Cancer-Testis Antigens and INGI Tumor Suppressor Gene Product Are Breast Cancer Antigens: Characterization of Tissue-specific INGI Transcripts and a Homologue Gene

Dirk Jäger, Elisabeth Stockert, Matthew J. Scanlan, Ali O. Güre, Elke Jäger, Alexander Knuth, Lloyd J. Old, and Yao-Tseng Chen


ABSTRACT

SEREX (serological analysis of recombinant tumor cDNA expression libraries) has been applied to several different tumor types and has led to the identification of a wide range of tumor antigens. In this study, a breast cancer library and a normal testicular library were analyzed using autologous and allogeneic breast cancer sera. Thirty genes were isolated, including 27 known genes and 3 previously unknown genes. Among the known genes, two cancer-testis (CT) antigens, NY-ESO-1 and SSX2, previously defined by SEREX analysis, were found. In addition, INGI, a candidate breast cancer suppressor gene, was isolated. This INGI gene product was also recognized by 2 of 14 allogeneic sera from breast cancer patients but not 12 normal adult sera. Comparison of INGI cDNA from normal and tumor tissues showed no mutation in the index breast cancer case and revealed the presence of at least three different mRNA transcripts with variable transcription initiation sites and exon usage. Tissue-specific expression of these transcripts was found in normal tissues and tumor cell line mRNAs. Furthermore, a novel gene, designated as ING2, sharing 76% nucleotide homology with INGI was identified in the breast cancer cDNA library. The basis of the immunogenicity of INGI and the biological role of INGI and ING2 need further exploration.

INTRODUCTION

Whether immunological factors play a role in the development, growth, and progression of human breast cancer remains a critical unresolved issue. The lymphocyte infiltrates frequently associated with breast cancer (1–5), particularly the intense T- and B-cell infiltrates in medullary breast cancer (6–8), and the reactive changes in the draining lymph node of breast cancer patients (9, 10) are consistent with the idea of immune recognition in breast cancer. However, efforts to relate the lymphocyte infiltrate and lymph node changes with prognosis have not yielded conclusive evidence for such an association (11). The search for breast cancer antigens that elicit humoral or cellular immune reactions in breast cancer patients also has a long history, from evidence for immune responses against the murine mammary tumor virus (12) and delayed hypersensitivity and humoral immunity against T/Tn antigens (13), to more recent findings with breast cancer (1–5), particularly the intense T- and B-cell infiltration and lymph node changes. This article must therefore be hereby marked as originating in the study of an unresolved issue. The lymphocyte infiltrates frequently associated with breast cancer (1–5), particularly the intense T- and B-cell infiltration and lymph node changes, are involved in breast cancer (25).

The recent development of a general method to analyze the humoral immune response of cancer patients that does not require tumor cell lines provides a powerful new way to dissect the immune response to breast cancer. This approach is called SEREX (20). It has now been applied to a wide range of different tumor types (21, 22). In the present study, we applied SEREX analysis to breast cancer and have identified a panel of antigens through autologous and allogeneic screening, including two previously isolated CT antigens, NY-ESO-1 (23) and SSX2 (24), and INGI, a tumor suppressor gene suggested to be involved in breast cancer (25).

MATERIALS AND METHODS

Cell Lines and Tissues. Breast cancer and melanoma cell lines were established previously (26, 27) or obtained from American Type Culture Collection. Specimens of normal and tumor tissues were obtained from Krankenhaus Nordwest and from the Departments of Pathology at the New York Hospital-Cornell Medical Center and Memorial Sloan-Kettering Cancer Center. The tumor specimen for constructing the expression cDNA library was obtained from a cutaneous metastasis of a breast cancer patient BR11 (NW349). This patient had metastatic breast carcinoma with an exceptionally favorable clinical course, characterized by several long-lasting remissions after palliative chemotherapy.

RNA Extraction and Construction of cDNA Expression Library. Total RNA was extracted from the BR11 tumor sample by the conventional CsCl-guanidine thiocyanate gradient method (28). A cDNA library was constructed in a ZAP Express vector, using a commercial cDNA library kit (Stratagene).

