Molecular classification of spontaneous endometrial adenocarcinomas in BDII rats

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Abstract

Female rats of the BDII/Han inbred strain are prone to spontaneously develop endometrial carcinomas (EC) that in cell biology and pathogenesis are very similar to those of human. Human EC are classified into two major groups: Type I displays endometrioid histology, is hormone-dependent, and characterized by frequent microsatellite instability and *PTEN*, *K-RAS*, and *CTNNB1* (β -*Catenin*) mutations; Type II shows non-endometrioid histology, is hormone-unrelated, displays recurrent *TP53* mutation, *CDKN2A* (*P16*) inactivation, over-expression of *ERBB2* (*Her2/neu*), and reduced *CDH1* (*Cadherin 1* or *E-Cadherin*) expression. However, many human EC have overlapping clinical, morphologic, immunohistochemical, and molecular features of types I and II. The EC developed in BDII rats can be related to type I tumors, since they are hormone-related and histologically from endometrioid type. Here, we combined gene sequencing (*Pten*, *Irf1*, and *Ctnnb1*) and real-time gene expression analysis (*Pten*, *Cdh1*, *P16*, *ErbB2*, *Ctnnb1*, *Tp53*, and *Irf1*) to further characterize molecular alterations in this tumor model with respect to different subtypes of EC in humans. No mutation in *Pten* and *Ctnnb1* was detected, whereas three tumors displayed sequence aberrations of the *Irf1* gene. Significant down regulation of *Pten*, *Cdh1*, *p16*, *ErbB2*, and *Ctnnb1* gene products was found in the tumors. In conclusion, our data suggest that molecular features of spontaneous EC in BDII rats can be related to higher-grade human type I tumors and thus, this model represents an excellent experimental tool for research on this malignancy in human.

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Introduction

Endometrial cancer is the most frequently diagnosed gynecological malignancy in the western world with an estimated incidence of 15–20 cases per 100 000 women per year (Esteller *et al.* 1999, Ryan *et al.* 2005). Endometrial carcinoma (EC) is the prevalent subtype, accounting for ~75% of the reported cases (Cavanagh *et al.* 1999). Most of the ECs are sporadic, but an inherited genetic predisposition has been demonstrated in about 5% of cases, as the risk for a woman to develop EC is tripled when there is an affected first-degree relative (Sandles *et al.* 1992, Gruber & Thompson 1996, Sandles 1998). Based on the biology and clinical features, two different pathways are distinguished for tumorigenesis of sporadic EC, ~80% are designated as type I, including those with endometrioid histology, mostly of low

grade, occur between the ages of 20–54 years old, follow the estrogen-related pathway, and have a favorable outcome. Type II tumors comprise about 10–20% of sporadic cases, show non-endometrioid histology (usually papillary serous or clear cell, arising occasionally in endometrial polyps or from precancerous lesions that develop in atrophic endometrial), usually occur at latter age (5–10 years later than type I tumors). This type follows the estrogen-unrelated pathway and is characterized by an aggressive clinical course and poor prognosis (Lax 2004, 2007, Ryan *et al.* 2005, Hecht & Mutter 2006, Liu 2007). Using molecular analysis, these two pathways are further characterized by distinctive types of genetic instability and molecular alterations, including frequent microsatellite instability accompanied by *PTEN*, *K-RAS*, and *CTNNB1* (β -*Catenin*) mutations in type I, and *TP53*

mutation, *CDKN2a* (*P16*) inactivation, over-expression of *ERBB2* (*Her2/neu*), and reduced *CDH1* (*Cadherin 1* or *E-Cadherin*) expression in type II tumors (reviewed in (Lax 2007, Liu 2007) and summarized in Table 4). However, the boundary between the two types is blurry. In fact, there is evidence for a third and larger group of EC with overlapping clinical and molecular features of both types I and II endometrial cancers. For instance, many of low-grade type I endometrioid carcinoma occur unrelated to estrogen and are found in the background of atrophic endometrium. On the other hand, it has been clearly shown that papillary serous carcinomas may occasionally develop from a pre-existing endometrioid carcinoma as a result of tumor progression, giving rise to mixed endometrioid-non-endometrioid carcinomas (Carcangiu & Chambers 1992, Catusus et al. 1998). Accordingly, these tumors may share the pathologic and molecular features of types I and II endometrial cancers.

