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Cloning a cDNA encoding an alternatively spliced protein of BRCA2-associated factor 35

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Abstract

Inheritance of mutations in the breast cancer susceptibility gene, BRCA2, predisposes humans to breast and ovarian cancers. Inherited mutations in the BRCA2 gene are also known to cause susceptibility to prostate cancer. BRCA2 protein exists in a large multi-protein complex from which a novel structural DNA binding protein BRCA2-associated factor 35 (BRAF35) has been isolated. We have cloned a novel cDNA encoding an alternatively spliced protein of BRAF35, designated as BRAF25. BRAF25 transcript is present in various human cells. We have precisely mapped the BRAF25 cDNA sequence to the genomic chromosome 19 sequence. Analysis of the predicted sequence of BRAF25 identified a protein of 215 amino acids. BRAF25 contains a truncated high mobility group domain, a kinesin-like coiled-coil domain and multiple Src homology 2 (SH2) motifs. Western blot analysis using antibodies specific for BRAF25 revealed the presence of BRAF25 in human prostate cancer cells.

Keywords: BRCA2 complex; Cancer predisposition; Breast cancer; Ovarian cancer; Prostate cancer; Alternatively spliced BRAF25 protein; Truncated HMG domain; Chromosome 19; Immunoprecipitation

The breast cancer susceptibility genes, BRCA1 and BRCA2, are identified by the linkage analysis of families with breast cancer [1–3]. Germline mutations in the BRCA1 and BRCA2 genes predispose humans to breast, ovarian, and prostate cancers [1–5]. These genes are believed to be tumor suppressors because breast and ovarian tumors from predisposed individuals often exhibit loss of heterozygosity in the non-mutated alleles while retaining the mutant alleles [6,7]. Although the precise biochemical functions of the BRCA1 and BRCA2 proteins are unknown, there is considerable evidence that both proteins have been implicated in homologous recombination and double-strand DNA repair to maintain the chromosomal integrity [8–15].

BRCA2 gene has a complex genomic structure with 27 exons, encodes a very large protein of 3418 amino acids, and is expressed in a wide variety of adult tissues [1–5]. Recently, it has been demonstrated that the BRCA2 protein exists in a large multi-protein complex from which a novel structural DNA binding protein BRCA2-associated factor 35 (BRAF35) has been isolated [16]. BRAF35 interacts with BRCA2 protein in early phase of mitotic cell cycle progression. BRAF35 is expressed in various adult tissues with highest levels in testis and ovary. The authors have further demonstrated that BRAF35 and BRCA2 co-localize to condensing chromosomes, and micro-injection of anti-BRCA2 or anti-BRAF35 antibodies delays metaphase progression [16].

Here, we report the detailed cloning and characterization of a novel cDNA encoding an alternatively spliced protein of BRAF35, termed BRAF25. To confirm the presence of the alternatively spliced BRAF25 transcript, we isolated and sequenced 34 cDNA clones from various human cell lines and normal human lung tissue. We have precisely mapped the BRAF25 cDNA sequence to the genomic chromosome 19 sequence. We demonstrate that anti-BRAF25 antibody specific for the

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BRAF25 protein immunoprecipitates the BRAF25 protein from prostate cancer cell lines.

Materials and methods

Cell lines and RNA isolation. Human PPC-1, DU145, LNCap, and PC3 prostate cells, HeLa S3 and HEC1B cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS). Human K562, U266, ML-2, Daudi, Raji, U937, Molt16, and SCLC cells were maintained in Roswell Park Memorial Institute1640 medium with 10% FBS. Total RNA was isolated from cells by the guanidinium thiocyanate/cesium-chloride method [17]. RNA concentration and purity were determined on the basis of optical density measurements at 260 and 280 nm after chloroform/phenol extraction (twice) and ethanol precipitation as described previously [18]. Total RNA from normal human lung tissue was purchased from Ambion (Austin, TX).

