LOSS OF HETEROZYGOSITY ON CHROMOSOME 9 IN HUMAN BREAST CANCER: ASSOCIATION WITH CLINICAL VARIABLES AND GENETIC CHANGES AT OTHER CHROMOSOME REGIONS

Gudny EIRIKSDOTTIR¹, Asgeir SIGURDSSON¹, Jon Gunnlaugur JONASSON¹, Bjarni A. AGNARSSON¹, Helgi SIGURDSSON², Julius GUDMUNDSSON¹, Jon Thor BERGTHORSSON¹, Rosa Björk BARKARDOTTIR¹, Valgardur EGILSSON¹ and Sigurdur INGVARSSON^{1,3}

¹Department of Pathology and ²Department of Oncology, University and National Hospital of Iceland, IS 121 Reykjavik, Iceland.

Primary breast tumors were tested for loss of heterozygosity (LOH), on chromosome 9p with microsatellite markers restricted to a 28 cM region including the MTS1 gene. LOH was found with at least 1 marker in 38% of the 201 cases analyzed. A high frequency of deletions was detected at the 9p23-p21 region, indicating a tumor suppressor gene(s) important for breast cancer tumorigenesis. Tumors with and without LOH on 9p were compared with respect to clinico-pathological factors using χ^2 analysis. Tumors with 9p LOH were significantly associated with high S-phase status and aneuploidy, but not with type, node status, estrogen and progesterone receptor content or age of the patients at diagnosis. Survival analysis showed that LOH at 9p did not significantly affect the survival rate of breast cancer patients. Our results indicate that the aberrations on 9p detected in this study are not of independent prognostic value. A significant association was found between LOH at 9p and LOH at chromosomal arms 3p and 6q, which is an additional contribution toward understanding the genetic events in breast tumor pathogenesis.

© 1995 Wiley-Liss, Inc.

The accumulation of genetic changes in somatic cells can disrupt the control of cell division and lead to cancer. The inactivation of several tumor suppressor genes is a molecular mechanism that can contribute to tumorigenesis. Studies designed to detect loss of heterozygosity (LOH) have shown that many regions in the genome are affected and consequently may harbor tumor suppressor genes that are implicated in the progression of invasive carcinomas in various tissues.

Abnormalities of chromosome 9p21 have been observed in human malignant cell lines, and the most frequently deleted region included the p16 inhibitor (Nobori *et al.*, 1994) of cyclin-dependent kinase 4/cyclin D catalytic activity in the cell cycle (Serrano *et al.*, 1993). Mutations in the MTS1 gene that encodes for the p16 inhibitor were detected in 9p21-linked families with predisposition to melanoma (Hussussian *et al.*, 1994). Deletion studies have shown that the short arm of chromosome 9 is frequently lost in primary tumors in various tissues, *i.e.*, brain (Jen *et al.*, 1994), bladder (Devlin *et al.*, 1994), renal cell (Cairns *et al.*, 1995), head and neck (van der Riet *et al.*, 1994), nasopharynx (Huang *et al.*, 1994), pancreas (Caldas *et al.*, 1994), lung (Merlo *et al.*, 1994), hematopoietic cells (Ogawa *et al.*, 1994), mesothelial cells (Cheng *et al.*, 1994) and esophagus (Aoki *et al.*, 1994). These studies provide evidence that the 9p21 region could harbor a tumor suppressor gene(s) involved in many cancer types.

In this study, we analyzed sporadic breast tumors for deletions on chromosome 9p using a panel of highly informative microsatellite markers. We tested the results for association with clinico-pathological factors in an attempt to evaluate the prognostic value of genetic changes on the short arm of chromosome 9. The results were also compared with aberrations at other chromosomal regions to evaluate the cooperative effect of other putative tumor suppressor genes in the multi-step pathogenesis of breast cancer.

