Model for the Molecular Genetic Diagnosis of Endometrial Cancer Using K-ras Mutation Analysis

Rajai Munir Al-Jehani, Arjun Ravindran Jeyarajah, Bjorn Hagen, Estrid Vilma Hogdall, David Howard Oram, Ian Jeffrey Jacobs*

Endometrial cancer is the most common female genital tract cancer in Western societies (1). The prognosis for endometrial cancer is closely associated with tumor stage and histology (2). Currently, almost all cases are diagnosed following presentation with postmenopausal bleeding. Although the value of screening women in the general population for endometrial cancer is questionable, there are arguments for screening selected populations, such as women who belong to cancer-susceptible families [e.g., those with the Lynch II cancer family syndrome (3)] and women who are taking tamoxifen (4).

New technologies such as the polymerase chain reaction (PCR) offer exciting opportunities for the molecular detection of cancer. For example, K-ras and p53 gene mutations have been identified in the stools of patients with colorectal cancer (5–8) and pancreatic cancer (9), the sputum of patients with lung cancer (10), and the urine of patients with bladder cancer (11), in some cases up to 1 year prior to clinical diagnosis (8,10).

The aim of this study was to develop a model for the application of molecular diagnostic techniques to a gynecological cancer. We have chosen endometrial cancer because the endometrium is easily accessible for biopsy sampling, detection of poor-prognosis endometrial cancers at an early stage could conceivably result in an improvement in cause-specific mortality, and K-ras (Kirsten-RAS) mutations are known to occur in endometrial cancer (12–14). Although K-ras mutations are relatively infrequent in this type of cancer, they are a suitable target for study because the mutations are mostly confined to codons 12 or 13 of the gene (15–18) and they occur relatively early in the process of endometrial carcinogenesis (13,18–20). In this study, we tested the hypothesis that K-ras mutations could be detected in DNA samples prepared from cells in endometrial aspirates and cervical smear specimens taken from patients with endometrial cancer.

Tissue specimens were collected at the time of surgery from 42 endometrial cancer patients aged 42 years through 93 years. These samples included a cervical smear (taken with a cotton swab), an endometrial aspirate biopsy specimen (taken with a pipelle suction curette), and a small portion of the primary tumor. All samples were placed in sterile Eppendorf tubes, snap-frozen in liquid nitrogen, and stored at −80°C.

The 42 tumors examined by histologic methods were classified by tumor type, stage, and grade as follows: 30 were adenocarcinomas, seven were endometrioid carcinomas, two were carcinosarcomas, two were clear-cell adenocarcinomas, and one was a müllerian mixed tumor; 33 were International Federation of Gynecology and Obstetrics (FIGO) stage I, three were FIGO stage II, two were FIGO stage III, two were FIGO stage IV, and two were not staged (21); 16 were grade 1, 15 were grade 2, eight were grade 3, and the two clear cell carcinomas and the one müllerian mixed tumor were not graded (21). Cell lines with known K-ras mutations (SW480 human colon carcinoma cell line and Hecl-A human endometrial carcinoma cell line) and without K-ras mutations (HEK293 normal human embryonic kidney cell line) were used as positive and negative controls, respectively.

DNA was extracted from all tissue specimens and cultured cell samples by use of standard techniques. Tumor samples were minced by use of a sterile scalpel and then digested overnight in 5–10 mL of lysis buffer (150 mM NaCl, 10 mM Tris–HCl, 10 mM EDTA, 0.5% sodium dodecyl sulfate, and 250 µg/mL proteinase K) at 37°C. The blood sample, which was collected in an EDTA-containing tube, was first treated with distilled water to lyse the red blood cells and then centrifuged at 2500 rpm at room temperature for 10 minutes to concentrate the lymphocytes. The supernatant was discarded, and the pellet was resuspended in lysis buffer. DNA was prepared from the cells by phenol–chloroform–isoamyl alcohol extraction (25:24:1) and ethanol precipitation. The DNA precipitate was dissolved in sterile water. DNA was prepared from cells in endometrial aspirate specimens in the same way as for the tumor specimens but with the use of a smaller volume (2–3 mL) of lysis buffer. We extracted DNA from the cells in the cervical smear specimens by first washing the cotton swab in 0.5 mL of lysis solution before continuing with the rest of the extraction procedure as described above. Confluent cultured cells were first washed in phosphate-buffered saline and then placed in lysis buffer before the rest of the extraction procedure was continued as described above. So that we could minimize the risk of contamination, extractions of DNA from cells in the cervical smears, endometrial aspirates, and in vitro cell cultures were performed in a separate laboratory where no DNA or PCR work had previously been performed. All reagents, tools, and equipment used for DNA extraction were handled with a strict protocol to eliminate the possibility of contamination of PCR reactions. Samples were processed separately with the use of a fresh change of gloves, a bench cover, and a polystyrene tube rack for each preparation. Sterile disposable, plastic Pasteur pipettes were used for all steps.

