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Advances in Brief

NBS1 Is a Prostate Cancer Susceptibility Gene

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Abstract

To evaluate whether an inactivating mutation in the gene for the Nijmegen breakage syndrome (*NBS1*) plays a role in the etiology of prostate cancer, we compared the prevalence of the 657del5 *NBS1* founder allele in 56 patients with familial prostate cancer, 305 patients with nonfamilial prostate cancer, and 1500 control subjects from Poland. Loss of heterozygosity analysis also was performed on DNA samples isolated from 17 microdissected prostate cancers, including 8 from carriers of the 657del5 mutation. The *NBS1* founder mutation was present in 5 of 56 (9%) patients with familial prostate cancer (odds ratio, 16; $P < 0.0001$), 7 of 305 (2.2%) patients with nonfamilial prostate cancer (odds ratio, 3.9; $P = 0.01$), and 9 of 1500 control subjects (0.6%). The wild-type *NBS1* allele was lost in seven of eight prostate tumors from carriers of the 657del5 allele, but loss of heterozygosity was seen in only one of nine tumors from noncarriers ($P = 0.003$). These findings suggest that heterozygous carriers of the *NBS1* founder mutation exhibit increased susceptibility to prostate cancer and that the cancers that develop in the prostates of carriers are functionally homozygous for the mutation.

Introduction

Prostate cancer is among the leading causes of morbidity and mortality from cancer in men. Relatively little is known about the genetic determinants of this disease, but epidemiologic data suggest that dominant susceptibility genes may be responsible for up to 5% of all of the cases (1, 2). A recent Scandinavian study of twins suggests that the heritability of prostate cancer may be as high as 42% (3). The genetic basis of prostate cancer is complex and appears to involve multiple susceptibility genes. Through linkage analysis, numerous chromosomal loci have been identified, but no clear prostate susceptibility gene has emerged. Three candidate susceptibility genes have been positionally cloned—*HPC1*, *HPC2/ELAC2*, and *MSR1*—but a clear role for any of these genes in hereditary prostate cancer has not been established (4–9). There also is evidence that mutations in *BRCA2* or *CHEK2* predispose to prostate cancer, but the contribution of these two genes to prostate cancer etiology is relatively small (10–13).

The DNA damage signaling pathway plays a crucial role in the maintenance of the integrity of the genome in response to DNA damage and has been implicated in the pathogenesis of prostate cancer (10–16). Individuals with inherited recessive clinical syndromes, such as Nijmegen breakage syndrome (NBS), Bloom syndrome, Fanconi anemia, and ataxia telangiectasia, which are characterized by spontaneous chromosomal instability, immunodeficiency, and a predisposition to cancer, carry a mutation in one of the genes in the DNA damage signaling pathway (17, 18). The gene for NBS is situated on chromosome 8q21 (19). The product of the *NBS1* gene (nibrin, also referred to as p95) is a component of the hMRE11/hRAD50/NBS1 nuclease complex (20). This complex is part of the BRCA1-associated genome surveillance complex, which is responsible for DNA damage repair (18). A 5-bp deletion in exon 6 of *NBS1* (657del5) is present in the majority of NBS patients from eastern Europe (21).

It has been suggested that heterozygous carriers of the founder mutation of the NBS (657del5 allele) may be at increased risk of cancer, but prostate cancer specifically has not been studied to date (22–27). In this study, we compared the frequency of the 657del5 mutation in unselected patients with sporadic and familial prostate cancer with that of a control group to determine whether *NBS1* plays a role in the development of prostate cancer.

