Amplification in Human Breast Cancer of a Gene Encoding a c-myc mRNA-binding Protein

Glenn A. Doyle, Jeanne M. Bourdeau-Heller, Stephanie Coulthard, Lorraine F. Meisner, and Jeffrey Ross

Abstract

The coding region determinant-binding protein (CRD-BP) binds in vitro to c-myc mRNA and is thought to stabilize the mRNA and increase c-Myc protein abundance. The CRD-BP gene has 15 exons and 14 introns, and its presence should increase CRD-BP also protects c-myc from rapid degradation and thereby to prolong its half-life. If the CRD-BP functions as a shield to protect c-myc in vitro have purified and characterized a c-myc mRNA. We clones containing the human or mouse CRD-BP gene were nick translated in reactions containing fluorescein-12-dUTP. Each probe was suspended in 10 µl of Oncor probe or Hybrisor VII (Oncor) and placed on dehydrated metaphase slides, which were sealed, heated to 75°C for 3 min to denature the probes, and hybridized at 37°C for 16–20 h in a humidified chamber. Slides were washed three times (5 min each) in 0.1% NP40, pH 7.4). Slides were counterstained with 4',6-diamidino-2-phenylindole and analyzed on an Olympus B-MAX fluorescent microscope equipped with a filter wheel and Applied Imaging software.

To analyze paraffin-embedded breast tumors, three sections of 4 µm were cut from paraffin blocks and mounted on Superfrost Plus Microscope Slides (Fisher). One slide from each specimen was stained with H&E to identify cancer and normal cells. The other slides were baked on a 65°C hot plate for 18 h and then deparaffinized in three changes (5 min each) of Hemo-De (Fisher), followed by two changes (2 min each) of absolute ethanol. Slides were baked on a 65°C hot plate for 18 h and then deparaffinized in three changes (5 min each) of Hemo-De (Fisher), followed by two changes (2 min each) of absolute ethanol. Slides were then treated with 1 µm sodium thiocyanate at 45°C for 15 min, rinsed in water, incubated in 0.125 mg/ml Protein Digesting Enzyme (Ingen) in 2× SSC at 37°C for 30–45 min, washed in 2× SSC, and dehydrated. Probe (10 µl) was placed onto the specimen, which was then covered with an 18 × 18-mm coverslip and sealed. DNA was denatured at 75°C for 15 min, and hybridization, washing, and counterstaining were performed as above. Fluorescent signals per nucleus were counted in at least 40 tumor cells and 40 normal cells per case. CRD-BP gene amplification was based on an average of 2.5 or more signals per cell; HER-2/neu amplification was based on 3.5 or more signals per cell. When both CRD-BP and HER-2/neu gene copies exceeded 2.5, ploidy was assessed using the chromosome 10 centromere probe, and gene signal counts were corrected for polyploidy, when indicated by increased copies of chromosome 10.
Results and Discussion

The mouse CRD-BP gene is ~40 kb in length and contains 15 exons and 14 introns (Fig. 1). The CRD-BP itself contains three motifs that are present in other RNA-binding proteins: two RRMs, an RGG box, and four KH domains (3). None of the RRM or KH motifs is encoded by any single exon, although the boundaries of the first RRM and of KH domain 3 coincide with intron-exon boundaries. The size of intron 2 has not been defined but is at least 16 kb. By comparing Southern blots of DNA from 129/SvJ and C3H mice, the

Fig. 1. Organization of the mouse CRD-BP gene. A, gene map. A mouse BAC clone was analyzed by Southern blotting and PCR to generate the map. Lines, flanking DNA and introns; rectangles, exons. A, Avr II; B, BamHI; Bg, BglII; E, EcoRI. ATG and TGA designate the translation start and stop sites, respectively. The sizes of exon 1 and intron I have not been determined. Dagger symbols, polymorphic BamHI sites. B, mRNA map. The relationship between exons and CRD-BP mRNA is indicated. Lines, 5' and 3' untranslated regions; rectangles, coding region. Exon 1 contains at least 174 bp. C, protein map. The abbreviations denote RNA-binding motifs that are characteristic of this and other RNA-binding proteins. M, the start methionine; *, COOH terminus.

