

Overrepresentation of the *EBAG9* Gene at 8q23 Associated with Early-Stage Breast Cancers¹

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ABSTRACT

EBAG9, an estrogen-responsive gene located at 8q23 was identified in an effort to clone CpG-binding sites. Its product was later found to be identical to *RCAS1*, a cancer cell-surface antigen implicated in immune escape. We determined the sequence of the complete cDNA and the genomic structure for *EBAG9*. *EBAG9* gene copy number in 21% (27 of 129) primary breast cancers we examined; *EBAG9* mRNA was consistently expressed in cancer cell lines. Detailed physical mapping of the 8q arm, including polymorphic markers for *EBAG9* and the *CMYC* loci, revealed allelic gain of either *EBAG9*, *CMYC*, or both, in 45% (58 of 129) of the breast cancers we examined. The *EBAG9* gene was increased exclusively in 16 of the 27 tumors showing gain at that locus; the other 11 showed gain of a larger chromosomal region containing both *EBAG9* and *CMYC*. Analysis of subsequent series of 144 primary breast cancers for allelic gain at *EBAG9* and *CMYC* locus showed a similar degree of gain at *EBAG9*, *CMYC*, or both. When a total of 273 breast cancers from two series were combined and analyzed for clinicopathological correlation, almost all of the tumors with *EBAG9* increased but not those with *CMYC*. Twenty-eight of 29 were T1/T2 stage carcinomas (<5 cm in diameter), whereas one third (21 of 61) of the tumors in which *CMYC* was increased but *EBAG9* was not, were advanced T3-stage

tumors ($P = 0.0012$). These data suggest that *EBAG9* and *CMYC* gene are independent targets of gain and that overrepresentation of *EBAG9* may play a specific role in early stages of breast carcinogenesis.

INTRODUCTION

Numerous genetic alterations identified to date in human breast cancers include gains of specific chromosomal regions (some containing known proto-oncogenes) and/or loss of DNA at other loci in tumor cells. Other changes can include allelic losses (LOH),³ which imply inactivation of tumor suppressor genes. Frequent LOH for certain polymorphic DNA markers has been well documented in primary breast cancers on chromosomes 1p, 3p, 7p, 11p, 13q, 16q, 17, 18q, and 22q; however, little is known about other types of chromosomal alterations that may be involved in breast carcinogenesis (1–9). Attempts to study large numbers of primary breast cancers by cytogenetic methods have been limited because of technical difficulties involved in the direct preparation of chromosomes in solid tumors.

Recently the usefulness of polymorphic markers for detecting both LOH and modest increases of chromosomal copy number in solid tumor tissues was proven by careful comparative studies of karyotypic and RFLP analyses. Partial trisomy of chromosome 5q in renal cell carcinomas (10) and trisomy of chromosome 7 in malignant mesotheliomas (11) were unambiguously demonstrated in autoradiographies of hybridization experiments. The degrees of chromosome-specific numerical changes and the sizes of the regions involved indicate that the chromosomal changes in those tumors represent trisomy, tetrasomy, or polysomy of all or parts of a chromosome, rather than the “gene amplification” phenomenon represented by double-minutes or heterogeneously staining regions in which a minute segment of a chromosome containing a drug resistance gene or an oncogene, for example, is amplified up to several hundred fold (12, 13).

Through a CpG-binding site cloning approach, we previously cloned a partial cDNA for *EBAG9*, a novel estrogen-responsive gene (14). *EBAG9* mRNA is up-regulated by estrogen treatment in MCF-7 breast cancer cells within 6 h (14). We subsequently located the estrogen-responsive element (ERE) in the 5' regulatory region of *EBAG9*, and localized the gene to 8q23 by fluorescence *in situ* hybridization (15). One year after the previous work was published, Nakashima *et al.* (16) recently

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³ The abbreviations used are: LOH, loss of heterozygosity; RACE, rapid amplification of cDNA end; SSCP, single-strand conformational polymorphism; BAC, bacterial artificial chromosome; RT-PCR, reverse transcription-PCR; ER, estrogen receptor; PgR, progesterone receptor; CHLC, Cooperative Human Linkage Center; UTR, untranslated region; IL, interleukin.

