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PRINCIPAL INVESTIGATOR: Dr. Rita Kandel

CONTRACTING ORGANIZATION: Mount Sinai Hospital
Toronto, Ontario, Canada M5G 1X5

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6. AUTHOR(S)
Dr. Rita Kandel

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Mount Sinai Hospital
Toronto, Ontario, Canada M5G 1X5

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We recently completed a case-control study nested within a cohort of 4,888 women with benign breast disease (BBD) who participated in the National Breast Screening Study (NBSS). This showed that accumulation of p53 protein was associated with an increased risk of progression to breast cancer (adjusted odds ratio (OR) 2.55; 95% confidence interval (CI) 1.01-6.40), whereas c-erbB-2 protein overexpression was not (adjusted OR 0.65; 95% CI 0.27-1.53). In this grant, we proposed to: (1) collect paraffin-embedded breast tissue from the remaining 4,333 (that is 4,888-552) cohort members; (2) enlarge our ongoing case-control study with an additional 38 cases (and their controls) which we anticipate will be identified as a result of a linkage of the NBSS database to the Canadian Cancer Database; (3) examine whether cyclin D1 overexpression determined immmunohistochemically is a biomarker of increased risk of breast cancer. To date, we have updated the data base and initiated block collection of the remaining cohort. Our initial analysis suggests that cyclin D1 overexpression, as detected immunohistochemically, does not appear to be associated with increased risk of developing breast cancer (OR 1.2; CI 0.65-2.29) in the individuals studied to date. This study will be expanded when the additional cases are identified by the linkage described above.

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Rita Kendall Oct 6/98

PI - Signature Date

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INTRODUCTION

We have recently completed a study of the associations between c-erbB-2 protein overexpression and p53 protein accumulation in benign breast tissue and the risk of subsequent breast cancer (copy of paper enclosed in appendix). The study was conducted as a case-control study nested within the cohort of 4888 women in the National Breast Screening Study of Canada (NBSS) who were diagnosed with benign breast disease during active follow-up. Case subjects were the women who subsequently developed breast cancer (ductal carcinoma in situ (DCIS) or invasive carcinoma). Control subjects were matched to each case subject by NBSS study arm, screening center, year of birth, and age at diagnosis of benign breast disease. Histologic sections of benign and cancerous breast tissues were analyzed immunohistochemically. Information on potential confounding factors was obtained by use of a self-administered lifestyle questionnaire completed at the time of enrolment. Accumulation of p53 protein was associated with an increased risk of progression to breast cancer (adjusted odds ratio (OR) = 2.55; 95% confidence interval (CI) = 1.01-6.40), whereas c-erbB-2 protein overexpression was not (adjusted OR = 0.65; 95% CI = 0.27-1.53). The findings for c-erbB-2 and p53 did not differ among stratas defined by menopausal status, allocation within the NBSS, history of breast disease, and whether the benign breast disease was detected at a scheduled screen or between screens. The results were also similar after exclusion of case subjects whose diagnosis of breast cancer occurred within 1 year of their diagnosis of benign breast disease and after exclusion of subjects with DCIS. In summary, p53 protein accumulation, but not c-erbB-2 protein overexpression, appears to be associated with an increased risk of progression to breast cancer in women with benign breast disease. (That study was funded by the National Cancer Institute of Canada.) The work funded by the U.S. Army Medical Research and Materiel Command will allow us to enlarge this case-control study, study cyclin D1 expression in the same individuals, and to create a tissue bank for further studies.

The purpose of this project is three-fold. Specifically, we will:

(1) collect paraffin-embedded benign breast tissue from the remaining 4,336 cohort members who are not part of the case-control study. This will establish a bank of paraffin-embedded tissue for a cohort of women on whom there is extensive documentation of risk factor information. With further follow-up of the cohort, it will be possible to enlarge the above described case-control study, and to undertake additional studies of newly identified molecular markers of risk of progression to breast cancer.

(2) enlarge our recently completed case-control study of p53 with an additional 38 cases (and 5 matched controls per case) which we anticipate will be identified as a result of a linkage of the NBSS database to the Canadian Cancer Database which is scheduled for late 1998. We propose to examine biopsies from these subjects for evidence of c-erbB-2 overexpression and p53 protein accumulation. Addition of these cases and controls to the previous study will increase its statistical power.