The genetic events underlying susceptibility to hereditary endometrial cancer are still poorly understood. Familial clustering of EC is reported to be the most common extra-colonic malignancy in hereditary non-polyposis colorectal cancer (HNPCC), a multi organ cancer syndrome with mismatch repair (MMR) deficiency (Miyaki et al. 1997, Peltomaki & Vasen 1997, Lynch & de la Chapelle 1999, Ollikainen et al. 2005). This group of EC are related to type I tumors, since they occur at young age and are histologically of mucinous or endometrioid type (Parc et al. 2000), but their pathway is driven by germ line mutations and is, thus, distinctive (Liu 2007). In addition to endometrial cancer arising from HNPCC, occasional families show clustering of endometrial cancer alone, without colon or other cancers. This group was termed as familial site-specific endometrial cancer (Sandles 1998). Inactivation of MMR seems not to be of central role in tumorigenesis of these tumors (Table 4), suggesting another as yet unknown etiology in the most familial site-specific endometrial cancer (Ollikainen et al. 2005).

It appears that much work still remains to be done to clearly understand the biologic processes behind the development of different groups of EC. For this purpose, an animal model would be convenient to use. Females of the inbred BDII/Han (here after BDII) rat strain are genetically prone to spontaneously develop EC (Deerberg & Kaspareit 1987, Kaspareit-Rittinghausen et al. 1987). These tumors display large similarities in cell biology and pathogenesis to those of human (Deerberg & Kaspareit 1987). Endometrial carcinogenesis in female BDII rats is hormone dependent (Deerberg & Kaspareit 1987, Deerberg

et al. 1995) and are the only early-onset tumors in the animals (Kaspareit-Rittinghausen et al. 1987). Thus, in terms of an endometrial cancer model, the female rats of this strain are unique (Vollmer 2003). Over the past decade, we have subjected this model to genetic characterizations and in the present study, we combined gene expression, allelic imbalance (AI), and mutation studies to investigate the extent of similarity of tumors developed in the BDII model to those of human EC. We investigated the expression levels of *Pten*, *Tp53*, *Irf1*, *Ctnnb1* (β -catenin), *Cdkn2a* (*P16*), *Cdh1* (*Cadherin 1*, *E-cadherin*), and *ErbB2* (*Her2/neu*) in rat endometrial adenocarcinoma compared with non-malignant endometrium (NME) in quantitative real-time PCR. A selected number of tumors were screened for mutations in the *Pten*, *Irf1*, and *Ctnnb1* genes using direct sequencing as well as for (AI, sometimes referred to as LOH analysis) at the *Pten* locus.

Materials and methods

Animal crosses and tumor material

Animals of the inbred BDII rat strain are genetically predisposed to spontaneous endometrial adenocarcinoma (EAC), with an incidence of more than 90% in virgin females as early as 13 months of age (Deerberg & Kaspareit 1987, Kaspareit-Rittinghausen et al. 1987). BDII females were crossed to males from two non-susceptible strains, BN/Han, and SPRD-Cu3/Han. The F1-animals were crossed to each other or crossed back to BDII females to generate F2 and backcross (N1) populations. Spontaneously arising tumors developed in a proportion of F1, F2, and N1 animals. Tumors in the progeny were pathologically classified mainly as EAC or other uterine tumors (Roshani et al. 2001, 2005). In some cases, no malignant cells were detected in the removed cell mass when pathologically characterized. However, numerical chromosomal changes were detected in these samples (unpublished data). We consider these tissues represent normal or pre-malignant endometrium and are therefore of great importance in the present study. Herein, these cell lines are referred to as NME samples. At necropsy, tumor specimens and matched liver and/or spleen tissue samples were collected from animals for DNA extraction and cell culture establishment (Helou et al. 2001). Small pieces of fresh tumor tissues were used to set up primary cell cultures and a total of 40 cultures were established, representing 33 EAC tumors and seven NME samples (Table 1). A panel of 29 EAC and seven NME samples were selected for quantitative Real-time PCR analysis. Based on the results, we

Table 1 Panel of cell cultures for the present study

Cross	Generation ^a	Tumor designation	Tumor type ^b	Character number ^c
BDIIxBN	F1	RUT7	EAC	43
	F1	RUT12	EAC	51
	F2	RUT25	EAC	48
	F2	RUT30	EAC	61
	N1	NUT6	EAC	60
	N1	NUT16	EAC	42
	N1	NUT51	EAC	71
	N1	NUT52	EAC	42
	N1	NUT81	EAC	42
	N1	NUT97	EAC	52
	N1	NUT99	EAC	67
	N1	NUT100	EAC	67
	N1	NUT127	EAC	70
	N1	NUT128	EAC	64
	N1	NUT31	EAC	67
	N1	NUT46	EAC	56
	N1	NUT50	EAC	64
	N1	NUT76	EAC	43
	N1	NUT82	EAC	78
	N1	NUT98	EAC	52
	N1	NUT118	NME	nd
	N1	NUT122	NME	nd
	N1	NUT123	NME	nd
	N1	NUT129	NME	nd
BDIIxSPRD	F1	RUT2	EAC	38
	F2	RUT3	EAC	59
	F2	RUT6	EAC	58
	F2	RUT13	EAC	62
	N1	NUT4	EAC	62
	N1	NUT7	EAC	46*
	N1	NUT12	EAC	59
	N1	NUT47	EAC	48
	N1	NUT55	EAC	44
	N1	NUT84	EAC	41*
	N1	NUT39	EAC	39
	N1	NUT42	EAC	44
	N1	NUT14	EAC	44
	N1	NUT89	NME	nd
	N1	NUT58	NME	nd
	N1	NUT18	NME	nd

Tumors selected for gene mutation sequencing are shaded grey. *Additional tetraploid subpopulation found in the tumors.

