Pathol. Oncol. Res. (2013) 19:495–499
DOI 10.1007/s12253-013-9607-3

RESEARCH

Primary Squamous Cell Carcinoma of the Endometrium Unrelated to Human Papilloma Virus: A Molecular Study

Giovanna Giordano · Silvia Pizzi · Cinzia Azzoni · Lorena Bottarelli · Tiziana D'Adda

Received: 3 April 2012 / Accepted: 4 February 2013 / Published online: 21 February 2013

Abstract In this paper we report a molecular study of a case of Primary Endometrial Squamous Carcinoma (PESC), in which a Human Papilloma Virus (HPV) infection had been previously excluded by Polymerase Chain Reaction (PCR). The studies performed in an effort to explain the carcinogenesis included immunohistochemical over-expression of p53 and p16 proteins as previously observed in our own papers, plus microsatellite analysis of D10S1765 at 10q23.3 (PTEN) and TP53 at 17p13.1 (P53) as well as the methylation status of the of BRCA1 and p16 promoters using specific PCRs. In this rare malignancy, we found allelic imbalance (AI) at 17p13.1 (P53). Instead, AI at D10S1765 (PTEN) gene was absent. The genetic alteration of p53, with hyper-expression of p53 protein and an absence of abnormalities in the PTEN gene are consistent with the similarities between Uterine Serous Carcinoma (USC) and our case of PESC. The aberrant methylation of both p16 and BCAR1 promoters was not detected in our case. This finding too could imply that ESC is more similar to Uterine Serous Carcinoma than Uterine Endometrioid Carcinoma (UEC). Moreover, the lack of aberrant methylation of p16, which is in accordance with over-expression of p16 immunoreactivity, in the absence of HPV infection may be related to other unknown genetic alterations. In our opinion, it is hard to reach any definite conclusion concerning the carcinogenesis of PESC, because of its rarity and the very few molecular studies reported in the literature. Further studies with more numerous cases and larger molecular analyses are mandatory for this malignancy, to confirm whether it is more closely related to papillary endometrial cancer than to endometrioid carcinoma.

Keywords Endometrial squamous carcinoma · Allelic imbalance · Aberrant methylation of promoters

G. Giordano (✉) · S. Pizzi · C. Azzoni · L. Bottarelli · T. D'Adda
Department of Biomedical, Biotechnological & Translational Sciences, Pathological Anatomy & Histology Unit, University of Parma, Faculty of Medicine, via Antonio Gramsci, 14 43126 Parma, Italy
e-mail: giovanna.giordano@unipr.it

S. Pizzi
e-mail: silvia.pizzi@unipr.it

C. Azzoni
e-mail: cinzia.azzoni@unipr.it

L. Bottarelli
e-mail: lorena.bottarelli@unipr.it

T. D'Adda
e-mail: tiziana.dadda@unipr.it

Introduction

The most frequently found squamous cell carcinoma of the female genital tract involves the cervix and is usually due to persistent, sexually-transmitted infection. The etiologic agent is a DNA virus, the Human Papilloma Virus (HPV).

P16ink4a over-expression is an indicator of an aberrant expression of viral oncogenes and can serve as a marker for early diagnosis of cervical cancer on immunohistochemical analysis [1].

Primary squamous cell carcinomas in the organs of the upper female genital tract are very rare malignancies and their association with HPV DNA has been demonstrated in only a few cases [2–4].

Mutationof p53 gene in Primary Endometrial Squamous Carcinoma (PESC) with human papillomavirus type 31 had been reported in only one case [2].

In cases unrelated to HPV infection, precisely because of their rarity, there is a few studies capable of demonstrating the carcinogenesis of these rare malignancies [5]. Immunohistochemical features too have only been reported in a few papers [5, 6].

In this paper, we report a molecular study of a case of (PESC), in which an HPV infection had previously been excluded by Polymerase Chain Reaction (PCR) analysis [7]. Additionally, the immunohistochemical over-expression of p53 and p16 proteins observed in our earlier papers [7, 8] and new molecular studies such as microsatellite analysis of D10S1765 at 10q23.3 (PTEN) and TP53 at 17p13.1 (P53) and the methylation status of BRCA1 and p16 promoters, were used in an effort to explain the carcinogenesis of this rare malignancy.

