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# Immunohistological detection of BRAF25 in human prostate tumor and cancer specimens

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**Abstract**

BRAF25 is an alternatively spliced protein of BRAF35 (see associated paper). We have mapped the BRAF25 gene to chromosome sub-band 19p13.3, a region where loss of chromosomal heterozygosity has been reported in about 50% of ovarian cancers. Because of the high incidence of genetic links of prostate cancer to breast and ovarian cancers, we investigated the BRAF25 expression in the prostate specimens. Immunohistochemical analysis using antibodies specific for BRAF25 revealed a strong immunostaining in sections of the benign prostatic hyperplasia (BPH). The staining was concentrated on the nuclei of cells facing the lumen of prostatic glands, even though the sporadic nuclei of cells in stromas were also stained. However, the expression of BRAF25 was dramatically reduced in intermediate prostate cancer and absent in advanced prostate cancer. Preincubation of the antibody with the immunizing peptide abolished immunostaining in BPH specimens. Therefore, the expression of BRAF25 was gradually lost in prostate cancer. 

*Keywords:* BRCA2 complex; Cancer predisposition; Alternatively spliced BRAF25 protein; Prostate cancer; Breast cancer; Ovarian cancer; Chromosome 19; Immunohistochemical staining

The breast cancer susceptibility genes, BRCA1 and BRCA2, are identified by the linkage analysis of families with breast cancer [1–3]. Inherited mutations in the BRCA2 gene predispose humans to breast and ovarian cancers [2–6]. Inheritance of mutations in BRCA2 is also known to cause susceptibility to prostate cancer [1–10]. It was reported about 10 years ago that the risk of prostate cancer among relatives of the patients with breast cancer was significantly raised for all relatives [11]. A significant clustering of prostate cancer with breast cancers was found 20 years ago in the Utah Mormon case of 2824 men with prostate cancer [12]. Eight cases of prostate cancer were found about 30 years ago among relatives of women with breast cancer (six fathers, one uncle, and one brother) [13]. Recently, a high incidence of prostate cancer has been found in Icelandic families with a history of breast cancer. In some families, men with germline mutations in the BRCA2 gene are highly predisposed to prostate cancer [14]. Most of these mutations are frameshift mutations, which are predicted to result in truncation of the encoded protein. However, somatic mutations in the BRCA1 and BRCA2 genes are rare in sporadic breast cancer [15–18]. This is completely contrary to the wide anticipations.

The BRCA genes that are relevant to cancer predisposition come from the studies of human and mouse cells deficient in wild type BRCA1 and BRCA2 [19–21]. Mouse cells harboring mutations in the Brca1 or Brca2 genes suffer from chromosomal abnormalities. These include spontaneous accumulation of broken chromosomes and chromatids that lead to a proliferative impediment [22,23], multiple centrosome amplification that results in unequal chromosome segregation [24], and micronuclei that are formed through chromosome missegregation [25]. These observations are concurrently observed in patient tumor specimens [26,27] and human cancer cells [28]. These studies have demonstrated that both BRCA proteins are required to maintain the chromosome integrity.

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Recently, it has been demonstrated that the BRCA2 protein exists in a large multi-protein (2 MDa) complex, from which a novel structural DNA binding protein, BRAF35, has been isolated [29]. Several lines of evidence suggest a role for the BRAF35/BRCA2 complex in the control of cell cycle progression. We have cloned a cDNA encoding an alternatively spliced BRAF25 protein (see associated paper). We have precisely mapped the BRAF25 gene to chromosome sub-band 19p13.3, a region where loss of chromosomal heterozygosity has been reported in about 50% of ovarian cancers [30,31]. Because of the high incidence of the genetic links of prostate cancer to breast and ovarian cancers [1–14], we investigated the BRAF25 expression in the prostate specimens. We provide evidence that BRAF25 protein is present in the benign prostate tumor but absent in advanced prostate cancer.