Cloning of the BRAF25 cDNA. The 3′ and 5′ RACE systems for amplification of cDNA ends and the pAMP1 system for cloning of amplification products (Gibco) were used to clone the 3′ and 5′ end sequences. The first-strand cDNAs were amplified by the polymerase chain reaction (PCR) using (T1) 5′-$(CAU)_4$ ATC CCG GGA CCC GGT CGC CA-3′ and (T2) 5′-$(CAU)_4$ TCG CCA CTA CAG ACC CCG GCT-3′ [19]. 5′ End sequence was amplified using 5′-TTC TTC TGG GTG TAT CGC GCA-3′ (located in the nucleotides 1136–1156 in the cDNA sequence) and a nested primer, 5′-$(CAU)_4$ GCG AGT GCT TTT TCC GGG TTC-3′ (located in the nucleotides 1114–1135). The full-length cDNA was amplified using 5′-$(CAU)_4$ AAG AAG ATT CTG CCG AAT GGG C-3′ (located in the nucleotides 161–182) and universal amplification primer (UAP). The amplified clones were cloned into the pAMP1. cDNA clones were sequenced with ABI 373 sequencer using ABI prism dye terminator cycle sequencing ready kit. All the primers used for cloning and sequencing were purchased from IDT (Coralville, IA).

Antibody raised against the full-length BRAF25 protein. The cDNA encoding the full-length BRAF25 was amplified by PCR. The PCR products were cloned into the pCR 2.1 (Invitrogen) and then cloned in-frame into pTrcHis A vector (Invitrogen). The plasmid construct was expressed in the TOPO10F′ from which the histidine–BRAF25 fusion protein was isolated and purified by ProBond nickel-chelating resin column. The rabbit polyclonal antibody was raised against the histidine–BRAF25 fusion protein. Purified fusion protein (100 μg/ml) was well mixed with the adjuvant Imject Alum (Pierce) and injected intramuscularly into the hind legs of three rabbits. Booster injections were given at weeks 4 and 8. Sera were collected every 4 weeks. Preimmune sera were obtained prior to the initial immunization as described previously [20]. An enzyme-linked immunosorbent assay (ELISA) using the BRAF25 fusion protein as the solid phase was used to assess the immune responses to the BRAF25 fusion protein.

Antibody raised against the BRAF25 peptide. The immunizing peptide used to generate the anti-BRAF25 antibody was designed to the amino acid residues 173–187 of BRAF25. The immunizing peptide was custom-synthesized by Sigma Genosys (Woodland, TX). The anti-BRAF25 antibody was raised against the synthetic peptide (Sigma Genosys). ELISA using the immunizing peptide as the solid phase was used to assess the immune responses to the BRAF25 peptide antigen.

Methods used to determine the specificity of the anti-BRAF25 antibodies. Competitive ELISA and Western blot analysis were used to determine the specificity of the anti-BRAF25 antibodies. The competitive ELISA and Western blot analysis were performed as described previously [20,21] and detailed in Fig. 3.

Immunoprecipitation and Western blot analysis. Cells were incubated at 1×10^6 per ml in culture medium. Cells were harvested, washed with cold PBS, and lysed for 30 min at 4 °C in cold lysis buffer as described previously [21]. Cell lysates were immunoprecipitated overnight with the anti-BRAF25 antibody (1:500) and the preimmune serum (1:500), respectively. The immuno-complexes were incubated with protein A–Sepharose CL-4B beads (Pharmacia) at 4 °C for 3 h and collected by centrifugation at 1600 rpm for 1 min at 4 °C. The beads were washed with the ice-cold lysis buffer four times and collected by centrifugation. Proteins were eluted in sample buffer at 110 °C for 10 min. Eluted proteins were electrophoresed through 12% SDS–PAGE, transferred onto membrane, and analyzed with the anti-BRAF25 antibody as described in Fig. 4.

Results and discussion

Screening the 3′ and 5′ RACE clones to clone BRAF25 cDNA from PPC-1

We have been engaged in studying the expression of the Bcl-2 in the prostate cancer cell lines. Ab-1 antibody (Oncogene Res.) against amino acids 61–76 of Bcl-2 immunoprecipitated a protein besides the Bcl-2 protein from PPC-1 and LNCap (data not shown). Therefore, two primers (T1 and T2) were designed to nucleotides 183–201, and 208–228 of Bcl-2 cDNA, respectively [19]. These primers were paired with the UAP for PCR to amplify cDNAs derived from the 3′ RACE. Electrophoresis of PCR products from the T1 primer revealed six major bands and some minor bands that were cloned into the pAMP1. 318 cDNA clones were isolated. cDNA inserts in 318 clones were characterized by *Eco*R1 and *Bam*H1 mapping. 50 cDNA clones (BR1–BR50, BR stands for the Bcl-2 related clones) of the 318 clones were sequenced. The 5′ sequences of BR8 and BR33 from PPC-1 showed 15 nucleotides identity compared to the T1 Bcl-2 sense primer used for cloning the cDNA.