^aF1, strain intercross first generation animals; F2, intercross second generation animal; N1 = F1 animal back-crossed to BDII female.

^bEAC, endometrial adenocarcinoma; NME, non-malignant endometrium.

^cAverage of chromosome number in tumor.

selected a second panel of 14 EAC tumors for mutation sequencing (*Cttnb1*, *Ifr1*, and *Pten*) as well as AI analysis of *Pten* (Table 1).

Real-time quantitative PCR

Expression of seven genes (*Pten*, *Cttnb1*, *P16*, *Tp53*, *ErbB2*, *Irf1*, and *Cdh1*) was analyzed in 36 samples using quantitative Real-time PCR.

cDNA preparation

Two microgram total RNA of each sample was treated with DNaseI (Life Technologies) for 15 min at room temperature to remove any trace of genomic DNA; DNaseI was inactivated by adding Stop buffer and incubated at 70 °C for 10 min. The treated RNA was then reverse-transcribed in reactions containing, Buffer RT, Oligo-dT primer, dNTP-mix, RNase inhibitor, and Ominiscript Reverse Transcriptase at 37 °C for 1 h

according to the protocol provided by manufacturer (Qiagen).

Endogenous control

We used data from an earlier cDNA oligo array analysis (Karlsson et al. 2007) on this material to select the most appropriate endogenous controls, (i.e. *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase), β -actin, and *Rps9* (ribosomal protein S9)), for the Real-time PCR quantification analysis.

Real-time quantitative PCR with TaqMan

Real-time PCR was performed in 384-well plates using an ABI PRISM 7900HT Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA). TaqMan primers and probes (Table 2) were derived from the commercially available ‘TaqMan Gene Expression Assays’ (<http://products.appliedbiosystems.com/>). A keyword search for each gene name or accession number was performed, and the respective assay kit was ordered directly from the website. Amplification reactions were carried out in triplicate with 0.1 µl template cDNA, 1 µl TaqMan Universal PCR Master Mix (Applied Biosystems) and 1 µl FAM-labeled Assay-on-Demand Gene expression Assay Mix (Applied Biosystems; Table 2). Thermal cycling was initiated with a 2 min incubation at 50 °C followed by a first denaturation step of 10 min at 95 °C, and then by 40–50 cycles of 15 s at 95 °C and 1 min at 60 °C. In each assay, a standard curve with six cDNA dilutions was recorded, and four no-template controls were included.

Quantification and normalization

Quantification was performed by the standard-curve method. In summary, a standard curve was prepared in each PCR assay for all genes using serial dilutions (1:1, 1:3, 1:9, 1:18, 1:36, and 1:72) of calibrator cDNA (rat reference RNA, Stratagene, La Jolla, CA, USA). The mean *C_t*-value for triplicates was calculated, and the gene concentration (or gene copy number) of test

samples was determined, based on standard curves. Expression from target genes was then normalized using the expression from an endogenous control gene.

Statistical analysis

The normalized expression levels were log transformed and the Welch’s *t*-test was used to compare EAC with NME. For all samples, gene expressions are presented as fold changes, calculated as the ratio between the expression levels in tumor samples and the geometric mean of expression levels in the NME group.

AI scoring at the *Pten* locus

Analysis of AI at the *Pten* locus in the tumor material was performed by allelotyping using a polymorphic intragenic *Pten* marker (Sjöling et al. 2001) as well as four other markers (D1Rat79, D1Rat81, D1Rat169, and D1Rat300), located close to the *Pten* gene. Primer pair sequences for these markers were obtained from public databases. PCR amplification, scoring the AI and calculation of the allelic imbalance ratio (AIR) was performed essentially as described previously (Behboudi et al. 2001).

