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Analysis of the 10q23 chromosomal region and the *PTEN* gene in human sporadic breast carcinoma

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Summary We examined a panel of sporadic breast carcinomas for loss of heterozygosity (LOH) in a 10-cM interval on chromosome 10 known to encompass the *PTEN* gene. We detected allele loss in 27 of 70 breast tumour DNAs. Fifteen of these showed loss limited to a subregion of the area studied. The most commonly deleted region was flanked by *D10S215* and *D10S541* and encompasses the *PTEN* locus. We used a combination of denaturing gradient gel electrophoresis and single-strand conformation polymorphism analyses to investigate the presence of *PTEN* mutations in tumours with LOH in this region. We did not detect mutations of *PTEN* in any of these tumours. Our data show that, in sporadic breast carcinoma, loss of heterozygosity of the *PTEN* locus is frequent, but mutation of *PTEN* is not. These results are consistent with loss of another unidentified tumour suppressor in this region in sporadic breast carcinoma.

Keywords: breast carcinoma; chromosome 10q; Cowden disease; *PTEN*

Breast cancer affects one in nine women in their lifetime (Kelsey, 1979). In more than 90% of cases, the disease arises sporadically, with no previous associated family history. Several genes (e.g. *BRCA1*, *BRCA2*) have been shown to have a role in susceptibility to inherited breast cancer. Because of this association, such genes have been extensively investigated in sporadic breast tumours (reviewed in Smith and Ponder, 1993; Stratton, 1996); however, the genetic events occurring in the majority of these tumours still remain to be identified. Additional candidate genes for involvement in sporadic breast disease are those implicated in cancer syndromes associated with an increased risk of breast cancer. One such syndrome is Cowden disease (CD), or multiple hamartoma syndrome, an autosomal dominantly inherited cancer syndrome characterized by oral and cutaneous lesions and an increased risk of follicular thyroid tumours and breast carcinomas (Brownstein et al, 1978; Starink et al, 1986). The incidence of breast cancer among women with CD is estimated to be 30–50% (Starink et al, 1986).

The gene for CD was initially mapped by linkage analysis to 10q22–23, within a 5-cM interval flanked by *D10S215* and *D10S564* (Nelen et al, 1996). Loss of heterozygosity (LOH) of this interval was also observed in some tumours from CD patients (Marsh et al, 1997), suggesting the CD gene was a tumour suppressor. In addition to those tumours associated with CD, other human cancers have been shown to have losses of this region of chromosome 10 (Moschonas et al, 1996). A candidate tumour-suppressor gene for glioblastoma multiforme has been mapped to 10q24–qter (Karlbom et al, 1993; Pershouse et al, 1993; Rasheed et al, 1995; Albarosa et al, 1996), whereas a region in 10q23–q26 is frequently deleted in endometrial cancers (Simon et al, 1990; Peiffer et al, 1995). Common deleted regions have also been noted in renal cell carcinoma at 10q21–q22 (Morita et al, 1991), at 10q22–qter in malignant melanoma (Parmiter et al, 1988; Herbst et al, 1994), at 10q22.3–q23.1 in muscle-invasive bladder cancers (Kagan et al, 1998) and at 10q23–q24 in prostate cancer (Gray et al, 1996; Ittmann, 1996; Trybus et al, 1996; Feilotter et al, 1998).

Germline mutations of the *PTEN*/*MMAC1* tumour-suppressor gene (Li et al, 1997; Steck et al, 1997), which lies within the 10q22–23 critical interval, have been identified in the majority of CD families (Liaw et al, 1997; Lynch et al, 1997; Nelen et al, 1997; Tsou et al, 1997; Marsh et al, 1998*a*). However, mutations are rare or absent in breast cancer families without other typical CD stigmata (Tsou et al, 1997; Chen et al, 1998; FitzGerald et al, 1998). Somatic mutations of this gene have been found at varying frequencies in some of the tumour types described above. *PTEN* mutations are frequent in endometrial carcinoma (Kong et al, 1997; Risinger et al, 1997; Tashiro et al, 1997), malignant melanoma (Guldberg et al, 1997), high-grade gliomas (Rasheed et al, 1997; Wang et al, 1997; Bostrom et al, 1998) and metastatic prostate cancer (Cairns et al, 1997; Suzuki et al, 1998). However, mutations are rare in unselected prostate carcinoma (Feilotter et al, 1998), meningioma (Bostrom et al, 1998), low-grade gliomas (Rasheed et al, 1997) and unselected breast carcinoma (Rhei et al, 1997). Thus, it appears that the *PTEN* tumour suppressor may play a role in a subset of the advanced human cancers noted above, but not in all of these neoplasms. It is still not clear whether other tumour-suppressor genes which contribute to multiple tumour types lie close to *PTEN* on 10q23. In this study, we have investigated LOH in the 10q22–23 region in a series of primary breast carcinomas. We have further analysed those tumours with LOH in

