RASSF1A methylation and K-ras and B-raf mutations and recurrent endometrial cancer

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Background: Aberrations in mediators of Ras signaling may increase the risk of developing recurrent endometrial carcinoma.

Patients and methods: Primary tumors of patients with (n = 44) and without (n = 44) recurrent stage I endometrioid endometrial carcinoma were compared regarding the presence of K-ras mutations (codons 12 and 13), B-raf mutations (V599), and RASSF1A gene promoter methylation.

Results: K-ras mutations were present in 15% of the patients independent of recurrent disease. No B-raf mutations were found. RASSF1A methylation was demonstrated in 85% of endometrial carcinomas, independent of recurrence. The presence of K-ras mutations and RASSF1A promoter methylation were not related, either directly or inversely. Analysis in premenopausal endometrial carcinomas demonstrated K-ras mutations in 40%, no B-raf mutations, and RASSF1A promoter methylation in 70% of the cases. RASSF1A methylation was also observed in samples of cyclic (n = 14), hyperplastic (n = 8), and atrophic (n = 13) endometrial tissues in 21%, 50% and 38%, respectively.

Conclusions: RASSF1A methylation was observed in a high frequency in endometrioid endometrial carcinoma whereas K-ras and B-raf mutations were observed in a low frequency. No association was observed with the development of recurrent disease. High-frequency RASSF1A methylation in premenopausal carcinomas and an increased frequency in endometrial hyperplasia indicate that this may be an early event in endometrial carcinogenesis.

Key words: B-raf, endometrial carcinoma, K-ras, mutation, RASSF1A

Introduction

Endometrial carcinoma is one of the most common malignancies of the female tract [1]. At least two different types of endometrial carcinoma with distinctive molecular characteristics and tumor behavior can be distinguished: the endometrioid- and the serous-type endometrial carcinoma [2–5]. The serous type is the most aggressive variant and accounts for <10% of the endometrial tumors [3]. The endometrioid type occurs most frequently and accounts for 57%–80% of all cases [6, 7]. This tumor type is characterized by mutations in the K-ras and PTEN gene, microsatellite instability, and the expression of steroid receptors [4, 5]. Although the prognosis of the endometrioid type is relatively good, some patients present with recurrent disease shortly after treatment [8, 9]. Metastases are the major cause of death of carcinoma patients. The increase in cellular mobility of the carcinoma cells is usually the result of a phenotypic conversion, which is referred to as epithelial to mesenchymal transition (EMT). The hallmarks of EMT are the loss of cell–cell adhesion and cytoskeletal rearrangements, by which epithelial cells acquire migratory behavior and escape from the well-organized tissue structures [10]. Activation of the Ras signaling cascade is required for the accomplishment of these processes. Ras is known to participate in various signaling pathways one of which is the Raf/mitogen-activated protein kinase (MAPK) pathway. Overexpression of Ras and Raf results in increased cell mobility and dispersion [11]. Therefore, mutations in the Ras and Raf genes that result in proteins that are continuously in an activated stage may lead to a migratory phenotype of subsets of carcinoma cells.

The K-ras gene is the locus for the c-K-ras proto-oncogene, lying on chromosome 12p12, and is ~45 000 bp in length. It encodes for a 2.0-kb transcript that is highly conserved across species, and is translated into the p21-ras protein. These proteins are located in the plasma membrane and transduce growth and differentiation signals from activated receptors to protein kinases within the cell [12]. The K-ras proteins are in a weak guanosine triphosphate (GTP)-bound, active state, thereby altering transduction into the cell. The majority of mutations have been found at K-ras codons 12 and 13, and to a lesser extent at codon 61 [13]. These mutations are somatic rather than in the germline, and consist of single-base-pair

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substitutions which lead to the change of one amino acid in the protein. These mutations result in a K-ras protein that remains in the GTP-bound, activated state. Mutations in codons 12 and 13 of K-ras are found in 10%–30% of the endometrial carcinomas [14–17], and have been associated with a more aggressive behavior [15], a finding which has not been confirmed by others [18, 19].

Mutations in B-raf, which encodes for a serine/threonine kinase, have been reported in several human cancers. Most of these mutations occur within the kinase domain, resulting in elevated kinase activity and transforming capacity in NIH3T3 cells [20]. A single-base substitution (T→A change at nucleotide 1796) resulting in an amino acid substitution (V600E) accounts for 80%–90% of these mutations [20]. B-raf, but not K-ras, mutations occur more frequently in mismatch repair-deficient colon tumors and are correlated with hMLH1 hypermethylation [21, 22]. So far, the presence of B-raf mutations has not been reported in endometrial carcinoma.