Immunoscreening of the cDNA Library. The BR11 cDNA expression library was amplified once and screened with the autologous patient serum at 1:200 dilution or, alternatively, with an allogeneic pooled serum sample derived from seven different breast cancer patients, at a final dilution of 1:1000 for each serum. The screening procedure was as described previously (20, 23). Briefly, the serum was diluted 1:10, preabsorbed with transfectected Escherichia coli lysate, further diluted to 1:200 (autologous screening) or 1:1000 dilution (allogeneic screening), and incubated overnight at room temperature with the nitrocellulose membranes containing the phage plaques at a density of 4000–5000 pfu per 130-mm plate. After washing, the filters were incubated with alkaline phosphatase-conjugated goat antihuman Fc secondary antibodies, and the reactive phage plaques were visualized by incubating with 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium. In addition to the BR11 cDNA library, a commercially obtained testicular cDNA library (Clontech) was also screened with the BR11 serum in an identical fashion.

Sequence Analysis of the Reactive Clones. The reactive clones were subcloned, purified, and in vivo excised to pBK-CMV plasmid forms (Stratagene). Plasmid DNA was prepared by using the Wizard Miniprep DNA Purification System (Promega). The inserted DNA was evaluated by EcoRI-Xbal restriction mapping, and clones representing different cDNA inserts were

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The abbreviations used are: SEREX, serological analysis of recombinant tumor cDNA expression libraries; pfu, plaque-forming unit(s); CT, cancer-testis; RT-PCR, reverse transcription-PCR; RACE, rapid amplification of cDNA ends; EST, expressed sequence tag; ORF, open reading frame.

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1.12 × 10^6 pfu from the BR11 cDNA library was then screened using a pooled serum sample derived from seven different breast cancer patients at a final dilution of 1:1000 for each serum. Twenty-three positive clones were isolated, derived from nine known genes (Table 2). All known genes were widely expressed in normal tissues, demonstrated by the existence of multiple EST entries from normal somatic tissues in the EST database. Of the three unknown genes, two shared identical sequences to ESTs derived from various normal tissues. Gene-specific primers were designed to evaluate the third unknown gene because no EST sequences were found in the database. Results showed universal expression in all normal tissues tested (brain, kidney, liver, colon, and testis). With the exception of ING1, a gene previously implicated as a tumor suppressor gene in breast cancer (25), none of the other 13 genes were further investigated.

**Allogeneic Screening of BR11 cDNA Library.** A total of 8 × 10^5 pfu from the BR11 cDNA library was then screened using a pooled serum sample derived from seven different breast cancer patients at a final dilution of 1:1000 for each serum. Twenty-three positive clones were isolated, derived from nine known genes (Table 2). Of 23 clones isolated, 4 were derived from the NY-ESO-1 gene. Other CT antigens, including SSX2, were not identified in this pooled screening. The seven sera had been tested previously for anti-CT antibody by ELISA and lacked antibodies to NY-ESO-1 and SSX2. The isolation of NY-ESO-1 in this screening attests to the high sensitivity of SEREX methodology. All genes isolated from this allogeneic screening, except for NY-ESO-1, showed universal expression in normal tissues.

Comparison of the genes identified in autologous and allogeneic screening showed that NY-ESO-1 and human keratin 10 gene were the only two genes isolated in both analyses. Some other genes identified in the autologous or allogeneic screening were isolated previously by SEREX, including aldolase A from a lung cancer library (35), U1snRNP from esophageal (23) and colon cancer libraries (36), poly(ADP-ribose) polymerase and adenylosuccinate lyase from colon cancer libraries (36), and alanyl-t-RNA synthetase from renal cancer libraries.4

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**Table 1** SEREX-defined genes identified by autologous screening of BR11 cDNA library

