

Allelic loss at the *SEP15* locus in breast cancer

Research Article

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Summary

Selenium is currently being considered as a promising chemopreventive agent, although the mechanisms responsible for the suppression of tumorigenesis by this nutrient remain unknown. *Sep15* is a selenium-containing protein whose gene is mapped on human chromosome 1 at position 117-123 cM on the human transcript map, corresponding approximately to 1p31, a common position of chromosomal loss in breast cancer and other solid tumors. The coding sequence for *Sep15* includes two polymorphic sites located at positions 811 (C/T) and at 1125 (G/A) within the 3'-untranslated region. Previous work has implicated *Sep15* in cancer etiology by demonstrating significant differences in *Sep15* allele frequencies between the DNAs of certain tumors as compared to DNA from cancer-free individuals, although this study was unable to distinguish between alleles being associated with cancer risk or allelic loss during tumor development. In this study, four highly polymorphic microsatellite markers on chromosome 1, spanning the region of the chromosome including the *Sep15* gene, were used to assess differences in the heterozygosity index at these loci in the DNA from 61 breast cancer samples as compared DNA obtained from cancer-free individuals. Significantly fewer heterozygotes (28%) at the *DIS2766* locus, which is tightly linked to *Sep15*, were observed in the breast cancer DNA samples examined. Similar analysis of other microsatellite markers on 1p failed to detect significant difference in heterozygosity indices between tumor and control DNAs, suggesting that loss of *Sep15* or another tightly linked gene was a common event in these samples. These results support a role for *Sep15* allelic loss with the development of breast cancer.

I. Introduction

The essential trace element selenium is effective in the reduction of tumor frequency when provided to animals at doses only 5-10 fold above the nutritional requirement (Ip, 1986). It is effective in the protection against cancer in a wide variety of tissues, and against many different types of carcinogens (El-Bayoumy et al, 1995). In humans, supplementation with non-toxic doses of selenium has been reported to reduce the incidence of several types of cancers (Clark et al, 1996; Yu et al, 1997). In addition to studies suggesting benefits to selenium supplementation, other data has shown an inverse relationship between dietary selenium levels and cancer risk at several sites (Knekt et al, 1998; Yoshizawa et al, 1998; Ghadirian et al, 2000) While the mechanism of protection offered by selenium remains unknown, genetic data implicating specific selenoproteins in cancer etiology would support the possibility that a particular selenoprotein was involved in the protective effects provided by selenium consumption.

Sep15 is a selenoprotein of unknown function (Gladyshev et al, 1998; Kumaraswamy et al, 2002),

although it has been shown to be associated with UDP-glucose: glycoprotein glucosyltransferase, a protein involved with proper protein folding (Korotkov et al, 2001). While expressed to varying degrees in several tissue types, highest levels of *Sep15* expression were seen in the thyroid and prostate (Gladyshev et al, 1998; Kumaraswamy et al, 2002). *Sep15* maps to human chromosome 1, at position 117-123 cM on the human transcript gene map corresponding to approximately 1p31 (Gladyshev et al, 1998). Analysis of the EST database has shown two polymorphic positions within the 3'-untranslated region of the *Sep15* gene at nucleotide positions 811 and 1125 in the human cDNA (Gladyshev et al, 1998). This analysis indicated that C⁸¹¹ was exclusively associated with G¹¹²⁵, while T⁸¹¹ was exclusively associated with A¹¹²⁵ in each EST sequence that contained both polymorphic sites, indicating the presence of two haplotypes, C⁸¹¹/G¹¹²⁵ and T⁸¹¹/A¹¹²⁵. The *Sep15* haplotype frequency differs significantly between Caucasians and African Americans, and examination of haplotype frequencies between DNA obtained from either breast cancers or tumors of the head and neck indicated fewer heterozygotes in these tumor DNAs as compared to