Primary tumors were initially screened for mutations in codons 12 and 13 of the K-ras gene by use of the

*Affiliations of authors: R. M. Al-Jehani, A. R. Jeyarajah, D. H. Oram, I. J. Jacobs, Gynaecology Cancer Research Unit, Department of Gynaecological Oncology, St. Bartholomew’s Hospital, London, U.K.; B. Hagen, Department of Obstetrics and Gynaecology, Trondheim University Hospital, Norway; E. V. Hogdall, Laboratory for Molecular Biology, Statens Serum Institut, Copenhagen, Denmark.

Correspondence to: Rajai Munir Al-Jehani, Ph.D., Department of Gynaecological Oncology, St. Bartholomew’s Hospital, West Smithfield, London, EC1A 7BE, U.K. E-mail: RALJEHANI@mds.qmw.ac.uk

See “Notes” following “References.”

© Oxford University Press
primer-mediated restriction fragment length polymorphism approach as previously described (7). K-ras mutations were identified in seven (17%) of the 42 tumors (Table 1). DNAs from six of the corresponding endometrial aspirates and from three corresponding cervical smears were also found to harbor K-ras mutations by this method. When a more sensitive mutant-enriched PCR approach (7) was used, K-ras mutations were detected in an additional endometrial aspirate and an additional three cervical smears (Fig. 1). All mutant fragments were isolated from the agarose gels, purified with use of the Wizard PCR Preps DNA purification kit (Promega Corp., Southampton, U.K.) and then sequenced by use of the Taq Dideoxy Terminator Cycle Sequencing Kit (Perkin-Elmer Applied Biosystems, Warrington, U.K.) with the reverse primer 5′-ATT CGT CCA CAA AAT GAT TC-3′ on an automated sequencer (Perkin-Elmer Applied Biosystems). To avoid the possibility of introducing bias into the results, a researcher who was blinded with respect to the sources of the DNA samples performed the sequencing reactions. Nucleotide substitutions identical to those found in the K-ras genes of the corresponding tumor DNA samples were found in all seven endometrial aspirate DNA samples and all six cervical smear DNA samples (Table 1). K-ras mutations were not detected in any of 13 cervical smears corresponding to K-ras-negative tumors analyzed by the mutant-enriched PCR technique. These results support the conclusion that the K-ras mutations detected in the endometrial aspirates and cervical smears were derived from tumor cells that had been shed from the primary tumor and not from any extraneous source.

To assess the sensitivity of conventional cytology for detection of endometrial cancer, we reviewed the cytology reports on cervical smears taken within 30 days of endometrial cancer diagnosis from 40 other patients chosen at random from our database. Only nine (23%) of 40 smears showed any evidence or suspicion of cancer. To assess the sensitivity of our molecular genetic approach for the detection of malignant cells in cervical smears, as compared with conventional cytological analysis, we re-examined the cytology of cervical smears taken from patients with K-ras-positive primary tumors. Cervical smears obtained from three patients, 2, 3, and 5 months before hysterectomy, did not exhibit any cytological abnormality. However, DNA samples prepared from cells in cervical smears from two of the three patients were K-ras positive.

The sensitivity of the mutant-enriched PCR technique (7) was improved by use of the primer-mediated RFLP assay. A pair of primers in which a mismatched forward primer, K-ras (12)-5′ or K-ras (13)-5′, is used to introduce a base substitution into codon 12 or 13 of the K-ras oncogene, respectively. Subsequent digestion with Mva I (for codon 12 mutations) or Bgl I (for codon 13 mutations) restriction enzyme results in digestion of product from wild-type, but not mutant, fragments (7). Samples were visualized by electrophoresis on agarose gels. Fragments of 192 base pairs (bp) indicate mutant alleles, and fragments of 163 bp or 160 bp represent wild-type alleles. Both mutant and wild-type alleles are seen in the tumor DNA samples (t) from patients Pi, 20, and De and in the aspirate DNA samples (a) from patients Pi, 20, Ha, De, and 16. The tumor from patient Go is homozygous for a K-ras codon 12 mutation. Control samples (c) include the SW480 human colon carcinoma cell line DNA (SW), which is homozygous for a K-ras codon 12 mutation (positive control) and normal human lymphocyte DNA (Bi), which harbors only wild-type K-ras alleles (negative control). Panels (C) and (D) demonstrate K-ras codon 12 and codon 13 gene mutations, respectively, in endometrial aspirates and cervical smears detected by the mutant-enriched PCR technique. This is a semi-nested PCR approach in which the products of the primer-mediated RFLP technique are subjected to a second round of PCR and enzymatic digestion leading to enrichment of mutant alleles. The normal pattern of bands can be seen in the lymphocyte control sample (Bi) and is represented by a wild-type band of 128 bp (for codon 12) or 125 bp (for codon 13) which is more intense than the upper, 157-bp, mutant band. Mutant fragments are identified in all the aspirate (a) and smear samples (s) from patients Sm, Ha, 16, and De, where the intensity of the upper, 157-bp, mutant band is equal to or greater than the intensity of the lower, 128-bp or 125-bp, wild-type band. The smear from patient 20 shows the normal pattern of bands, whereas the aspirate DNA from this patient shows the mutant pattern.