MATERIAL AND METHODS

Patients and tumor material

Fresh biopsies from primary breast tumors from patients diagnosed with breast cancer in Iceland are routinely sent to our laboratory for estrogen and progesterone receptor (ER and PgR) analysis. Blood samples from the patients are collected in EDTA and if not processed immediately the tumors and blood are quick frozen at -70°C . Two hundred and one breast normal/tumor pairs were analyzed.

DNA extraction and analysis

Genomic DNA was extracted from whole blood (normal DNA) and from tumor tissue using standard procedures. The nuclear pellet remaining after cytosol removal for the hormone receptor analysis was also used for tumor DNA extraction. Paired blood and tumor DNA was subjected to PCR analysis using DynaZyme polymerase (Finnzymes Oy, Espoo, Finland) in the buffer solution provided by the manufacturer. The markers used (*D9S168*, *D9S156*, *D9S157*, *D9S162*, *IFNA*, *D9S171*, *D9S126* and *D9S104*) were obtained from the Nordic primer resource in Uppsala (C. Wadelius, Department of Clinical Genetics, University Hospital, Uppsala, Sweden) and Research Genetics (Huntsville, AL, USA) (Table I). The markers were elongated using terminal-transferase in 40 mM K-HEPES/1 mM CoCl_2 buffer, pH 7.2, overnight at 37°C . The PCR products were subjected to denaturing gel electrophoresis, transferred to Hybond- N^+ membranes (Amersham, Aylesbury, UK) and hybridized for at least 3 hr at 42°C with the elongated markers covalently labelled with peroxidase (ECL kit Amersham). The membranes are washed once in $3\times$ SSC/0.1% SDS at $37-42^{\circ}\text{C}$, then twice in $0.2\times$ SSC, bathed in a detection reagent containing H_2O_2 , luminol and an enhancer (ECL kit, Amersham) for 1 min and finally detected by chemi-luminescence on DUPONT Cronex-4 film (Wilmington, DE). Autoradiograms were inspected visually, comparing the intensity of alleles from normal and tumor DNA. Any absence or significant decrease of one allele relative to the other was considered LOH.

Flow cytometry

A representative paraffin block containing adequate amount of tumor cells was selected from each tumor. The DNA content of tumor cells was analyzed using a single cell suspension prepared according to a modified method described by Jonasson and Hrafnkelsson (1994). Nuclear DNA was measured using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). A total of 20,000 cells was analyzed for each tumor. The Cell Fit system (Becton Dickinson) was used for S-phase analyses and calculated with a

³To whom correspondence and reprint requests should be sent at the Department of Pathology, University and National Hospital of Iceland, P.O. Box 1465, IS 121 Reykjavik, Iceland.

TABLE I - CHARACTERISTICS OF MARKERS USED¹

Marker	Distance (cM)	Cytological location	Number of samples	Number of heterozygotes (%)	Number of LOH tumors (%)
D9S168	10	9p23-p22	145	120 (83)	17 (14)
D9S156	1	9p23-p22	154	126 (82)	30 (24)
D9S157	1	9p23-p22	157	133 (85)	30 (23)
D9S162	6	9p23-p22	138	90 (65)	10 (11)
IFNA	2	9p22-p21	139	89 (64)	21 (24)
D9S171	2	9p21	175	139 (79)	28 (20)
D9S126	2	9p21	138	80 (58)	15 (19)
D9S104	8	9p21	151	117 (77)	20 (17)

¹Cytological information and distances between markers were obtained from the Genome Data Base (Baltimore, MD). The Table shows the number of tumors tested at each marker, the number of informative tumors (heterozygotes) and the number of tumors where LOH was detected.

planimetric method. The tumor ploidy was defined as diploid if only one stem cell population was detected on the histogram and as aneuploid if two or more stem cell populations were detected.

Statistical analysis

Prognostic factors. A χ^2 test was used to assess the relationship between 9p LOH and other prognostic variables. Prognostic factors were categorized using commonly applied cut-off levels (Table II). Survival curves were calculated according to the method of Kaplan and Meier. Tests of difference between curves were made with the log-rank test for censored survival data. The Statistica/Mac package (Statsoft, Tulsa, OK) was used for the statistical analyses.