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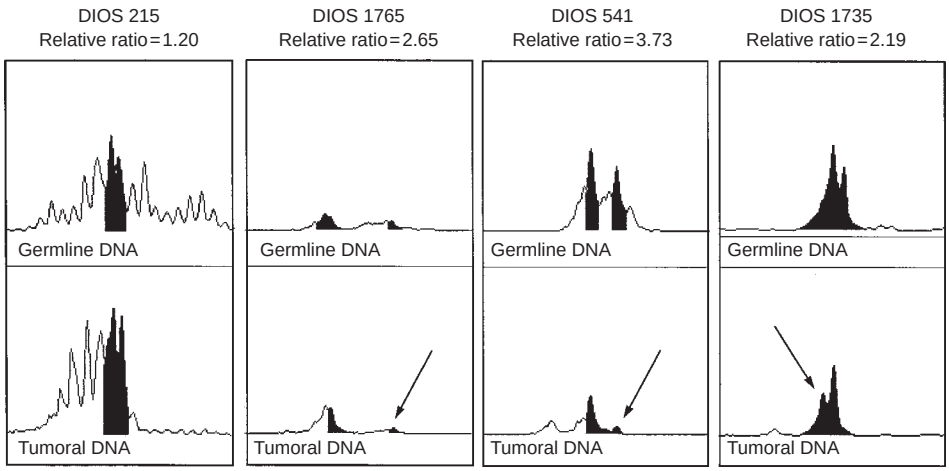

Figure 1 Loss of heterozygosity analysis in tumour 22 for four informative markers. For each marker, the alleles present in normal (top) and tumour (bottom) tissue are shown, with the ratio of normal/tumour allele peak heights given above. Ratios equal to or greater than 1.6 were scored as allele loss. A ratio of less than 1.5 was scored as no loss. Thus, the figure depicts allele loss at *D10S1765*, *D10S541* and *D10S1735* and no loss at *D10S215*. The arrows indicate the deleted alleles in the tumour samples

this critical region for mutations of the *PTEN* gene to clarify the role of *PTEN*, or closely linked tumour-suppressor genes, in this disease.

MATERIALS AND METHODS

Samples

We used a panel of 70 unselected sporadic primary breast cancers and matched normal tissue from two series for LOH on chromosome 10q, with particular attention to a 10-cM interval at 10q23 which spans the *PTEN* locus. Sixty-eight of the 70 samples were infiltrating ductal carcinomas, whereas the remaining two were infiltrating lobular carcinomas. The selected cases did not have a history of familial breast cancer or other cancer-related syndromes. Samples collected at the Institut Bergonié (cases 3–36) were isolated from fresh surgical material and snap-frozen in liquid nitrogen before DNA extraction. Peripheral blood leucocytes were obtained as a source of matched constitutional DNA. Genomic DNA purification from blood and snap-frozen tissue samples was according to published protocols (Dorion-Bonnet et al, 1995). DNA from samples collected at Kingston General Hospital (cases 37–72) was extracted from formalin-fixed paraffin-embedded archival material according to published protocols (Wright and Manos, 1990). Before each DNA extraction, tumour tissues were microdissected from contaminating normal cells using a haematoxylin phloxine saffron-stained section for reference. Control blood DNAs from a panel of 60 unrelated individuals were extracted using established protocols and have been previously described (Feilotter et al, 1998).