Material and Methods

Clinicopathological Data of the Patient

The patient was a 72-year-old woman admitted to the Department of Obstetrics and Gynaecology for weight loss and a pelvic mass. She had no history of IUD use, or pyometra.

Pathological examination revealed, only in the endometrium, neoplastic tissue that extended to the serosa which corresponded to a deeply invasive, moderately differentiated squamous cell carcinoma with keratinisation The neoplasm had entirely replaced the myometrium and appeared on the serosal surface, where perforation was observed macroscopically. The squamous cells showed numerous mitotic figures, which were often atypical. The cervix, which was entirely sampled, showed chronic inflammation. Multiple metastases were present in the omentum.

On PCR analysis, the neoplasm revealed no HPV DNA [7], while on immunohistochemical analysis, the neoplasm showed over-expression of p53 and p16 proteins [7, 8].

DNA Extraction

Normal and tumour tissues were manually micro-dissected from multiple 4 μm-thick histological sections stained with haematoxylin and incubated overnight at 56 °C in Tris EDTA lysis buffer, pH9, containing 5 % Chelex 100 (Bio-Rad Laboratories, Hercules, CA) as chelating resin and 0.4 mg/ml Proteinase K (Sigma-Aldrich, St. Louis, MO, USA). After Proteinase K heat inactivation, the crude DNA extract was directly submitted to microsatellite and methylation analyses.

Microsatellite Analysis

Microsatellite markers D10S1765 at 10q23.3 (PTEN) and TP53 at 17p13.1 (P53) were PCR amplified in normal and tumour DNA, while fragment analysis was performed as previously described [9]. In the tumour DNA, a disappearance or significant reduction in one of the two alleles observed in normal DNA was defined as allelic imbalance (AI), a mechanism commonly involved in tumour suppressor gene inactivation [9, 10].

Methylation Analysis

The methylation status of the promoters of BRCA1 and p16 genes, known to be epigenetically inactivated in human cancerogenesis, was evaluated. The tumour DNA underwent chemical modification with sodium bisulphite, followed by methylation-specific PCR.

Bisulphite Modification Treatment with sodium bisulphite induces chemical modifications in DNA sequences, converting unmethylated cytosines to uracil, while methylated cytosines remain unaltered [11]. DNA modification was performed with the EpiTect bisulphite kit (QIAgen Inc., Valencia, CA), following the manufacturer's protocol.

Methylation-Specific PCR (MSP) Bisulphite-induced DNA modifications were revealed by PCR amplification, using primers specifically designed to distinguish methylated from unmethylated cytosines in the promoters of BRCA1 [12] and p16 [11] genes. Two parallel MSP reactions (called "U" and "M") were performed in a 25 μl mix containing 1× PCR buffer [14], 1.0 mM of each dNTP (Promega, Madison, WI, USA), 0.4 μM of each primer pair (specific for unmethylated and methylated DNA in the "U" and "M" reaction, respectively), 1U of Thermo-Start *Taq* DNA Polymerase (Abgene Limited, Epsom, UK) and 2 μl of bisulphite-modified DNA. Each MSP reaction was performed in an AB2720 thermal cycler (Applied Biosystems, LifeTechnology, Carlsbad, CA, USA) and included appropriate positive and negative controls. Twenty μl of each MSP reaction were loaded on a 2.5 % agarose gel (EuroClone, Pero (MI), Italy), electrophoresed and visualized under UV illumination in a Gel Doc XR system (Bio-Rad Laboratories, Hercules, CA, USA). Promoter hypermethylation was assessed when an amplification product of the expected size was observed in the "M" lane reaction.

Results

Microsatellite Analysis

In neoplastic tissue, AI was observed at TP53 (Fig. 1), but was absent at D10S1765 (PTEN).

Methylation Analysis

Aberrant methylation of both p16 and BRCA1 promoter was not found in the neoplastic tissue (Fig. 2).