Gene sequencings

PCR primers were designed (Table 3) based on the available sequences in GenBank for amplification of the entire coding sequences of the *Pten*, *Irf1*, and *Ctnnb1* genes using tumor cDNA as template. The entire coding region of *Pten* consisting of 1212 bp (NM_031606) and the entire coding sequence of *Irf1* consisting of 987 bp (NM_012591) plus 195 bp in 5’UTR, and 617 bp in the 3’UTR were amplified in single fragments. The entire coding sequence of the *Ctnnb1* gene is 2353 bp long (NM_053357) and was amplified in two separate overlapping segments: segment A, from nucleotide 2–1317 (1315 bp) and segment B from nucleotide 1202–2353 (1151 bp). PCR amplification products were purified using GFX PCR

Table 2 TaqMan assays used in quantitative real-time PCR

Gene	Assay	Accession numbers	Exon boundary	Assay location	Gene location (characters)
<i>Pten</i>	Rn00477208_m1	NM_031606.1	4–5	nt 255	1q52
<i>Cdkn2b (P16)</i>	Rn00676469_m1	NM_031550.1	–	nt 168	5q32
<i>E-cadherin (Cdh1)</i>	Rn00580109_m1	NM_031334.1	3–4	nt 526	19q12
β -catenin (<i>Ctnnb1</i>)	Rn00584431_g1	NM_053357.2	11–12	nt 1957	8q32
<i>Irf1</i>	Rn00561424_m1	NM_012591	1–2	nt 189	10q22
<i>ErbB2</i>	Rn00690259_m1	NM_017003.2	1–2	nt 186	10q32.1
<i>Tp53</i>	Rn00755717_m1	NM_030989	8–9	nt 1175	10q24
<i>Rps9</i>	Rn01530912_g1	NM_031108.2	3–4	nt 318	1q12

Table 3 Primers used for sequencing of the entire coding sequences of the *Pten*, *Ctnnb1*, and *Irf1* genes

Gene	Primer designation	Primer sequence (5' to 3')	Reference sequence
<i>Pten</i>	exon1F	TGACAGCCATCATCAAAGAG	NM_031606
	exon6F	GGAGTAACTATTCCCAGTCAGAG	
	exon7R	CTTTTGGAGCATCTTGTTCTGTT	
	exon9R	TCAGACTTTTGTAAATTTGTGAAT	
<i>Ctnnb1</i> part A	AF	CAATGGCTACTCAAGCTGACC	NM_053357.2
	2F	GCCATGTTCCCTGAGACACT	
	3F	CGCACCATGCAGAATACAAA	
	4F	TTGCTCAACAAAACAAACGTG	
	2R	GCATGCCCTCATCTAGTGTC	
	AR	ACCATCATCTTGTTTTTGTAAATTATTG	
<i>Ctnnb1</i> part B	BF	AAGGCCTCCTTGGGACTCTA	NM_053357.2
	5F	TTGGATTGATCCGAAACCTT	
	6F	CCACTGTTTGTGCAGTTGCT	
	7F	CCAATGGCTTGGAATGAGAC	
	BR	TATCAAACCAGGCCAGCTGAT	
		CGACGAAGGAGTAGGACGAG	
<i>Irf1</i>	1F	CCGATACAAAGCTGGGGAAAAAG	NM_012591
	2F	TCGTCAGCAGCAGTCTCTCT	
	3F	GGATTGGCGTTCTGAGGTTA	
	4F	TTTGTATCGGCCTGTGTGAA	
	1R	CTATCTCGATGTCCCCTCCA	
	2R	TGGTGAGACCCTCTCTAAGCA	
	3R	CAGAAATGTGGCAAAGTCCA	
	4R		

F, forward primer; R, reverse primer.

DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech). The purified fragments were subjected to sequencing using a second set of primers (Table 3) as described previously (Nordlander *et al.* 2007). Briefly, using ABI PRISM BigDye1 Terminator v1.1 Cycle Sequencing Kit (PE Applied Biosystems), the purified DNA fragments were subjected to sequencing according to the protocol provided by the manufacturer. Sequencing products were then denatured, cooled on ice and 1.5–1.8 µl of each sample was separated in a pre-warmed 5% denaturing polyacrylamide gel in 1xTBE buffer (89 mM Tris/ 89 mM boric acid/2 mM EDTA, pH 8.3) using an automated ABI Prism 377 Genescan Analyzer (PE Applied Biosystems). The sequence data obtained were subjected to BLAST searches (<http://www.ncbi.nlm.nih.gov/BLAST/>) for comparison with published sequences and inspected visually in the Sequencing Analysis software (PE Applied Biosystems).

Results

Real-time quantification PCR

Gene expressions of *Pten*, *Ctnnb1*, *Tp53*, *P16*, *Cdh1*, *ErbB2*, and *Irf1* were examined in a panel of 29 rat EAC and seven NME cell cultures. *Rps9* showed the lowest

variations in ΔC_t levels, and was expressed at similar levels in all samples regardless of the cell type (i.e. EAC or NME). Thus, *Rps9* was selected and used as an internal reference for normalization in the quantitative Real-time PCR analysis.

