Separation and direct quantification of PCR products can be performed automatically and requires approximately 3 h without additional staining steps. This allows quantification of as many as 40 individual samples simultaneously and to run two or three gels per day on a routine basis. (2) Fluorescent labelled primers and PCR products can be stored, since labelling for both primers and products is stable for several months at − 20°C. (3) The enhanced sensitivity of the fluorescent detection method requires only 25–30 PCR cycles to achieve detectable results. Linearity of fluorescence detection covers a much wider range than scanning of autoradiograms or ethidium bromide or silver stained gels, resulting in an improved quality of data. Therefore, this approach is particularly suitable for the analysis of large series of samples and routine clinical use.

In spite of numerous reports about molecular factors in endometrial cancer [6–14], to date there have been no conclusive prospective randomised trials that would offer definitive guidelines for therapeutic decisions based on molecular tumour biological factors. None of these molecular factors proved to be of better prognostic or predictive clinicopathological findings, which currently form the basis for individualised treatment [2–4, 15].

Cytogenetic and molecular genetic analyses of endometrial cancer samples have focused on few genes and LOH of more or less defined chromosomal regions. Gene amplification has been detected in the *Her-2/neu* oncogene [10, 11], mutations in the c-Ki-*Ras* [17, 18] and the *Tp53* genes [13, 14]. Mutational inactivation of *Tp53* is often accompanied by allelic deletion [13, 16]. Additional chromosomal regions that have sustained allelic deletion, implicating location of TSGs at the affected loci, have been defined. LOH could be detected on chromosomes 1p, 3p, 8p, 9p, 10q, 14q, 16q, 17q, and 18q with allelic loss frequencies mostly far below 30% [16, 20–23].

In sporadic breast cancer, compiled allelotype data have demonstrated an overall 4% background incidence of LOH and defined a non-specific/specific arbitrary 10% cut-off [30]. An LOH rate below this value is not expected as indicative for a TSG important for breast tumour development, but merely results from genetic instability associated with tumour development and progression. For endometrial cancer, we showed a similar background level of allele loss with the highest LOH rate for the chromosome 11q22 marker *D11S35*. Loss rates of the markers analysed were significantly above this cut-off value, with a rate of 26.9% for the *TP53* marker. LOH rates of various markers in endometrial cancer samples are not extensive, indicating that a relatively large number of different chromosomal loci are likely to play a role in the aetiology and progression of this tumour type, due to a relatively high genetic instability in endometrial cancer. Still, the assessment of a specific marker loss at a certain stage or the combination of LOH of various markers may be of prognostic or predictive value and consequently helpful in the clinical setting.

The prognostic value of classical parameters, like grading or PgR expression, was confirmed in this study group [2, 3, 6]. These findings, in combination with the distribution of clinical stages, define this routine study population. LOH may indicate the inactivation of a TSG or a putative TSG locus. For *TP53* protein expression, as well as gene mutation, data are available [12–14], but for *BRCA1* or *TCRD* those data are lacking. Furthermore, due to the size of the *BRCA1* gene (24 exons), a routine mutation analysis is not yet feasible. Immunohistochemical detection of the *TP53* protein (overexpression/mutated) showed alterations in 20–48% of the tumours, mostly based on *TP53* gene mutations [6, 12–14]. Here, LOH of *TP53* was found in 26.9% of cases. This finding confirms published *TP53* gene alteration frequency data in endometrial cancers, but contradicts *TP53* LOH data published by Fujino and colleagues [21] who reported a *TP53* LOH rate of 11%. In this paper, an allelotype analysis was undertaken with 70 polymorphic markers distributed among all chromosome arms with the highest LOH rates (> 15%) on chromosomes 3p, 8p, 9p, 14q, 16q and 18q. For the *TCRD* marker, a lower percentage of LOH (19%) was shown compared with our results (26.3%) whereas the LOH rate on chromosome 16q (22–23%) was much higher than the LOH of marker *D16S511* (5.3%) used in our study as a control locus for background level of LOH in endometrial carcinomas. Differences in sample size, tumour histology or technical variations may account for this discrepancy.

Mutation analyses or protein expression of *TP53* were not assessed in this study. In breast [32] and endometrial carcinomas [16], however, LOH only correlated with a point mutation of the corresponding allele and/or expression of the mutant p53 protein in 60–75% of cases. Mutations of the

TP53 gene without loss of the corresponding wild-type allele as well as LOH of *TP53* without any mutation in the *TP53* coding sequence, were reported. The latter may hint to another TSG on chromosome 17p. So the assessment of each molecular alteration may add a different perspective to tumour development. Here, *TP53* LOH correlated with high grade and loss of PgR expression, both findings indicating a poor prognosis.

Even though the loss rate at the *TCRD* locus was high, except for positive cancer family history, no statistical significance with any of the other parameters analysed could be found, implying an undefined role of this gene locus in the development of endometrial cancer. Positive cancer family history was also found with LOH at the *BRCA1* locus. The families analysed here showed one or two cases of breast or ovarian cancer below the age of 60 years and at least another case of other cancer type. At present, the explanation for this phenomenon is not straightforward. Classical *BRCA1* families do not correlate with an increased risk of endometrial cancer. The definition of family history applied here may be too simplistic and does not define high risk *BRCA1* families. Still, *BRCA1* is an oestrogen regulated gene, which may be important in tumour development in the endometrium, one of the major oestrogen regulated tissues. Otherwise, unknown concomitant genetic alterations may be responsible for an increased familial risk of endometrial cancer [33–35]. LOH of the *BRCA1* marker correlated with positive ER expression, increased tumour grade and decreased OS. Whether direct alteration of the *BRCA1* gene or of another gene in the vicinity of *BRCA1* also affected by LOH is responsible for these findings cannot be concluded from this study. Based on evidence from sporadic breast cancer, a chromosomal region extending *BRCA1* may be affected by LOH [28]. The *17HSD* gene encoding 17β-hydroxysteroid dehydrogenase (EDH17B2) is located in the vicinity of *BRCA1* [25, 26]. EDH17B2 catalyses the interconversion of oestradiol and the less active oestrogen, oestrone. Therefore, genetic alteration of the 17HSD locus may affect local oestrogen metabolism and, directly or indirectly, lead to an increased local proliferative stimulus [26, 35] and an increased risk of cancer in hormone sensitive tissues. This is demonstrated by the significant correlation of *BRCA1/EDH17B2* allele loss and positive family history. The increased number of tumours in hormone sensitive tissues in these families may indicate that the candidate gene implicated in LOH 17q21 in endometrial cancer is not *BRCA1* but *EDH17B2*.

Here, we report for the first time the use of LOH as a prognostic factor in endometrial cancer. Although several molecular alterations have been identified, the molecular pathogenesis of endometrial cancer remains poorly understood. Whether losses of *TP53* or *BRCA1* loci are key steps in multistep carcinogenesis and whether LOH analysis results in more relevant prognostic information than classical parameters, has to be verified in a larger study group including precursor lesions of sporadic endometrial cancer.

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