LOH analyses

Seven microsatellite markers, known to map to the 10q23 interval, were analysed. The order of these markers from centromere to telomere was *D10S579*, *D10S215*, *D10S1765*, *D10S541*, *D10S1735*, *D10S1739*, *D10S564* based on published maps (Gyapay et al, 1994; Chumakov et al, 1995; Moschonas et al, 1996; Nelen et al, 1996; Gray et al, 1997; Marsh et al, 1997) and data from the Center for Genome Research at the Whitehead Institute for Biomedical Science (www-genome.wi.mit.edu). Primer sequences for markers were obtained from the Genome Data Base. Polymerase chain reactions (PCR) were carried out according to published protocols using either fluorescently or radiolabelled primers. For fluorescent products, allele loss was scored when the ratio of peak heights of the tumour to the leucocyte alleles, as determined by Genescan 672 software (Applied Biosystems), exceeded 1.6 (Figure 1). This cut-off value represents a conservative scoring of allele loss, and is consistent with that used in other studies of LOH (Gray et al, 1996; Trybus et al, 1996; Marsh et al, 1997). Samples with a ratio of peak heights between 1.5 and 1.6 were considered ambiguous and were not scored. For radiolabelled products (samples 37–72), allele loss was scored by eye by two independent observers.

Single-strand conformation polymorphism (SSCP) analysis

SSCP analysis of *PTEN* was performed using previously described PCR conditions and intronic primers flanking each of the gene's nine exons (Feilotter et al, 1998). Aliquots of the labelled PCR products were electrophoresed on non-denaturing acrylamide/glycerol gels at 14°C for 16 h using a variety of polyacrylamide and glycerol concentrations, ranging from 6% to 8% acrylamide and from 0% to 10% glycerol. Gels were dried and exposed to film.

Denaturing gradient gel electrophoresis (DGGE) analysis

DGGE was performed according to the methods described by Stoppa-Lyonnet et al (1997). DGGE electrophoretic conditions (optimized using Meltmap and SQHTX programs) and primer sequences have been previously described (Laugé et al, 1998).

Sequencing analysis

Electrophoretic variants predicted by SSCP or DGGE were confirmed by direct sequencing. PCR products were amplified

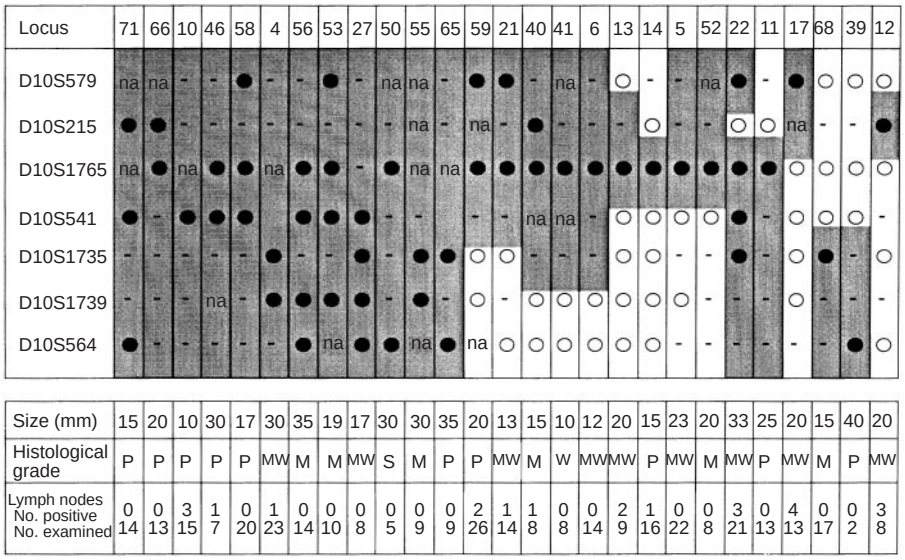

Figure 2 Regions of allele loss for the 27 tumours showing LOH. Open circles = no LOH; filled circles = LOH, – = non-informative; na = not available. The shading represents the maximum possible interval of loss for each tumour. Grade for each tumour is given as W, well differentiated; M, moderately differentiated; MW, moderately/well differentiated; P, poorly differentiated, and S, special type, not graded (medullary carcinoma). Lymph node involvement is indicated as number of axillary lymph nodes with metastatic cancer over the total number of nodes examined

essentially as described above (Feilotter et al, 1998; Longy et al, 1998) except without the addition of isotope. Products were purified using the Wizard PCR Preps DNA purification system (Promega) and sequenced using the Thermosequenase Sequencing Kit (Amersham-Life Science) or the ABI Prism Dye Terminator Sequencing Ready Reaction Kit (Perkin Elmer) according to the manufacturer's instructions.