Recently, a new ras effector, RASSF1A, was characterized by Dammann et al. [23]. The gene is located at chromosome 3p21.3, a region which frequently shows allelic loss in many cancers [24]. RASSF1 encodes for more than seven isoforms including RASSF1A, RASSF1B, and RASSF1C, derived from alternative messenger RNA (mRNA) splicing and promoter usage [25]. RASSF1A is thought to be responsible for the Ras-dependent growth inhibition through its proapoptotic function [26], since elimination of Ras inhibits apoptosis induced by transient transfection of RASSF1 into 293-T cells. In addition, Shivakumar and co-workers [27] showed that RASSF1A can induce cell cycle arrest by inhibiting accumulation of cyclin D1, thus preventing G1/S-phase cell cycle progression. Loss of RASSF1A expression by methylation may shift the balance towards a growth-promoting effect without the necessity of activating Ras mutations. Methylation of the RASSF1A gene promoter has been observed in many human tumors and is an important mechanism for inactivation of the RASSF1A [28–32].

In the present study, we investigated whether activation of the ras signaling pathway as a result of activating aberrations in mediators of this pathway is associated with recurrent endometrial carcinoma. To this end, we carried out a case–control study and evaluated whether activating mutations in K-ras, B-raf, and promoter hypermethylation of RASSF1A are related to recurrent disease.

patients and methods

patients and tissue specimens

Patients with recurrent endometrioid endometrial carcinoma were selected from the Dutch National Pathology Register. Forty-four patients treated for stage I disease, classified according to the International Federation of Gynecology and Obstetrics criteria [33], with recurrent disease within 3 years of diagnosis, were included. Patients were treated by hysterectomy and bilateral salpingo-oophorectomy. Postoperative radiotherapy was applied when tumor invasiveness was >50% of the myometrial wall and/or when the tumor was poorly differentiated. Forty-four control patients were selected and matched for tumor stage, tumor differentiation, age at diagnosis, and therapy. Patients with recurrent endometrial carcinoma presented with local recurrence at the vagina vault (n = 35), pelvic wall (n = 1), distant metastases (n = 5), or both local recurrence and distant metastases (n = 3). A pathologist revised the histopathology and the endometrioid-type adenocarcinoma was confirmed in all cases. All patients (n = 88) were postmenopausal, and no significant differences were found in clinicopathological characteristics and treatment between patients with and without recurrence. In the patient group with recurrence, the recurrence was diagnosed after a median of 12.5 months after primary treatment (range 2–32 months).

Cyclic endometrial tissue was obtained from healthy women who underwent laparoscopic surgery for benign indications, not related to endometrium pathology (median 34, range 30–49 years). Postmenopausal atrophic endometrial tissue was collected from 13 women who underwent a hysterectomy for a genital prolapse (median 63, range 47–83 years). Hyperplastic endometrial tissue of eight patients was obtained from the pathology archive (median 57, range 46–73). Tumor tissue of 10 patients with premenopausal stage I endometrial carcinoma was subjected to analysis as well (median 47, range 43–52 years). The Medical Ethical Committee of the University Hospital of Maastricht approved the study.

DNA isolation

After identification of the tumor area by a pathologist, tumor tissue was manually dissected from five consecutive 20-μm sections of the paraffin-embedded tissue. Genomic DNA was extracted using a proteinase K (Qiagen, Hilden, Germany) digestion followed by DNA isolation using the Puregene DNA Isolation Kit (Gentra Systems, MN, USA). In addition, DNA was extracted from three endometrial carcinoma cell lines (EC1, AN3CA, and RL95.2).

mutation analysis of K-ras

A K-ras codon 12 and 13 PCR product of 179 bp was amplified and used as a template for the amplification of a 114-bp fragment. Genomic DNA (250 ng) was added to 50 mmol/l KCl, 10 mmol/l Tris–HCl (pH 8.3), 2.0 mmol MgCl2, 250 μmol/l deoxynucleoside triphosphate (dNTP) (Pharmacia, Uppsala, Sweden), 200 nmol/l of the flank primers, 0.5 U Taq polymerase (Invitrogen, Breda, The Netherlands) (annealing temperature 55°C).