Table 1 Oligonucleotide used to define exon/intron junction of human *EBAG9* gene

Exon	Oligonucleotide sequence		Nucleotide position in cDNA (forward/reverse)
	Forward	Reverse	
1	5'-TGCGTGACCTTACTTACTGCCCTCCGTCT-3'		95-119/
2	5'-ACCATGGCCATCACCCAGTTTCGGTTA-3'	5'-TCTTTAGGAATGAGAATACTGTTGCT-3'	290-317/338-363
3	5'-AGAGGACGGAAATTAAGTGGAGACCA-3'	5'-TTAGGAAGTATGAAATACTCACTGT-3'	383-409/426-451
4	5'-ATGTTGAAGAGTGGACTTCCTGGGA-3'	5'-GTTGGTGTTCATGTCCTTAAAATAGT-3'	459-484/571-595
5	5'-TATTAAGAAGAGAGAACCATTGAAT-3'	5'-AGATCTTGTGTAGCTGCTAATCTAC-3'	620-644/679-703
6	5'-TGACTTAGATACCTGGCAGAAAATA-3'	5'-ACTTCTTCTGCTTGCCAGGCTGCAT-3'	734-759/783-808
7		5'-TCCTACTGGCATGATAAAATTTGAA-3'	/942-966

described cloning of a partial cDNA for *RCAS1*, as a cancer cell-surface antigen implicated in immune escape. This *RCAS1* cDNA was then found to be identical to previously described *EBAG9* cDNA. In the work reported here, because only partial cDNA sequence was described in either report, we cloned the complete structure of the *EBAG9/RCAS1* cDNA by 3'RACE experiments. We also cloned and determined the genomic structure of the *EBAG9* gene. Because previous reports have shown that a large chromosomal region containing *CMYC*, 8q22-8qter, was frequently amplified in various types of tumors including breast cancers (12, 13, 17-19), we constructed a detailed physical amplicon map of the 8q arm that encompasses the *EBAG9* and *CMYC* loci.

MATERIALS AND METHODS

Cloning a Full-Length cDNA. To obtain the full-length cDNA sequence of the human *EBAG9* cDNA, we carried out 3' RACE experiments using the partial cDNA sequence that we had characterized earlier and a SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA). In brief, polyadenylate RNA from human breast (Clontech), was reverse-transcribed with Superscript II reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) using an oligo(dT) primer. First-strand cDNA was subjected to two rounds of PCR, and the products were resolved by electrophoresis in a 1% agarose gel. The specific PCR product visualized by ethidium-bromide staining was purified with a Gel Extraction kit (Qiagen) and subcloned using an AdvanTage PCR cloning kit (Clontech). The nucleotide sequence of the cDNA was determined by the BigDye Terminator cycle sequencing method using a 377 ABI Prism automated DNA sequencer (Perkin-Elmer, Norwalk, CT).

Genomic Cloning of the Human *EBAG9* Gene. A BAC library containing human DNA, pooled in a three-dimensional structure (Genome Systems, St. Louis, MO), was used to isolate genomic clones containing the human *EBAG9* gene sequence. The BAC library was screened with two oppositely oriented oligonucleotides (forward, 5'-GGAGGGCAGTAAAGTCACGC-3'; reverse, 5'-ACCTCTGGGGTGAGGAAACT-3'). *Escherichia coli* cells containing the positive clone were cultured in the presence of chloramphenicol, and BAC DNA was isolated as described previously (20).

Determination of Genomic Sequence. Nucleotide sequences of exon/intron junctions, their boundaries, and the size of each intron were determined by directly sequencing BAC clones or PCR products by LA Taq (TAKARA, Tokyo, Japan), using oppositely oriented primers located in the exons flanking

each intron. The primers were designed on the basis of the partial *EBAG9* cDNA sequence determined earlier (Ref. 14; Table 1). Sequencing was performed by the BigDye Terminator cycle-sequencing method, using a 377 ABI Prism automated DNA sequencer (Perkin-Elmer).

Cell Culture. The 94 cancer-cell lines analyzed were derived from 13 hepatocellular carcinomas (SK-HEP-1, C-HC-4, Hep-KANO CL-2, WRL68, Chang liver, Hep-TA-BATA, HuH7, Hep G2, HT17, Li7, PLC/PRF/5, Hep3b, and C3A), 10 breast cancers (MDA-MB-453, CRL1500, YMB-1-E, MCF7, HBL100, OCUB-M, BT-20, BT-474, MDA-MB-435S, and SK-BR-3), 10 uterine cancers (SIHA, HT-3, D98-AH-2, HeLaTG, HeLa, CaSki, ME-180, HeLaP3, HEC1-A, and SK-UT-1B), 7 lung cancers (RERF-LC-AI, LU65, Lu99, PC14, A549, EBC-1, and LK-2), 7 brain tumors (TE671 subNO.2, U-373 MG, T98G, u87 MG, u251 MG, SNB19, and uw228), 6 thyroid cancers (WRO, NPA, 8305c, ARO, FRO, and 8505c), 6 gastric cancers (HuGC-OOHIRA, AZ521, H-111-TC, SH-10-TC, MKN-7, and NUGC-4), 5 ovarian cancers (CAOV-3, SK-OV-3, NIH OVCA-3, OV-1063, and OVK18), 4 renal cell carcinomas (OS-RC-2, VMRC-RCW, RCC10RB, and Caki-1), 4 urinary bladder cancers (5637, T24, EJ-1, and Shimura), 4 osteosarcomas (MG-63, Saos-2, Hu0-3N1, and U-2 OS), 4 neuroblastomas (IMR-32, NH-12, SCCH-26, and NB-1), 4 colon cancers (DLD-1, SW480, HCT-15, and WiDr), 2 tongue cancers (HSC-3 and HSC-4), 2 bile ductal cancers (HuH-28 and TFK-1), 2 oral cancers (KB and HO-1-u-1), 2 pancreatic cancers (MIA Paca 2 and PK-8), 1 laryngeal cancer (HEp-2), and 1 gingival cancer (Ca9-22). The cell lines were either donated by the Cell Resource Center for Biomedical Research at the Institute of Development, Aging and Cancer of Tohoku University or by Dr. Baba at Mie University (Tsu City, Mie, Japan), or were purchased from the American Type Culture Collection (Manassas, Virginia). Each cell line was cultured in optimized conditions recommended by its respective distributor.