ethnicity matched cancer-free individuals (Hu et al, 2001) These studies, however, could not distinguish between a particular haplotype being associated with increased risk of cancer, or LOH occurring during tumor development. In addition, this study was unable to determine whether chromosome 1 loss in these samples was extensive or restricted to the immediate vicinity of *Sep15*. In this study, we have extended the original observation that there are fewer heterozygotes in breast cancer samples than is represented in an ethnicity-matched control population. In order to determine whether the loss of genetic material was localized to the *Sep15* gene, heterozygosity analysis was conducted on DNA obtained from 61 breast cancer samples and 50 blood samples obtained from cancer-free individuals. Using 4 frequently heterogeneous DNA microsatellite markers that span chromosome 1, significant reduction in heterozygosity was observed only for the marker in the immediate vicinity of *Sep15*. These data indicate that allelic loss of *Sep15* or another tightly linked gene is a common event in breast cancer development and further suggests that *Sep15* is a candidate mediator for the protective effects of selenium.

II. Materials and methods

A. Blood and tissue specimens

Breast cancer samples were obtained from the Tissue and Sera Bank of the Department of Surgical Oncology at University of Illinois, Chicago, IL under an institutionally approved IRB protocol. Fresh tissue samples were collected from the hospitals of diagnosis, and paraffin sections were mounted on microscopic slides and stained with haematoxylin and eosin. A pathologist identified areas containing tumor tissue and those containing normal breast tissue, which were then microdissected and immediately frozen in liquid nitrogen then stored at -70°C . Blood derived from a panel of 50 normal volunteers (free of cancer) were obtained from Loyola Medical Center, Maywood, IL under an approved protocol from that institution. Given previous data indicating differences in *Sep15* allele frequencies between Caucasians and African Americans (Hu et al, 2001), the studies presented herein were restricted to samples obtained from African Americans. Obtaining samples from all patients, as well as the analysis described in this manuscript, were conducted under approved institutional protocols.

B. DNA isolation

DNA was isolated from the frozen-fresh tumor tissue samples and from blood as described earlier (Hu et al, 2001) using the protocols and procedures included in the Puragene DNA Purification Kit, Gentra System, (Minneapolis, Minnesota, US).

C. Genotyping

DNA from both breast cancer tissue and bloods were genotyped for 4 highly polymorphic microsatellite markers on chromosome 1. Primer pairs used to analyze microsatellite markers were obtained from ResGen (Huntsville, AL USA). DNA was used as a template for amplification in a 25 μl reaction volume containing 0.25 mM each of dATP, dGTP, dCTP, and dTTP, 5 pmol of each primer and 4 units of Taq DNA polymerase (Invitrogen). The thermocycling conditions (Eppendorff/Brinkmann Mastercycler gradient, Westbury, NY) consisted of initial denaturation of 3 min at 94°C , followed by 50

cycles of denaturation at 94°C for 30 sec, annealing at $55-62^{\circ}\text{C}$ for 1 min, elongation at 72°C for 90 sec, with a final extension at 72°C for 10 min. PCR products were electrophoresed on 10% polyacrylamide gels and visualized by ethidium bromide staining. Heterozygosity was defined in this study as the presence of two discernable bands observed following gel electrophoresis of the amplification products, with one no less than 50% the intensity of the other. All PCR experiments included an amplification reaction without DNA template as a control for contamination and the analysis of each DNA sample was repeated at least three times.

D. Haplotype analysis

Haplotype analysis was performed to determine the nucleotide identity of polymorphic positions 811 and 1125 of the *Sep15* gene as previously described (Hu et al, 2001). In short, PCR was performed to amplify a 413 bp region of the 3'-UTR of the *Sep15* gene including both polymorphic positions. Differential restriction enzyme digestion was performed with *Dra1* to distinguish between a C and a T at position 811, and cleavage with *Bfa1* was performed to distinguish between a G and an A at position 1125. All samples were analyzed at both positions without detecting any exceptions to the TA or CG association.

E. Statistical analysis

Statistical differences in heterozygosity frequencies obtained between cancer-free individuals and tumor samples were calculated by χ^2 . p -values were two-sided. Only informative samples were included in the analysis and p -values less than 0.05 were considered to be significant.