BRAF25 cDNA (895) AAT CCT GGC CCA GGT CGC CA (914)

Bcl-2 cDNA (183) ATC CCG GGA CCC GGT CGC CA (201)

These cDNA clones were 625 base pairs (bp) in length and corresponded to the nucleotides 895–1520 in the cDNA sequence (Fig. 1). Two Bcl-2 cDNA clones (BR44 and BR49) were amplified (data not shown). Two novel cDNA clones (GenBank Accession Nos.: AF261072 and AF439744) were amplified using the T2 primer, which map to human chromosome 19. Twenty-five clones were isolated from 5′ RACE. Six clones contained the same size insert. Clone (3C13b11) had the overlapping sequence with clones BR8 and BR33. The other five clones (3C13b1, 3C13b4, 3C13b10, 3C13b12, and 3C13b13) were also sequenced and had the overlapping sequences with clone BR8 and BR33. The cDNA sequences obtained from these clones corresponded to the nucleotides 1–1156 in the cDNA sequence (Fig. 1). Therefore, a full length of cDNA was obtained.

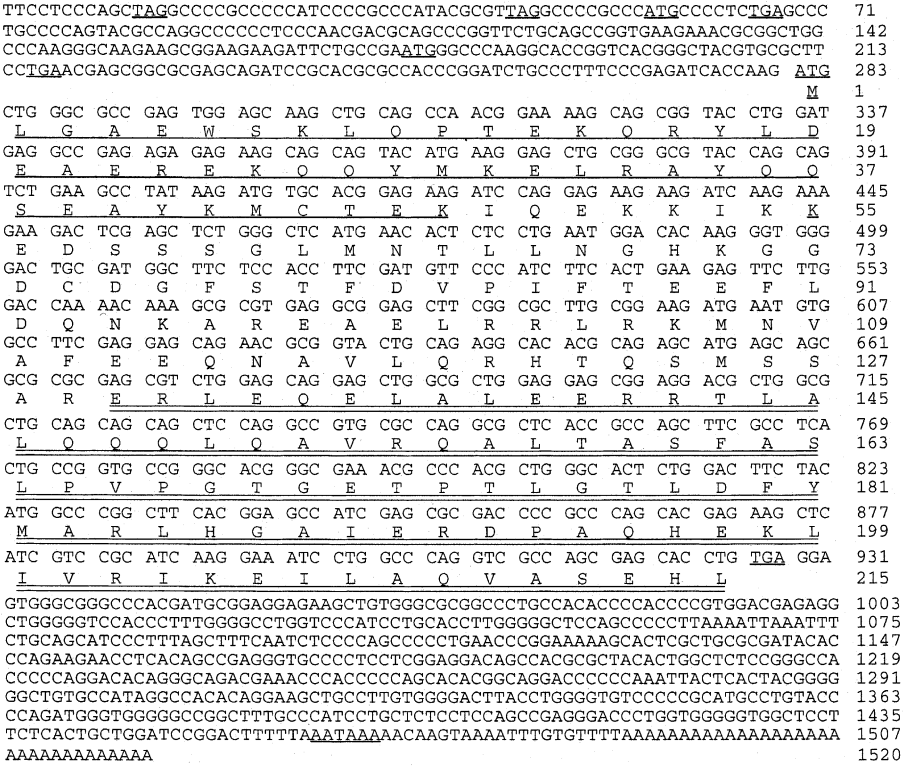

Fig. 1. The complete nucleotide sequence (GenBank Accession No.: AF072165) encoding BRAF25 and the predicted amino acid sequence of BRAF25. TAG, ATG, TGA, and polyadenylation signal (AATAAA) in the cDNA sequence are underlined. The conserved HMG-h domain is single underlined and kinesin-like domain is double underlined in the amino acid sequence of BRAF25. A short ORF located in the nucleotides 177–216 represents a minicistron. The presence of the minicistron and ORF in the cDNA sequence was confirmed in 34 cDNA clones from 13 human cancer cell lines and normal human lung tissue. The numbers beside the sequences indicate the positions of the nucleotides and the amino acids in the sequences.