Chromosomal regions. The same normal/tumor pairs have been screened in our laboratory for LOH using markers at other chromosomal regions. The results from the studies on chromosome 3p, 6q, 11 and 16q have been published (Eiriksdottir *et al.*, 1995; Bragadottir *et al.*, 1995; Gudmundsson *et al.*, 1995; Skirnisdottir *et al.*, 1995). The markers used in the deletion studies on chromosome 17 were: 17p—*TP53*; 17q—*THRA*, *D17S800*, *D17S855*, *D17S579* (all from the *BRCA1* region). A χ^2 analysis was used to assess the relationship between 9p LOH and genetic changes at the other loci.

RESULTS

The breast tumors were analyzed for LOH using 8 markers mapping to chromosome 9p. A representative autoradiogram is shown in Figure 1. LOH was observed in 77 of the 201 (38%) informative tumors analyzed. Eighteen (23%) tumors with deletions showed LOH at all markers tested, indicating total loss of the short arm of chromosome 9. A homozygous deletion was detected at the *IFNA* marker in 1 of the tumors, which otherwise showed an apparent retention of this single locus within a large region of deletion. The highest frequency of deletions at individual loci was observed with the markers *D9S156* and *IFNA* (24% of informative samples for both markers). Figure 2 shows the deletion pattern in 24 tumors where partial or interstitial deletions were observed. In an attempt to map further the smallest region of overlapping deletions, we observed 2 regions on 9p to be more frequently deleted than others depicted by groups A and C. Both regions are deleted in tumors in group B. In group B the region at marker *D9S162* is retained. A number of tumors were tested for homozygous deletions. Homozygous deletions at marker *D9S162* were not detectable in tumors 1206, 1290, 570 and 795 (autoradiograms not shown).

The results of the χ^2 analysis comparing LOH on 9p with clinico-pathological factors are shown in Table II. A significant association was found between LOH and high S-phase frac-

FIGURE 1 - Dinucleotide repeat polymorphisms for microsatellite markers *D9S156* (a) and *IFNA* (b) in selected matched normal (N) and tumor (T) tissue from breast cancer patients. Case numbers are shown at the top and allele sizes (in nucleotides) are indicated to the right. Cases 1225 and 1210 are homozygotes, LOH is seen in cases 1216, 1220, 1206 and 1208. Retention of alleles is observed in cases 1215, 1218 and 1207.

tion, aneuploidy and tumor size. A significant correlation was found between individual markers in both regions on 9p of high deletion frequency and high S-phase fraction, although only between individual markers in the more centromeric deletion region and aneuploidy. Survival analysis with a median of 3 years follow-up showed that LOH on 9p in tumors did not significantly affect the survival rate of the breast cancer patients ($p = 0.236$).

The comparison of LOH between chromosomal regions is shown in Table III. A significant association was found

TABLE III - CHI-SQUARE ANALYSIS FOR SIGNIFICANCE BETWEEN LOH AT 9p AND LOH AT 7 OTHER CHROMOSOMAL REGIONS¹

Chromosome region	Number of patients			<i>p</i>	
	Sample size	LOH	9pLOH and region		
3p	110	36	45	21	.010 ²
6q	141	49	55	30	.000 ³
11p	52	9	23	5	.452
11q	65	20	29	11	.262
16q	147	100	58	42	.357
17p	58	20	25	12	.059
17q	77	21	33	11	.301

between LOH on 9p and LOH on 3p and 6q. However, no association was found between LOH on 9p and LOH at chromosomal regions 11p, 11q, 16q, 17p and 17q (the *BRCAl* region).