III. Results

A. *Sep15* haplotype frequencies in breast cancer samples vs. blood samples obtained from cancer-free individuals

We previously examined the frequency of *Sep15* alleles representing either the TA or CG haplotype in DNA obtained either from breast tumors or bloods obtained from cancer-free individuals, and this analysis indicated significant differences in haplotype distribution (Hu et al, 2001). These data have been extended in (Table 1) with the analysis of additional tumor samples, and it is apparent that there is a trend towards fewer heterozygotes in the tumor samples. We therefore used frequently heterozygous microsatellite markers on chromosome 1 to assess whether genetic loss in the vicinity of *Sep15* was restricted to that locus or spanning a large region of the chromosome.

B. Heterozygosity analysis of chromosome 1 microsatellite markers

Given the differences observed in *Sep15* allele frequencies in ethnicity matched control and tumor samples, a strategy was used to investigate allelic loss at or near the *Sep15* locus. To accomplish this, microsatellite markers with high heterozygosity indices located on chromosome 1, the same chromosome as *Sep15*, were analyzed in the sample sets. The identity of each of these markers, their locations, reported heterozygosity index and

primer sequences were retrieved from Genome Data Base (GDB) <http://www.gdb.org>, Marshfield clinic (<http://research.marshfieldclinic.org>), and Stanford G3 radiation panel (<http://www.shgc.stanford.edu/Mapping/rh/Maps>). The selected markers, the sequence of PCR primers used to amplify these regions, and the anticipated size range of the PCR products are presented in (Table 2), and their relative position on the chromosome, along with that of *Sep15*, is presented schematically in (Figure 2).

The reported heterozygosity indices for each of the selected microsatellite markers are presented in (Table 3).

To validate these indices, 50 DNA samples derived from cancer-free African American women were analyzed with all 4 markers and the results, indicating good agreement with the provided data, are also included in the table (Table 3). Subsequent analyses used the indices determined in our laboratory. Examples of the banding patterns obtained on ethidium bromide stained gels representing the amplification of the selected microsatellite markers, indicating either heterozygosity (2 bands) or homo/hemizyosity (1 band) are presented for each of the markers in Figure 1.

Table 1: Allelic distribution of *Sep 15* haplotypes in DNA from breast tissue vs. lymphocytes from cancer free women. All samples were obtained from African Americans.

Genotype	Cancer free (n=490)	Breast Cancer (n=76)	95 % confidence limit	Odd ratio	p- value
CG/CG	81(16%)	21(28%)		1	
CG/TA	259 (53%)	33(43%)	0.02-0.287	0.491	0.0206
TA/TA	150 (31%)	22(29%)	0.294-1.090	0.566	0.0888

Table 2. Primers pair sequences for microsatellite markers and their accession numbers retrieved from GDB Data Base.

Accession Number	Locus	Primer Name	Primer Sequence	PCR Product
GDB: 199861	<i>DIS481</i>	AFM294wg1a AFM294wg1m	ATGTCCATGTTTTACCTAATTGTCC AGGTTTGCTGGTGCATNTCT	235-255
GDB: 200204	<i>DIS488</i>	AFM299ze9a AFM299ze9m	GCAAAACAGAGACTTCACCT CTTCCAGGGACTAGAATGG	181-205
GDB: 610932	<i>DIS2766</i>	AFMb320yf1a AFMb320yf1m	CTCAGCCTAGTGCAGCC GCTTAAACCCATGATTGGTAT	183-195
GDB: 613626	<i>DIS2865</i>	AFMa050ta5a AFMa050ta5m	AGTGCCATGTACTGCC GGCTCCATAATTCTGGTAGAAG	221-233

