Genetic and Epigenetic Analysis of the Putative Tumor Suppressor km23 in Primary Ovarian, Breast, and Colorectal Cancers

Ian G. Campbell,1,3 Wayne A. Phillips,2,4 and David Y. H. Choong1

Abstract

Purpose: A very high frequency of somatic mutations in the transforming growth factor-β signaling component km23 has been reported in a small series of ovarian cancers (8 of 19, 42%). Functional studies showed that some mutations disrupt km23 function, resulting in aberrant transforming growth factor-β signaling and presumably enhanced tumorigenicity. If verified, this would elevate mutation of km23 as the single most frequent somatic event in ovarian cancer.

Experimental Design: We sought to verify the frequency of silencing of km23 among 104 primary ovarian cancers (49 serous, 18 mucinous, 29 endometrioid/clear cell, and 8 undifferentiated) as well as 72 breast and 61 colorectal cancers by undertaking both somatic mutation and promoter methylation analyses. All four exons of km23 were individually amplified from genomic DNA with primers complementary to surrounding intronic sequences and analyzed by single-stranded conformational polymorphism analysis.

Results: Two germ line polymorphisms were identified, but none of the 237 tumors analyzed harbored somatic km23 mutations. In addition, promoter methylation analysis showed that in all cases, the 5′ CpG island was unmethylated.

Conclusions: Our data suggest that silencing of km23, either through somatic genetic mutation or promoter hypermethylation, is rare in ovarian, breast, and colorectal cancers.

Materials and Methods

Tumor samples. One hundred four primary ovarian cancer (49 serous, 18 mucinous, 29 endometrioid/clear cell, and 8 undifferentiated) biopsies were obtained from hospitals in the south of England. All DNA was extracted from fresh frozen specimens and, where necessary, microdissected to contain >80% tumor cells. Normal DNA was extracted from matching peripheral blood samples. Matching tumor and normal DNA from 72 primary breast cancers was provided by the Peter MacCallum Cancer Centre tissue bank or by Dr Nick Hayward (Queensland Institute for Medical Research, Brisbane, Australia). Sixty-one colon carcinomas were obtained from patients undergoing elective surgery at Western Hospital (Victoria, Australia). Normal DNA was extracted from normal-appearing mucosa >5 cm from the margins of the carcinoma. Appropriate institutional ethics committees approved the collection and use of tissues for this study.

Screening for somatic genetic mutations. All four exons of km23 were individually amplified from genomic DNA with primers complementary to surrounding intronic sequences (Table 1). PCR was carried out using 10 ng of genomic DNA in a reaction volume of 10 μL, with the inclusion of 0.5 μCi of [α-32P]dATP and 0.1 unit Hot Star Taq DNA polymerase (Qiagen, Inc., Valencia, CA). Following an initial denaturation step of 95°C for 10 minutes, a “touchdown” program was used, consisting of two cycles of amplification at annealing temperatures of 63°C to 59°C followed by 30 amplification cycles at an annealing temperature of 58°C and a final extension cycle of 72°C for 5 minutes.

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The methylation status of the 5′-flanking intronic sequence of the km23 gene was assessed using methylation-sensitive single-strand conformation analysis (MS-SSCA) and DHPLC (Varian-Helix DHPLC, Varian, Inc., Palo Alto, CA). Samples were prepared for single-strand conformational polymorphism analysis and separated on 0.5% agarose gel containing 0.3 mg/mL ethidium bromide. The primers amplify a 301-bp fragment of the km23 gene containing the CpG island between residues –182 to +119 bp, relative to the km23 translation start site.

### Results and Discussion

A total of 237 primary ovarian, breast, and colorectal cancers were screened for somatic mutations in all four exons of km23. To enhance the mutation detection sensitivity, we analyzed all tumors using both single-strand conformational polymorphism and DHPLC analyses. An A → G substitution (53 nucleotides downstream of exon 2) was identified in 20% of samples (Fig. 1A), and a G → T substitution (17 nucleotides downstream of exon 2) was detected in a single breast cancer sample (Fig. 1B). However, comparison with matching normal DNA showed that both these variants were present in the germ line. Despite using these rigorous mutation analysis techniques, no