Analysis of *EBAG9* and *CMYC* Expression. We used RT-PCR experiments to examine levels of *EBAG9* expression in 56 cell lines that originated from 7 kinds of cancers (13 hepatocellular carcinomas, 10 uterine cancers, 10 breast cancers, 7 lung cancers, 6 gastric cancers, 6 thyroid cancers, and 4 renal cell carcinomas) using primers *EBAG9* (forward, exon 3 forward; reverse, exon 4 reverse; identified as such in Table 1). *CMYC* expression was also examined in all 10 breast cancer cell lines using primers *CMYC* (forward, 5'-TGAAAGGCTCTCCT-TGCAGC-3'; reverse, 5'-TCGCAGTAGAAATACGGCTG-3'). To quantify expression levels relative to other genes, we

carried out duplex PCR experiments using primers to amplify G3PDH (forward, 5'-ACCACAGTCCATGCCATCAC-3'; reverse, 5'-TCCACCACCCTGTTGCTGTA-3') as an internal control. Cycling conditions were 94°C for 4 min, then 27 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, with a final extension step of 5 min at 72°C. The PCR products were electrophoresed in 1.5% agarose gels. Enough quantity of mRNA was unavailable for all of the 10 cell lines to perform Northern blot analysis.

Isolation of DNA from Normal and Tumor Tissues.

Tumors and corresponding normal tissues were obtained from 129 patients who underwent surgery for primary ductal breast cancers at the Cancer Institute Hospital, Tokyo. Resected tissue samples were immediately frozen and stored at -80°C. DNA was extracted from frozen tissues according to methods described previously (21). Informed consent was obtained from all of the participating patients prior to surgery. For additional genotyping of *EBAG9* and *CMYC* locus in an independent panel of 144 breast cancer patients, genomic DNA was isolated from tumor and corresponding normal tissues in a procedure as described above.

SSCP Analysis. To look for alterations in the *EBAG9* gene in tumor cells, we divided the coding region into six fragments and amplified each exon and its flanking sequences with appropriate primers. PCR experiments were performed with genomic DNA from the 94 tumor cell lines described above. For each reaction, about 10 ng of PCR product was heat-denatured in the presence of 80% of deionized formamide and electrophoresed in 8% polyacrylamide gels, with or without 10% glycerol, in 0.5× TBE at 8 V/cm for 10 h at room temperature. DNA fragments were visualized by silver staining using the Plus One DNA silver staining kit (Pharmacia Biotech, Tokyo, Japan). Each fragment showing that an altered SSCP band pattern was subcloned with an AdvanTAge PCR cloning kit (Clontech) and sequenced.

Microsatellite Analysis by the Differential-PCR Method.

DNA from 129 primary breast tumors was examined for allelic gain or loss at eight highly polymorphic microsatellite loci ordered along the distal long arm of chromosome 8 according to the comprehensive human linkage map (22-24). The linear order of these markers is (centromere)-CHLC. GATA26A08-CHLC. ATA23G06-*EBAG9*-AFM200VC7-CHLC. GATA72D08-D8S522-D8S1179-D8S1128 (telomere). Marker D8S522 is in the vicinity of the *CMYC* gene on 8q24.1. A microsatellite marker for the *EBAG9* region on 8q23, described previously, was also included (25). The IL-1β (2q13-q21) gene was chosen as a single-copy control marker to obtain a reference for equal quantities of DNA, because this region of chromosome 2 has never shown chromosomal alterations in breast cancers. Primers IL1B.PCR2.1 and IL1B.PCR2.2 (26) were used to amplify this control gene.