## Materials and methods

*Human prostate tumor and cancer tissues*. The University of Tennessee Department of Urology Tissue and Specimen Bank (UTSB) has been banking prostate tissues since 1995. The current inventory consists of approximately 300 prostatectomy specimens. Most of these specimens were radical prostatectomy specimens performed for cancer. Approximately 40 specimens were obtained from cystoprostatectomies for bladder cancer and simple prostatectomies for prostatic hyperplasia. In addition, the UTSB has metastatic tumors from patients with advanced disease and several specimens obtained during routine autopsy. The processing methodologies used consist of snap freezing tissues and processing for routine histology. This study was approved by the University Institutional Review Board. Informed consent was obtained from all patients.

*Anti-BRAF25 antibodies*. Anti-BRAF25 antibodies were raised against the full length of BRAF25 protein and BRAF25 peptide. The specificity of these antibodies for BRAF25 has been thoroughly characterized by the methods of competitive enzyme-linked immunosorbent assay (ELISA) and Western blot analysis (see associated paper) and competitive immunohistochemistry (see below). However, because BRAF25 is an alternatively spliced protein of BRAF35, the anti-BRAF25 antibodies may react to BRAF35 in immunohistochemical staining.

*Preparation of prostate tumor and cancer tissues for immunohistochemistry*. Human prostate tumor and cancer tissues in more than 30 cases with BPH, or prostate cancers, were randomly selected from the files of the Tissue and Specimen Bank. These tumor samples included 11 BPH specimens, 10 specimens with the intermediate Gleason grade, and 10 specimens with the high Gleason grade. The standard procedure was used to prepare prostate specimens on slides. Briefly, the fresh patient tissue was cut into proper thickness and fixed in 10% neutral buffered formalin at room temperature for 24 h to preserve the tissue for optimal immunohistochemical preservation of proteins. The tissue was completely dehydrated with 50%, 70%, 95%, and three times of 100% alcohol, cleared with xylene, infiltrated with paraffin for 2 h, and embedded into paraffin blocks. The blocks were sectioned and floated on a water bath. The flattened sections were picked up on glass slides and dried overnight at room temperature.

*Immunohistochemical staining of the prostate tumor and cancer tissues*. Immunohistochemical staining was performed using the HistoMark Biotin Streptavidin–HRP systems (KPL, Gaithersburg, MD). All reactions were carried out in a humid chamber at room temperature and slides were washed between reactions with PBS, pH 7.4. Tissue sections were deparaffinized, rehydrated with Trilogy (Cell Marque, Austin, TX), immersed in 3% hydrogen peroxide for 30 min to block endogenous peroxidase activity, incubated for 20 min with normal goat serum (KPL) as blocking agent to reduce non-specific binding, and further blocked for 15 min each with avidin D and biotin (Vector Laboratories, Burlingame, CA). Tissue sections were then incubated for 1 h with the anti-BRAF25 antibody (1:1000 dilution) (Sigma Genosys). Sections were subsequently incubated for 30 min with biotinylated goat anti-rabbit antibody (KPL) and then for 30 min with streptavidin-peroxidase (KPL). Sections were developed for 10 min with true blue peroxidase substrate (KPL) as chromagen and counterstained for 3 min with either the contrast red (KPL) or briefly with eosin (KPL). Preimmune sera (1:1000) were used as control antibody. The intensity of immunoreactivity was rated as strong, weak, or absent. For comparison, serial sections were stained with hematoxylin and eosin.

*Competitive immunohistochemistry assay*. The procedure for the peptide competition assay (Sigma Genosys) was followed to determine the specificity of the immunohistochemical staining obtained above. Briefly, the immunizing peptide (2 mg/ml) was reconstituted in PBS. The anti-BRAF25 antibody (1:1000) was pre-incubated with the reconstituted immunizing peptide at concentrations of 80 and 160 μg/ml, respectively, at room temperature for 2 h. This accommodated the conditions used in the competitive ELISA (see associated paper). The absorbed antibody was directly used in the competitive immunohistochemical staining to confirm the specific staining.