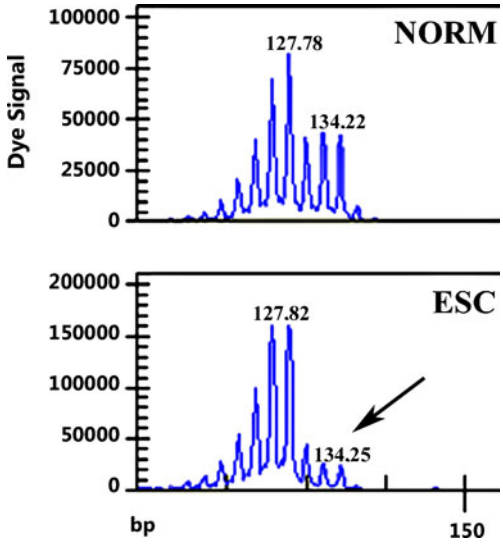

Fig. 1 Electrophoretic profiles for the TP53 microsatellite at 17p13.1 showing AI, indicative of loss of the larger allele (*arrow*), in endometrial squamous carcinoma (ESC). X axis: size of the PCR fragments in base pairs (bp); Y axis: intensity of fluorescence (peak heights); NORM: normal tissue

Discussion

Unlike cervical squamous carcinoma, which is the second most common female malignancy in the world and affects early age and women with multiple sexual partners and HPV infections, Primary Endometrial Squamous Carcinoma (PESC) is very rare neoplasm, observed in nulliparous post-menopausal women with a history of chronic pyometra of pelvic radiation [7].

Moreover, HPV infection in (PESC) has only been demonstrated in a few cases [2].

To date, because of its rarity, molecular alterations in unrelated HPV PESC are as yet unconfirmed.

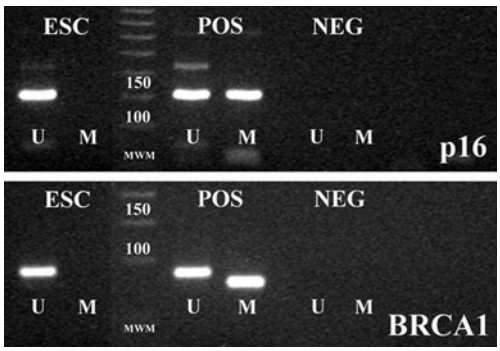

Fig 2 Absence of aberrant methylation of p16 and BRCA1 gene promoters in endometrial squamous carcinoma (ESC), as indicated by the lack of amplification product in the "M" lane. MSP reactions with primer sets for unmethylated (U) and methylated (M) sequences; POS: positive controls; NEG: negative controls; MWM: 50 bp-spaced molecular weight marker

In an effort to explain the carcinogenesis of this rare malignancy, we made use of immunohistochemical over-expression of p53 and p16 proteins, previously observed in our unrelated HPV PESC [7, 8], and new molecular studies including microsatellite analysis and the methylation status of BRCA1 and p16 promoters.

Microsatellites are stretches of DNA in which a short motif (usually one to five nucleotides long) is repeated several times. Microsatellites are dispersed throughout the genome and are usually non-coding. These repeated sequences of DNA are routinely present but highly variable from person to person. In the cells with mutations in DNA repair genes however, some of these sequences accumulate errors and become longer or shorter. The appearance of abnormally long or short microsatellites in an individual's DNA is referred to as microsatellite instability (MSI) [12, 13].

MSI is a key factor in several cancers, a defective DNA mismatch repair (MMR) gene being thought to promote tumourigenesis by accelerating the accumulation of mutations in oncogenes and tumour suppressor genes.

Microsatellite instability (MSI) is a form of genetic instability observed in virtually all tumours in patients with hereditary cancer [14] and in a subset of various sporadic tumours, including colorectal, gastric and endometrial cancer [15–17].

In our study, the microsatellite markers evaluated were TP53 at 17p13.1 (P53) and D10S1765 at 10q23.3 (PTEN) in a case of ESC. The *p53* gene at 17p13.1, has attracted wide attention as a tumour suppressor gene, transcription factor and mediator of apoptosis in many types of cancer [18, 19].

In this rare malignancy, we found AI at 17p13.1 (P53).

In our opinion, this finding, in this case of PESC, may explain the immunohistochemical over-expression of the p53 protein already reported in our previous study [7], and this is in accordance with other studies which have demonstrated that mutant p53 proteins generally have a longer half-life than wild-type p53 proteins and lead to nuclear accumulation [20, 21] which often reaches immunohistochemically detectable levels [22, 23].