(3) examine whether cyclin D1 overexpression, determined immmunohistochemically, is a molecular marker of risk of progression from BBD to breast cancer in the enlarged case-control study. We hypothesize that cyclin D1 overexpression in benign breast disease is associated with increased risk of progression to breast cancer. Multiple molecular markers are being examined, since progression to cancer probably results from the accumulation of several genetic events.

Summary of proposed work - We propose to collect paraffin-embedded benign breast tissue from the remaining 4,336 women in the NBSS (including the 38 cases and 190 controls who we anticipate will be added to the case-control study - see Table 1) who were diagnosed with BBD. Our approach to collecting this tissue will be the same as that already employed for the completed investigation.

The collection of these blocks will allow us to enlarge the completed study. We will examine the additional cases and controls for c-erbB-2 overexpression and p53 protein accumulation. For all subjects in the case-control study nested cohort study, we will determine which of them display immunohistochemical evidence of overexpression of cyclin D1 in their benign breast tissue. We wish to add assessment of cyclin D1 overexpression to the study because recent evidence suggests a potential role for cyclin D1 in mammary carcinogenesis and because it is likely that multiple genetic events are involved in carcinogenesis.

Table 1. Numbers[a] of subjects by funding agency, component of project, and case/control status

Project Component	NCIC[b]		US Army (proposed)	
	Cases	Controls	Cases	Controls
Cyclin D1 staining			130	650
C-erb-2 and p53 analysis	92	460	38	190
Tissue bank		552		4,888

[a] Numbers of blocks requested. Please note we do not expect to obtain all blocks (see page 11).
[b] National Cancer Institute of Canada

SUMMARY OF LITERATURE: A history of benign breast disease is associated with a two-fold increase in risk of subsequent breast cancer (1,2). However, risk of breast cancer in women with BBD differs according to the histological characteristics of the BBD - risk is higher for women with atypical proliferative forms of BBD, in whom the relative risk (versus that for women without proliferative disease) is of the order of four, while the relative risk for women with proliferative disease without atypia is about two (2-8). There is some evidence to suggest that there are interactions between atypical hyperplasia and family

history of breast cancer (3,4), nulliparity (5), and menopausal status (6) in determining risk. In some studies, women with fibroadenomas or non-proliferative forms of BBD have been observed to have a small increase in risk of breast cancer (2,8,9).

The transition from regulated cell turnover in breast tissue to unregulated proliferation and the development of carcinoma in-situ and subsequent tumour invasion and metastasis appears to result from a variety of genetic events, including oncogene(s) activation, as well as inactivation of tumour suppressor gene(s) (reviewed in 10,11,12). For example, amplification of DNA, with or without overexpression of oncogenes such as erbA, erbB-2, c-myc, H-ras, hst and int 2, has been detected in breast cancers (10,13-15). Cyclin D1 overexpression has also been implicated in the development of breast cancer (16-21). Additionally, changes in the tumour suppressor genes p53 and Rb, and changes in or loss of heterozygosity at the chromosomal loci 11q, 17q, 17p, 1p, 16q, and 13q have been detected (10,11,22), as have mutations in the HRAS1 mini-satellite locus (23).

Recently, the BRCA1 gene was identified and shown to be mutated in kindred with hereditary breast cancer (24). Decreased BRCA1 mRNA expression has been detected in sporadic breast cancer in both the in-situ and invasive forms (25).

Other factors might also be involved in the development of malignancy: growth factors such as fibroblast growth factor(s), insulin like growth factor 1, epidermal growth factor and the ligands for erbB-2 have been implicated in the transformation of normal epithelium to a malignant phenotype (reviewed in 10, 26,27); and cellular changes, including changes in steroid hormone receptor levels and cellular proteases, and changes in the levels and types of membrane adhesion molecules (integrins)(26,28,29). It is possible that these interact to maintain cancer growth.

At the later stages of carcinogenesis, increased angiogenesis has also been shown to occur (26,30). As well, lower levels of nm23 (metastasis suppressor gene) mRNA correlate with decreased disease-free and overall survival (10), although the exact role of nm23 in breast cancer has not been elucidated (31).