## Results and discussion

To investigate the BRAF25 protein expression in prostate tumor and cancer tissues, we used two approaches to raise the antibodies against the BRAF25 protein. First, to raise the anti-BRAF25 antibody against the full length of BRAF25 protein, we made a plasmid construct. This plasmid construct was expressed in bacteria. The BRAF25 fusion protein was isolated and used to raise the antibody against the full-length BRAF25. Second, we designed a specific BRAF25 peptide sequence that was used by Sigma Genosys to raise the anti-BRAF25 peptide antibody.We have characterized the specificity of the anti-BRAF25 antibodies, prior to use in the immunoprecipitation (see associated paper) and immunohistochemical staining.

We performed the competitive ELISA assays. Preincubation of the anti-BRAF25 peptide antibody with the immunizing peptide resulted in the suppression of binding to the immunizing peptide (see associated paper). The suppression was dramatic when compared with the results obtained in the absence of the immunizing peptide in preincubation with the antibody.

To further test the specificity of the anti-BRAF25 antibodies, we performed the Western blot analysis. Western blot analysis using the anti-BRAF25 antibodies showed that the antibodies against the full length of BRAF25 protein and BRAF25 peptide specifically detected the BRAF25 fusion protein (see associated paper). These results together with the results from the competitive ELISA have confirmed that the anti-BRAF25 antibodies are specific. We then used these

antibodies in the immunohistochemical staining of the prostate tumor and cancer tissues.

*Anti-BRAF25 peptide antibody strongly stained BRAF25 in BPH specimens*

We first examined the BRAF25 protein expression in the non-cancerous prostate tumor tissues. We selected 11 tumor specimens with the low Gleason grade (patient ID: 2000-001, 2000-003, 2000-010, 2000-013, 2000-016, 99-002 #1, 99-008 #1, 99-010 #1, 99-011 #1, 99-012, and 99-023 #1). In this group of non-cancerous specimens, the expression of BRAF25 was detected in all those tumor specimens with the BPH grade. Eleven specimens (100%) displayed extremely strong immunoreactivity (Fig. 1A). Virtually every nucleus of luminal epithelial cells displayed intense immunoreactivity for anti-BRAF25 antibody (Fig. 1A). In particular, in specimens containing the benign glandular morphology, all the nuclei of all luminal epithelial cells, including both basal and superficial epithelial cells, were stained with the anti-BRAF25 antibody. The distribution of staining of the nuclei was homogeneous, both within an individual gland and among glands in the same tissue specimen. The intensity of staining of all nuclei was also homogeneous, even among glands in different tissue specimens. The distribution of staining benign epithelial cells was predominantly concentrated on the nuclei of the cells facing the lumen of the prostatic glands, even though the sporadic nuclei of the cells in the stromas were also intensely stained with this antibody (Fig. 1A). In all the benign specimens analyzed in this study, all the nuclei whether of epithelial or stromal origin were stained strongly with this antibody. In contrast, the preimmune serum as control antibody did not stain the BRAF25 protein in the non-cancerous specimens from the patients with BPH (Fig. 1B).

*Anti-BRAF25 antibody weakly stained BRAF25 in specimens with intermediate Gleason grade*

We then examined the BRAF25 protein expression in the prostate cancer tissues. We selected 10 cancer specimens with the intermediate Gleason grade (patient ID: 2000-018, 99-001 #2, 99-002 #2, 99-008 #2, 99-009, 99-010 #2, 99-011 #2, 99-023 #2, 99-038, and 99-039). In this group of intermediate cancer specimens, the expression of BRAF25 was weakly detected in all the specimens. The staining of nuclei of epithelial cells was completely irregular. In particular, the staining of nuclei was either extremely weaker or became lightly stained with hollows in the nuclei where no staining can be seen (Fig. 2A). The distribution of this light staining was also concentrated on the nuclei of epithelial cells, including cells in the well-differentiated areas and in the diffused areas of the intermediate prostate cancers, with literally no background staining with the preimmune serum (Fig. 2B). These results have shown that the expression level of BRAF25 was reduced in the specimens from the patients with intermediate prostate cancer.