Materials and Methods

Patients. All of the 359 men diagnosed with prostate cancer at the University Hospital in Szczecin, Poland between 1999 and 2002 were invited to participate in this study. Of these, 340 (95%) agreed to participate. All of the subjects were recruited within 6 months of the date of diagnosis. Family histories of cancer were obtained from each subject. Thirty-five patients (10.3%) had one or more first- or second-degree relatives with prostate cancer (familial cases). We also included a second set of 21 familial cases of prostate cancer from men who were referred for evaluation at the Hereditary Cancer Center by family doctors or urologists because of familial aggregation of prostate cancers. In total, there were 56 familial cases (Table 1) and 305 nonfamilial cases. The familial cases from the incident sample contained, on average, 2.1 cases of prostate cancer (mean age of onset, 67.3 years), and the familial cases from the Hereditary Cancer Center sample contained 2.6 cases of prostate cancer (mean age of onset, 63.3 years).

There were 1500 unaffected control subjects. One thousand control subjects were selected at random from the computerized patient lists of three family practices in Szczecin (508 women and 492 men; age range, 26–89 years). In addition, a second control group comprised 500 newborns from Szczecin for whom a sample of umbilical cord blood was available.

Mutation Detection. Allele-specific PCR was used to detect the *NBS1* founder mutation using DNA isolated from peripheral blood leukocytes. The PCR reaction was carried out in ThermalCycler 9600 (Perkin-Elmer, Boston, MA) with Nbsex6f primer, Nbsex6r primer, and Nbsdel5 primer (as described in Ref. 24). Detailed experimental conditions are available on request. PCR products were separated in 1.5% agarose gel and visualized in UV light. When a shorter PCR product was observed, a separate DNA sample was sequenced using BigDye Terminator Ready Reaction Kit v3.0 (Applied Biosystems, Foster City, CA) to confirm the presence of the *NBS1* founder mutation. Sequencing products were analyzed in ABI PRISM 377 DNA Sequencer (Applied Biosystems).

Loss of Heterozygosity Analysis. For each of the nine prostate cancers in men with an *NBS1* mutation, a single noncarrier control tumor was selected. The control subject was born within 2 years of the patient and had a tumor of the same Gleason score as the matched patient. DNA of sufficient quality for PCR amplification was obtained from eight of the nine paraffin-embedded, microdissected tumors from *NBS1* mutation carriers and from all of the nine noncarrier control subjects. Five-ìm sections of formalin-fixed, paraffin-embedded tissues were cut onto slides. Tumors were sectioned onto six slides. One was stained with H&E. The remaining five slides were used for microdissection. Sections were deparaffinized in two changes of xylene for 5 min.

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Table 1 *Clinical characteristics of familial prostate cancer cases*

Number of men affected with prostate cancer	Number of families	Mean age of diagnosis (range)
6	1	64.8 (55–70)
5	2	62.6 (44–81)
4	1	61.7 (53–74)
3	13	67.7 (50–90)
2	39	65.4 (43–90)

Sections were hydrated through a series of graded alcohols [96% ethanol (2×), 70% ethanol, and dH_2O in each for 5 min]. Slides were stained in hematoxylin. Using the light microscope, homogenous fields of cancer cells were chosen in H&E-stained sections. Those fields were microdissected carefully using needle from slides stained only with hematoxylin under light microscope, avoiding contamination with nonmalignant cells. In parallel, normal tissues were cut from the same slides. Microdissected tissues were digested in 1 ml digestion buffer [50 mM TrisHCl and 1 mM $CaCl_2$ (pH 8.0) with 20 μl 10% SDS and 500 μg proteinase K]. In each series, negative controls without tissue were used. Enzymatic digestion was carried out at 55°C for 2 weeks. After digestion, proteinase was heat inactivated at 96°C for 10 min. Digestion product was purified in Microcon-100 tubes (Millipore, Billerica, MA) according to manufacturer's procedure. After purification, solution containing DNA was diluted in 50 μl dH_2O. In such a way, DNA from prostate cancer tumor tissue and DNA from normal tissue were obtained from mutation carriers and matched control subjects.