Each microsatellite was coamplified with the single-copy control in the PCR using 20 ng of genomic DNA, 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 0.01% of gelatin, 200 mM dNTPs, 2.5 pmol each of [γ-³²P]ATP-end-labeled primer and nonlabeled primer, 0.8 to 2.5 pmol each of [γ-³²P]ATP-end-labeled IL1B.PCR2.1 and nonlabeled IL1B.PCR2.2, and 0.25 units of Taq polymerase in a volume of 10 μl. The amount of end-labeled control primer was adjusted to

1	ATTCGCCATGCTCCGCTCACGCGTGGGAGACTGGGCTGTGGGGTACCGGCCGGGAAAGCA	60
61	CGCAGCCTCCAAAGCCGCTTCTCAGGGAAATTTGCGTGACCTTACTGCCCTCCGCTTA	120
121	CAGGCCCTGTACCTCTCCAGGCCGATTTTCCACAATTTAAATCCCAAGTTCCACCTGGTAT	180
181	CCAGCTCCAGCAACTTAGAGCGTTTCACTCACCGCCGGCCAGGCGTCCGCTTGATATA	240
241	ACCTGAAAACCGCTCTGTTTTTCTCATCTGTGCGAGTGGTTTTGATTTCCACCATTGGCCA	300
	M A	3
301	TCACCCAGTTTCGGTTATTTAAATTTTGTACCTGCCTAGCAACAGTATTCTCATTCTTAA	360
	3 I T Q P R L F K F C T C L A T V F S F L	23
361	AGAGATTAAATATGCAGATCTGGCAGAGGACGAAATTAAGTGGAGACCAAATAACTTTGC	420
23	K R L I C R S G R G R K L S G D Q I T L	43
421	CAACTACACTTGATTATTCATCAGTTCCTAAGCAGACAGATGTTGAAGAGTGGACTTCTCT	480
43	P T T V D Y S S V P K Q T D V E E W T S	63
481	GGGATGAAGATGCACCCACCACTGTAAGATCGAAGAGGGAATGGGAATTTGGCAACAC	540
63	W D E D A F T S V K I E G G N G N V A T	83
541	AACAAAATCTTTGGAAACAACCTGAACTGACTATTTAAGGACATGACACCAACTATTA	600
83	Q Q N S L E Q L E P D Y F K D M T P T I	103
601	GGAAAACCTCAGAAAATGTTATTAAGAAGAGAGAACCACTGAATTTTGGCATCCACAGATG	660
103	R K T Q K I V I K K R E P L N F G I P D	123
661	GGAGCAGAGCTTCTCTAGTAGATTAGCAGCTACACAGATGCTGCCCTTTATTCATCAGT	720
123	G S T G F S S R L A A T Q D L F F I H Q	143
721	CTTCTGAATAGTGTACTTAGATACCTGGCAGGAAATACCAATGCATGGGAAGAAGAAG	780
143	S S E L G D L D T W Q E N T N A W E E E	163
781	AAGTGCAGCCTGGCAAGCAGAAGAAGTCTGAGACAGCAGAAACTAGCAGACAGAGAAA	840
163	E D A A W Q A E E V L R Q Q K L A D R E	183
841	AGAGAGCAGCCGAACAACAAAGGAAATGGAAGAGGAAAGCACAACCGCTAATGAAGA	900
183	K R A A E Q Q R K K M E K E A Q R L H K	203
901	AGGAACAAAACAAAATGCTGTGAACCTTCATAACACATGTTCAAATTTTATCATGCCA	960
203	K E Q N K I G V K L S *	214
961	GTAGGAGAAATCTCAGCTCCACAACCCAAGCAACATTTGTATGGATTAAAGATATTTTA	1020
1021	AGAAGACATACTGCTTGAATTTAATACATTTGATCAGGCCATCCAGGACACCAGATTCTC	1080
1081	CCAAAGTACCTTGAACCTTCTAGTATTGAGACTCAAAAACAAAAGACTTGAGACAA	1140
1141	TGTTTTCTCAACATGCTCCAAATAAAGACATTTGTTGCTGTACAGAAAGTATCACAA	1200
1201	ATGGAATATATCAGTACCTCTCAAGCTAGTGTCTTAGCTAAATAAATGGGTGTATATAA	1260
1261	TTTTATGGTGAAGAAGAACTGTACTGTCTGTATGATTTCTCTCAAGTGCATTAATGATA	1320
1321	AAATAAATAAATTTAATATCTTTTGTTCATCGTTTACCTGACCTAAATAGATAAAT	1380
1381	GTAGGCTTTAGCTTTCTTATTTTGTCAAAAGTTGGTGTGACATACATCTCCCTAAT	1440
1441	TTGAACCTGATTTGTTTACGTTTGATCAACATTAAGGAATTTGATGATTTTCTATTCTAT	1500
1501	GAAAATGACATTAATGCATAATTTTACTTATC	