Moreover, these data, in this example of PESC, demonstrates that p53 immunohistochemical over-expression could be related to mutation at AI TP53 at 17p13.1, similarly demonstrated by other Authors regarding other neoplasms [24].

Instead, AI at D10S1765 (PTEN) gene was absent in this case of PESC.

P-TEN is a candidate tumour-suppressor gene identified on chromosome 10, also known as MMAC1 (Mutated in Multiple Advanced Cancers 1).

P-TEN was isolated from a locus on chromosome 10, 10q22-23 and is deleted in a large number of tumours [25, 26].

Mutations of the PTEN gene have been detected more frequently in endometrioid endometrial carcinoma than in non-endometrioid carcinomas [27]. Moreover, these

alterations have been observed in endometrial hyperplasia, suggesting that these genetic alterations are early events in the development of endometrioid endometrial carcinoma [28, 29].

Thus, this case of PESC could be considered different from the common endometrioid endometrial carcinoma in which the most frequent genetic alteration involves the PTEN gene [28–30].

In this study, we also evaluated the methylation of p16, and BRCA1 gene promoters.

P16 is a tumour suppressor gene contributing to cell cycle arrest as a member of the cyclin-dependent kinase-inhibitor family. P16 regulates the G1-S cell cycle transition by inhibiting the cyclin D-cyclin-dependent kinase (CDK) 4/CDK6-mediated phosphorylation of retinoblastoma protein [31].

DNA methylation plays various critical roles in the control of gene expression [11, 32]. In humans, DNA methylation occurs at cytosines located 5′ to guanosines (CpG dinucleotides), which are abundant in the promoters of many genes; these CpG-rich regions ("CpG islands") are usually unmethylated in normal cells [11, 12]. Aberrant methylation of "CpG islands" in promoter regions is a major event in human cancerogenesis, leading to transcriptional inactivation mechanism for the silencing of tumour suppressor genes [11].

Many studies have demonstrated that methylation of the p16INK4A gene is present in a proportion of primary gynaecological malignancies and this alteration can be associated with poor prognosis [33]. In this case of PESC, we did not find aberrant methylation of p16 promoter. This finding is in accordance with over-expression of p16 immunoreactivity, which, in the absence of HPV infection [1], could be related to other unknown genetic alterations.

In this particular PESC we did not observe aberrant methylation of the BCRA1 promoter, either.

The *BRCA1* gene was first located in 1994 on 17q21 by positional cloning techniques [34].

The gene encodes a 1863 amino acid protein that is expressed in numerous tissues and may act at various points in nuclear function and cell growth control. The evidence chiefly points to roles in DNA repair and transcription [35, 36].

Cells from patients with *BRCA1* mutations and knock-out/partial knock-out mice, demonstrate gross chromosomal abnormalities which are hypersensitive to ionising-irradiation or X-rays, suggesting a defect in double-stranded break repair [37–39].

Hyper-methylation of the *BRCA1* promoter has been detected in sporadic breast [40] and ovarian cancer samples [32, 41].

In conclusion, this neoplasm revealed features that might imply that it is more similar to Uterine Serous Carcinoma (USC) than common Uterine Endometrioid Carcinoma (UEC).

The genetic alteration of p53, with hyper-expression of p53 protein and an absence of abnormalities in the PTEN gene are consistent with the similarities between USC and our case of PESC.

In fact, Risinger et al., have demonstrated that alteration of the PTEN gene was more closely correlated with endometrioid than serous histology [42]. In addition, these Authors observed that there was an inverse relationship between p53 over-expression and PTEN abnormalities [42].

The aberrant methylation of both p16 and BCAR1 promoters was not detected in our case. This finding too could suggest that ESC is more similar to uterine serous carcinoma than uterine endometrioid carcinoma (UEC). In fact, in accordance with the recent study by Seeber et al., which demonstrated that aberrant methylation was lower in USC than in UEC [43], also the carcinogenesis of PESC may be less dependent upon aberrant methylation of the promoters.

Moreover, both USC and PESC present similar clinical features, such as post-menopausal status and aggressive biological behaviour [6, 7].

In our opinion, for the time being, it is hard to accept any definite conclusion concerning the carcinogenesis of PESC, because of its rarity and the very few molecular studies reported in the literature.

Further studies with more numerous cases and larger molecular analyses are mandatory for this malignancy, to confirm whether it is more closely related to papillary endometrial cancer than to endometrioid carcinoma.