*Anti-BRAF25 antibody did not stain BRAF25 in specimens with high Gleason grade*

We then selected 10 cancer specimens with the high Gleason grade (patient ID: 99-016 #2, 99-026 #1, 99-026 #2, 99-028 #2, 99-029, 99-034 #2, 99-044 #2, 00-053 #2, 99-075 #2B, and 2000-011). In this group of cancer specimens with high Gleason grade, the nuclei of malignant cells were not stained at all (Fig. 3A). The preimmune sera as control antibody did not result in any

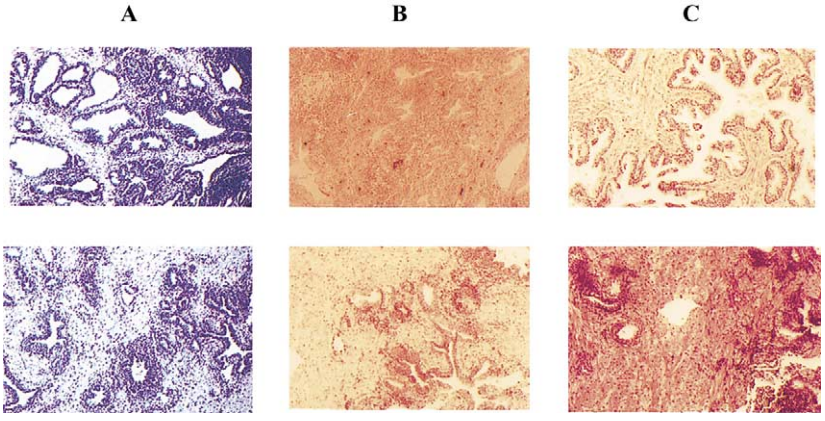


Fig. 1. Immunohistochemical detection of BRAF25 in the sections of BPH specimens is shown (original magnification, 100×). Immunohistochemical staining was performed using the HistoMark Biotin Streptavidin–HRP systems (KPL, Gaithersburg, MD). (A) The tissue sections of the BPH specimens were stained with the antibody raised against the BRAF25 peptide and counterstained with the contrast red. (B) Sections of the BPH specimens were stained with the preimmune serum and counterstained with the contrast red. (C) Sections of the BPH specimens were stained with hematoxylin and eosin. Two BPH specimens [patient ID: 2000-003 (top panel) and patient ID: 2000-013 (lower panel)] from the first group patients are shown.

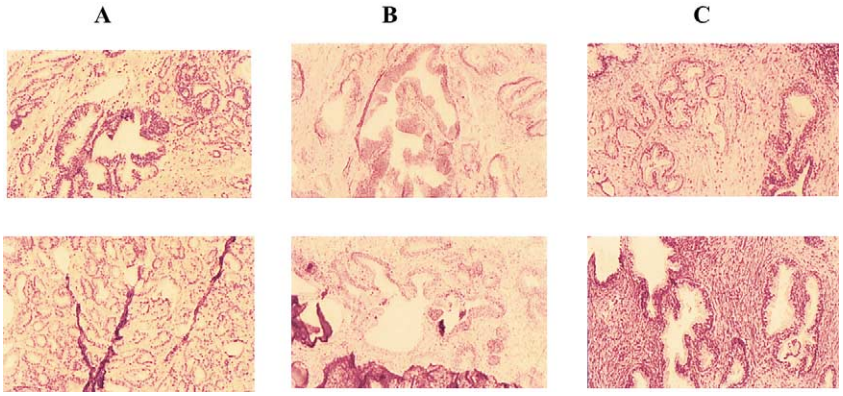


Fig. 2. Immunohistochemical detection of BRAF25 in the sections of the intermediate-grade prostate cancer specimens is shown (original magnification, 100×). Immunohistochemical staining was performed using the HistoMark Biotin Streptavidin–HRP systems (KPL, Gaithersburg, MD). (A) Sections of specimens were stained with the antibody raised against the BRAF25 peptide and counterstained with the contrast red. (B) Sections of specimens were stained with the preimmune serum and counterstained with the contrast red. (C) Sections of specimens were stained with hematoxylin and eosin. Two intermediate cancer specimens [patient ID: 99-002 #2 (top panel) and patient ID: 99-008 #2 (lower panel)] from the second group patients are shown.