The contribution of each of these genetic changes to the development of the malignant phenotype is not clear, because breast cancers exhibit genotypic and phenotypic heterogeneity (32-34). Of the genes mentioned above, p53, c-erbB-2, and cyclin D1 are amongst those which most frequently show alteration in breast cancer (observed frequencies vary from 13% to 46% for p53, 20% to 50% for c-erbB-2, and 40% to 81% for cyclin D1) (10,35-37). Alterations in these genes have been shown to be present in the earliest stages of human breast cancer (carcinoma in-situ) (38,39).

Cyclin D1: It has only recently become evident that cyclin D1 might contribute to the development of breast cancer (17-21,40,41). Cyclin D is involved in regulating cell cycle progression from G_1 into the S phase (42,43). There are three types of cyclin D, namely D1, D2 and D3, each having its own pattern of tissue-specific expression. These cyclins

can form complexes with cdk4 or cdk6 which then phosphorylate the retinoblastoma (RB) protein and allow the cells to pass into the S phase. Cells which overexpress cyclin D1 show reduced exit from G_1 to G_0 suggesting a role for cyclin D1 at this regulatory point (44). Cyclin D1 can be induced by estrogens, suggesting a potential mechanism through which estrogens influence the risk of developing breast cancer (18,45). Transfection of cells with vectors expressing cyclin D1 has shown that overexpression of cyclin D1 accelerates G1/S transition (46,47). Studies in mice have indicated a role for cyclin D1 in breast development as cyclin D1 knockout mice do not undergo the normal breast changes during pregnancy (48).

Several observations have led to the suggestion that cyclin D1 amplification and/or overexpression are tumorigenic. Firstly, cell transformation results when cyclin D1 is transfected with the adenovirus E1A oncogene into BRK cells (49); secondly, rat fibroblasts transfected with cyclin D1 have a shortened G_1 phase and form tumours when injected into nude mice (50); and thirdly, mammary hyperplasia and breast cancer develop in transgenic mice which overexpress cyclin D1 (51). It has been suggested that cyclin D1 is not a dominant oncogene but requires the presence of other oncogenes to induce tumours (52). Other investigators have suggested that cyclin D1 overexpression enhances gene amplification and may contribute to genomic instability (53).

Cyclin D1 has been studied extensively in breast cancer. Cyclin D1 amplification has been observed in up to 33% of breast cancer cell lines and between 11 and 23% of human breast cancers (54-59). Cyclin D1 accumulation as detected immunohistochemically occurs in up to 81% of breast cancers, although the frequency appears to be dependent on the antibody used (54,55,59). However, there have been relatively few studies of cyclin D1 in normal breast tissue and in breast lesions associated with increased risk of developing invasive breast cancer. Some immunohistochemical studies have shown that normal human breast epithelium and breast tissue adjacent to breast cancers demonstrate, at most, occasional cells which express cyclin D1 protein (57,60,61). Recently Alle et al. (62) showed that there was cyclin D1 overexpression in normal breast epithelium and that the proportion of tissues showing immunopositivity increased with increasing cellular proliferation and cellular atypia. There was a significant difference in expression between normal tissue and proliferative disease and between proliferative disease and ductal carcinoma in situ. We (63) have demonstrated that 15% of those with normal breast tissue, 19% of those with epithelial hyperplasia without atypia, 27% those with atypical ductal hyperplasia, 35% of those with ductal carcinoma in situ, and 25% of those with invasive ductal carcinoma have cyclin D1 gene amplification; the corresponding figures for protein overexpression were 13%, 13%, 57%, 50%, and 64%, respectively. The results of this study suggest that cyclin D1 amplification and protein overexpression can occur before histological alterations are seen, and that the frequencies of these changes are higher in histological lesions with cellular atypia (atypical hyperplasia and DCIS) reaching frequencies similar to those observed in invasive carcinoma. Simpson et al. also has shown that cyclin D1 gene amplification and overexpression as well as protein accumulation occur in ductal carcinoma in situ (64). An in-situ hybridization study

demonstrated that 18% of benign breast lesions showed cyclin D1 mRNA overexpression (65).