For the loss of heterozygosity (LOH) studies, two primer pairs were used, corresponding to the polymorphic microsatellite markers D8S88 and D8S1811. Each of the 17 patients and control subjects was informative for at least one of the two markers. PCR was performed using fluorescent primers (as described in Ref. 24). PCR products were separated in ABI PRISM 377 DNA Sequencer (Applied Biosystems). Data collection and analysis were performed using ABI PRISM 377 Collection Software and GenScan Analysis Software Version 3.0 (Applied Biosystems). A signal reduction in one allele of at least 70% was taken as the threshold of recognition for LOH. The *NBS1* mutant allele is five nucleotides shorter than the wild-type *NBS1* allele. For the LOH analysis of mutation-positive cases, additional primers were designed specifically to amplify exon 6 of *NBS1*, which contains the deleted sequence. PCR conditions using this primer set were as for allele-specific PCR. This primer set generates two distinct fragments from constitutional DNA from men with an *NBS1* deletion.

Results and Discussion

The *NBS1* mutation was present in 9 of 340 unselected patients with prostate cancer (2.6%) compared with only 9 of 1500 (0.6%) control subjects from the general population (odds ratio, 4.5; 95% confidence interval, 1.7–11.5; $P = 0.002$). The 657del5 germline mutation was present in 5 of the 56 (9%) familial cases (odds ratio, 16; 95% confidence interval, 5.2–50; $P < 0.0001$).