specific staining (Fig. 3B). Therefore, as prostate tumors worsened, the BRAF25 protein was either very lightly stained or completely absent in the specimens from the patients with intermediate prostate cancer or advanced prostate cancer. To determine the expression of BRAF25 in other tissues, the autopsy specimens of testicle, adrenals, and lymph nodes were also stained with the anti-BRAF25 antibody. The BRAF25 protein was uniformly detected with the anti-BRAF25 antibody in all these tissues (data not shown).

*Preincubation of the anti-BRAF25 antibody with the immunizing peptide abolished immunostaining in BPH specimens*

As described in the results of the competitive ELISA assay (see associated paper), the binding of the anti-BRAF25 antibody to the immunizing peptide was dramatically suppressed when compared with the immunizing peptide which was omitted. Here, to determine the specificity of immunostaining obtained in Fig. 1A, we performed a peptide competition assay at the immunohistochemical level that adopted the conditions used in the competitive ELISA assays. During preincubation of the anti-BRAF25 antibody with the immunizing peptide, the strong immunostaining patterns observed in the sections of the BPH specimens had disappeared (Fig. 4B) compared with the strong immunostaining patterns observed in the sections of the BPH specimens where the immunizing peptide was omitted (Fig. 4A). These results further confirmed a true anti-BRAF25 peptide response present in the sera and the specificity of the anti-BRAF25 antibody, demonstrating that the expression of BRAF25 detected by the anti-BRAF25 antibody in the BPH

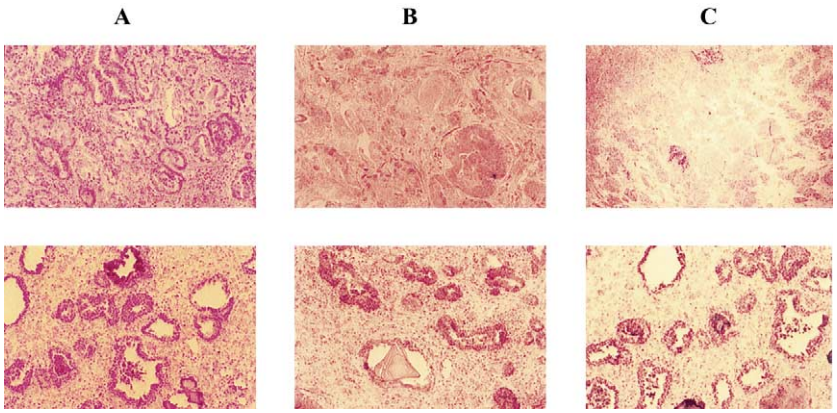


Fig. 3. Immunohistochemical detection of BRAF25 in the sections of the high-grade prostate cancer specimens is shown (original magnification, 100×). Immunohistochemical staining was performed using the HistoMark Biotin Streptavidin–HRP systems (KPL, Gaithersburg, MD). (A) Sections of specimens were stained with the antibody raised against the BRAF25 peptide and counterstained with the contrast red. (B) Sections of specimens were stained with the preimmune serum and counterstained with the contrast red. (C) Sections of specimens were stained with hematoxylin and eosin. Two high-grade prostate cancer specimens [patient ID: 99-026 #2 (top panel) and 99-029 (lower panel)] from the third group patients are shown.