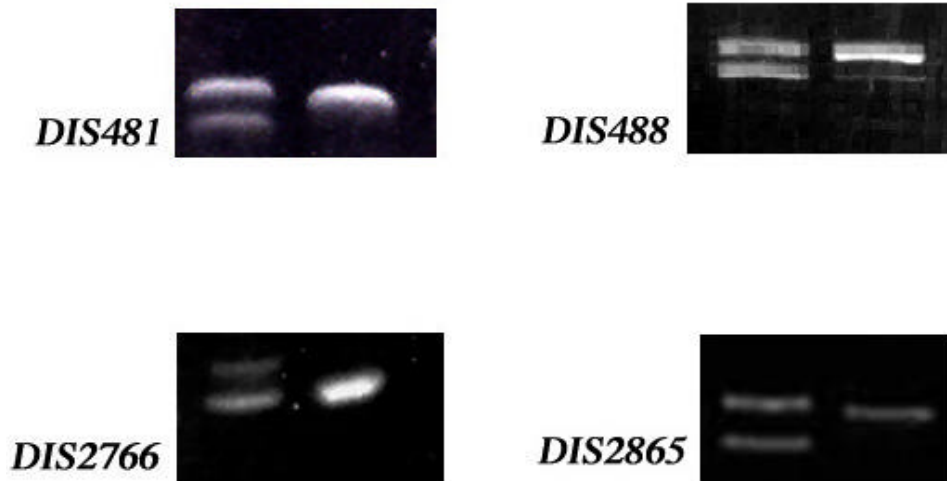


Figure 1. Examples of genotype analysis use the *DIS481*, *DIS488*, *DIS2766* and *DIS2865* microsatellite markers. Genotype analysis was performed on frequently heterogeneous microsatellite markers using primers obtained from Resgen (<http://www.resgen.com>). For each pair of DNAs presented in the Figure, the sample on the left is heterozygous as determined by the observation of 2 PCR bands, while the sample on the right is either homozygous or hemizygous at that position.

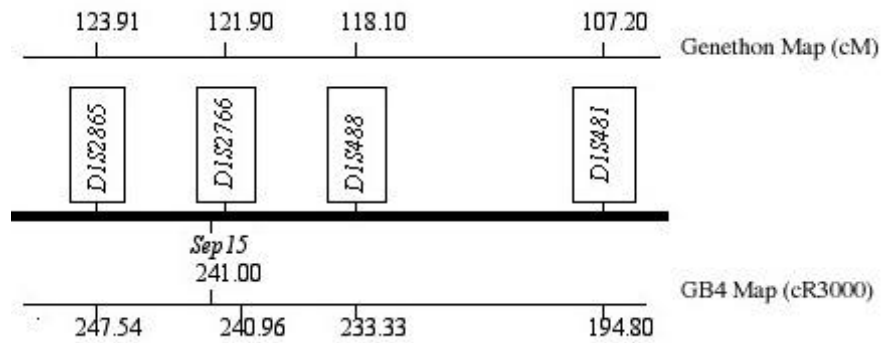


Figure 2. Physical map: The location of 4 microsatellite markers in the vicinity of *Sep15* gene on chromosome 1, top and bottom lines are Genethon (cM) and GeneMap99 Genebridge 4 hybrid maps (cR3000), respectively. Marker locations obtained from Genethon (<http://www.genethon.fr>), the National Center of Biotechnology Information (NCBI) GeneMap99-GB4 (<http://www.ncbi.nlm.nih.gov/genemap99/loc.cgi>), locations were confirmed using other available web sites, including the National Cancer Institute (NCI) (<http://gai.nci.nih.gov>).

Table 3. Genotyping analysis, reported heterozygosity indices, calculated heterozygosity indices in DNA samples obtained from cancer-free individuals and breast cancers.

Locus	Het. Index (%) (Reported) ¹	Het. Index (%) (Cancer-Free) ²	Het. Index (%) (Breast Cancer) ²	<i>p</i> -value ³
<i>DIS481</i>	86	71	72	0.914
<i>DIS488</i>	76	72	74	0.852
<i>DIS2766</i>	75	68	41	0.004
<i>DIS2865</i>	62	61	61	0.982

Sample analysis was restricted to those obtained from African American women because of the differences in haplotype frequency reported between Caucasians and African Americans (Hu et al, 2001) Almost all cases were informative for the four loci examined, and the experimentally determined heterozygosity indices in these two populations are presented in **Table 3**. Examination of the data in that Table indicate that the heterozygosity indices determined for markers *DIS481*, *DIS488* and *DIS2865* were statistically indistinguishable in tumor samples vs. controls. In contrast, significantly fewer heterozygotes were present for marker *DIS2766* in the tumor samples than the controls (41% vs. 68%). The genomic position of the *DIS2766* locus is in close proximity of *Sep15* at 121.9 cM, (**Figure 2**).