Water was added to a final volume of 50 μl. The subsequent PCR was carried out in a final volume of 50 μl, containing 5 μl of a 1:100 dilution of the flank product; 50 mmol/l KCl, 10 mmol/l Tris–HCl (pH 8.3); 1.5–2.5 mmol MgCl2; 250 μmol/l dNTP; 200 mmol/l nested primers, 0.5 U Taq polymerase (annealing temperature 58°C). Amplification was carried out using one standard and one biotinylated primer. DNA isolated from the colorectal cancer cell line (CaCo2) was used as positive control. In the negative controls no DNA was added. The size and amount of the PCR products were checked by electrophoresis on 2% agarose gels. The sequencing reactions of the biotinylated products were carried out using the autoload solid-phase sequencing kit (Amersham Pharmacia, Uppsala, Sweden) with an extended Cy5-labeled primer. The biotinylated PCR products were captured on a sequencing comb coated with streptavidin, and the nonbiotinylated strand was removed by alkaline denaturation. The remaining strand served as a template for dideoxy sequencing reactions using a Cy5-labeled primer (5'-Cy5-CTCTATTGTTGGATCATATTCCGAC-3') and T7 polymerase. The PCR products were separated on the ALF express DNA Analysis System and analyzed by ALFwin software (Amersham Pharmacia Biotech, Uppsala, Sweden).

B-raf mutation detection

The presence of the mutation in B-raf (V600E) was determined by seminested restriction fragment length polymorphism analysis (RFLP) on

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B-raf mutation detection

The presence of the mutation in B-raf (V600E) was determined by seminested restriction fragment length polymorphism analysis (RFLP) on
the basis of the protocol described by Davies et al. [20], resulting in a 255-bp product. Primer sequences are 5'-CTCAATGCTGCTGATTAGGA (flank forward, nested forward), 5'-ATGACTTTCTAGTAACCTGAGC (flank reverse), and GGCACAAAAGTTTATCGTGGCA (nested reverse). The PCR mix (50 μl final volume) contained 150 ng of genomic DNA, 0.5 μl (10 pmol) of each primer, 0.5 μl (1 U) of platinum-Taq DNA polymerase (Invitrogen) and 1.5 mmol MgCl₂. The mix for the nested PCR (50 μl final volume) consisted of 5 μl of 1 : 100 diluted flank PCR product, 0.5 μl (10 pmol) of each primer, 0.5 μl (25 mmol) dNTPs, 0.2 μl (1 U) of platinum-Taq DNA polymerase (Invitrogen), and 1.5 mM MgCl₂.

The wild-type allele contains a TspRI (New England Biolabs, Beverly, MA) restriction site. Thus, upon TspRI digestion, PCR products derived from a wild-type allele generate a single fragment of 224 bp. Digestion was carried out with 15 μl PCR product for 3 h at 65°C according to the manufacturers instructions. Subsequently, 5 μl sample buffer (98% formamide by vol., 0.025% mass/vol. bromophenol blue, 0.025% mass/vol. xylene cyanol FF) was added to the digestion mixture, which was heated to 95°C for 3 min and placed on ice directly afterwards. The digested PCR products were separated on a nondenaturing 6% polyacrylamide gel, and visualized by UV illumination after staining with ethidium bromide.

methylation analysis of RASSF1A and cell culture
DNA methylation in the CpG island of RASSF1A was determined as described previously [34]. A nested approach was chosen above a direct methylation specific PCR (MSP) on DNA isolated from paraffin-embedded tissues, because a direct MSP did not yield any amplicons (results not shown).

To assess whether the methylated promoter region has functional consequences in cells, endometrial carcinoma cell lines (ECC1 and RL95.2) were cultured for 3 days in the presence of the demethylating agent 5-aza-2'-deoxycytidine (DAC) (200 nm). RNA was isolated and RT-PCR was carried out with the RSF-4 and RSF-3 primers as described by Lee et al. [32]. The colorectal cancer cell line SW480 was used as a positive control for RASSF1A, GAPDH was used as internal control. Subsequently, DNA isolated from both untreated (control) and treated endometrial carcinoma cell lines was subjected to bisulfite treatment and MSP for the detection of RASSF1A gene promoter methylation (Figure 3).

bisulfite sequencing
To determine whether the methylated amplicons are partially or completely methylated, the PCR fragments were subjected to bisulfite sequencing. First the 144-bp fragment resulting from the flanked (outside) PCR was cut out of the gel, purified with the PCR cleanup kit (Qiagen), and then cloned in the pGEM®-T Easy vector (Promega, Leiden, The Netherlands) according to the manufacturer’s protocol. The vector with the insert was transformed into JM 109 competent cells with the heat-shock method.