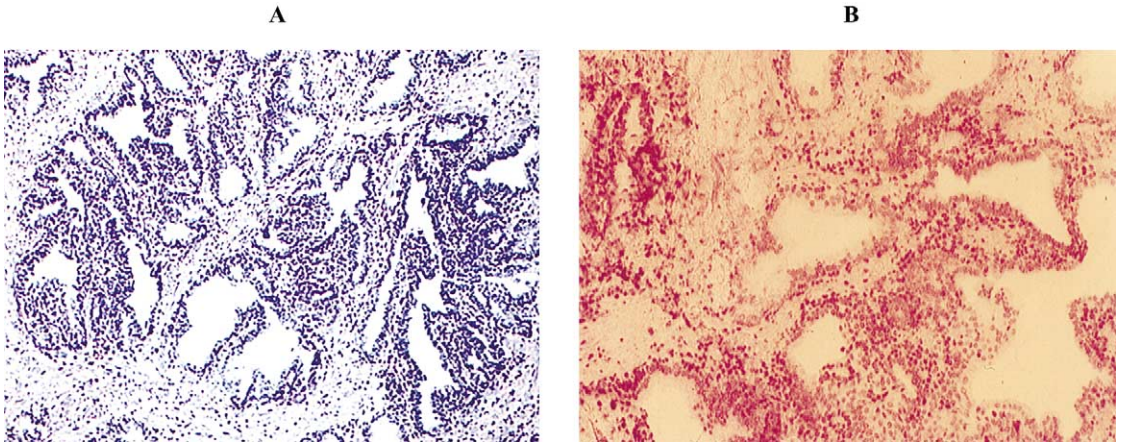


Fig. 4. Competitive immunostaining of the BPH specimens is shown (original magnification, 100×). Immunohistochemical staining was performed using the HistoMark Biotin Streptavidin–HRP systems (KPL, Gaithersburg, MD). (A) The BPH specimen (patient ID: 2000-003) was stained with the antibody raised against the BRAF25 peptide in the absence of immunizing peptide is shown. (B) Specimen as shown in A was stained with the antibody raised against the BRAF25 peptide in the presence of immunizing peptide.

specimens and other tissues (data not shown) is specific (Figs. 1A and 4A). However, as described above because BRAF25 is an alternatively spliced protein of BRAF35, the anti-BRAF25 antibody may react to BRAF35 in the immunohistochemical staining. Nevertheless, the strong immunostaining detected in the BPH specimens with the anti-BRAF25 antibody is specific for the BRAF25/BRAF35 proteins.

In summary, the expression of BRAF25 has been examined in a wide range of benign and malignant prostate tissues. The data reveal that the expression of BRAF25 is conversely associated with the tumor grade. Eleven tumor specimens from the patients with BPH express BRAF25. This extremely strong immunoreactivity occurs in the nuclei of cells facing the lumen of the prostate glands. The nuclei of cells in the stromas of the BPH specimens are also stained with the anti-BRAF25 antibody. Conversely, 10 prostate cancer specimens with the intermediate Gleason grade show much weaker staining with the anti-BRAF25 antibody. In particular, the well-differentiated areas in these intermediate-grade cancers are not stained. Likewise, 10 prostate cancers with the high Gleason grade do not react with the anti-BRAF25 antibody. These results indicate that the expression of BRAF25 is gradually lost in the prostate cancer tissues.

The BRAF25 gene maps to chromosome sub-band 19p13.3, a region where loss of chromosomal heterozygosity has been reported in about 50% of ovarian cancers [30,31]. Loss of chromosomal heterozygosity places the matching chromosome at risk of mutational events that may contribute to the formation of cancer cells [32]. Loss of heterozygosity involving chromosome sub-band 19p13.3 has not been reported in prostate cancer. However, current literature indicates frequent allelic imbalance in prostate cancer on chromosomes 7, 8, 10, 13, 16, 17, and 18 [32]. The allelic loss has been found on 8p, 10q, 16q in 29% of cases of prostatic intraepithelial neoplasia (PIN). The regions such as 8p22, 8p21, and 8p12 on the chromosome 8p are the most frequently deleted regions in prostate cancer. In particular, the tumor suppressor gene RB maps to chromosome 13q14.1 and loss of chromosomal heterozygosity involving the long arm of chromosome 13 has been reported in one-third of prostatic neoplasms [32]. Our results have identified the chromosome sub-band 19p13.3 as a risk region in the prostate cancer.

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