Fig. 1 Nucleotide sequence of human *EBAG9* cDNA. Bold, the novel sequence of the 3'-UTR determined in the present study; underlined, the polyadenylation site.

roughly equalize signal intensities of both target and control markers. Cycle conditions were 94°C for 4 min, then 25 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 1 min, with a final extension step of 5 min at 72°C, in a GeneAmp PCR 9600 System (Perkin-Elmer Corp. Instruments, Norwalk, CT). PCR products were electrophoresed in 0.3 mm-thick denaturing 6% polyacrylamide gels containing 36% formamide and 8 M urea, at 2000 V for 2-4 h. Gels were transferred to filter papers, dried at 80°C, and exposed to autoradiographic film at room temperature for 16-20 h.

Determination of Allelic Dosage. Methods of measuring allelic copy number gain using polymorphic microsatellite markers had been established previously (12, 13). In brief, signal intensities of the control marker and each polymorphic allele of the microsatellites were quantified by a Hoefer GS-300 scanning densitometer; peak areas corresponding to each signal were calculated by electronic integration using the GS-370 electrophoresis data system (Hoefer Scientific Instruments, San Francisco, CA). An analysis of allelic dosage consisted of comparing signal intensities of four bands, two derived from the

Fig. 2 Schematic structure of the human *EBAG9* gene. Shading within the exons, coding sequence. A single BAC clone (above the map) covers the entire gene.

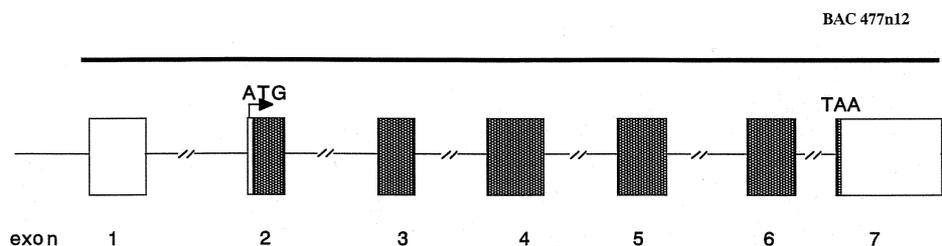


Table 2 Exon/intron organization of the human *EBAG9* gene

The sequences of exon/intron boundaries were determined as described in "Materials and Methods." The lowercase letters refer to intron sequences and the uppercase letters refer to exon sequences.

Exon number	Exon size (bp)	Sequence at exon/intron junction	
		3' splicing acceptor	5' splice donor
1	278		TGTGCAGTGGgtatgatttt
2	98	tcttccacagTTTTGATTC	TAATATGCAGgtaaataagt
3	79	tttgtttcagATCTGGCAGA	TCCTAAGCAGgtaggttttt
4	159	ttaaatttagACAGATGTTG	AACTCAGAAAgtagttaaag
5	108	aatctttcagATTGTTATTA	TCATCAGTCTgtaagtatgt
6	92	ttcattgtagTCTGAATTAG	AAGTCTGAGgtatattgagt
7	720	tcttttcagACAGCAGAAA	TTTACTTATCataaacattt

polymorphic alleles on 8q in normal DNA and the other two derived from the same pair of alleles in tumor DNA. They were normalized to the signal intensity of the control marker to adjust for subtle differences in PCR amplification efficiency between normal and tumor samples. The extent of increase of an allele that had gained signal was calculated by dividing the ratio of the intensity of the increased allele:the intensity of the normal allele in tumor DNA by the corresponding ratio measured in normal DNA; when the results exceeded 1.8, the allele was considered to be overrepresented.

Clinicopathological Parameters. The following parameters were examined: histological type, tumor size, lymph-node metastasis (status), and status of ER and PgR in each of the 129 breast cancer patients. The tumors, all of them ductal carcinomas, were classified by pathologists according to the histological WHO classification and the histological typing scheme of the Japanese Breast Cancer Society (27). Tumor size and lymph-node metastasis (status) were classified by pathologists according to UICC TNM classification. ER and PgR were measured by radioreceptor assay according to a standard dextran-coated charcoal (DCC) method, using [¹²⁵I]-labeled estradiol as labeled ligand on homogenates of fresh-frozen tissue (Otsuka Pharmaceutical, Tokushima, Japan). All samples containing >5 fmol of ER or PgR per mg protein were considered receptor positive. The χ^2 test and Fisher's exact test were used for statistical analysis. One-tailed *P*s of <0.05 were considered statistically significant.

RESULTS

Cloning and Structural Analysis of the *EBAG9* Gene.

