Relevance of the Viral RAK Alpha Gene in Diagnosis of Malignant Versus Nonmalignant Tumors of the Ovary and Uterus

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Cancers of the reproductive organs are the most common malignancies in women (7, 13, 19). Prognostic factors for those patients are directly dependent on the stage of the cancer at diagnosis. Earlier detection of breast, ovarian, cervical, or endometrial cancer would significantly increase survival rates of female patients (9, 20, 30). Only 10% of breast and 5% of ovarian cancer cases have a documented inherent pattern, associated mainly with the mutated genes BRCA (15, 18, 34) or the oncogene Her-2/Neu (31).

Except for cervical cancer, which is predominantly associated with human papillomavirus (HPV) (2, 8, 14, 17), a viral etiology of other types of female reproductive tract cancers remains unverified. However, the fact that the majority of papillomavirus-infected women do not develop cervical cancer strongly implies that HPV may be an important cofactor, but not the direct cause, of that cancer. Recent studies of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma (3) strongly suggest that the role of retroviruses in human cancer was somehow underestimated for a long time.

Viral particles (4, 5, 6, 10, 11, 16) and DNA sequences (1, 32, 33) with homology to the mouse mammary tumor virus (MMTV) have been observed in breast cancer for some time. Since MMTV-like sequences are present in cancer patients, as well as in healthy patients, an involvement of this virus in the etiology of human cancer remains controversial. Our recent studies indicated that breast, ovarian, uterine, cervical, and vulvar cancers in women (22–26, 28, 29) and prostate cancer in men (21) express unique antigens, named after the investigator E. M. Rakowicz-Szulczynska (RAK). RAK markers react with the epitope-specific monoclonal antibody (MAb) directed against human immunodeficiency virus type 1 (HIV-1) envelope protein gp120 (27). Another MAb (MAb RAK BrI), developed against RAK antigens, cross-reacts with the GRAF (glycine-arginine-alanine-phenylalanine) linear epitope of the variable loop V3 of the HIV-1 envelope protein gp120, which confirms the strong homology of HIV-1 and RAK proteins. RAK markers p120, p42, and p25, which exhibit molecular weight correlation with the major proteins encoded by HIV-1, have been detected in 100% of breast cancer cases and in 100% of prostate cancer cases. The RAK antigen p160, which presumebly represents a precursor of RAK p120 and p42, was also found in the serum of the majority of ovarian and cervical cancer patients (25, 29).

In addition to HIV-1-like antigens, DNA segments (142 bp) with 90 to 96% homology to the HIV-1 gene encoding the transmembrane protein gp41 have also been identified in breast (22, 24) and prostate cancers (21). The DNA sequences with homology to HIV-1 have been deposited in the GenBank as the breast and prostate cancer-associated RAK alpha genes.

The viral origin of RAK markers is strongly supported by the finding of viral particles immuno reactive with MAb anti-HIV-1 gp120 in breast and cervical cancer cells (22). Antisense oligonucleotides complementary to the HIV-1-like sequences of cancer DNA were found to affect the activity of the viral reverse transcriptase and to inhibit the growth of cancer cells in vitro (22). We suggest that the long-postulated breast cancer virus in fact exists but is related to lentiviruses (HIV-1) and not to the MMTV as was postulated by some researchers (22). Recent studies revealed viral particles in ovarian cancer cells isolated from the abdominal ascites fluid produced by patients with ovarian carcinoma (27). It is noteworthy that the production of viral particles was synchronized with syncytium forma-
tion by these cells. The isolated ovarian cancer virus transformed normal cells (27). Production of viral proteins and syncytium formation were inhibited by MAb anti-HIV-1 gp120, which suggests that antigen RAK p120 may play a similar role as the gp120 protein in HIV-1-infected T lymphocytes. Syncytia were not formed by ovarian benign tumor cells, which also did not produce viral particles. Since similar RAK antigens and related genes RAK alpha were found in breast, gynecological, and prostatic cancers, the identified cancer virus seems to belong to a broad family of lentiviruses, which might be involved in the etiology of certain prostatic malignancies.

The current study concentrated on gynecological cancer, which was found previously to contain HIV-1-like antigens (26, 29). RAK antigens were found to be expressed by 95% of ovarian, uterine, and cervical cancer cases, and the HIV-1-like segments of uterine and ovarian cancer DNA exhibit over 90% homology to HIV-1. Frameshift mutations detected in benign tumors of the ovary and uterus strongly suggest that different variants of the novel lentivirus might lead to benign versus malignant tumors.