In all but two of the genes tested (i.e. *Irf1* and *Tp53*), the expression levels were lower in the EAC compared with the NME samples. The results revealed that *Pten*, *Cdh1*, *P16*, *ErbB2*, and *Ctnnb1* differed significantly in expression levels (nominal *P* value <0.05, Fig. 2, Table 4). In addition, when removing one outlier for *Tp53* in the NME group, this gene also showed significant difference in expression level between the two sample groups (*P*=0.001, fold=2.1).

Allelic imbalance analysis and mutation sequencing of the *Pten* gene

To investigate the status of *Pten* in the EAC susceptible rat model, the region encompassing this tumor suppressor gene (located at RNO1q52) was screened for indications of AI. The *Pten* intragenic marker D1Lev1 (Sjöling *et al.* 2002) is informative between the BDII and SPRD strain animals, but not between BDII and BN rats. The D1Lev1 marker was used in conjunction with another informative microsatellite marker (D1Rat81) for AI scoring of *Pten* locus in

Table 4 Summary of molecular analysis of EACs developed in the BDII rat tumor model compared with different subgroups of human ECs

Gene alteration	Pathway	Human endometrial carcinoma ^c				Endometrial carcinoma in BDII rats ^d
		Type I	Type II	Arised from HNPCC	Familial site-specific	
Microsatellite instability	MMR	20–45%	0–5%	90–95%	8–9%	0–5%
<i>PTEN</i> mutations	Akt	50–80%	10%	60–85%	N	No mutation, but 2.3 fold down-regulation ^a
<i>K-RAS</i> mutations	Akt	10–30%	0–5%	10%	N	No
<i>ERBB2</i>	Akt	10–30%	45–80%	N	N	No, to the contrary 4.5 fold down-regulation ^a
over-expression						
<i>TP53</i> mutations	P53	10–20%	90%	Low	N	67% mutations ^{a,b}
<i>P16</i> inactivation	P53	10%	40%	N	N	57% deletion and 28 fold down-regulation ^a
<i>CDH1</i> down-regulation	Wnt	10–20%	80–90%	N	N	Yes, 23 fold ^a
<i>CTNNB1</i> mutations	Wnt	20%	0–5%	N	N	No, to the contrary 2.2 fold down-regulation ^a

N, not known or no information available.
^aPresent work.
^bAfter removing one outlier from the NME samples.
^cKariola et al. 2005, Lax 2007, Liu 2007, Ollikainen et al. 2005.
^dBehboudi et al. 2001, Helou et al. 2001, Nordlander et al. 2005, Adamovic et al. 2008.

animals of the SPRD cross. AI analysis of the tumors developed in the BN cross was performed using D1Rat81 plus three additional informative markers, D1Rat169, D1Rat300, and D1Rat79, spanning a region of 6.5 cM at the *Pten* locus. In the SPRD cross, among three tumors heterozygous in this region, one (RUT6) showed AI at *Pten*. In animals from the BN cross, two out of four heterozygous tumors at *Pten*, displayed AI (RUT12 and RUT30). Taken together, three out of seven informative tumors exhibited AI/LOH at the *Pten* locus. Furthermore, cDNA of the entire coding region (1212 bp) of *Pten* was screened for mutations. As comparison, cDNA from normal tissue was subjected to sequencing as well. No mutation was detected anywhere in the coding sequence of the *Pten* gene in any of the samples.

Mutation sequencing of the *Ctnnb1* gene

The cDNA sequence for *Ctnnb1* gene was divided into two parts, A and B, for PCR amplification. Furthermore, these segments were PCR amplified in smaller fragments of 400–500 bp for direct sequencing (Table 3). cDNA from the three parental strains (i.e. BDII, SPRD, and BN) and 14 EAC tumors were sequenced. A difference in nucleotide 1109 in exon 6 (GenBank-our data, C-T), compared with the available GenBank sequence for rat *Ctnnb1* mRNA (NM_053357) was found. The new data will cause

codon 368 to be changed from Prolin (CCT) to Leucine (CTT). Since we found the same sequence in all the three parental strains, we believe that this sequence may be representative for an important subset of inbred rat strains. This finding is supported by the fact that both humans and mice have a Leucine, rather than a Prolin, at the corresponding position in the homologous proteins. We have submitted the sequence to GenBank. No further sequence aberrations or mutations were found.