Bacteria were grown in SOC medium for 1 h at 37°C and then plated on Lennox L Broth Base/ampicillin/Isopropyl-D-thiogalactopyranosid/X-Gal plates and incubated overnight at 37°C. White colonies, indicative of inserted sequences, were picked and a M13-PCR was carried out. The PCR products of the positive colonies (revealed by a 245-bp amplicon) were purified with the PCR purification kit (Qiagen) and sequenced with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Genome Center, Maastricht, The Netherlands).

data analysis
The SPSS software program (11.5) was used for statistical analysis. Median values were calculated for the patient’s age and follow-up time. To test whether the differences in both patient groups were significant, Students’ t-test for paired analysis was carried out for continuous variables (age, follow-up).

McNemar’s test for paired analysis was carried out for dichotomous variables (K-ras and B-raf mutations, RASSF1A methylation, tumor differentiation, and tumor invasion). The t-test was carried out for the comparison of the patient’s age at diagnosis for RASSF1A-methylated and unmethylated cases, and K-ras-mutated and nonmutated cases. Odds ratios (OR) were calculated for the presence of K-ras mutations in patients with recurrence compared with patients without recurrence, and in premenopausal compared with postmenopausal endometrial carcinomas. To determine the statistical significance, 95% confidence intervals (CI) were calculated. All tests of statistical significance were two-sided, and a P value of 0.05 was considered significant.

results
K-ras mutations
K-ras mutations were detected in 15 out of 85 (18%) endometrial carcinomas (Table 1). In three cases, K-ras mutation analysis could repeatedly not be carried out, probably due to poor DNA quality. K-ras mutations were detected in five patients with recurrence and in 10 control patients. This frequency difference was not statistically significant (OR 0.42; 95% CI 0.13–1.36). In premenopausal endometrial carcinomas, K-ras mutations were detected in four out of 10 patients (40%). Although the presence of K-ras mutations in premenopausal endometrial carcinomas was much higher compared with postmenopausal endometrial carcinomas, 40% versus 18%, the difference was not statistically significant (OR 3.1; 95%)

### Table 1. Prevalence of K-ras and B-raf mutations and RASSF1A gene promoter methylation in benign and malignant endometrial samples

<table>
<thead>
<tr>
<th></th>
<th>K-ras mutations (%)</th>
<th>B-raf mutations (%)</th>
<th>RASSF1A methylation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U</td>
<td>M</td>
<td>U</td>
</tr>
<tr>
<td>Postmenopausal endometrial</td>
<td>15/85 (18)</td>
<td>0/85 (0)</td>
<td>13/85 (15)</td>
</tr>
<tr>
<td>carcinomas</td>
<td>5/43 (12)</td>
<td>0/42 (0)</td>
<td>9/43 (21)</td>
</tr>
<tr>
<td>With recurrent disease</td>
<td>10/42 (24)</td>
<td>0/43 (0)</td>
<td>4/42 (10)</td>
</tr>
<tr>
<td>Without recurrence</td>
<td>4/10 (40)</td>
<td>0/10 (0)</td>
<td>3/10 (30)</td>
</tr>
<tr>
<td>Premenopausal endometrial</td>
<td>0/14 (0)</td>
<td>0/14 (0)</td>
<td>11/14 (79)</td>
</tr>
<tr>
<td>carcinosomas</td>
<td>0/13 (0)</td>
<td>0/13 (0)</td>
<td>8/13 (62)</td>
</tr>
<tr>
<td>Endometrial tissue</td>
<td>0/82 (0)</td>
<td>0/8 (0)</td>
<td>4/8 (50)</td>
</tr>
<tr>
<td>Cyclic endometrium</td>
<td>0/14 (0)</td>
<td>0/14 (0)</td>
<td>3/14 (21)</td>
</tr>
<tr>
<td>Atrophic endometrium</td>
<td>0/13 (0)</td>
<td>0/13 (0)</td>
<td>8/13 (62)</td>
</tr>
<tr>
<td>Hyperplastic endometrium</td>
<td>0/8 (0)</td>
<td>0/8 (0)</td>
<td>4/8 (50)</td>
</tr>
</tbody>
</table>

U, unmethylated alleles; M, methylated alleles.
endometrial carcinomas showed an equally high percentage of RASSF1A methylation, 70% (7/10). There was no difference between the age at diagnosis of patients with RASSF1A gene promoter methylation (69.7 years) compared with those without RASSF1A gene promoter methylation (70.6 years).