Screening of cDNA clones to clone the full length of cDNA

Thirty-six clones were isolated from PPC-1. Three clones (1,11/16/97; 7, 11/16/97; and 14, 11/16/97) from PPC-1 had the identical sequences at their 5′ and 3′ ends compared to the 5′ end and 3′ end clones obtained above. To confirm the cDNA sequence from other cell lines, total RNAs from 13 cell lines and normal human lung tissue were used as the templates in the 3′ RACE. Two-hundred and sixty one clones were isolated and characterized by *Eco*R1 and *Bam*H1 mapping. Twenty-three cDNA clones contained the predicted cDNA insert. These clones were as follows: A6 and A7 from HeLa S3; B8 from SCLC; C1 from LNCap; D2 from K562; E6 and E9 from HEC1B; F36 from U937; G35 from Daudi; H11 from ML-2; I13 from Raji; J5 from PC3; K2 and K9 from DU145; Topo 3, Topo 5, and (1,12/10/97) from PPC-1; L3, L4, and L6 from Molt16; and M5, M7, and M8 from normal human lung. Identical sequences were obtained from all these clones. A major ORF encoding BRAF25 was found in the cDNA sequence (Fig. 1). This ORF started with an ATG codon at position 281 and terminated with a TGA codon at position 926. Other than this ORF, a short ORF located in the nucleotides 177–216 represented a minicistron that preceded the major ORF. This short ORF encoded a 13 amino acid peptide. Both the minicistron and major ORF were all preceded by the in-frame termination codons. A 5′ end proximal ATG codon was located at position 56 and followed by a TGA codon at position 65. This termination codon is in-frame with the initiation codon for the major ORF. Two additional TAG termination codons occur at positions 12 and 44 in the cDNA sequence. The former termination codon is in-frame with the ATG for the minicistron. The latter termination codon is in-frame with the ATG codon for the major ORF. A polyadenylation signal, AATAAA, was located in 27 nucleotides upstream of the long poly(A) tail at the 3′end of the cDNA sequence. A homology research of the recent computer databases revealed that the BRAF25 cDNA shared homology with cDNA sequences encoding HMG20B [22], BRAF35 [16], and Smarce1 [23]. Data from all these studies indicated the presence of alternatively spliced transcripts. The spliced variants have been observed in the RACE experiments by Sumoy et al. [22].

BRAF25 cDNA precisely maps to chromosome 19

The BRAF25 gene maps to chromosome sub-band 19p13.3 (AC005786), a region where loss of chromosomal heterozygosity has been reported in about 50% of ovarian cancers [24,25]. The BRAF25 gene consists of 7 exons and 6 introns. The predicted protein contained 215 amino acids with a predicted molecular weight of 25,820 Da (Fig. 1). A homology search of databases revealed that the amino acid sequence of BRAF25 was identical in sequence to amino acids 103–317 of human HMG20B and BRAF35 [16,22] and almost identical in sequence to amino acids 103–317 of mouse Smarce1r [23]. Therefore, we have designated the protein encoded by the cDNA as BRAF25. BRAF25 contains a truncated HMG domain and a region with two-heptad repeats similar to the coiled-coil domain of kinesin (Fig. 1). HMG domain is composed of about 85 amino acids [26]. Because the truncated HMG domain of BRAF25 contained 47 amino acids that account for only half of the amino acids found in other HMG domains, this truncated HMG domain was termed the HMG-h domain of BRAF25 (Fig. 2). Despite of truncation of the HMG-h domain of BRAF25, all the specified residues [26] at positions 41 (W), 49 (K), 52 (Y), 56 (A), 63 (Y), and 70 (Y) are conserved in the HMG-h domain of BRAF25. These residues that are involved in binding to DNA are highly conserved in various HMG domains. The two-heptad repeats with the sequence similarity to the coiled-coil domain of kinesin exhibit a repetitive pattern as found in the leucine zipper motifs, suggesting a similar function [27]. Multiple Src homology 2 (SH2) motifs occur in the positions of tyrosines (Y) 17 (Y17), 28 (Y28), 35 (Y35), 41 (Y41), and 181 (Y181) in the amino acid sequence of BRAF25 (Fig. 1). SH2 motifs are characterized by the presence of tyrosine-containing short amino acid sequence in protein sequence [28].