The human and experimental data suggest that cyclin D1 amplification and/or protein overexpression might have a role not only in breast cancer but also in the putative early stages of breast neoplasia such as epithelial hyperplasia, a histopathological change known to be associated with increased risk of progression to breast cancer.

p53: p53 is also involved in regulating progression of cells from the G1 to the S phase as well as G1 to M phase of the cell cycle (66). However, in contrast to cyclin D1, wild-type p53 protein prevents cells from progressing into S phase (66,67). One mechanism by which p53 exerts this effect is by inducing p21, a cyclin-dependent kinase inhibitor (68-73). Other genes with p53 responsive elements which might also be involved in cell proliferation include GADD45 (74) and mdm-2 (75). Alternatively, under the appropriate stimulus, wild-type p53 can also initiate apoptosis (66,76). Wild-type p53 has also been shown to be involved in promoting chromsomal stability (66).

Overexpression of wild-type p53 can cause immortalization of cells (77,78), but p53 gene mutations with or without loss of heterozygosity are usually required for tumor formation. In its mutant form, p53 can stimulate proliferation or act as a dominant transforming oncogene, as has been shown by Lane and Benchimol in transfection studies (79). As well, mutant p53 expressed in cell lines lacking p53 can enhance tumorigenic potential in nude mice (80). Cell lines with mutations in the p53 gene show an increased rate of aneuploidy and gene amplification (81). In experimental studies, mice deficient in p53 are susceptible to forming tumors (82).

Mutations in the p53 tumor suppressor gene have been linked to a variety of malignancies including those of the breast (12,66,83-85). The observed frequency of mutations in the sporadic forms of breast cancer has been observed in 15 to 30% of cancers but can be as high as 50% (86).

Mutant and wild-type p53 protein half-life can be altered by interaction with the E6 protein from human papillomavirus, or with heat shock protein 70 or mdm-2 (87-90). Additionally, Moll et al. (91) have shown that in some breast cancers, p53 protein is present in the cytoplasm and excluded from the nucleus. This process would prevent the wild-type protein from exerting its regulatory effect. Cytoplasmic localization of p53 has been identified in other tumors (92).

The presence of p53 mutations and immunohistochemical detection of p53 protein accumulation have been detected in 16% to 33% of intraductal carcinomas (38,93,94). In addition, there is also a literature to suggest that p53 may play a role in the development of breast cancer. p53 protein accumulation has been demonstrated immunohistochemically in the benign breast tissue of patients with the Li-Fraumeni syndrome, who are at high risk of developing breast cancer (94), and in a cancer family who developed breast cancer (95).

Five reports have shown positive immunostaining for p53 in benign breast disease (96-100), and one has shown gene amplification (100). In the latter study, the DNA was not analyzed for the presence of mutations. Mutated p53 and overexpression of wild type p53 occurs in the preneoplastic stage of mouse mammary tumor development (101). p53 has also been detected in precursor lesions of other cancers. For example, p53 accumulation was present in dysplastic squamous epithelium of bronchi in 53% of patients with squamous cell carcinoma of the lung (102), and in 23% of biopsies of Barrett's esophagus (103).

C-erbB-2: c-erbB-2 (neu or HER 2) is a normal cellular gene present on chromosome 17q21 (15). It encodes for a membrane protein (p185) which is tyrosine phosphorylated following interaction with its ligands (15). c-erbB-2 overexpression occurs either through changes in amplification and/or mRNA overexpression, perhaps due to OB2-1 overexpression (10,104). Several experimental studies have suggested that c-erbB-2 may play a role in the pathogenesis of breast cancer. Di Fiore et al (105) showed that five to ten-fold overexpression of normal human c-erbB-2 following transfection resulted in transformation of NIH 3T3 cells. The observation that the activated version of the c-erbB-2 gene is detected in the breast cancer that develops in transgenic mice also suggests that this gene may have a role in mammary tumorigenesis (106,107). In one transgenic strain, high levels of expression of the activated gene in the mammary epithelium resulted in the one-step acquisition of the transformed phenotype (107). Moreover, high levels of the transgene transcript could be detected in the earliest stages of mammary tumour formation, suggesting that no further genetic events were required for the conversion of the primary epithelial cell to the malignant phenotype.

c-erbB-2 overexpression has also been examined in several studies of benign and malignant human breast tissue. Overexpression of the c-erbB-2 gene has been noted in early stage human carcinomas (108) and is most frequently detected in ductal carcinoma in-situ (10, 108-110). Immunohistochemical studies demonstrate c-erbB-2 overexpression more consistently in the in-situ component (approximately 50%) than in the infiltrating malignant cells in breast cancer (approximately 20%) (111,112). Several studies have examined c-erbB-2 overexpression in biopsies of benign breast tissue (100,109,113-115). These showed various findings ranging from no staining to positive immunostaining in up to 25% (115) of cases. Tsutsumi et al. (116) observed that the adjacent normal epithelium in some breast cancer resections showed stronger c-erbB-2 immunostaining than the cancer. These results raise the possibility that c-erbB-2 expression may be involved at a relatively early stage of tumour formation.