Since methylation of RASSF1A was so common in endometrial cancers, we examined the methylation status of the nonmalignant endometrial tissue as well. RASSF1A gene promoter methylation was present in 21% (3/14) of cyclic endometrial tissue and in 38% (5/13) of postmenopausal atrophic endometrial tissue. Hyperplastic endometrial tissue demonstrated RASSF1A promoter methylation in 50% (4/8) of the samples (Table 1). All three endometrial carcinoma cell lines were fully methylated for RASSF1A. The presence of RASSF1A gene promoter methylation was not (inversely) correlated with the presence of K-ras mutations.

Bisulfite sequencing was successfully carried out on 10 methylated clones from three hyperplasias and 11 methylated endometrial carcinomas. At least 25 bp of the promoter region were sequenced in all clones.

Overall, RASSF1A gene promoter methylation was present in 85% (72 of 85) of the endometrial carcinomas (Table 1). In three cases, results of the MSP were not conclusive. Representative results are shown in Figure 2. Patients with recurrence demonstrated RASSF1A gene promoter methylation in 79% compared with 90% in patients without recurrence, which was not significantly different (Table 1). Premenopausal endometrial carcinomas showed an equally high percentage of RASSF1A methylation, 70% (7/10). There was no difference between the age at diagnosis of patients with RASSF1A gene promoter methylation (69.7 years) compared with those without RASSF1A gene promoter methylation (70.6 years).

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Figure 1. Representative results of the restriction fragment length polymorphism for the detection of B-raf (V600E) mutations of eight samples. Colorectal cancer cell lines CaCo2 and HT29 were used as negative and positive controls, respectively.

Figure 2. Representative examples of RASSF1A MSP reactions of six endometrial carcinoma patients. The presence of a visible PCR product in those lanes marked U indicates the presence of unmethylated alleles. The presence of PCR product in the M-marked lanes indicates the presence of methylated alleles. Normal lymphocytes (NL) and in vitro methylated DNA (IVD) were used as negative and positive controls.

Figure 3. (A) Expression of RASSF1A in untreated endometrial carcinoma cell lines (control) and reexpression of RASSF1A by treatment with 5-aza-2’-deoxycytidine (DAC) in two endometrial carcinoma cell lines (ECC1, RL95.2). The cell lines were treated for 3 days with 200 nM of DAC. RASSF1A was analyzed by RT-PCR. Expression of GAPDH was determined as a control for RNA integrity. (B) MSP reactions of the untreated (control) endometrial carcinoma cell lines (ECC1, RL95.2) and of the cell lines treated with 200 nM DAC. The presence of a visible PCR product in those lanes marked U indicates the presence of unmethylated alleles. The presence of PCR product in the M-marked lanes indicates the presence of methylated alleles. Normal lymphocytes (NL) and in vitro methylated DNA (IVD) were used as negative and positive controls. The H2O control was included in both the flanking PCR and in the MSP. SW480 was included as a positive control.
of activating mutations of K-ras and B-raf or methylation of the RASSF1 promoter are associated with aggressive tumor behavior.

*RASSF1* promoter methylation was demonstrated in 85% of the primary tumor tissue of patients with endometrioid endometrial carcinoma, independent of recurrent disease. The first short report of *RASSF1A* methylation in endometrial carcinoma was published in 2004, but analysis was carried out in 15 cases only and the frequency of *RASSF1A* methylation was not mentioned [35]. Recently, Kang et al. [36] described *RASSF1A* hypermethylation in 33.3% of adenocarcinomas of the cervix compared with 81% of endometrial carcinomas, which correspond with our frequency. It is unlikely that the high frequency of *RASSF1A* methylation is due to the nested PCR procedure, since the same detection was carried out on the paraffin-embedded tissue of colorectal cancers in which a frequency of 20% *RASSF1A* methylation was found, which is in accordance with other reports [34, 37]. The fact that a direct MSP on these samples did not result in detectable PCR products, further justifies the use of a nested approach.