By carrying out 3'-RACE experiments, we determined the complete sequence of the 3'-UTR of the human *EBAG9* cDNA (Fig. 1). The full-length cDNA for human *EBAG9* consists of a

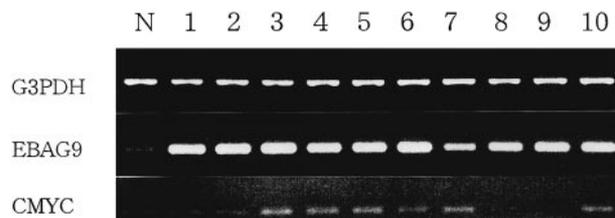


Fig. 3 *EBAG9* gene and *CMYC* gene expression in the cell lines by RT-PCR. *N*, normal breast; *Lanes 1-10*, breast cancer cell lines (*Lane 1*, MDA-MB-453; *Lane 2*, CRL1500; *Lane 3*, YMB-1-E; *Lane 4*, MCF7; *Lane 5*, HBL100; *Lane 6*, OCUB-M; *Lane 7*, BT-20; *Lane 8*, BT-474; *Lane 9*, MDA-MB-435S; *Lane 10*, SK-BR-3).

293-bp 5'-UTR, 642 bp of coding elements, and 599 bp of 3'-UTR.

After obtaining the genomic clone containing the *EBAG9* gene, BAC477n12, we found the entire gene to consist of seven exons interrupted by six introns (Fig. 2). The boundary sequences are shown in Table 2. Exon 3 was the smallest at 79 bp; other exons ranged from 92 to 720 bp. Sequences at all exon-intron boundaries were compatible with the consensus sequence for splicing junctions (28). Comparison of genomic with cDNA sequence revealed that exon 1 and the 5' portion of exon 2 encode the 5'-UTR. The translated region is encoded by the 5' portion of exon 2 through exon 7, and the remainder of exon 7 encodes the 3'-UTR. Exon 7 possesses a polyadenylation site preceded by a polyadenylation signal.

Analysis of *EBAG9* and *CMYC* Expression and SSCP.

Results of comparative *EBAG9* and *CMYC* expression analysis measured by RT-PCR experiments showed that *EBAG9* gene was overexpressed in all 10 of the breast cancer cell lines,

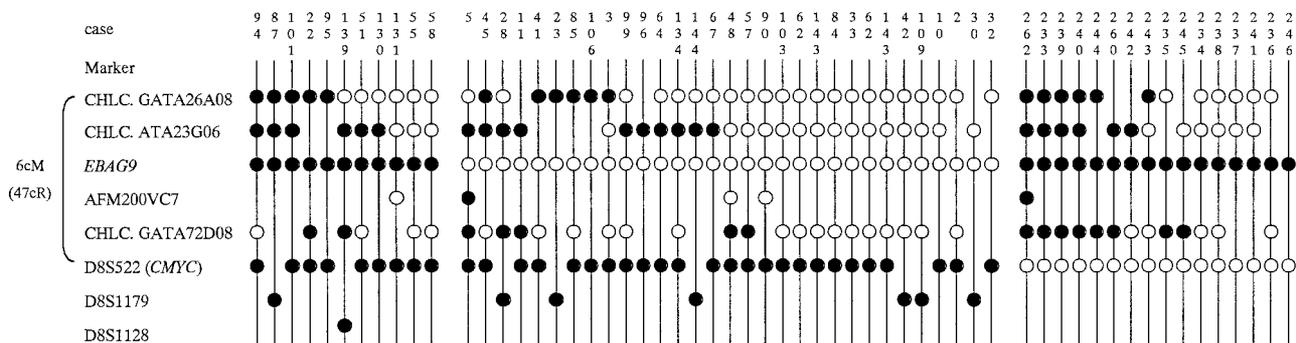


Fig. 4 Physical mapping in 58 breast cancers that showed gains of DNA at some loci on chromosome 8q23–24. Locations and order of the microsatellite markers were derived from published linkage information. At the top of each column, tumor-identification numbers. O, retention of heterozygosity; ●, increase; gaps reflect uninformative for specific markers.

whereas *CMYC* gene was overexpressed in only a fraction of breast cancer cell lines (Fig. 3). A correlation between gene expression and gene copy number could not be determined because normal counterpart cells necessary for this comparison were unavailable among these cell lines. To screen cancer cells for sequence variations in the *EBAG9* gene, we amplified by PCR its entire coding region in DNA from each of the 94 cell lines and analyzed the products for SSCP. Products with potential variations were sequenced directly by automated instruments. No somatic mutations or polymorphisms were observed in any of those tumor cells.

Physical Mapping around the *EBAG9* Gene. The signal intensity of at least one marker on chromosome 8q was increased 2- to 3-fold in 58 (45%) of the 129 breast tumors examined in this study. The frequencies of allelic gain observed at each of the eight tested markers described above are shown, in descending order from proximal to distal (Fig. 4). Allelic gains involving both *CMYC* and *EBAG9*, indicating polysomy, were observed in 11 tumors; the other 47 showed gain of only part of the examined portion of 8q. LOH was rare at any locus on 8q.