Acknowledgements The Authors wish to thank Professor Alex Gillan for his help with the English translation and Mrs Emilia Corradini for technical assistence.

References

1. Klaes R, Friedrich T, Spitkovsky D et al (2001) Overexpression of $p16^{ink4a}$ as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri. Int J Cancer 92:276–284
2. Kataoka A, Nishida T, Sugiyama T, Hori K, Honda S, Yakushiji M (1997) Squamous cell carcinoma of the endometrium with human papillomavirus type 31 and without tumor suppressor gene p53 mutation. Gynecol Oncol 65:180–184
3. Pins MR, Young RH, Crum CP, Leach IH, Scully RE (1997) Cervical squamous cell carcinoma in situ with intraepithelial extension to the upper genital tract and invasion of tubes and ovaries: report of a case with human papilloma virus analysis. Int J Gynecol Pathol 16:272–278
4. Wang Z, Dong J, Eyzaguirre EJ, Tang WW, Eltorky MA, Qiu S (2009) Detection of human papilloma virus subtypes 16 and P16(ink4a) in invasive squamous cell carcinoma of the fallopian tube and concomitant squamous cell carcinoma in situ of the cervix. J Obstet Gynaecol Res 35:385–389
5. Bifulco G, Giampaolino P, Mandato VD, Morra I, Nappi C, Insabato L (2011) Primary squamous cell carcinoma of the endometrium: a case report. Eur J Gynaecol Oncol 32:350–352