Sequencing the *Irf1* gene

The entire coding region of rat *Irf1* gene is 987 bp. Using primers 1L and 1R (Table 3), a fragment of 1799 bp of the *Irf1* gene was amplified from cDNA. This fragment consists of 195 bp of the 5'UTR, the entire coding sequence and 617 bp of the 3'UTR. Using a set of eight primers (Table 3), the fragment was fully sequenced in the tumors and in normal cDNA from the parental strains (i.e. BDII, BN, and SPRD). The sequence obtained for *Irf1* normal cDNA for each strain was identical, but they differed in six nucleotides compared with the available GenBank reference sequence (NM_012591). The differences were (GenBank-our data): nucleotides 324 (T-A), 325 (T-A), 338 (T-C), and 398 (T-A) in the coding sequence, whereas the nucleotides C and A, which we found at positions 1521 and 1530 respectively, both inside the

3'UTR, seem to be missing in GenBank sequence. We have submitted the sequence to GenBank. The new data for nucleotides 324 and 325 will cause codon 43 to be changed from Leucine (UUG) to Lysine (AAG). Since we found the same sequence in all three parental strains, we believe that this sequence may be representative for an important subset of inbred rat strains. This is supported by the fact that both humans and mice have a Lysine, rather than a Leucine, at the corresponding position in the homologous proteins. The base substitutions at positions 338 and 398 represent third nucleotide changes in codons 47 and 67, respectively, and were silent, causing no amino acid substitution. Subsequently, *Irf1* was amplified and sequenced in 14 tumors samples. Three EAC tumors (RUT3, NUT4, NUT12) displayed variants in the gene sequence, compared with the normal cDNA sequence. RUT3 that contained two copies of proximal RNO10 (Nordlander *et al.* 2007) displayed a deletion of three nucleotides in exon 9 at codon 312 (1131-1133, NM_012591), resulting in deletion of one amino acid, Alanine, from the *Irf1* activator domain. Possibly, this change occurred because of nucleotide slippage, since the result of the mutation is that a double CTG triplet (CTGCTG) becomes a single CTG (Fig. 1A). The cDNA from NUT12 (showing two copies of proximal RNO10 (Nordlander *et al.* 2007)) displayed deletion of 146 nucleotides (nucleotides 349-495, NM_012591) encoding 50 amino acids (codon 49 to 99) of the *Irf1* DNA binding domain (Fig. 1B). The deletions resulted in the omission of 34 and 112 nucleotides from the end of exon 2 and the beginning of exon 3, respectively. Analyzing the deletion site, it was found that the break points occurred within a motif of seven nucleotides, AACAAGG, which was present in both exons in the intact allele, whereas in the deleted allele only one motif was retained. The third tumor (NUT4TC; 2–3 copies of proximal RNO10)

displayed deletion of 318 bp (nucleotides 1310–1628, NM_012591) from 3'UTR of the gene. In summary, only three EAC samples showed sequence aberrations of the *Irf1* gene in this investigation, and the expression level did not differ significantly in the EAC versus the NME samples (Fig. 2).

Discussion

Human type I ECs are characterized by frequent microsatellite instability as well as *PTEN*, *K-RAS*, and *CTNNB1* mutations, whereas *TP53* mutation, *P16* inactivation, over-expression of *ERBB2*, and low *CDH1* expression are common features of type II tumors. EC arising in HNPCC are characterized by inherited mutation in the MMR genes, while molecular data are scarce for the familial site-specific subtype.

BDII rat spontaneous EC are largely similar to human type I tumors in their histology and cellular aspects of their pathogenesis (Vollmer 2003). Frequent *TP53* mutations (Nordlander *et al.* 2007) and lack of *K-ras* mutation (Helou *et al.* 2001) were shown in BDII rat EACs. Additionally, neither microsatellite instability (Behboudi *et al.* 2001, Adamovic *et al.* 2005, Nordlander *et al.* 2005) nor *ErbB2* amplifications (Helou *et al.* 2001) were previously found among BDII rat tumors. Expression of Estrogen receptor α (*ER1*) was detected in the majority and progesterone receptor (*Pgr*) in a subset of these tumors (Karlsson *et al.* 2001). Here, we combined gene expression, AI, and gene mutation analysis to further characterize molecular features of endometrial carcinogenesis in the BDII rat model with respect to available molecular data for different subtypes of human ECs (Table 4).

The *PTEN* tumor suppressor gene has been described as one of the most frequently altered tumor suppressor genes in human cancer (Steck *et al.* 1997, Li & Sun 1998, Planchon *et al.* 2008). *PTEN* exhibits combined LOH of one allele and mutation in the other allele at frequencies up to 50% in human endometrial tumors. *PTEN* mutations have even been detected in 33–55% of the endometrial precancerous lesions (Risinger *et al.* 1997, 1998, Tashiro *et al.* 1997, Konopka *et al.* 2002, Latta & Chapman 2002). We found no sequence alterations in the entire coding region of the *Pten* gene in any of the BDII rat tumors. However, we found that AI at the *Pten* locus was present in about 50% of these tumors. We also found significant lower levels of mRNA (2.3 fold) in the EAC tumors compared with the NME samples. Taken together, these results suggest that *Pten* is likely involved in tumorigenesis of BDII rat EAC. Similar conclusions regarding *Pten* functioning in a

Figure 1 *Irf1* transcript aberrations. (A) Deletion of *Irf1* codon 312 in RUT3 (cDNA template); (B) Absence of 146 bp in the *Irf1* cDNA from NUT12. The lost segment lies between two identical 7-base sequences situated in exons 2 and 3 (cDNA template).