IV. Discussion

The data present here extends our previous observations indicating differences in *Sep15* haplotype frequencies in breast cancer samples as compared to that obtained from blood samples of individuals of the same ethnicity. This experimental design, involving differential restriction enzyme digestion of PCR products containing the polymorphic sites, cannot distinguish between homozygosity and hemizyosity resulting from loss of one of two *Sep15* alleles. In addition, the heterozygosity frequency for *Sep15* in cancer-free samples is approximately 50%, making this a difficult locus to use for examining differences in heterozygosity frequencies among different sample sets. To address this issue, polymorphic microsatellite markers with high frequencies

of heterozygosity were examined along chromosome 1. Of the four markers examined, only one of these exhibited a significantly lower heterozygosity frequency in tumor samples. That marker, *DIS2766*, is tightly linked to *Sep15* and there were 27% fewer heterozygotes in tumors (from 68% to 41%, $p < 0.05$). The lower heterozygosity frequency of these tightly linked sequences indicates that loss of *Sep15* or another unidentified tightly-linked gene is an important event in breast cancer development.

Sep15 is a highly conserved selenoprotein, with homologous genes found in mice, rat, *B. malayi*, and other animals (Gladyshev et al, 1998). Several observations suggest that lower levels of *Sep15* may be significant in promoting carcinogenesis. *Sep15* has been shown to be expressed at reduced levels in liver tumors that developed in a TGF β -c-myc hepatocellular carcinogenesis animal model as compared to adjacent, normal liver tissue obtained from the same animals (Kumaraswamy et al, 2000). In this same study, virtually undetectable levels of *Sep15* were reported in prostate cancer cell lines while the normal prostate usually contains high *Sep15* levels. These observations, the studies reported here for breast cancer, and the likelihood that *Sep15* protein levels might be reduced in individuals with sub-optimal selenium intake, raise the possibility that the *Sep15* gene product provides an anti-cancer protective role and may mediate some of the protective effects associated with selenium adequate or supplemental intake.

LOH on the short arm of chromosome 1 has been reported in several cancer types, including breast cancer, melanoma, intestinal cancer, thyroid cancer, liver cancer, and stomach cancer (Kubo et al, 1991; Bardi et al, 1993;

Yeh et al, 1994; Nagai et al, 1995; Ezaki et al, 1996; Bieche et al, 1999; Ragnarsson et al, 1999; Igarashi et al, 2000). Chromosome 1p has been reported as one of the most involved chromosome arms in breast cancer (Nagai et al, 1995; Loupart et al, 1995; Hoggard et al, 1995). Furthermore, chromosome arm 1p is one of the most commonly altered regions in breast cancer (Callahan, et al, 1992; Bieche and Lidereau, 1995; Weith et al, 1996). Frequent LOH (21%) at the *DIS488* locus has been previously observed in 8 studied breast cancer samples (Peng et al, 2000) although we have failed to detect a significant allelic loss at this position in the studies presented here. It is unclear why there is a difference between these observations, although some possibilities include the origin of the clinical samples, ethnicity of individual's genotyped, sample size, and the clinical classification of tumors.

In summary, the reduced heterozygosity frequency observed at both the *Sep15* locus and a tightly linked, microsatellite marker suggest that allelic loss of *Sep15* may promote breast cancer development. A putative tumor suppressor role for this selenoprotein may help to explain how selenium supplementation could be effective in reducing cancer incidence. Future studies examining LOH in this region of chromosome 1 using matched pairs of tumor and normal tissue from the same individuals, as well as examination for associations of polymorphisms within the *Sep 15* gene and cancer risk will be required to gain a better appreciation for the role of this gene in carcinogenesis.

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