RESULTS

Of the seventy pairs of matched breast carcinoma and germline genomic DNAs examined, all were informative (heterozygous) for at least one of the markers analysed. In total, 27 tumours (39%) showed LOH at one or more marker loci. Twelve of these (71, 66, 10, 46, 58, 4, 56, 53, 27, 50, 55 and 65) exhibited allele loss for all informative markers over the entire 10-cM region examined (Figure 2). Thus, LOH in these tumours spanned extensive regions of 10q and was not useful for further defining the critical deleted interval or intervals. In the remaining 15 cases, LOH was detected at only a subset of the informative loci within the 10q23 region.

The most frequent allele loss was at *D10S1765*, although loss was common between *D10S215* and *D10S541*. Thirteen out of the 15 tumours which showed loss at only a subset of loci had LOH within this interval (59, 21, 40, 41, 6, 13, 14, 5, 52, 22, 11, 17 and 12) (Figure 2). This commonly deleted interval spans the *PTEN* locus.

In addition to the interval described above, a distinct non-overlapping region of allelic loss was seen in two samples (Figure 2). Tumours 68 and 39 showed allele loss only for markers telomeric of *D10S541*. Further, tumour 68 showed LOH at the more distal locus *D10S574* (data not shown), suggesting that the deleted interval spanned markers well telomeric of *D10S564* (Figure 2).

PTEN mutation analyses

To investigate whether *PTEN* was the target of allele loss in tumours where LOH was detected, we examined all nine exons of the *PTEN* gene in each of the 27 tumours which showed allele loss at 10q23 loci. For each tumour sample and control, *PTEN* exons were amplified using intronic primers. PCR products with altered mobility (SSCP) or denaturation profiles (DGGE), suggesting the presence of mutations, were directly sequenced. SSCP and DGGE variants were detected with PCR products corresponding to exons 2, 4 and 6. Sequencing of these variants confirmed that these products represented previously reported polymorphic alleles present in the normal population (Rhei et al, 1997; Feilotter et al, 1998). We did not detect mutations of the *PTEN* gene in any of the 27 breast carcinomas examined in this study. We conclude that mutations of *PTEN* are not common in breast cancer.

DISCUSSION

The *PTEN/MMAC1* gene lies between *D10S1765* and *D10S541* (Gray et al, 1996) within the major region of loss detected in this study. The gene encodes a molecule with homology to two cytoskeletal proteins, tensin and auxilin (Li et al, 1997; Steck et al, 1997). PTEN has demonstrated dual specificity phosphatase activity (Li and Sun, 1997; Myers et al, 1997), which suggests a role in both tyrosine and serine/threonine phosphatase-mediated signal transduction.

Although frequent mutations of *PTEN* in a variety of tumour types was predicted from early studies of tumour cell lines, this has not been generally confirmed. Analyses of *PTEN* in primary tumours have identified mutations in approximately one-third of high-grade gliomas (Rasheed et al, 1997; Wang et al, 1997; Bostrom et al, 1998) and about 50% of endometrial tumours (Kong et al, 1997; Risinger et al, 1997; Tashiro et al, 1997). However, mutations are common only in metastatic prostate