Conclusion: Epithelial cancers appear to be the result of an accumulation of multiple genetic events (117,118). To date, in relation to breast cancer, cyclin D1 and c-erbB-2 overexpression, and p53 mutations are amongst the most frequent genetic changes detected. While cyclin D1, c-erbB-2, and p53 appear to play an important part in mammary carcinogenesis, their precise role in this process is unclear. For example, it is unclear whether they are involved in the initiation of transformation or at a subsequent stage, or

whether they are just indicators of increased risk of developing breast cancer as they may be markers of genetic instability.

The preceding human and experimental evidence suggests that cyclin D1 and c-erbB-2 overexpression, and p53 protein accumulation with or without gene mutation, may be present in benign breast disease. Since they may also be present in breast cancer, they might either influence progression from BBD to breast cancer or be markers of increased cancer risk.

BODY

(1) STUDY DESIGN:

Our study uses paraffin-embedded discard breast tissues which have been obtained from the cohort of women enrolled within the National Breast Screening Study (NBSS) who received a diagnosis of benign breast disease during the active follow-up phase of the study. In the ensuing paragraphs, we describe the NBSS first, and then present details of our ongoing and proposed investigations.

(a) The National Breast Screening Study: The NBSS is a multi-centre randomized controlled trial of screening for breast cancer in Canadian women aged 40 to 59 at recruitment (119,120). The study involves 89,835 women who were recruited at 15 screening centres across Canada. Recruitment commenced in 1980 and ended in 1985. Women were eligible to participate in the study if they had no history of breast cancer, were not currently pregnant, and had not had a mammogram in the preceding 12 months.

Women aged 40-49 years were randomized either to have annual mammography plus physical examination, or to have initial physical examination only, and women in both the intervention and the control group were taught breast self-examination. Randomization in the 50-59 year age-group was either to annual mammography plus physical examination, or to annual physical examination alone (women in this arm of the 50-59 year age-group were also taught breast self-examination).

(b) Diagnosis of BBD and breast cancer in the NBSS: At each visit, study participants had a physical examination. For those who were randomized to the intervention group, physical examination was followed by mammography, the films from which were read by a study radiologist who was unaware of the physical examination results. If the examiner or the radiologist reported an abnormality requiring further assessment, a referral was made to a review clinic where the participant was seen by a study surgeon. If, on review, a recommendation was made for biopsy, this recommendation was conveyed to the participant's family physician, and the participant was contacted and asked to visit her family physician for further management.

Women in both control groups were referred for mammography if either they or their primary care physician discovered an abnormality for which referral was warranted. Staff in each screening centre identified the pathology laboratory in which biopsies were examined, and they obtained slides or blocks for review by a locally designated reference pathologist. Results of the histological review of the biopsies were forwarded to the coordinating centre.

(c) Follow-up in the NBSS: Active follow-up continued until 1988. During this phase of the NBSS (when the study participants underwent the screening schedule corresponding to their allocation, as described in (b) above), there was in each study centre a coordinator

(usually a nurse) who had experience in clinic or study management. The coordinators were responsible for ascertaining whether the recommended diagnostic procedures had been carried out and for collecting reports of the surgical and pathological procedures from the institutions where they had been performed. Procedures performed independently of the screening process were identified through annual questionnaires sent to study subjects, and reports of these procedures were then obtained from the relevant institutions. Study records for women who moved out of their original area were transferred to the centre nearest their new residence. Following completion of their screening schedule, direct follow-up stopped for those with no diagnosis of breast cancer. However, until 1988-1990 (depending upon the province) information about new diagnoses of breast cancer was obtained by linkage with the provincial cancer registries (cancer is registered in each province in Canada, and, for Ontario at least, registration is essentially complete (121)). Subsequently, new diagnoses of cancer will be ascertained by linkage to the Canadian Cancer Database, which is operated by the Canadian Centre for Health Information at Statistics Canada, and consists of registration data reported annually by the provincial registries. A linkage yielding incidence data to the end of 1993 was completed recently, and we propose that another linkage take place in late 2000 to yield a further few years of follow-up data.