6. Goodrich S, Kebria-Moslemi M, Broshears J, Sutton GP, Rose P. Primary squamous cell carcinoma of the endometrium: two cases and a review of the literature. Diagn Cytopathol. 2012 Jan 12. doi:10.1002/dc.22814. [Epub ahead of print]
7. Giordano G, D'Adda T, Merisio C, Gnetti L (2005) Primary squamous carcinoma of the endometrium: a case report with immunohistochemical and molecular study. Gynecol Oncol 96:876–879
8. Giordano G, Azzoni C, D'Adda T, Merisio C (2007) P16INK4a overexpression independent of Human Papilloma Virus (HPV) infection in rare subtypes of endometrial carcinomas. Pathol Res Pract 7:533–538
9. D'Adda T, Bottarelli L, Azzoni C et al (2005) Malignancy-associated X chromosome allelic losses in foregut endocrine neoplasms: further evidence from lung tumors. Mod Pathol 18:795–805
10. Naidoo R, Chetty R (1998) The application of microsatellites in molecular pathology. Pathol Oncol Res 4:310–315
11. Herman JG, Graff JR, Myöhänen S, Nelkin BD, Baylin SB (1996) Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 93:9821–9826
12. Esteller M (2008) Epigenetics in cancer. N Engl J Med 358:1148–1159
13. Weissenbach J, Gyapay G, Dib C et al (1992) A second-generation linkage map of the human genome. Nature 359:794–801
14. Peltomaki P, Lothe RA, Aaltonen LA et al (1993) Microsatellite instability is associated with tumors that characterize the hereditary non-polyposis colorectal carcinoma syndrome. Cancer Res 53:5853–5855
15. Ionov Y, Peinado MA, Malkhosyan S, Shibata D, Perucho M (1993) Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. Nature 363:558–561
16. Wirtz HC, Muller W, Noguchi T et al (1998) Prognostic value and clinicopathological profile of microsatellite instability in gastric cancer. Clin Cancer Res 4:1749–1754
17. Duggan BD, Felix JC, Muderspach LI, Tourgeman D, Zheng J, Shibata D (1994) Microsatellite instability in sporadic endometrial carcinoma. J Natl Cancer Inst 86:1216–1221
18. Steele RJC, Thompson AM, Hall PA, Lane DP (1998) The p53 tumour suppressor gene. Br J Surg 85:1460–1467
19. Ziyaie D, Hupp TR, Thompson AM (2000) p53 and breast cancer. Breast 9:239–246
20. Rogel A, Popliker M, Webb CG, Oren M (1985) p53 cellular tumor antigen: analysis of mRNA levels in normal adult tissues, embryos, and tumors. Mol Cell Biol 5:2851–2855
21. Finlay CA, Hinds PW, Tan TH, Eliyahu D, Oren M, Levine AJ (1988) Activating mutations for transformation by p53 produce a gene product that forms an hsc70-p53 complex with an altered half-life. Mol Cell Biol 8:531–539
22. Lakshmi S, Nair MB, Jayaprakash PG, Rajalekshmy TN, Nair MK, Pillai MR (1997) p53 protein and tumorigenesis in the uterine cervix. Gen Diagn Pathol 142:281–287
23. Reihsaus E, Kohler M, Kraiss S et al (1990) Regulation of the level of the oncoprotein p53 in non transformed and transformed cells. Oncogene 5:137–145
24. Munn KE, Walker RA, Menasce L, Varley JM (1996) Mutation of the TP53 gene and allelic imbalance at chromosome 17p13 in ductal carcinoma in situ. Br J Cancer 74:1578–1585
25. Steck PA, Perhouse MA, Jasser SA et al (1997) Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nat Genet 15:356–362
26. Li J, Yen C, Liaw D et al (1997) PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science 275:1943–1946
27. Bussaglia E, del Rio E, Matias-Guiu X, Prat J (2000) PTEN mutations in endometrial carcinomas: a molecular and clinicopathologic analysis of 38 cases. Hum Pathol 31:312–317
28. Levine RL, Cargile CB, Blazes MS, van Rees B, Kurman RJ, Ellenson LH (1998) PTEN mutations and microsatellite instability in complex atypical hyperplasia, a precursor lesion to uterine endometrioid carcinoma. Cancer Res 58:3254–3258
29. Maxwell GL, Risinger JI, Gumbs C et al (1998) Mutation of the PTEN tumor suppressor gene in endometrial hyperplasias. Cancer Res 58:2500–2503
30. Mutter GL (2001) PTEN, a protean tumor suppressor. Am J Pathol 158:1895–1898
31. Omura-Minamisawa M, Diccianni MB, Chang RC et al (2001) p16/p14(ARF) cell cycle regulatory pathways in primary neuroblastoma: p16 expression is associated with advanced stage disease. Clin Cancer Res 7:3481–3490
32. Esteller M, Silva JM, Dominguez G et al (2000) Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. J Natl Cancer Inst 92:564–569
33. Wong YF, Chung TK, Cheung TH et al (1999) Methylation of p16INK4A in primary gynecologic malignancy. Cancer Lett 136:231–235
34. Miki Y, Swensen J, Shattuck-Eidens D et al (1994) A strong candidate for the breast and ovarian cancer susceptibility gene *BRCA1*. Science 266:66–71
35. Venkitaraman AR (2002) Cancer susceptibility and the functions of BRCA1 and BRCA2. Cell 108:171–182
36. Kerr P, Ashworth A (2001) New complexities for BRCA1 and BRCA2. Curr Biol 11:R668–R676
37. Shen S-X, Weaver Z, Xu X et al (1998) A targeted disruption of the murine *BRCA1* gene causes gamma-irradiation hypersensitivity and genetic instability. Oncogene 17:3115–3124
38. Foray N, Randrianarison V, Mrot D, Perricaudet M, Lenoir G, Feunteun J (1999) Gamma-rays-induced death of human cells carrying mutations of BRCA1 or BRCA2. Oncogene 18:7334–7342
39. Xu X, Wagner KU, Larson D et al (1999) Conditional mutation of BRCA1 in mammary epithelial cells results in blunted ductal morphogenesis and tumour formation. Nat Genet 22:37–43
40. Bianco T, Chenevix-Trench G, Walsh DC, Cooper JE, Dobrovic A (2000) Tumour-specific distribution of BRCA1 promoter region methylation supports a pathogenetic role in breast and ovarian cancer. Carcinogenesis 21:147–151
41. Baldwin RL, Nemeth E, Tran H et al (2000) BRCA1 promoter region hypermethylation in ovarian carcinoma: a population-based study. Cancer Res 60:5329–5333
42. Risinger JI, Hayes AK, Berchuck A, Barrett JC (1997) PTEN/MMAC1 mutations in endometrial cancers. Cancer Res 57:4736–4738
43. Seeber LM, Zweemer RP, Marchionni L et al (2010) Methylation profiles of endometrioid and serous endometrial cancers. Endocr Relat Cancer 17:663–673