### Table 1. Primer sequences used for PCR amplification and sequencing

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSCP and DHPLC primers*</td>
<td></td>
</tr>
<tr>
<td>Exon 1</td>
<td></td>
</tr>
<tr>
<td>km23ex1-F</td>
<td>CTCACGCTGGCTCTGATA</td>
</tr>
<tr>
<td>km23ex1-R</td>
<td>CACCTCGGGAAGACTGAAGG</td>
</tr>
<tr>
<td>Exon 2</td>
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<tr>
<td>km23ex2-F</td>
<td>CTTGGGATGAGGAAGTTAAC</td>
</tr>
<tr>
<td>km23ex2-R</td>
<td>TCTAGAAGGCTTTAAGTAGA</td>
</tr>
<tr>
<td>Exon 3</td>
<td></td>
</tr>
<tr>
<td>km23ex3-F</td>
<td>AGAACGGGCTCCACCTCT</td>
</tr>
<tr>
<td>km23ex3-R</td>
<td>GTCGACATGAGGTCTGT</td>
</tr>
<tr>
<td>Exon 4</td>
<td></td>
</tr>
<tr>
<td>km23ex4-F</td>
<td>CCCCCCTCTCATTGTATGTTAG</td>
</tr>
<tr>
<td>km23ex4-R</td>
<td>TGTCGATTCCACTGACATGATT</td>
</tr>
<tr>
<td>MS-SSCA primers†</td>
<td></td>
</tr>
<tr>
<td>km23meth-F</td>
<td>GGGTGGAAAGTAGATTTTTTAAAGATT</td>
</tr>
<tr>
<td>km23meth-R</td>
<td>CCAACACACCAACATCCTAT</td>
</tr>
</tbody>
</table>

Abbreviations: F, forward; R, reverse; MS-SSCA, methylation-sensitive single-strand conformation analysis.

* Primers complementary to flanking intronic sequence were designed to individually amplify the four coding exons of the km23 gene. For DHPLC analysis, temperatures required for the successful resolution of heteroduplex molecules were determined by the use of the DHPLC melting algorithm (http://insertion.Stanford.edu/melt.html).

† The primers amplify a 301-bp fragment of the 5′ CpG island between residues –182 to +119 bp, relative to the km23 translation start site.
it seems unlikely that this could be due to chance sampling errors, particularly, as we had a good representation of the major histologic subtypes of ovarian cancer (49 serous, 18 mucinous, 29 endometrioid/clear cell, and 8 undifferentiated). The discrepancy is also unlikely to be due to a failure of our mutation detection methodology because we employed both single-strand conformational polymorphism and DHPLC, which have been shown to detect 95% to 100% of mutations (6). Furthermore, the ovarian, breast, and colorectal cancers analyzed in this study are known to contain minimal normal tissue contamination, and mutations in other genes have previously been identified in these samples using the same methodology (7–11).

The reasons for the discrepancy between our data and that of Ding et al. (1) are not clear, but some of the technical aspects of their study warrant closer scrutiny. One possible source of erroneous mutations may stem from the fact that the mutation studies were done on RNA extracted from laser capture microdissected tissue followed by reverse transcription and amplification of target sequences using nested primers. In particular, the reliance on a nested PCR strategy is likely to greatly increase the risk of generating bogus Taq DNA polymerase-induced sequence alterations. Although Ding et al. did use a high-fidelity Pfu polymerase with 3′ to 5′ exonuclease activity, this reduces rather than eliminates PCR errors. To counter this possibility, Ding et al. reasoned that because no mutations were identified among 15 control normal ovarian tissue samples (of which only three were matched to the tumor), whereas eight mutations were detected among 19 tumor samples, the mutations must be tumor specific (P ≤ 0.005). However, in only three of the controls were the RNA derived from laser-captured material and RNA extracted in the same manner as for the cancer tissues. The remaining 12 normal tissues were extracted from larger biopsies and using a different RNA extraction procedure. If these 12 are excluded from the statistical calculation, the difference in the mutation frequency between the tumor and normal tissue becomes nonsignificant (P = 0.27). Analysis of the relevant genomic DNA sequences, which could have circumvented these difficulties, was not reported.

In summary, we have found no evidence that transforming growth factor-β signaling component km23 is either genetically or epigenetically inactivated in ovarian, breast, or colorectal cancers. Although we cannot discount the possibility that some of the sequence variations reported previously in ovarian cancer may represent bona fide somatic mutations, our data suggest that if km23 mutations do exist, they occur at a very low frequency.

References


Fig. 2. Methylation-sensitive single-strand conformation analysis of primary ovarian cancers. Methylation-sensitive single-strand conformation analysis was done as described previously (3, 4). Representative examples of the methylation-sensitive single-strand conformation analysis of the km23 CpG island in primary ovarian tumor samples. The controls are two normal genomic DNA samples (obtained from lymphocytes) that were treated with bisulfite and confirmed by direct sequencing to be unmethylated for all CpGs. The methylation positive controls (Sss1) are normal genomic DNA samples that were Sss1 methyltransferase treated before bisulfite conversion. Direct sequencing of the Sss1-treated samples confirmed that all CpGs were fully methylated. The Sss1 treated samples show clear band shifts compared with the unmethylated controls. All primary cancers show identical banding pattern to the unmethylated controls.
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