<table>
<thead>
<tr>
<th>Gene group</th>
<th>No. of clones</th>
<th>Comments</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT genes</td>
<td>10</td>
<td>NY-ESO-1</td>
<td>Tumor, testis</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>SSX2</td>
<td>Tumor, testis</td>
</tr>
<tr>
<td>Non-CT genes</td>
<td>5</td>
<td>Nuclear receptor corepressor</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Poly(ADP-ribose) polymerase</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Adenylosuccinate lyase</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>CD 151 (transmembrane protein)</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Human HRY Gen</td>
<td>RT-PCR: multiple normal tissues</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Alanyl-t-RNA synthetase</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>NAD(+) ADP-ribosyltransferase</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Human keratin 10</td>
<td>ESTs: multiple normal tissues</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Human epidermal growth factor receptor kinase substrate</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>ING1 tumor suppressor gene</td>
<td>RT-PCR: multiple normal tissues</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Unknown gene; NCI_TGAP_P11 cDNA clone</td>
<td>ESTs: pancreas, liver, spleen, uterus</td>
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<td></td>
<td>1</td>
<td>Unknown gene</td>
<td>ESTs: multiple normal tissues</td>
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<tr>
<td></td>
<td>1</td>
<td>Unknown gene</td>
<td>RT-PCR: multiple normal tissues</td>
</tr>
</tbody>
</table>

**RESULTS**

**Autologous Screening of BR11 cDNA Library.** A total of 1.12 × 10^6 pfu from the BR11 cDNA library was screened, and 38 positive clones were identified. These 38 clones were purified, in vivo excised, and converted to pBK-CMV plasmid forms. cDNA inserts were analyzed and grouped based on a combined strategy of restriction mapping and DNA sequencing. Of the 38 clones analyzed, 15 were identical to either NY-ESO-1 or SSX2, two recently identified members of the CT antigen family. The other 23 clones were derived from 14 genes, 11 known and 3 unknown (Table 1).

**CT Antigens.** The single most dominant group (15 of 38 clones) were CT antigens, 10 clones from NY-ESO-1 and 5 clones from SSX2. No other CT antigens were identified. By RT-PCR analysis, the BR11 tumor specimen expressed a broad range of CT antigens, including MAGE-1, MAGE-3, MAGE-4, BAGE, SSX2, NY-ESO-1, and CT7 but not SCP-1, SSX1, SSX4, or SSX5. ELISA analysis of the BR11 serum showed high-titer antibody against NY-ESO-1 and SSX2 recombinant proteins but no detectable antibody titers against the other two CT antigens tested (MAGE-1 and MAGE-3). Thus, the RT-PCR and ELISA data correlate with the SEREX findings.

**Non-CT Genes.** In addition to CT antigen genes, 14 distinct gene products (11 known and 3 unknown genes) were identified in the remaining 23 clones. All known genes were widely expressed in normal tissues, demonstrated by the existence of multiple EST entries from normal somatic tissues in the EST database. Of the three unknown genes, two shared identical sequences to ESTs derived from various normal tissues. Gene-specific primers were designed to evaluate the third unknown gene because no EST sequences were found in the database. Results showed universal expression in all normal tissues tested (brain, kidney, liver, colon, and testis). With the exception of ING1, a gene previously implicated as a tumor suppressor gene in breast cancer (25), none of the other 13 genes were further investigated.

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*4 M. J. Scanlan, personal communication.*
SEREX. All genes, except screening, including colon cancer (36). In addition, the gene homologous to NY-ESO-1 cDNA (750 bp; Ref. 23). A comparison with clones isolated by BR11 autoologous screening showed two genes isolated from both libraries, i.e., the poly(ADP-ribose) polymerase and the tumor suppressor gene INGI. Poly(ADP-ribose) polymerase has also been identified from other SEREX screening, including colon cancer (36). In addition, the gene homologous to TITIN was isolated previously from prostate cancer SEREX. All genes, except SSX2, were universally expressed, demonstrated by comparison with EST databanks and/or RT-PCR with gene-specific primers.