Representative autoradiograms are presented in Fig. 5. In cases 106, 94, and 48, for example, marker CHLC. GATA26A08, *EBAG9*, and CHLC. GATA72D08 revealed increased intensity of alleles in tumor DNA compared with its counterpart in normal DNA. Densitometric quantification showed that the intensities of the alleles in tumor DNA were increased about 2- to 3-fold compared with normal DNA, although the intensities of the other alleles and the single-copy control marker in tumor DNA were almost equal to those of normal DNA.

Of the 58 breast cancers showing a gain in copy number of loci on 8q, we observed an increase of the *EBAG9* locus in 27 (20.9%) informative tumors. Copy number gain at the *CMYC* locus (D8S522) was observed in 42 (32.6%) of the 129 tumors. Fig. 4 displays a physical map of the distal 8q region for the 58 tumors having partial allelic gain of the region. Among them, 16 (28%) showed gain of at the *EBAG9* locus but not *CMYC* (Fig. 4, left panel). Eleven (19%) of these 58 tumors showed allelic gain at both *EBAG9* and *CMYC* loci (Fig. 4, middle panel). Increased copy number of *CMYC* but not *EBAG9* was observed in 31 tumors (Fig. 4, right panel). These data clearly demon-

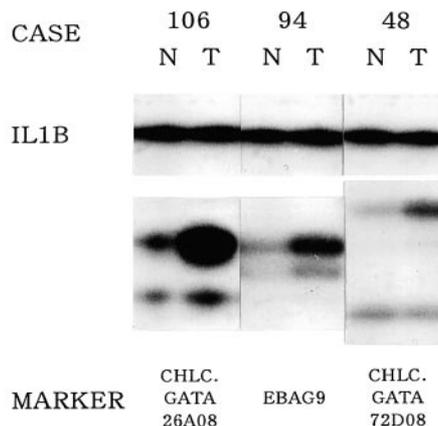


Fig. 5 Representative autoradiograms revealing allelic gain on chromosome 8q23 in primary breast carcinomas. *N* and *T*, DNA samples isolated from normal and tumor tissues respectively. On top of the panel, case number. On the bottom of each autoradiogram, polymorphic markers. IL-1β (*IL1B*) is control marker.

strate that *EBAG9* and *CMYC* are independent targets of gene amplification in breast cancers that contain chromosomal gains on the 8q arm.

Correlation of the physical mapping data with clinicopathological parameters for those tumors is summarized in Table 3. All of the tumors with increase of *EBAG9* copy number but without gain of *CMYC* were T1- or T2-stage tumors (<5 cm in diameter). No tumors with this combined genotype were advanced T3-stage tumors, whereas one-third of the tumors in which *CMYC* was overrepresented but *EBAG9* was not, were advanced (T4-stage) tumors ($P = 0.0204$; Table 4, Initial panel). No other clinicopathological parameter showed significant association with changes on chromosome 8.

Subsequently, we took independent series of additional 144 primary breast cancers and analyzed for allelic gain at *EBAG9* and *CMYC* loci with respective polymorphic markers to confirm our initial finding of independent increase of *EBAG9* as well as of *CMYC*. Of the 31 tumors with *EBAG9* increase, 15 showed gain at *EBAG9* but not at *CMYC*. The remaining 16 showed gain of *EBAG9* as well as of *CMYC*. Of the 48 tumors with *CMYC*

Table 3 Correlation between allelic gain at *EBAG9* region and *CMYC* and clinicopathological parameters in primary breast cancers

	<i>EBAG9</i> increase (+)	<i>CMYC</i> increase (+)
	<i>CMYC</i> increase (-)	<i>EBAG9</i> increase (-)
	16	31
Histological type		
WHO classification		
Invasive ductal carcinoma	13	26
Apocrine carcinoma	1	0
Invasive lobular carcinoma	0	2
ND ^a	2	3
Classification of the Japanese Breast Cancer Society		
Invasive ductal carcinoma		
Papillotubular carcinoma	3	4
Solid-tubular carcinoma	4	12
Scirrhus carcinoma	6	10
Special type		
Apocrine carcinoma	1	0
Invasive lobular carcinoma	0	2
ND	2	3
Tumor size, UICC TNM classification		
T1	2	1
T2	12	19
T3	0	9
ND	2	2
Lymph node, UICC TNM classification		
pN0	8	8
pN1	7	21
pN2	0	0
ND	1	2
ER		
Positive	8	12
Negative	6	11
ND	2	8
PgR		
Positive	9	15
Negative	5	9
ND	2	7

^a ND, not determined.

gain, 32 showed gain at *CMYC* but not at *EBAG9*. When a total of 273 breast cancers from initial and second panels were analyzed together for clinicopathological correlation, almost all (28 of 29) of the tumors with *EBAG9* gain but not *CMYC* were T1/T2 stage carcinomas (<5 cm in diameter), whereas one-third (21 of 61) of the tumors in which *CMYC* was increased but *EBAG9* was not, were advanced T3-stage tumors ($P = 0.0012$; Table 4, *Total panel*).