We investigated the segregation of the *NBS1* mutant allele with

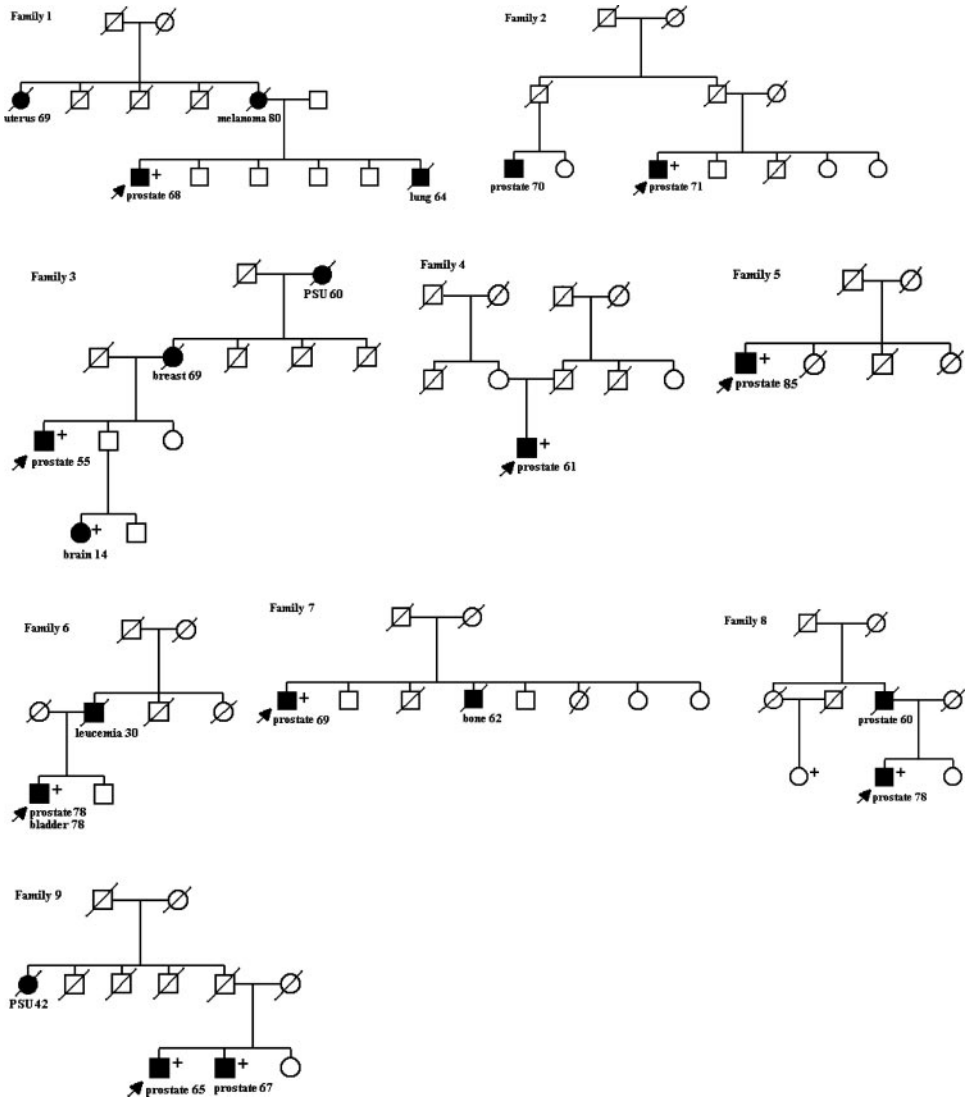

Fig. 1. Pedigree of nine *NBS1* mutation-positive families from a series of 340 consecutive unselected patients with prostate cancer. Solid squares (■) indicate affected men; solid circles (●) indicate affected females. The type of cancer and age of diagnosis are indicated next to the symbol. A + at the upper right corner of the symbol indicates the presence of the *NBS1* founder mutation, and a − symbol indicates a negative result. *PSU*, primary cancer site unknown.

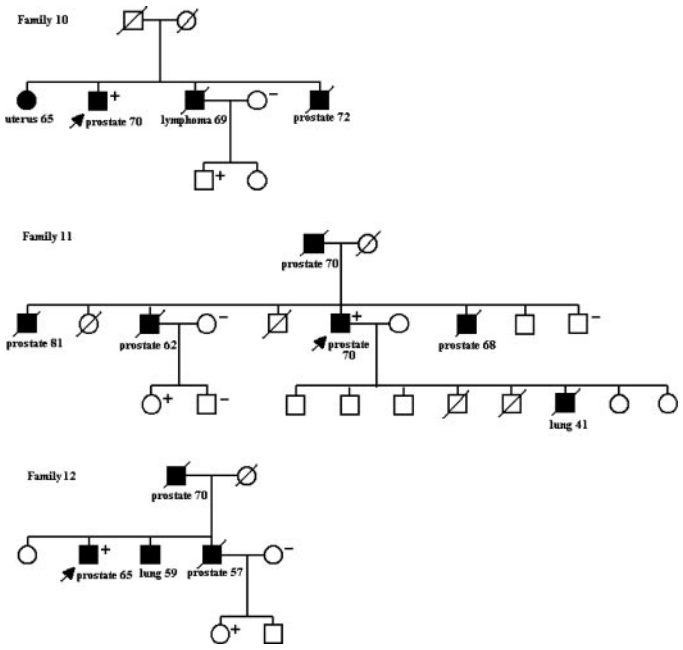

Fig. 2. Pedigrees of three additional *NBS1* mutation-positive familial prostate cancer cases.

prostate cancer in four families. We were able to establish the mutation status in two affected males from each family; in each family, the *NBS1* mutation was present in both affected members (Figs. 1 and 2).

To analyze whether the wild-type allele of *NBS1* is lost in prostate cancer, we performed LOH analysis of microdissected prostate tumors from eight patients who carried the *NBS1* mutation and from nine patients who were found not to carry the *NBS1* mutation. Carriers and noncarriers were matched for age at onset (within 2 years) and Gleason score. LOH at *NBS1* was observed in seven of eight tumors from men who carried a germline *NBS1* deletion (Fig. 3) compared with only in one of nine *NBS1* mutation-negative prostate tumors ($P = 0.003$). Additional primers were used, which allowed us to distinguish between the wild-type *NBS1* allele and the 5-bp deletion. A comparison of the DNA fragments generated from the tumors and corresponding normal tissue from each of the seven men who showed LOH indicated that the wild-type allele was invariably lost.

Our report is the first to document the role of the *NBS1* gene in the etiology of prostate cancer. The prevalence of the mutant founder allele was high particularly among men with familial prostate cancer (9%), and within these families, the *NBS1* mutation segregated with the disease. We also show a statistically significant excess of the *NBS1* founder mutation in unselected prostate cancer cases compared with the general public (odds ratio, 4.5; $P = 0.002$), and we document the loss of the wild-type *NBS1* allele in the majority (88%) of the prostate cancers from 657del5 mutation carriers.