**ING1 Not Mutated in BR11.** Two clones isolated above were identified as tumor suppressor gene candidate INGI (37), one from the BR11 cDNA library (clone BR11–74d), and the other from the testicular library (clone TB-32). Comparison of the BR11–74d (partial cDNA clone, 878-bp insert) with the published INGI sequence revealed differences in six residues (positions 818, 836, 855, 861, 866, and 874 of full-length INGI sequence; see variant A in Fig. 1). To evaluate whether any of these differences represented mutation in the BR11 tumor sample, a short PCR fragment containing the 6-bp differences was amplified from a panel of allogeneic normal tissue cDNA and subcloned into the PCR-direct cloning vector pGEMT. Sequencing analysis of the subcloned fragment showed DNA sequence identical to the BR11–74d clone in all six bases and different from the sequences deposited in GenBank, ruling out the possibility of a mutation in the BR11 cDNA clone. This conclusion was subsequently confirmed by sequencing the testicular clone TB-32 and by restriction analysis of several different normal tissue INGI cDNA, using enzymes that would distinguish these sequence differences.

**Multiple Splicing Variants of INGI.** To exclude the possibility that genetic variations might exist in the 5’ segment of the INGI gene that was absent in clone BR11–74d, an attempt was made to obtain full-length INGI cDNA sequence from the BR11 tumor library and the testicular library, using BR11–74d as a nucleotide probe. Four different clones were isolated from the testicular library. No positive clone was obtained from BR11 library, likely reflecting the lower INGI expression in BR11 tumor cells (see below). Sequencing data revealed that these four clones were derived from three transcript variants, designated as variants A, B, and C (Fig. 1). All three variants were identical in their sequences 3’ to nucleotide 586 (based on nucleotide numbering of A variant; Fig. 1) but differed in their 5’ regions, suggesting the likelihood of alternatively spliced variants involving the same exon-intron junction. Moreover, the original INGI sequence, published by Garkavtsev et al. (25), was also different in this 5’ region, representing a fourth variant (variant D; Fig. 1).

The ORFs of these variants were then analyzed. The translation initiation codons were defined in variants A, C, and D (Fig. 1), encoding polypeptides of 279, 235, and 294 amino acid residues, respectively, with 233 amino acids in the shared 3’ region. The amino acid sequences are shown in Fig. 2. No translational initiation site, however, was identified in the 5’ unique sequence of the B variant. To explore the possibility of additional ORFs in the 5’ end of variant B transcripts, 5’ RACE experiments were performed using variant B specific primers and testicular mRNA as the substrate. Cloning and sequencing of the RACE products revealed the variant C sequence 5’ to the original variant B sequence shown in Fig. 1, and the full-length variant B cDNA contained an additional exon of 609 nucleotides between the variant C sequence and the shared 3’ sequence. This additional exon of variant B, however, did not contain any ORF, and the first available initiation site for variant B would be the internal methionine at amino acid position 70 of the predicted INGI peptide of variant A. If expressed, variant B would thus be a truncated form of the INGI gene product (210 amino acid residues), with a 5’ untranslated region of 681 bp (Fig. 2).

**Tissue-specific Expression of INGI Transcripts.** The presence of transcript variants, having at least three different transcriptional initiation sites and possibly different promoters, raised the possibility that their mRNA expression is under different tissue-specific regulation. To evaluate this, the expression of variants A, B, and C were analyzed by RT-PCR using variant-specific primers.

Of five normal tissues and cultured melanocytes, only variant A is universally expressed in these tissues and cells. Variant B is expressed in testis, liver, and kidney, weakly expressed in colon and brain, and not expressed in normal breast and cultured melanocytes. Variant C is expressed only in testis and weakly in brain but not in breast, colon, kidney, or melanocytes. Expression of INGI in cancer tissues was confirmed by RT-PCR analysis of BR11 tumor RNA, six breast cancer cell lines, and eight melanoma cell lines. The signal intensity in BR11 is slightly weaker. Four of six breast cancer cell lines weakly expressed variant B, and all eight melanoma cell lines were negative. None of the breast or melanoma cell lines expressed variant C (Fig. 3).