Figure 2 Real-time RT-PCR analysis of seven genes in a panel of 29 rat endometrial adenocarcinomas (EAC) compared with seven pre-malignant (NME) samples. Expression level of each gene in each sample is presented as fold change. Significant down regulation of Pten, Cdh1, P16, ErbB2, and Ctnnb1 was observed among EAC tumors, where no significant changes were detected for Tp53, and Irf1. For Tp53, when one outlier was removed from the NME sample group, this gene also showed significant difference in expression between EAC and NME samples ($P=0.001$, Fold=2.1). NME, premalignant endometrium; EAC, rat EAC tumors; P , nominal P value.

haploinsufficient mode has been drawn in several other reports (Di Cristofano *et al.* 1998, Kwabi-Addo *et al.* 2001, Sjöling *et al.* 2002, Kolasa *et al.* 2006, Kwon *et al.* 2008). Decreasing level of PTEN expression was shown to correlate with the progressive outcome of solid cancers, including ovarian, prostate, and cervical cancers (Harima *et al.* 2001, Jiang & Liu 2008). Thus, in *Pten* inactivation, although through a different mechanism (i.e. haploinsufficiency/lower expression in BDII rat versus gene mutations in human), the BDII rat tumors can be related to human type I ECs.

CTNNB1 mutation is present in about 25–40% of human endometrioid (type I) carcinomas (Saegusa

et al. 2001, Lax 2004). *CTNNB1* is crucial in cell–cell adhesion through a complex with *CDH1*. In addition, it is an important member of the WNT signal transduction pathway that is required for adult tissue maintenance (Logan & Nusse 2004). The function of *CTNNB1* in endometrioid tumorigenesis is still unknown; no correlation to microsatellite instability, *K-RAS*, *PTEN*, and/or *CTNNB1* mutations have been found, suggesting that the WNT/*CTNNB1* pathway may play an independent role in endometrial cancer tumorigenesis (Palacios *et al.* 2001). In the present work, no mutation in the *Ctnnb1* gene was found in BDII rat EAC tumors. In the Real-time PCR analysis,

however, the expression level of *Ctnnb1* was found to be significantly lower (2.2-fold) in the EAC compared with the NME samples. ERBB2 oncogene codes for a transmembrane receptor tyrosine kinase involved in cell signaling. Over-expression add/or amplification of this gene seems to play a role in 10–30% of higher-grade human ECs (Liu 2007). Neither *ErbB2* amplification (Helou *et al.* 2001) nor over expression was found in BDII rat EAC tumors.

Cadherins are a family of adhesion molecules essential for tight connection between cells. *E-Cadherin* (*CDH1*) is mainly expressed in epithelial cells. Reduced *CDH1* expression is associated with dysfunction of cell–cell adhesion system and has been shown to promote tumor invasion and metastasis (Strauli & Haemmerli 1984, Volk *et al.* 1984). Decreased expression of *CDH1* correlates with high histological grade and aggressive behavior and has been reported in 10–20% of type I and in 80–90% of type II human endometrial tumors (reviewed in Lax (2004, 2007) and Liu (2007)). Here, we found *Cdh1* expressed to a significantly lower level in the EAC compared with the NME sample groups. Our results are congruent with previous reports on *CDH1*, and suggest a parallel expression pattern of the gene in the BDII EAC and human EC tumors.

P16 is a tumor suppressor gene encoding for a cell-cycle regulatory protein and inactivation of this gene is suggested to lead to cell anchorage independency, invasion, and metastasis. Loss of *P16* expression has been found more commonly in human serous (type II, about 45%) compared with endometrioid carcinomas (type I, less than 10% and mainly found in high-grade tumors; Salvesen *et al.* 2000). *P16* inactivation correlates with *K-RAS* mutations, high stage and high grading of tumors as well as poor survival (Salvesen *et al.* 2005). We found significant low expression level (28-fold) of *P16* in the EAC tumors, which is in accordance to result from a recent study indicating frequent hemi- or homozygous deletions of *P16* in BDII rat EAC tumors (Adamovic *et al.* 2008). However, due to lack of *K-ras* mutations in this model, no correlation between inactivation of *P16* and mutation of *K-ras* in the BDII rat tumor model can be concluded.