MATERIALS AND METHODS

Cancer tissue. The majority of gynecological cancer samples were provided by the National Cancer Institute Cooperative Human Tissue Network. Some samples were obtained during the standard surgical procedures performed by the Department of Obstetrics and Gynecology of the University of Nebraska Medical Center. Human samples were collected according to Internal Review Board protocols.

MAbs. MAb RAK Br1 has been directed against RAK antigens, and this MAb cross-reacts with HIV-1 gp160 or gp120 (22).

Cell fractionation. Sections of cancerous or normal tissues, obtained during standard surgical procedures, were homogenized in 0.35 M sucrose–10 mM Tris-HCl (pH 7.5) to 80% homology to HIV-1. Frameshift mutations detected in benign tumors of the ovary and uterus strongly suggest that different variants of the novel lentivirus might lead to benign versus malignant tumors.

Carcinoma association of RAK antigens. Western blot hybridizations of proteins from endometrial, cervical, ovarian, and vulvar cancer with MAb RAK Br1 revealed RAK antigens p120, p42, and p25 in the majority of cancer cases (Table 1). Examples of Western blot analysis of normal and cancer tissue proteins are shown in Fig. 1. Expression of all three RAK antigens was detected in 95.5% of ovarian cancer cases, 96.9% uterine cancer cases, 92.9% of cervical cancer cases, and 75% of vulvar cancer cases (Table 1). Only 1 of 15 normal ovarian cases, 1 of 15 normal fallopian tube cases, and 2 of 18 normal cervix cases tested positive. All normal ovary cases and all normal cervix cases originated from cancer patients; therefore, in the normal population of healthy women the expression of RAK antigens is expected to be much lower. Expression of RAK antigens in tissues adjacent to cancer varied from 25 to 36.7%. Only 3 of 12 benign ovary tumors tested RAK positive, while 33.3% of the uterine fibroid cases and 50% of the uterine leiomyoma cases tested RAK positive. In contrast to the strong expression of all three RAK antigens (RAK p120, p42, and p25) in cancer cases, benign ovary or uterine tumors either did not produce RAK antigens or were very low or only two antigens were present.

PCR amplification of cancer DNA with HIV-derived primers. PCR amplification of cancer DNA with HIV-1-derived primers revealed amplification bands of 142 bp in cervical cancer (Fig. 2A, lane 3), uterine cancer (Fig. 2A, lanes 4, 5, and 8; Fig. 2B, lane 4), ovarian cancer (Fig. 2A, lanes 6 and 7), and vulvar cancer (not shown). The majority of DNA from normal uterine (Fig. 2A, lane 1; Fig. 2E, lane 5), normal cervix (Fig. 2A, lanes 2, 2E, lane 7), normal ovary (Fig. 2B, lanes 2, 5, Fig. 1E, lane 5), normal vagina (Fig. 2E, lane 5), and normal vulva (Fig. 1E, lane 4), as well as from other normal tissue DNA, including muscle and skin (Fig. 2E, lanes 8 and 9), tested negative. Examples of PCR-positive but histologically normal vulvar and vaginal tissue adjacent to the cancer are shown in Fig. 2B, lanes 6 and 7. An example of PCR-positive leiomyoma of the uterus is shown in Fig. 1A, lane 9. PCR analysis of breast cancer DNA was described before (21, 22, 24), and all breast DNA samples tested in parallel to gynecological cancer represent positive and negative controls, respectively (Fig. 2B to E).

All breast cancer cases tested PCR positive (Fig. 2C, lanes 6 and 7; Fig. 2D, lanes 3 and 4). Tissue adjacent to cancer tested negative in some patients (Fig. 2C, lane 1) and positive in others (Fig. 2C, lane 3, and Fig. 2D, lanes 5 and 6). Breast tissue located at some distance from the cancer margin tested

<table>
<thead>
<tr>
<th>Tissue</th>
<th>RAK antigen</th>
<th>No. tested</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovarian cancer</td>
<td>89/85</td>
<td>95.9</td>
<td></td>
</tr>
<tr>
<td>Ovary “normal”</td>
<td>15/1</td>
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<tr>
<td>Ovarian ADT</td>
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<td>35</td>
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<td></td>
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<td>15/1</td>
<td>6.7</td>
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<td>Fallopian tube ADT</td>
<td>20/5</td>
<td>25</td>
<td></td>
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<tr>
<td>Uterine cancer</td>
<td>64/62</td>
<td>96.9</td>
<td></td>
</tr>
<tr>
<td>Muscle ADT</td>
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<td></td>
</tr>
<tr>
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<td>18/6</td>
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<td>40/20</td>
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<td>30</td>
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<td>Vagina ADT</td>
<td>25/9</td>
<td>36</td>
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</tr>
<tr>
<td>Vulvar cancer</td>
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<td>75</td>
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<td>0</td>
<td></td>
</tr>
<tr>
<td>Carcillate normal</td>
<td>6/0</td>
<td>0</td>
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</tbody>
</table>

* Normal means histologically normal tissue from cancer-affected organ.