DISCUSSION

Mutation studies of the *MTS1/CDK4I* gene support the existence of a susceptibility gene at the 9p21 region for esophageal squamous cell carcinoma (Mori *et al.*, 1994), pancreatic adenocarcinoma (Caldas *et al.*, 1994) and melanoma (Hussussian *et al.*, 1994). However, Kamb *et al.* (1994)

¹95% confidence interval (CI), -299% CI.

reported a low frequency of *MTS1* mutations in melanoma-prone kindreds, but no mutations of the gene were observed in breast tumors (Xu *et al.*, 1994). Another gene involved in cell cycle control, also located at the 9p21 region, *p15*, is deleted in brain tumors (Jen *et al.*, 1994). This putative tumor suppressor gene remains to be analyzed with respect to mutations in breast cancer development. The deletion frequency determined in this study, *i.e.*, 38% with at least one marker and 24% at the 9p22-p21 susceptibility locus which includes the *p16* gene, is considerably less than the deletion frequencies observed in other cancer types, *e.g.*, 72% in head and neck, 68% in non-small lung cell cancer and 61% in nasopharyngeal cancer. These results are, however, comparable with the deletion frequencies determined for the same patient series at several other chromosomal regions with the exception of higher frequency of deletions detected at 16q (Skirnisdottir *et al.*, 1995). The low frequency of deletions at many chromosomal regions in the breast tumors suggests that breast cancer development is more heterogeneous with respect to genetic changes than many other carcinomas.

LOH at 9p was compared with various prognostic variables. No association was found with conventional prognostic variables, such as positive node status, age of the patients or low ER or PgR content (Table II). 9p LOH was, however, associated with DNA aneuploidy, suggesting an association with an aberrant and unstable genome. Association of 9p LOH with high S-phase fraction suggests that genes on 9p can affect the rate of cell proliferation.

Our results show a significant association between 9p LOH and aberrations at 3p and 6q. These combinations of LOH events in tumor cells could result in a growth selection in cancer pathogenesis, and preferred combinations of tumor suppressor gene deletions might enhance tumor growth. Recent work in our laboratory has shown that LOH at 3p (Eiriksdottir *et al.*, 1995) and 6q (Bragadottir *et al.*, 1995) are independent prognostic variables for breast cancer patients. Patients with tumors including allelic imbalance at 3p are at a 4-fold relative risk of death and 2-fold risk if tumors have aberrations at 6q. Survival of patients with tumors altered at 9p did not differ significantly from the survival of patients in whom no 9p changes were found. We conclude that the genetic alterations at 9p studied here have no independent prognostic value for breast cancer patients. Multivariate analysis and survival curves indicate that studying tumors for LOH at 9p could provide additional prognostic information for patients in whom 3p and 6q aberrations have been detected and are included in the model. However, treatment effects may be influencing the analysis and have yet to be considered before

patients with tumors containing these multiple genetic alterations can be classified as a new high-risk group.

The χ^2 analysis showed no association between 9p LOH and aberrations on chromosomes 11, 16q and 17. These results suggest that deletions at some regions of the genome are independent events and reflect a multi-factorial tumor progression in these breast cancer cases.

The highest deletion frequencies in the sporadic breast tumors in this study were observed with markers *D9S156* (at 9p23-p22) and *IFNA* (at 9p22-p21). Homozygous LOH at *IFNA* and *D9S162* are detectable in breast tumors, as has been reported in various other tumor types using comparative competition experiments, *i.e.*, multiplex PCR amplification (Huang *et al.*, 1994; Devlin *et al.*, 1994; Merlo *et al.*, 1994; Caldas *et al.*, 1994; Ogawa *et al.*, 1994; Cairns *et al.*, 1995). However, homozygous deletions at *D9S162* were not detectable in a few tumors. The apparent retention of that single locus (*D9S162*) within a large region of deletion suggested interstitial deletions for breast tumors.

Our results show 2 deletion peaks on 9p in breast tumors. The distance between the markers showing these peaks is small, so further studies are necessary to confirm the separate existence of 2 regions of deletion. The results suggest that loss of these regions is important in breast cancer development, as has been shown in other tumor types, but that the deletions are of limited prognostic value for breast cancer patients. However, the fact that alterations at chromosome 9p are associated with aberrations at chromosomes 3p, 6q and factors that are indicators of poor prognosis supports the multi-step concept of cancer genesis.