Specificity of the antibodies raised against the full-length BRAF25 and BRAF25 peptide

The anti-BRAF25 antibody was raised against the BRAF25 fusion protein. We also designed a specific BRAF25 peptide sequence that was used by Sigma Genosys to raise the anti-BRAF25 peptide antibody. We performed the competitive ELISA to characterize the specificity of the anti-BRAF25 antibody. Preincubation of the anti-BRAF25 peptide antibody with the immunizing peptide resulted in the suppression of the binding to the immunizing peptide (Fig. 3A, lanes 2 and 3). The suppression was dramatic when compared with the results obtained in the absence of the immunizing peptide in the preincubation with the antibody (Fig. 3A, lane 4). These results not only confirmed a true anti-peptide response present in the sera but also determined the specificity of the anti-BRAF25 antibody. To further determine 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 BRAF25 protein and BRAF25 peptide specifically detected the BRAF25 (25 kDa) fusion protein (5 kDa) (Fig. 3B, lanes 3–6). In contrast, the Western blot analyzed with the preimmune serum as control antibody detected no specific band (Fig. 3B, lanes 1 and 2). These results together with the results from the competitive ELISA have confirmed that the anti-BRAF25 antibodies are specific.

Anti-BRAF25 antibody immunoprecipitated the BRAF25 protein

Immunoprecipitation of the cell lysates with the anti-BRAF25 antibody followed by the Western blot analysis using the antibody against the full-length BRAF25 protein showed that the anti-BRAF25 antibody specifically precipitated a 25 kDa protein from two prostate cancer cell lines (Fig. 4, lanes 2 and 4). In

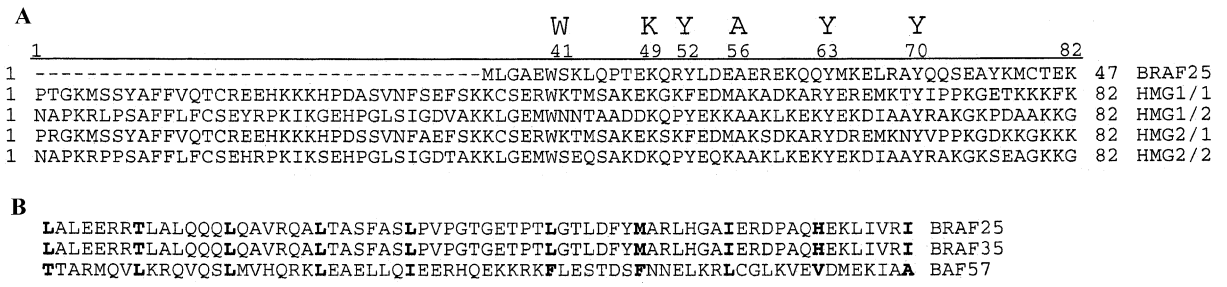

Fig. 2. Sequence analysis of BRAF25. (A) The amino acid sequence of the truncated HMG-h domain of BRAF25 was aligned with HMG1 (S29857) and HMG2 (P26583) domain proteins in GenBank and SwissProt databases. Amino acids Trp (W), Lys (K), Tyr (Y), Ala (A), Tyr (Y), and Tyr (Y) at the specified positions 41, 49, 52, 56, 63, and 70 are all conserved in the truncated HMG-h domain of BRAF25. The lines (-) represent the truncated region in the HMG-h domain of BRAF25. (B) Alignment of kinesin-like domains with those in BRAF35 (AF331191) and BAF57 (AF035262). The hydrophobic residues of the heptad repeats of the kinesin-like coiled-coil domain are bolded.