carcinoma (Cairns et al, 1997; Feilotter et al, 1998; Suzuki et al, 1998) and malignant melanoma (Guldberg et al, 1997). In recent studies, it has been shown that germline *PTEN* mutations are rare or absent in cases of familial breast cancer or early onset breast cancer without *BRCA1* mutations (Tsou et al, 1997; Chen et al, 1998; FitzGerald et al, 1998). Li et al (1997) identified mutations leading to truncation of the PTEN protein in 2 out of 20 (10%) breast tumour cell lines examined. Studies in unselected series of primary breast tumours have not identified high frequencies of *PTEN* mutations in sporadic tumours (Rhei et al, 1997). In this study, we examined a selected population of tumours already shown to have LOH for the *PTEN* region, which might be predicted to have a higher frequency of *PTEN* mutations. Interestingly, our data are very similar to results from Rhei et al (1997) who found only one *PTEN* mutation in 53 truly sporadic tumours. The low frequency of *PTEN* mutations in breast cancer cell lines and primary tumours, even in studies such as ours in which samples were preselected for LOH in the *PTEN* region, raises the possibility that another tumour-suppressor gene with a role in sporadic breast cancers may lie within this interval on 10q23.

Genome-wide allelotyping studies of sporadic breast cancers have not shown LOH above background levels on chromosome 10 (Sato et al, 1990; Devilee and Cornelisse, 1994; Bieché and Lidereau, 1995; Fujii et al, 1996; Kerangueven et al, 1996). However, these studies did not focus on the 10q23 region. In this study, we saw LOH for one or more loci in 10q23 in 27 out of 70 sporadic breast carcinomas. In >90% of these cases, deletion included the *PTEN* locus. Singh et al (1998) reported a similar frequency of LOH (41%) for this interval in sporadic breast tumours whereas Li et al (1997) saw LOH of *PTEN* in approximately 50% of primary tumours examined. Our data and those of Singh et al (1998) suggest that the most common interval of loss spans approximately 1 Mb of DNA which, although including the *PTEN* locus, could also include one or more further genes. As yet, additional loci within this interval, which might act as tumour-suppressor genes in sporadic breast cancer, have not been identified. Clearly, the existence of such candidate genes, which are lost in the samples studied here, remains to be determined.

Analysis of LOH in a variety of tumours has suggested that multiple discrete regions of chromosome 10 loss may occur in a single tumour or tumour type (Parmiter et al, 1988; Karlbom et al, 1993; Herbst et al, 1994; Peiffer et al, 1995; Albarosa et al, 1996; Ittmann, 1996; Trybus et al, 1996; Zedenius et al, 1996; Marsh et al 1998*b*). The precise physical localization of such deleted regions with respect to one another and the number of tumour-suppressor genes they represent has been difficult to determine, in part because of the variety of markers used in the different studies. Our identification of a second distinct deleted interval telomeric of the *D10S215*/*D10S541* interval is consistent with the suggestion that other genes on 10q23, outside of the *PTEN* interval, may contribute to some sporadic breast cancers. LOH analyses of a larger population of sporadic breast tumours may help to clarify this issue. The loss of multiple distinct regions on chromosome 10 may be analogous to the situation on chromosome 17 in sporadic breast tumours, in which at least five distinct deleted regions have been recognized (Nagai et al, 1994, 1995). In the case of chromosome 17, the pattern of LOH is significantly correlated with some clinical parameters of the breast tumours (Bevilacqua et al, 1989; Nagai et al, 1995; Midulla et al, 1996). Our data do not suggest any association of chromosome 10 allele loss with tumour size, stage or lymph node involvement, regardless of the deleted interval (Figure 2), although our numbers are too small for subset analyses. Singh et al (1998) has suggested a strong correlation of LOH in 10q23 with poorly differentiated cancers, although again the numbers examined were small and *PTEN* mutation status was not determined. Further study is required to confirm whether losses can be correlated with these or other clinical parameters.

In summary, we have identified two discrete regions of LOH on 10q in sporadic breast carcinomas. Losses of the major region identified in this study were found in 39% of the cases studied and spanned the locus of the *PTEN* tumour suppressor. Mutations of *PTEN* were not identified in any of these cases, suggesting other candidate tumour-suppressor genes, important in breast cancer, may lie within the same region. A tumour population with allele losses in the 10q23 region, such as we have described here, will provide a valuable tool for determining the relative contribution of inactivation of the *PTEN* gene, or a neighbouring tumour-suppressor gene, to breast tumorigenesis.

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