** ADT, tissue adjacent to cancer.
either negative in some patients or positive in others (Fig. 2B, lane 1; Fig. 2C, lanes 2, 4, 5, 8, and 9). Interesting examples are represented in Fig. 2C, lanes 4 and 5, where DNA extracted from one part of the breast tested PCR negative and from the other side tested positive. In another patient, both fragments of the tissue were PCR positive (Fig. 2C, lanes 8 and 9).

The data summarized in Table 2 indicate the PCR positivity of 100% of the tested cancer cases and of a significant percentage of the tissues isolated from the cancer-affected organs. Truly normal tissues obtained from cancer-free individuals were PCR negative.

Benign tumors of the ovary were PCR negative in most of the patients. PCR positivity was observed in 3 of 12 cases. Of 15 cases of benign tumor of the uterus (leiomyoma), 5 tested positive and 2 tested weakly positive.

To eliminate the possibility that the negative PCR with normal tissue DNA had been caused by nonspecific inhibition of the amplification reaction, each DNA sample was also amplified with globin primers. All SK68- SK69-negative samples were PCR positive with globin primer, as was the SK68- SK69-positive cancer DNA.

PCR-amplified fragments were isolated from the gel and subjected to DNA sequencing. Sequencing was done four to six times in both directions. Figure 3 exhibits sequences amplified in uterine cancer (patients 21, 28, and 32), benign tumor of the uterus (patient 30), ovarian cancer (patients 17, 18, and 23), and cancer of the omentum (patient 24) and in two different cases of benign tumor of the ovary (patients 19 and 20).

DNA sequences amplified in all cancer cases (patients 21, 28, 32, 17, 18, 23, and 24) exhibited more than 90% homology to the sequences amplified with HIV-1 DNA. Translation of the DNA sequences into amino acid sequences revealed peptides with >60% homology to the HIV-1 transmembrane protein gp41 (Fig. 4). It is noteworthy that the HIV-1-like peptides exhibited highly conserved mutations, which were present in different cancer patients. Specifically, methionine in position 11 of the HIV-1 peptide was replaced by isoleucine in all ovarian and uterine cancer cases. Specifically, methionine in position 11 of the HIV-1 peptide was replaced by isoleucine in all ovarian and uterine cancer cases. Glutamine in position 26 was replaced by asparagine in two of three uterine cancer patients and by lysine in one of four ovarian cancer patients. Glutamine in position 28 was replaced by proline in four of seven cancer patients. Glutamic acid in position 36 was replaced by aspartic...
acid in six of seven patients, and histidine in position 40 was replaced either by isoleucine or by leucine in six of seven patients. The fact that the same codons mutated in a highly conserved way in the majority of the ovarian and uterine cancer cases strongly suggests that these sequences might belong to a retrovirus, closely related to HIV-1, which potentially evolved from a common ancestor.

DNA sequences amplified in benign ovarian cancer cases are shown in Fig. 3; patients 19 and 20 and those amplified in leiomyoma of the uterus are represented by patient 30. It is noteworthy that the DNA fragment amplified in all benign tumors contained frameshift mutations. Specifically, sequences amplified in benign tumors of the ovary were one amino acid longer than fragments amplified in malignant tumors, a change which was secondary to the insertion of one adenine (A) in codon 14 in patient 19 or of one thymidine (T) in codon 22 in patient 20. Although all other codons showed perfect homology with the sequences amplified in cancer DNA, the insertion of each nucleotide resulted in the frameshift mutation, and the encoded peptide would contain a completely different composition from that found in cancer. In both benign tumors of the ovary, the translated peptide was only 35 amino acids long; this was due to the stop codon mutation replacing codon 36 (aspartic acid) (Fig. 4). In contrast to both benign ovary tumors, the uterine leiomyoma DNA sequences (patient 30) exhibited a deletion of the third nucleotide (guanine) in codon 33, and the gene containing that frameshift mutation (Fig. 3) would encode a completely different protein (Fig. 4). Other leiomyoma cases showed several mutations leading to the production of “nonsense” proteins (data not shown). This finding strongly suggests that benign tumors of the ovary and uterus may contain a defective retrovirus compared to malignant tumors, all of which have functional variants of the same viral sequences.