The *NBS1* founder allele appears to be responsible for ~1 in 11 families with two or more cases of prostate cancer in Poland. On the basis of a relative risk of 4.5 and a mutation prevalence of 1 in 167, we estimate that the gene is responsible for ~2% of prostate cancers in Poland. Given the geographic distribution of reported clinical cases of NBS, the 657del5 mutation also may be an important contributor to prostate cancer in patients of Slavic origin from other countries (the 657del5 allele is responsible for all of the cases of NBS in all of the Slavic populations reported to date).

Inherited alterations in the *NBS1* gene have been shown previously to be associated with other malignancies, including leukemia, malignant melanoma, breast cancer, and ovarian cancer (23–27). In our series, 657del5 mutation was present in 2.6% of the prostate cancer cases—the prevalence is similar to that seen in Poland for malignant melanoma (2.6%) and exceeds that reported for breast cancer (1.5%), stomach cancer (2.0%), colon cancer (1.2%), lung cancer (1.2%), and larynx cancer (1.0%; Refs. 23, 24, 26, 27).[4] These studies suggest the involvement of the mutant *NBS1* allele for cancers of various sites, but each study is small, and additional studies are needed to estimate accurately the full effect of this allele on the risk of cancer at all of the sites. In the 12 mutation-positive families in the present study, a range of cancer types was seen (Fig. 2), but none of these were clearly in excess. There were two cases reported of uterine cancer and lung cancer, and single cases were reported for cancer at seven other sites.

The data on the LOH suggest that *NBS1* functions as a classical tumor suppressor gene. Clinically, NBS is a recessive genetic condition. The heterozygote state may not be deleterious at the cellular level, but LOH renders cells hemizygous for the mutant allele. Cultured cells homozygous for the *NBS1* mutation are prone to chromosomal aberrations (28).

The classical two-hit model of tumorigenesis stipulates that both alleles of a tumor suppressor gene be inactivated before tumor formation (29). For some genes and cancer types, however, loss or mutation of a single allele may be sufficient to promote tumorigenesis (30–38). This phenomenon may be caused by either a gene-dosage effect (haploinsufficiency) or the inactivating property the mutant protein may have on a particular physiologic pathway (dominant-negative effect). The gene for Bloom syndrome (*BLM*) appears to act through haploinsufficiency (32). Mice heterozygous for a mutation in the *BLM* gene express genomic instability, and mice with mutations in the *APC* gene develop more intestinal tumors when they also carry a (heterozygous) mutation in *BLM* (33). Tumors from patients with a single truncating founder mutation in the *BLM* gene (BLM^{Ash}) do not demonstrate LOH (32) in contrast to *NBS1*. Similarly, tumors that develop in heterozygous carriers of an *ATM* mutation gene appear to be the consequence of a dominant-negative effect of the ATM protein (34, 35). As expected from the dominant-negative model, truncating mutations that lead to the absence of protein do not appear to be pathogenic; only missense mutations or in-frame deletions have been implicated in increasing the cancer risk. In contrast to these conditions, *NBS1* appears to act as a classical tumor suppressor gene because biallelic *NBS1* inactivation was observed in most tumors. However, some degree of haploinsufficiency and possible dominant-negative effect of *NBS1* mutations cannot be ruled out because it has not been established that *NBS1* heterozygous cells have impaired DNA repair capacity. The *NBS1* founder allele is predicted to result in a truncated protein of 219 of 754 amino acids (p26). p26 lacks crucial domain necessary for MRE11 interaction (39). It is not known whether this mutant protein possesses any residual activity or exerts a dominant-negative effect. However, the 657del5 allele also creates an aberrant translation initiation site, which generates a partially functional variant of the NBS1 protein (p70). p70 contains the MRE11 binding domain but does not confer full function within the MRE11 complex. In light of this, it is possible that p70 may produce a dominant-negative effect. Expression of p70 was detected in NBS1 657del5 lymphoblastoid cell lines but not in fibroblasts, and it is not known whether this variant is expressed in the prostate (39). It remains a possibility that *NBS1* allele is not the causative mutation but is in tight linkage disequilibrium with the true causative locus in this region. However, this is an unlikely possibility given the known function of this allele.