ACKNOWLEDGEMENTS

The authors thank Dr. J. Hallgrímsson and his staff for continuous support at the Department of Pathology. We also thank Ms. S. Björlling for the hormone receptor analyses, Ms. S. Kristjansdottir and Ms. S. Jonasdottir for flow cytometry, Ms. S. Skirnisdottir, Ms. G. Bragadottir and Mr. T. Baldursson for results from their deletion studies in the breast tumors and Dr. Ö. Olafsson for advice regarding the statistical analysis. We are grateful to Mr. A. Arason for the development of the ECL method of allele detection. This work was financially supported by the Nordic Cancer Union, Icelandic Cancer Society, The University of Iceland Research Fund, the Memorial Fund of Bergthóra Magnúsdottir and Jakob B. Bjarnason, the Science Fund of Iceland and the Science Fund of the University Hospital of Iceland. The Nordic primer resource in Uppsala, Sweden, is supported by the Nordic Council of Ministers.

REFERENCES

- AOKI, T., MORI, T., DU, X., NISHIHARA, T., MATSUBARA, T. and NAKAMURA, Y., Allelotype study of esophageal carcinoma. *Genes Chromosomes Cancer*, **10**, 177-182 (1994).
- BRAGADOTTIR, G., EIRIKSDOTTIR, G., SIGURDSSON, A., BARKARDOTTIR, R.B., GUDMUNDSSON, J., JONASSON, J.G. and INGVARSSON, S., Loss of heterozygosity at chromosome 6q correlates with tumor progression and patient survival. *Int. J. Oncol.*, **7**, 871-876 (1995).
- BRENNER, A.J. and ALDAZ, C.M., Chromosome 9p allelic loss and *p16/CDKN2* in breast cancer and evidence of *p16* inactivation in immortal breast epithelial cells. *Cancer Res.*, **55**, 2892-2895 (1995).
- CAIRNS, P., TOKINO, K., EBY, Y. and SIDRANSKY, D., Localization of tumor suppressor loci on chromosome 9 in primary human renal cell carcinomas. *Cancer Res.*, **55**, 224-227 (1995).
- CALDAS, C., HAHN, S.A., DA COSTA, L.T., REDSTON, M.S., SCHUTTE, M., SEYMOUR, A.B., WEINSTEIN, C.L., HRUBAN, R.H., YEO, C.J. and KERN, S.E., Frequent somatic mutations and homozygous deletions of the *p16 (MTS1)* gene in pancreatic adenocarcinoma. *Nature (Genet.)*, **8**, 27-32 (1994).
- CHENG, J.Q., JHANWAR, S.C., KLEIN, W.M., BELL, D.W., LEE, W.C., ALTOMARE, D.A., NOBORI, T., OLOPADE, O.I., BUCKLER, A.J. and TESTA, J.R., *p16* alterations and deletion mapping of 9p21-p22 in malignant mesothelioma. *Cancer Res.*, **54**, 5547-5551 (1994).
- DEVLIN, J., KEEN, A.J. and KNOWLES, M.A., Homozygous deletion mapping at 9p21 in bladder carcinoma defines a critical region within 2cM of *IFNA*. *Oncogene*, **9**, 2757-2760 (1994).
- EIRIKSDOTTIR, G., BERGTHORSSON, J.T., SIGURDSSON, H., GUDMUNDSSON, J., SKIRNISDOTTIR, S., EGILSSON, V., BARKARDOTTIR, R.B. and INGVARSSON, S., Mapping of chromosome 3 alterations in human breast cancer using microsatellite PCR markers: correlation with clinical variables. *Int. J. Oncol.*, **6**, 369-375 (1995).
- GUDMUNDSSON, J., BARKARDOTTIR, R.B., EIRIKSDOTTIR, G., BALDURSSON, T., ARASON, A., EGILSSON, V. and INGVARSSON, S., Loss of heterozygosity at chromosome 11 in breast cancer: association of prognostic factors with genetic alterations. *Brit. J. Cancer*, **72**, 696-701 (1995).
- HUANG, D.P., LO, K., VAN HASSELT, C.A., WOO, J.K.S., CHOI, P.H.K.,