**Immune Recognition of INGI Gene Product in Breast Cancer Patients.** To evaluate the presence or absence of antibodies against INGI gene product in normal and cancer patient sera, 14 breast cancer patient sera and 12 normal adult sera were tested by phage plaque immunoassay against the BR11–74d clone. Two of 14 allogeneic sera from breast cancer patients showed reactivity at 1:200 dilution. All normal sera were negative.

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3 Y. Obata, personal communication.
Cloning of an ING1 Homologue Gene. Screening the BR11 library using an ING1 cDNA probe derived from clone BR11–74d led to the identification of a novel cDNA (593 bp) with strong homology to the ING1 sequence. Genomic Southern blot analysis using ING1 probe showed two hybridizing DNA species, one of which also hybridized to the 593-bp ING1-like cDNA probe (Fig. 4), confirming the presence of two ING1-related genes in human genome. The transcriptional initiation site of this new ING1-like gene was defined by 5' RACE using normal fetus cDNA, and the full-length cDNA is of 771 bp in size excluding the poly(A) tail. This novel gene, designated as ING2, showed strong nucleotide homology to ING1 (Fig. 5), with strongest homology in the 5’ two-thirds of the sequence (76% identity, nucleotides 1–480). However, the longest ORF in this ING2 gene is only 129 bp in length and would encode a polypeptide of 42 amino acids (M, 5076), homologous to (76% amino acid identity) but much shorter than the ING1 products (210 amino acids to 294 amino acids for different variants). Excluding this coding region, the ING2 contains 203 bp of 5’ untranslated region and 439 bp of 3’ untranslated region.

Sequence comparison to DNA databanks showed no matches of this gene to known genes or EST sequences, and RT-PCR assays were used to evaluate ING2 mRNA expression in normal tissues and tumor
cell lines. All normal tissues (brain, colon, testis, kidney, liver, and breast) tested showed ING2 expression. Among cell lines, one of eight melanoma cell lines (SK-MEL-28) and two of six breast cancer cell lines (BT20 and 734B) showed no ING2 mRNA, whereas other cell lines showed variable levels of expression.

**DISCUSSION**

After the initial description of SEREX by Sahin et al. (20), this serological approach to the identification of human tumor antigens has been applied to several tumor types, and the number of antigens defined by this methodology has grown rapidly (20, 23, 29, 30, 35, 36, 38–40). More than 900 SEREX-identified gene sequences have now been deposited into the SEREX database developed by V. Jongeneel of the Ludwig Institute for Cancer Research.6 Of these SEREX-identified genes, approximately two-thirds represent previously known genes, whereas one-third are novel gene products. The key question related to SEREX-defined antigens are why these gene products are immunogenic, what role (if any) these antigens have in the cancer phenotype, and which SEREX antigens can serve as diagnostic reagents or as targets for vaccine-based therapies. The overall algorithm of SEREX analysis is to define the complete spec-

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trum of immunogenic gene products in cancer, a goal that we refer to as the “cancer immunome.”

Several categories of antigens have emerged from the SEREX studies to date (22, 41). These include differentiation antigens, mutational antigens, amplified/overexpressed antigens, retroviral antigens, splice variant antigens, and CT antigens. Tyrosinase, a classic example of a differentiation antigen in melanocytes, was recognized by an autologous melanoma patient serum in the initial SEREX analysis (20). Mutational antigens recognized by SEREX include mutated p53 in colon cancer (36) and possibly gene products encoded by chromosome 3p candidate tumor suppressor genes, isolated from lung cancer (35) and renal cancer (20). Overexpressed antigens include aldolase-A (35) and eIF-4g in lung cancer and carbonic anhydrase XII precursor in renal cancer (41). HERV-K10, an endogenous retroviral gene product, is the sole example of viral antigens identified by SEREX (20).