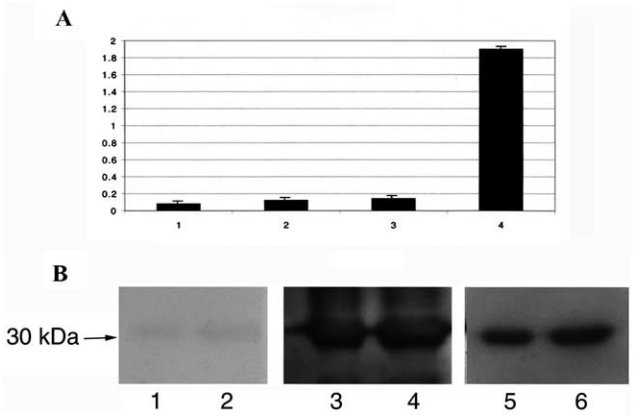

Fig. 3. Determination of the specificity of the antibodies raised against the full-length BRAF25 protein and BRAF25 peptide. (A) Result of competitive ELISA is shown. The wells of a plate were coated with the immunizing peptide at 10 μg/ml in buffer. The antibody raised against the BRAF25 peptide was preincubated with the immunizing peptide at 80 μg/ml (lane 2) and 160 μg/ml (lane 3), respectively, at room temperature for 1 h. The antibodies were serially diluted in buffer, added to the wells, and incubated at 37 °C for 2 h. The wells were washed with buffer; a diluted peroxidase-conjugated goat anti-rabbit immunoglobulin (Ig) was added and incubated at 37 °C for 2 h. After washing, 3,3′,5,5′-tetramethyl benzidine (TMB) was added, and the A_{450} was recorded after 15 min by an MR 600 microplate reader. The anti-BRAF25 antibody (lane 4) and preimmune serum (lane 1) were used as positive and negative controls, respectively. The column represents the mean value of optical density at A_{450} of four measurements (from four samples) and the bar represents the SD. (B) Western analysis to determine the specificity of the anti-BRAF25 antibodies. Three microgram (lanes 1, 3, and 5) and 5 μg (lanes 2, 4, and 6) of the BRAF25 (25 kDa) fusion protein (5 kDa) were separated by 10% SDS–PAGE. The blot (lanes 1 and 2) was analyzed with the preimmune serum. The blot (lanes 3 and 4) was analyzed with the antibody raised against the full-length BRAF25 protein, and blot (lanes 5 and 6) was analyzed with the antibody raised against the BRAF25 peptide, and followed by using the anti-rabbit Ig coupled to horseradish peroxidase (Amersham). Blots were developed with ECL Detection Kit (Amersham), and then exposed to Fuji X-ray film for 3–15 s as described previously [21].

contrast, the anti-BRAF25 Western blots of cell lysates immunoprecipitated with the preimmune sera as control antibody detected no specific bands (Fig. 4, lanes 1 and 3). The expression level of the BRAF25 was much higher in the primary prostate cells (PPC-1) (Fig. 4, lane 2) compared to that in the metastatic prostate cancer cells (LNCap) (Fig. 4, lane 4). Because BRAF25 is an alternatively spliced protein of BRAF35, the anti-BRAF25 antibody could react to BRAF35 and bring down BRAF35 in the immunoprecipitation. However, the antibody against the full-length BRAF25 protein did not precipitate the BRAF35 species from the PPC-1 and LNCap cell lines.

In summary, we have cloned a novel cDNA encoding an alternatively spliced BRAF25 protein. The cDNA contains an unusual structure with a minicistron motif and a major ORF (Fig. 1). Through the characterization of the BRAF25 cDNA sequence, we have cloned 34

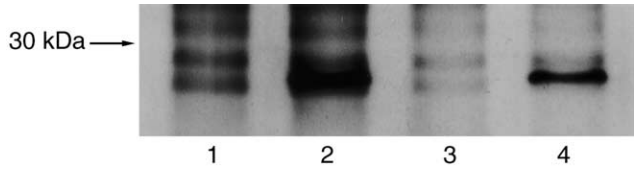

Fig. 4. Anti-BRAF25 antibody immunoprecipitated the BRAF25 in the cell lysates from PPC-1 and LNCap. Equal number of cells was lysed with the lysis buffer. Five-hundred microgram of protein was used in each immunoprecipitation. The cell lysates of PPC-1 and LNCap were immunoprecipitated with the antibody raised against the full-length BRAF25 protein, and the preimmune sera. Same amount of the protein was loaded in each lane. Proteins precipitated with the preimmune sera (lanes 1 and 3) and the antibody raised against the full-length BRAF25 protein (lanes 2 and 4) were separated by 12% SDS–PAGE. The Western blot was analyzed with the antibody raised against the full-length BRAF25 protein.