DISCUSSION

We have determined the complete cDNA sequence and the genomic structure of the human *EBAG9* gene. Although no inactivating intragenic alterations were detected among 94 cancers examined by SSCP, we identified frequent gains of DNA in the genomic region around the *EBAG9* gene (8q23) in primary breast cancers, as an event independent of *CMYC* amplification on the same chromosome arm.

Table 4 Allelic gain at *EBAG9* region and *CMYC*, and tumor stage in initial and secondary cancer panels of breast cancers

Gain	T1 + T2	T3	<i>P</i>	Total no. examined
Initial panel				
<i>EBAG9</i> (+)/ <i>CMYC</i> (-)	14/14	0/14	0.02	129
<i>EBAG9</i> (-)/ <i>CMYC</i> (+)	20/29	9/29		
Second panel				
<i>EBAG9</i> (+)/ <i>CMYC</i> (-)	15/15	0/15	0.0048	144
<i>EBAG9</i> (-)/ <i>CMYC</i> (+)	20/32	12/32		
Total panel				
<i>EBAG9</i> (+)/ <i>CMYC</i> (-)	29/29	0/29	0.0001	273
<i>EBAG9</i> (-)/ <i>CMYC</i> (+)	40/61	21/61		

Amplification of 8q, particularly involving the *CMYC* gene and its surrounding region at 8q24.1, has often been described in breast, lung, colon and gastric cancers, acute myeloid leukemias, and brain tumors (17, 29–33). In an earlier study, we also had detected frequent gains of chromosome region 8q24.1, centered by the *CMYC* gene, in primary breast carcinomas (13).

In the more focused study reported here, we demonstrated that increased copy number of the *EBAG9* gene at 8q23, which lies several centimorgans proximal to the *CMYC* gene, is an independent genetic event associated with primary breast cancers. We detected an increase in copy number of the *EBAG9* gene in 27 (21%) of the 129 primary breast cancers examined; 16 of those events involved the *EBAG9* gene and not *CMYC*. Our results clearly indicate that at least two target regions for amplification on distal 8q are somatically associated with the development of primary breast cancers.

We studied a total of 273 breast cancers from two series and analyzed for clinicopathological correlation. Almost all of the tumors with *EBAG9* increase but not *CMYC* (28 of 29) were T1/T2 stage carcinomas (<5 cm in diameter), whereas one-third (21 of 61) of the tumors in which *CMYC* was increased but *EBAG9* was not, were advanced T3-stage tumors ($P = 0.0012$). These data suggest that the *EBAG9* and *CMYC* genes are independent targets of DNA amplification, and that the gain of *EBAG9* may play a specific role in early stages of breast carcinogenesis.

In the present study, we observed consistent expression of *EBAG9* mRNA in cancer cell lines. Human breast cancer is a hormone-dependent tumor the growth of which is regulated by estrogen. In this connection, we note that human *EBAG9* was originally identified as an estrogen-responsive locus, that *EBAG9* mRNA was shown *in vitro* to be up-regulated by estrogen treatment in breast cancer cells, and that the 5' regulatory region of the *EBAG9* gene contains an identifiable estrogen-responsive element. No statistical association was observed between *EBAG9* copy number increase and responsiveness to ER and/or PgR, represented by ER/PgR positivity. Because *EBAG9* is a downstream effector of ER, the results appear to imply the presence of at least two distinct pathways for hormonal outgrowth of breast cancer cells: (a) activation of ER and up-regulated transcription of *EBAG9* gene without its genetic alteration; and (b) *EBAG9* copy number gain accompanied by its overexpression independent of ER signaling, in addition to other various hypothetical mechanisms. Furthermore, with respect to

immune escape mechanisms, it is relevant that the *EBAG9* gene product is identical to *RCAS1*, a cancer-surface antigen identified in a human uterine adenocarcinoma cell line and implicated in immune escape. Moreover, overexpression of *RCAS1* inhibits growth of tumor-stimulated host-immune cells and induces their apoptosis (16). These lines of evidence, together with the results obtained in the present study, imply that increased gene copy number and consequent overexpression of *EBAG9* may play an etiological role in the development and progression of breast cancers, especially in early stages.

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Overrepresentation of the *EBAG9* Gene at 8q23 Associated with Early-Stage Breast Cancers

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