Fig. 2. Localization by FISH of the CRD-BP gene in mouse and human chromosomes. A, mouse. Metaphase spreads from normal mouse embryo fibroblasts (Sv129 × C57B16 F1) were analyzed with a fluorescein-labeled mouse CRD-BP gene probe (green) and a rhodamine-labeled mouse chromosome 11 paint probe (red). B, human. Metaphase spreads from normal human blood lymphocytes were analyzed with a fluorescein-labeled human CRD-BP gene probe (green) and a digoxigenin-labeled RARA gene probe (red). This and other digoxigenin-labeled probes were detected with a rhodamine-conjugated anti-digoxigenin antibody. C, Top, detail of human chromosome 17 showing the CRD-BP gene signal (green) next to the same chromosome 17 banded with G1L and a chromosome 17 ideogram. Bottom, copies of chromosome 17 hybridized with the green CRD-BP gene probe and (from left to right) with red probes for HER-2/neu, MPO, and RARA genes, respectively.
CRD-BP gene contains at least two BamHI polymorphisms, one in intron 7 and the other in intron 9 (Fig. 1A). FISH analysis was performed to localize the CRD-BP gene in normal mouse and human cell chromosomes. In mouse cells, analysis with a chromosome 11 paint probe (red) plus a fluorescein-labeled CRD-BP probe (green) visualized the CRD-BP gene on chromosome 11 (Fig. 2A). In human cells, the CRD-BP gene resides on chromosome 17q21.3 (Fig. 2, B and C). The gene is distal to the HER-2/neu gene at 17q11.2–12 and to RARA at 17q21.1 and is proximal to MPO at 17q23.1 (Fig. 2C, bottom). The human CRD-BP gene was recently mapped to a region of chromosome 17 containing a gene thought to contribute to some cases of type 1 von Willebrand disease gene and is probably orthologous to the b-actin mRNA zipcode-binding protein gene (5, 14).

Three observations prompted us to use FISH to determine whether the CRD-BP gene is amplified in human tumors: (a) the proximal long-arm of human chromosome 17 contains several genes that are mutated, deleted, or amplified in cancer. BRCA-1 is located at 17q21, whereas HER-2/neu is located at 17q11; (b) genes encoding hKOC and p62, both of which are related to the CRD-BP gene, are overexpressed in some pancreatic cancers and hepatocellular carcinomas, respectively (7, 8); and (c) as determined by Western blotting, the CRD-BP is abundantly expressed in fetal tissues and in cancer cell lines but is undetectable in adult tissues (12). This latter observation suggests that the CRD-BP might be an oncofetal protein. If so, its overexpression in human cancers could contribute to cancer cell survival/replication, because the CRD-BP is thought to enhance c-Myc protein expression, and c-Myc protein promotes cell replication.

FISH was performed using 40 breast cancer samples, and the CRD-BP gene was amplified in 14 of these (Figs. 3 and 4). In two cases, the gene was highly amplified (14.4 and 20 copies) and appeared to be on double-minute chromosomes (Fig. 3). The significance of this observation is underscored by the fact that only the tumor cells in each sample contained amplified CRD-BP genes. For example, the large tumor cells shown in Fig. 3A have amplified CRD-BP genes (large arrow), whereas the surrounding smaller normal cells do not (small arrow). For comparison, an H&E section showing large, multinucleolated tumor cells surrounded by smaller nonneoplastic cells is shown in Fig. 3B. The CRD-BP gene was moderately amplified in another 12 breast cancer cases, when signal number was corrected for ploidy (Fig. 4). The HER-2/neu gene was coamplified in 7 of the 14 cases in which the CRD-BP gene was amplified, whereas the CRD-BP gene alone was amplified in the other seven cases. Therefore, amplification of these genes can occur independently, despite their proximity on chromosome 17. In both cases with highly amplified CRD-BP genes, the HER-2/neu gene was also amplified.

On the basis of these 40 cases, the CRD-BP gene is amplified in approximately one-third of human breast cancers. We do not yet know whether the CRD-BP itself is overexpressed in human breast cancer or not.
other cancers or whether CRD-BP overexpression increases c-Myc protein abundance. However, it seems significant that CRD-BP gene amplification can occur without concomitant HER-2/neu amplification. Future studies will need to address these issues, as well as whether CRD-BP overexpression, with or without amplification/overexpression of c-myc or HER-2/neu, affects the course of breast cancer, especially in those patients with poor outcomes despite otherwise favorable prognostic indicators. CRD-BP overexpression in cancer tissue, with little or no expression in normal surrounding tissue, could be a useful prognostic/diagnostic indicator, as well as a potential therapeutic target. Moreover, having mapped the structural features of the CRD-BP gene, it becomes feasible to design transgenic animal experiments to assess the in vivo function of the protein using transgenic and gene knockout animal models.

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