Several splice-variant antigens have been found by SEREX, including restin from Hodgkin’s disease (20) and NY-CO-38 from colon cancer (36). Given the multiple splicing isoforms of the candidate tumor suppressor gene ING1 identified in the current study, a splice variant of ING1 product may be the immunogenic stimulus leading to antibody formation.

The most frequently isolated genes in the present study are CT antigens, a special class of differentiation antigens. In normal tissues, CT expression is generally restricted to testis. In cancer, CT expression is found in a variable proportion of a wide range of different cancer types. Ten CT antigen genes or gene families have been defined to date: MAGE, BAGE, and GAGE as T-cell reactive tumor antigens (32, 33, 42, 43); NY-ESO-1, SSX, SCP1, CT7, and CT8 as SEREX-defined antigens (23, 24, 29, 38); CT9 as a testis-specific gene, and CT10 as a gene identified by representational difference analysis between a melanoma cell line and normal skin. Of these, MAGE-1, MAGE-4, NY-ESO-1, SSX2, SCP1, CT7, and CT8 have been found in SEREX (22). Our isolation of NY-ESO-1 and SSX2 is the first time that these two antigens were isolated from breast cancer by SEREX, indicating that these two antigens, among other CT antigens, may be suitable targets for breast cancer vaccines. This view is further supported by the recent studies demonstrating NY-ESO-1 expression in 24% of breast cancer and SSX (various gene family members) in 23% (41, 44).

The major focus of the present study was the ING1 gene, originally isolated by cloning of genes that were preferentially expressed in a normal breast epithelial cell line but not in breast cancer lines. By Western blotting with a polyclonal antibody, the product of the ING1 gene was shown to be a protein of Mr 33,000, designated p33ING1. By soft-agar and focus-forming assays, it has been shown that cells expressing ING1 in an antisense orientation would confer growth advantage (25). Acute transfection of ING1 sense construct into a breast cancer line led to growth inhibition, supporting the notion that

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**Fig. 5.** cDNA sequence comparison between ING2 and ING1 (variant D). Strong homology persists throughout the predicted 129-bp ORF of the ING2 gene (underlined), as well as the 5’ and part of the 3’ untranslated regions. This gene sequence has been deposited in GenBank (accession number AF149724).

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ING1 is a tumor suppressor gene (25). Subsequent studies showed this gene to be involved in the negative regulation of cell proliferation and in cellular aging and apoptosis (45, 46). The ING1 gene is mapped to chromosome 13q34 (47), one of the regions reported to be affected by loss of heterozygosity in breast cancer (48, 49). In a recent study by Garkavtsev et al. (37), the authors showed that the biological effects of ING1 and p53 are interrelated and require the simultaneous expression of both genes. This interaction was further supported by the physical association between p53 and p33ING1 shown by coimmuno-precipitation experiments using anti-p53 antibodies (37).

Comparison of the BR11–74d clone sequence with the ING1 sequence in the original cloning paper (Ref. 25; GenBank accession number AF001954) showed that BR11–74d is a partial cDNA clone of ING1 but with six scattered single-base differences. Repeated sequencing and restriction analysis of several normal tissues DNA showed no difference between normal ING1 sequence and the BR11–74d sequence, and the sequence in the GenBank entry was not observed. We have thus concluded that these single-base differences represent either sequencing errors in the GenBank entry or the presence of a rare allelic polymorphism.

Analysis of the ING1 cDNA clones from normal testicular library revealed the presence of three variants with different S’ sections (variants A, B, and C), which are all different from the originally cloned ING1 sequence (variant D; GenBank accession number AF001954). Further comparison showed variant A to be identical to a more recent entry of ING1 but with scattered single-base differences. Repeated sequencing and restriction analysis of several normal tissues DNA showed no difference between normal ING1 sequence and the BR11–74d sequence, and the sequence in the GenBank entry was not observed. We have thus concluded that these single-base differences represent either sequencing errors in the GenBank entry or the presence of a rare allelic polymorphism.

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REFERENCES

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