The studies of DNA damage signaling pathway exhibit an impor-

[4] Unpublished observations.

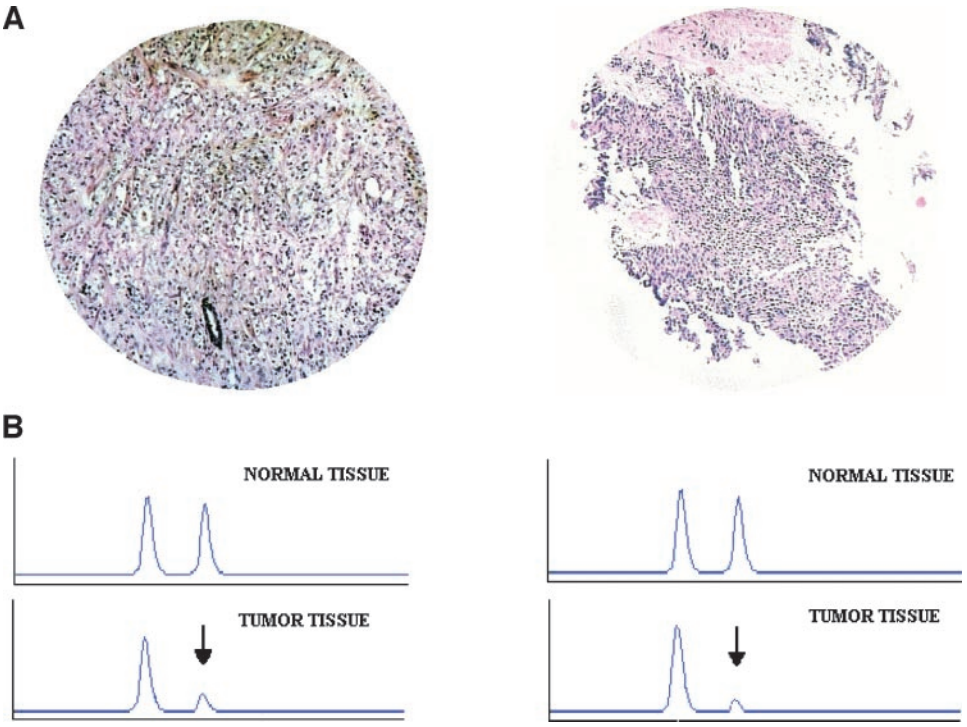

Fig. 3. Loss of heterozygosity (LOH) analysis in microdissected prostate cancer tissues from the proband of family 8 (*left*) and the proband of family 9 (*right*). *A*, representative homogenous fields of tumor cells that were microdissected for LOH analysis. *B*, exon 6-specific PCR. Loss of the wild-type *NBS1* allele in cancer tissue is indicated by arrow (↓).

tant role of BRCA1 multisubunit protein complex, referred to as the BRCA1-genome surveillance complex. This complex includes DNA repair proteins, such as MSH2-MSH6, and MLH1, ATM, NBS1, MRE11, and BLM. The product of the *NBS1* gene is an integral component of the Mre11/Rad50/NBS1 nuclease complex, which interacts with BRCA1. *NBS1* is phosphorylated directly by ATM, and ATM is required for phosphorylation of CHEK2 kinase (18–20, 40, 41). Some components of DNA damage signaling pathway, such as ATM, p53, BRCA1, BRCA2, and CHEK2, have been implicated in prostate cancer (10–16). Our data provide additional evidence for the involvement of DNA damage signaling pathway in the pathogenesis of prostate cancer.

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