- LEUNG, S., CHEUNG, S., CAIRNS, P., SIDRANSKY, D. and LEE, J.C.K., A region of homozygous deletion on chromosome 9p21-22 in primary nasopharyngeal carcinoma. *Cancer Res.*, **54**, 4003-4006 (1994).
- HUSSUSSIAN, C.J., STRUEWING, J.P., GOLDSTEIN, A.M., HIGGINS, P.A., ALLY, D.S., SHEAHAN, M.D., CLARK, W.H., JR., TUCKER, M.A. and DRACOPOLI, N.C., Germline p16 mutations in familial melanoma. *Nature (Genet.)*, **8**, 15-21 (1994).
- JEN, J., HARPER, J.W., BIGNER, S.H., BIGNER, D.D., PAPADOPOULOS, N., MARKOWITZ, S., WILLSON, J.K.V., KINZLER, K.W. and VOGELSTEIN, B., Deletion of p16 and p15 genes in brain tumors. *Cancer Res.*, **54**, 6353-6358 (1994).
- JONASSON, J.G. and HRAFNKELSSON, J., Nuclear DNA analysis and prognosis in carcinoma of the thyroid gland. *Virchows Archiv*, **425**, 349-355 (1994).
- KAMB, A. and 23 OTHERS, Analysis of the p16 gene (CDKN2) as a candidate for the chromosome 9p melanoma susceptibility locus. *Nature (Genet.)*, **8**, 22-26 (1994).
- MERLO, A., GABRIELSON, E., ASKIN, F. and SIDRANSKY, D., Frequent loss of chromosome 9 in human primary non-small cell lung cancer. *Cancer Res.*, **54**, 640-642 (1994).
- MORI, T., MIURA, K., AOKI, T., NISHIHARA, T., MORI, S. and NAKAMURA, Y., Frequent somatic mutation of the MTS1/CDK4I (multiple tumor suppressor/cyclin-dependent kinase 4 inhibitor) gene in esophageal squamous cell carcinoma. *Cancer Res.*, **54**, 3396-3397 (1994).
- NOBORI, T., MIURA, K., WU, D.J., LOIS, A., TAKABAYASHI, K. and CARSON, D.A., Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature (Lond.)*, **368**, 753-756 (1994).
- OGAWA, S., HIRANO, N., SATO, N., TAKAHASHI, T., HANGAISHI, A., TANAKA, K., KUROKAWA, M., TANAKA, T., MITANI, K., YAZAKI, Y. and HIRAI, H., Homozygous loss of the cyclin-dependent kinase 4-inhibitor (p16) gene in human leukemias. *Blood*, **84**, 2431-2435 (1994).
- SERRANO, M., HANNON, G.J. and BEACH, D., A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature (Lond.)*, **366**, 704-707 (1993).
- SKIRNISDOTTIR, S., EIRIKSDOTTIR, G., BALDURSSON, T., BARKARDOTTIR, R.B., EGILSSON, V. and INGVARSSON, S., High frequency of allelic imbalance at chromosome region 16q22-23 in human breast cancer: correlation with high PgR and low S-phase. *Int. J. Cancer*, **64**, 112-116 (1995).
- VAN DER RIET, P., NAWROZ, H., HRUBAN, R.H., CORIO, R., TOKINO, K., KOCH, W. and SIDRANSKY, D., Frequent loss of chromosome 9p21-22 early in head and neck cancer progression. *Cancer Res.*, **54**, 1156-1158 (1994).
- XU, L., SGROI, D., STERNER, C.J., BEAUCHAMP, R.L., PINNEY, D.M., KEEL, S., UEKI, K., RUTTER, J.L., BUCKLER, A.J., LOUIS, D.N., GUSELLA, J.F. and RAMESH, V., Mutational analysis of CDKN2 (MTS1/p16^{ink4}) in human breast carcinomas. *Cancer Res.*, **54**, 5262-5264 (1994).