Table 1. Tumor characteristics and K-ras codon 12 and 13 sequences of mutant DNAs from endometrial tumors, aspirates, and cervical smears

<table>
<thead>
<tr>
<th>Patient</th>
<th>Histology</th>
<th>Grade</th>
<th>Stage</th>
<th>Tumor</th>
<th>Aspirate</th>
<th>Smear</th>
</tr>
</thead>
<tbody>
<tr>
<td>de</td>
<td>Endometrioid carcinoma</td>
<td>1</td>
<td>I</td>
<td>GGTGAC</td>
<td>GGTGAC</td>
<td>GGTGAC</td>
</tr>
<tr>
<td>16</td>
<td>Adenocarcinoma</td>
<td>3</td>
<td>I</td>
<td>GGTGAC</td>
<td>GGTGAC</td>
<td>GGTGAC</td>
</tr>
<tr>
<td>Pi</td>
<td>Endometrioid carcinoma</td>
<td>2</td>
<td>I</td>
<td>GGTGAC</td>
<td>GGTGAC</td>
<td>GGTGAC</td>
</tr>
<tr>
<td>20</td>
<td>Adenocarcinoma</td>
<td>1</td>
<td>I</td>
<td>GGTGAC</td>
<td>GGTGAC</td>
<td>GGTGAC</td>
</tr>
<tr>
<td>Ha</td>
<td>Müllerian mixed tumor</td>
<td>—</td>
<td>—</td>
<td>GGTGAC</td>
<td>GGTGAC</td>
<td>GGTGAC</td>
</tr>
<tr>
<td>Sm</td>
<td>Adenocarcinoma</td>
<td>2</td>
<td>I</td>
<td>GGTGAC</td>
<td>GGTGAC</td>
<td>GGTGAC</td>
</tr>
<tr>
<td>Go</td>
<td>Endometrioid carcinoma</td>
<td>2</td>
<td>I</td>
<td>GGTGAC</td>
<td>GGTGAC</td>
<td>GGTGAC</td>
</tr>
</tbody>
</table>

*Tumors were graded and staged according to the International Federation of Gynecology and Obstetrics system (21).
†The normal K-ras sequence at codons 12 and 13 is GGTGGC. Mutant nucleotides are highlighted in bold.
enriched PCR was tested by a series of titration experiments. Cells from the Hec1-A human endometrial carcinoma cell line, which is heterozygous for a mutant K-ras codon 12 allele, were serially mixed at ratios from 1:10 to 1:10^13 with cells from the HEK293 normal human embryonic kidney cell line, which harbors only wild-type alleles. DNA was extracted from the different mixtures and analyzed for K-ras codon 12 mutations. The pattern of bands for mutant DNA was clearly distinguishable from the normal pattern, in up to a 1000-fold dilution of Hec1-A with HEK293 cells.

Our results demonstrate that molecular detection of gynecological cancer is feasible. First, we have shown that K-ras mutations can be detected in DNA samples prepared from cells in endometrial aspirate and cervical smear specimens. Second, false-positive results were not obtained in analyses of tissues without K-ras mutations. Third, we provided preliminary evidence that, at least for some specimens of K-ras-positive endometrial cancer, this molecular technique appears to be more sensitive for identification of cancer cells than conventional cytology. Even more sensitive strategies for detection of ras mutations based on the PCR and the ligase chain reaction techniques, which are capable of detecting mutated sequences in up to a 10^8-fold excess of wild-type DNA, have been described (22–25). It remains possible, therefore, that a more sensitive technique may be able to reveal a mutation in the one cervical smear that was found to be negative by our analysis.

In conclusion, we have established proof of the principle that molecular genetic techniques can be used to detect endometrial cancer. The methods developed in this study, involving use of the K-ras mutation as a tumor marker, provide a model that may be applicable to more frequent genetic alterations that may be subsequently identified in endometrial cancer. The availability of a highly specific molecular genetic marker could provide a useful screening tool that may be used to screen women who are at high risk of endometrial cancer. Alternatively, this technology could be used to augment current diagnostic procedures and/or to screen women for recurrence of endometrial cancer.

References


Notes

Supported by grants from the Joint Research Board, St. Bartholomew’s Hospital, and the Danish Cancer Society.

We thank Jane Bridges for providing some of the clinical material and Kirsten Lindboe for her technical assistance. We also thank Steve Jones and other members of the Department of Histopathology, St. Bartholomew’s Hospital, for their help.

Manuscript received August 29, 1997; revised January 20, 1998; accepted January 27, 1998.