### DISCUSSION

RAK antigens p120, p42, and p25 are expressed in cancers of the reproductive organs but are absent in normal tissues and in the majority of tissues adjacent to the cancer (21, 22, 24, 26, 28, 29). Why cancers of different histological structures, which originated from completely different tissues, all express these unique proteins remained for a long time a medical and biological puzzle. Cancer-limited expression of RAK antigens and the molecular and immunological similarity of these antigens to HIV-1 major proteins strongly suggested either that normal human genes are selectively transcribed in cancer or that a unique virus or family of viruses affects reproductive organs. The fact that HIV-1 gp41-derived primers PCR amplified breast cancer (22, 24) and prostate cancer (21) DNA but not normal tissue DNA, including DNA extracted from tissues adjacent to cancer, strongly supported an exogenous (viral) origin of RAK markers. The HIV-1-like segments of breast cancer DNA exhibited approximately 90% homology to HIV-1 (22), and the prostate cancer sequences exhibited almost 95%
homology to HIV-1 (21). If we assume that RAK antigens are encoded by a virus related to HIV-1, then different viruses belonging to the same family may lead to malignancies of various organs of the reproductive tract (21, 22).

The comparison of the HIV-1-like segments amplified in ovarian and uterine cancers (Fig. 3) indicated that conserved mutations are practically limited to three codons: codon 11, which in HIV-1 encodes methionine, is replaced in all cancers by the codon for isoleucine; codon 36 (glutamic acid) is replaced in the majority of cancer cases by the codon of aspartic acid; and codon 30 (histidine) is replaced by isoleucine or leucine. Three other codons that are mutated in several but not all cancer cases include three codons for glutamine in positions 26, 28, and 39. Comparison of the HIV-1 sequences of the RAK gene in uterine and ovarian cancers with previously published HIV-1-like sequences in prostate cancer (21) and breast cancer (22) indicated that mutations in codons 11, 26, 28, and 36 were common in all cancers, while other mutations were typical for specific types of cancer. This finding renders the possibility that a single, sexually transmitted virus might be implicated in various types of cancers very unlikely. Instead, a family of retroviruses must exist that have a predilection for different organs. The fact that the same codons were mutated in both ovarian cancer and uterine cancer DNAs strongly suggests that the RAK alpha gene evolved from a common ancestor.

Due to the strong homology of the RAK alpha gene to HIV-1 and of the RAK antigens to the HIV-1 proteins, I suggest that the cancer virus might also infect lymphocytes and remain inactive for a long time. Activation by hormones, carcinogens, or opportunistic infections might lead to activation of the virus and relocation to specific organs. The latter hypothesis is strongly supported by the finding of RAK p160 (potential precursor of RAK p120 and p42) in the serum of cancer patients (25, 29).

RAK antigens (p120, p42, and p25) were detected in 95 to 100% of breast, ovarian, uterine, and cervical cancer cases and in 75% of vulvar cancer cases (Table 1). Some benign tumors of the uterus and ovary also tested positive for the expression of RAK antigens.
of RAK antigens, but usually only one or two antigens were detected or else the expression of all three antigens was extremely weak. PCR with primers SK68 and SK69 was also positive in the same benign tumors. Sequencing of one HIV-1-like fragment amplified in a benign tumor of the uterus and of DNA sequences amplified in two cases of benign tumors of the ovary revealed frameshift mutations in all three tumors. Specifically, patient 30 exhibited deletion of the guanine in codon 33, patient 19 had an insertion of adenine in codon 14, and patient 20 had an insertion of thymidine in codon 22. The RAK gene with frameshift mutations would encode either truncated or nonfunctional proteins. DNA sequences amplified in DNA isolated from vaginal tissue of patients with benign tumors of the uterus all showed several frameshift mutations (not shown) coding for truncated proteins.

Although more studies have to be done with various benign versus malignant tumors, the fact that all ovarian and uterine cancers have HIV-1-like segments that are highly related to similar segments of DNA found previously in breast and prostate cancer strongly supports the diagnostic value of RAK genes. New data indicated that, in addition to gp41-like sequences, another segment of the HIV-1-like genome (Gag) can be detected in cancers of the reproductive tract (E. M. Rakowicz-Szulczynska, unpublished data). Inhibition of cancer cell growth, in parallel to the inhibition of the activity of reverse transcriptase in the presence of antisense oligonucleotides derived from the HIV-1-like segment, supports the viral nature of the RAK gene (22). The mechanism of cancer growth promotion by the novel virus remains to be further investigated. The growth factor-like character of RAK antigens might be suggested based on the previously described (28) cancer growth activation by MAb anti-HIV-1 gp120. Cloning the full genome of the putative virus might reveal additional critical differences between the virus associated with malignant and nonmalignant tumors. New therapeutic modalities, including anticancer vaccines, will have to be taken into consideration after the viral etiology of the reproductive tract cancers is verified.

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REFERENCES