Loss of *IRF1* has been reported to be a critical event in the development of human leukemia (Willman *et al.* 1993, Green *et al.* 1999). Additionally, frequent deletions in the *IRF1* locus have been detected in esophageal and gastric cancers (Ogasawara *et al.* 1996, Tamura *et al.* 1996). Several studies have suggested that post-transcriptional aberrations in the *IRF1* gene also may account for loss of *IRF1* function in tumors

(Green *et al.* 1999). In humans, the *IRF1* mRNA is expressed at a low basal level in a variety of cell types (Tanaka & Taniguchi 2000, Kroger *et al.* 2002). In the uterus, the site of expression of *IRF1* has been localized to the glandular epithelial compartment of the endometrium (Jabbour *et al.* 1999), i.e. the cell types from which EAC arises. Down-regulation of *IRF1* is a constant finding in endometrial tumorigenesis (Giatromanolaki *et al.* 2004) and expression of *IRF1* has been shown to be the highest in normal endometrium, and decrease with the grade of endometrioid adenocarcinoma (Kuroboshi *et al.* 2003). Earlier, in AI analysis of RNO10 in the BDII rat tumors (Behboudi *et al.* 2001, Nordlander *et al.* 2005) we identified three segments in the proximal part of the chromosome, which were commonly affected by AI. *Irf1* is a tumor suppressor gene located at RNO10q22 (Behboudi *et al.* 2002) and thus was selected for further analysis. In the present work, however, we found that the *Irf1* expression in the rat EAC did not significantly differ from the NME samples. Additionally, we detected aberration/mutation in the coding region of the *Irf1* transcript in only three samples, suggesting that *Irf1* is of less importance in the development of EAC tumors in this rat model.

In human malignancies, the *TP53* tumor suppressor gene is reported as the most commonly altered gene. The mutant p53 protein is non-functional, but resists degradation and accumulates in the cell. Mutations of the *TP53* gene are a frequent and characteristic finding in type II serous and are considered to be an early event in tumorigenesis (Ryan *et al.* 2005). In type I tumors, abnormalities of *TP53* are suggested as late events and are thought to be important step in the progression to high-grade tumors (Ryan *et al.* 2005). Earlier, we found recurrent *TP53* mutations (67%) in BDII rat EAC tumors (Nordlander *et al.* 2007). However, in majority of the mutations resulting in amino acid exchange, the hydrophobicity or hydrophilicity was maintained by equivalent substitutions. In the present work, we could show that *TP53* was expressed to a significantly lower level in the EAC than in the NME samples, only when one outlier in the NME group was removed from the analysis. Otherwise, there is no significant difference in expression between the two groups.

In conclusion, the EC developed in BDII rats are suggested to be related to human type I tumors, since they are hormone-related (Deerberg & Kaspareit 1987, Deerberg *et al.* 1995), express *Esr1* (Karlsson *et al.* 2001) and histologically from endometrioid type (Kaspareit-Rittinghausen *et al.* 1987). At the molecular level, it has been shown that *P16* deletions (Adamovic

et al. 2008) and *Tp53* mutations (Nordlander *et al.* 2007) are frequent. Furthermore, *ErbB2* amplification, *K-ras* mutation (Helou *et al.* 2001), and microsatellite instability (Behboudi *et al.* 2001, Adamovic *et al.* 2005, Nordlander *et al.* 2005) were not common features of BDII rat EAC tumors. Here, we combined gene expression, AI and gene mutation analysis to further characterize molecular alterations of endometrial carcinogenesis in this tumor model with respect to available molecular data for different subtypes of human ECs. No mutation in *Pten* and *Ctnnb1* was found. In gene expression and AI analysis, we found recurrent AI at the *Pten* locus accompanied by lower expression of the gene, substantial down-regulation of *P16* and *Cdh1* and slightly lower expression of *Ctnnb1* and *Tp53* in BDII rat EACs compared with the control samples (Table 4). In conclusion, our results suggest that endometrial carcinogenesis in BDII rats can be related to higher-grade human type I ECs.

It should be stressed that the rat disease genes identified so far have shown remarkable relevance to related human disease phenotypes (Aitman *et al.* 2008, Samuelson *et al.* 2008). Furthermore, there are instances that the rat model has inspired new therapeutic approaches (Gelderman *et al.* 2007, Pravenec & Kurtz 2007). The efficiency of translating results from rat models to the human thus seems to be high and rat model studies should thus provide important insights into disease genetics and mechanisms, as well as new therapeutic approaches. The present molecular data suggest that spontaneous endometrial carcinogenesis in the BDII rat tumor model can essentially be related to higher-grade human type I endometrioid carcinomas. Thus, in experimental setups characterizing the molecular mechanisms of human type I tumors, testing and developing new substances for therapeutic approaches, and examining safety of new drugs, in particular, the endocrine active substances intended for use in tumor therapy, this model is of potentially high value and should be considered as one of the most important contributing *in vivo* tools currently available for research on this malignancy.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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