*RASSF1A*-methylated endometrial carcinoma cell lines were treated with the demethylating agent DAC, resulting in a cell population unmethylated for *RASSF1A*. That the methylation of the investigated promoter region has functional relevance was demonstrated by the fact that after demethylation reexpression of *RASSF1A* did occur.

Gene promoter methylation is reported to be associated with aging. To exclude age as a confounder, a number of premenopausal endometrial carcinomas were tested as well. *RASSF1A* gene promoter methylation was also present in the majority of tested premenopausal endometrioid endometrial carcinomas and the frequencies were similar, given this small number of premenopausal tumors examined. This could indicate that *RASSF1A* methylation is a phenomenon correlated with carcinogenesis, rather than with age [38], and it may be an early change in endometrial tumor development. This is strengthened by the fact that *RASSF1A* gene promoter methylation was found in 50% of cases with endometrial hyperplasia, which is considered a precancerous state, and the fact that in hyperplasias the amplicon is already methylated to the same extent as in the carcinomas. Cellular atypia was present in only one case with endometrial hyperplasia, indicating that *RASSF1A* methylation was not restricted to those cases with cellular atypia.

The fact that a low frequency of *RASSF1A* methylation was also found in atrophic and normal cyclic endometrium indicates that *RASSF1A* methylation alone does not result in tumor development, but may increase susceptibility for malignant transformation [34]. Still, the presence of *RASSF1A* methylation could be a randomly assigned result, independent of cancer development. To confirm these present findings, analysis should be extended to a larger group of normal cyclic and atrophic endometrial tissues.

Loss of *RASSF1A* expression could shift the balance towards a growth-promoting effect as a result of the loss of the proapoptotic and cell cycle-suppressive actions, without the necessity of *RassF1*-activating mutations [27, 39]. Cells that have accumulated mutations may therefore not be adequately disposed of. In this regard, it is interesting to note that RASSF1 is a binding partner for the E1A-regulated transcription factor p120 (E4F), which also binds to p14ARF, Rb, and p53 [40]. In the presence of this transcription factor, RASSF1 induces G1 cell cycle arrest and S-phase inhibition, which makes it a putative tumor suppressor. Therefore, loss of *RASSF1* function may not
enhance the risk of developing cancer. The Ras-activating pathway may be disturbed by other recently described proteins. The RASSF1 homologue NORE1 shares almost 60% homology, at the amino acid level, with RASSF1A [41]. Methylation of NORE1A was shown to be present in a subset of breast, colorectal, and kidney tumor cell [42]. The presence of NORE1 hypermethylation was restricted to lung tumors with wild-type K-ras, this mutual exclusivity of the epigenetic and genetic alterations in the two genes of the Ras pathway indicates that NORE1 also may play a critical role in human tumorigenesis [43]. In colorectal cancer RASSF2A methylation equally demonstrated a mutual exclusivity with the presence of K-ras mutations [44].

K-ras mutations were detected in 18% of postmenopausal endometrial carcinomas, which corresponds with the frequency reported by others [18, 45, 46], and were not correlated with tumor relapse. In various tumors the presence of K-ras mutations and RASSF1A methylation were found to be mutually exclusive [34, 47]. This indicates that both epigenetic and genetic alterations of the ras pathway are involved in the formation or progression of different tumors. Our data do not support this mutual exclusivity in endometrial carcinoma.

Similarly, it has been described that mutations in B-raf and RASSF1A promoter methylation are mutually exclusive [48]. The fact that no B-raf mutations were detected in our patient groups is therefore probably due to the high prevalence of RASSF1A promoter methylation. In a recent study, Mutch et al. [49] found only one B-raf mutation in 146 mismatch-deficient endometrial tumors, which shows that B-raf mutations are not a frequent event in endometrial carcinomas. In this study, only the most frequently mutated nucleotide position of B-raf [20] was evaluated. Feng et al. [50] demonstrated in a recently published study the occurrence of B-raf mutations outside exons 11 and 15 by direct sequencing in endometrial carcinoma. Seven out of 21 mutations were found at the V600E allele. Due to the fact that RASSF1A promoter methylation, however, was not carried out, it remains unclear whether our K-ras mutations indicate that RASSF1A methylation or K-ras point mutations in human endometrial carcinomas: correlation with clinicopathological features and patients’ outcome. J Cancer Res Clin Oncol 1998; 124 (12): 695–700.


Furthermore, we would like to thank the Dutch National Pathology Register for their selection of patients with recurrent endometrial carcinoma.