BRAF25 cDNA clones from 13 established human cancer cell lines and normal human lung tissue. The BRAF25 cDNA sequence obtained from these cDNA clones confirms the presence of the minicistron motif and ORF in the BRAF25 cDNA sequence. We also determined the expression of the BRAF25 mRNA in various cell lines and normal human lung tissue using the real-time TaqMan RT-PCR. The BRAF25 gene was expressed in human cancer cells and normal human lung (data not shown).

More importantly, the BRAF25 cDNA sequence maps precisely to the genomic chromosome 19 sequence. It is noteworthy that the precise mapping of the BRAF25 cDNA sequence to the genomic chromosomal 19 sequence is directed by the joining rules of exon and intron structures under 100% assurance (Table 1). Likewise, the mapping of the BRAF35 cDNA sequence to chromosome 19 is also precise but the mapping extends further to the 5′ end of the chromosome 19 sequence (data not shown). BRAF25 gene consists of 7 exons and 6 introns. BRAF35 gene is composed of 9 exons and 8 introns. Both genes share identical sequence in 5 exons and 6 introns. These results provide the genetic evidence for the presence of the alternatively spliced BRAF25 encoded on chromosome 19 in various human cells.

To study the BRAF25 protein expression, we raised the antibodies against the full-length BRAF25 protein and BRAF25 peptide. We have characterized the specificity of the anti-BRAF25 antibodies and demonstrated that the anti-BRAF25 antibody specifically immunoprecipitates the BRAF25 protein in the cell lysates from prostate cancer cell lines (Fig. 4). BRAF25 contains the HMG-h domain. Two major types of HMG proteins have been characterized. Proteins that contain more than one HMG domains bind to DNA with low sequence specificity. These proteins are expressed in many tissues. Proteins with a single HMG domain interact with specific sequence in DNA. These DNA binding proteins regulate tissue-specific transcription [26].

Table 1
The gene encoding BRAF25 is composed of 7 exons and 6 introns

Exon no.	Sizes (bp)	Exon/intron 5′ Splicing donor	Intron/exon 3′ Splicing acceptor
1.	(325)	AAGCAG/gtgggc—(953)—ccccag/CGGTAC	
2.	(121)	AGAAAG/gtggga—(600)—cgccag/AAGACT	
3.	(47)	CACAAG/gtaagc—(245)—tcttag/GGTGGG	
4.	(73)	ACAAAG/gtgagc—(266)—gcgcag/CGCGTG	
5.	(216)	TGCCGG/gtgcgg—(873)—ccccag/GCACGG	
6.	(133)	CGCCAG/gtgtgt—(395)—tttcag/CGAGCA	
7.	(535)	CGGACTTTTTAAATAAA—poly(A) tail	

The sizes of the exons and introns are given in the brackets. The sequences in exons are in upper case letters and introns in lower case letters. The 12 nucleotides at the 5′ splicing donor and 3′ splicing acceptor junctions are shown. The slashes (/) denote junction at the exons and introns. The lines (—) represent the sequence in the introns and exons.

Consistent with these observations, BRAF35 has been found to bind to the branched DNA structure in the BRCA2 complex [16]. Since the alternatively spliced BRAF25 transcript remains in frame and is present in a wide variety of human cells, it is conceivable that the translation of this alternative form may have a function in the BRCA2 pathway.

Recently, Marmorstein et al. [16] have demonstrated that the BRCA2 protein exists in a large multi-protein complex. Since the BRCA2 gene was identified about six years ago, this is the first report that has indicated that the BRCA2 protein may interact with many proteins in the BRCA2 pathway. This emerging evidence indicates that identification of the protein components in the multi-protein BRCA2 complex could lead to answer some critical questions about cancer predisposition associated with mutations in the BRCA2 gene. For example, the BRCA2 gene is expressed in a wide variety of adult tissues and participates in universal cellular pathways. However, cancer predisposition associated with BRCA2 gene mutations manifests mainly in the breast, ovary, or prostate epithelial tissues [4,5]. Yet we have no answers and are still in search of reasons. An alternatively spliced BRCA1 transcript has been found in many tissues [1] but its function is unknown. Truncated BRCA2 is cytoplasmic and nonfunctional [29]. It has become more and more clear that further studies to characterize the functions of these truncated or alternatively spliced proteins, including BRAF25, will be essential to understand cancer predisposition associated with mutations in the BRCA genes.

Acknowledgments

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