(d) Design of the ongoing study: The investigation currently underway is being undertaken within the cohort of 4,888 women within the NBSS who received a histopathologic diagnosis of BBD during the active follow-up phase of the NBSS (99,100). In order to reduce costs substantially while having relatively little impact on the precision of the estimates of association (122), the study is being conducted as a case-control study nested within this cohort. Cases are women who subsequently developed breast cancer, while controls are women who had not developed breast cancer by the date of diagnosis of the corresponding case. Five controls were selected for each case, and they are matched to the corresponding case on study arm within the NBSS, screening centre, year of birth, and age at diagnosis of BBD.

(e) Case definition: Cases are women with a history of BBD detected during the course of the NBSS who subsequently developed breast cancer. By this definition, 92 cases were identified by previous linkages. We have collected the benign tissue for 74 cases. As described below, as a result of the forthcoming linkage, we expect to identify an additional 38 cases, and on the basis of our experience to date we would expect to obtain 30 (74/92 x 38) of these cases. This would yield a total of 104 cases for the ongoing case-control study.

The number of additional cases of breast cancer which we expect to be identified as a result of the forthcoming linkage was calculated as follows. To the end of 1987, 820 incident cases had been identified in the NBSS, of which 67 (8.2%) were in the cohort of women with benign breast disease. The linkage to the Canadian Cancer Database will yield follow-up data to the end of 1993, and it is expected (see below) that 1600 breast cancer cases will have occurred in the NBSS by this time (by the end of 1993 the study

participants had each accrued an average of approximately 10.4 years of follow-up). Assuming that the proportion of this total number of cases which is in the cohort of women with BBD has remained stable, we would anticipate that the number of women with BBD who had developed breast cancer by the end of 1993 will be 130 (i.e. 1600 x 67/820). The number of existing cases (and controls), and the number of additional cases that we expect to obtain, are summarized in Table 2 below. [The estimate of 1600 cases of breast cancer in the NBSS by the end of 1993 was derived using standard life table techniques, together with age-specific Canadian cancer incidence rates, and Canadian all-cause mortality rates for women for the years 1985-1986 (123,124). It has been observed previously that in the control groups in the NBSS, the observed age-specific breast cancer incidence rates to the end of 1987 were very similar to those expected from national rates. As expected, an excess of breast cancer was observed in the mammography arms, particularly at first screening, which presumably represents the effect of early detection, and possibly also the effect of detection of minimal lesions which might otherwise never have come to diagnosis. In calculating the expected numbers, the excess occurring in the mammography arms was ignored since there is no way of determining if and when this excess will disappear. Additionally, it is clear that removal of prevalent cases at baseline will have reduced the subsequent incidence rate for some period of time, but it is not easy to allow for this. Nevertheless, this probably makes little difference overall, especially given the large expected number of cases of breast cancer.]

(f) Definition of controls: Controls are women who had not developed breast cancer by (but were alive at) the date of diagnosis of the corresponding case (they will, of course, have a diagnosis of BBD). Since there are no estimates of the likely magnitude of the effects of interest on risk of progression from BBD to breast cancer, we select 5 controls for each case in order to maximize statistical power. Controls are matched to cases on study arm within the NBSS, screening centre, year of birth, and age at diagnosis of BBD (and implicitly on the interval between diagnosis of BBD and the date of diagnosis of breast cancer in the corresponding case). These matching criteria are chosen either because the factors of interest are related to breast cancer risk (age, and possibly age at diagnosis of BBD) or because they are related to the risk of disease detection (allocation and screening centre). It is also conceivable that at least some of these factors are related to the exposures of interest. However, it should be noted that little is known about the "epidemiological" correlates of cyclin D1 and c-erbB-2 overexpression, and p53 protein accumulation. Additionally, the implicit matching on duration of follow-up (as well as age) means that the controls have had the same opportunity (at least, in terms of the elapse of time) to develop breast cancer as the cases.

For each case, the controls were randomly selected from eligible subjects in the subgroup defined by the characteristics of the case. (Procedures for the ascertainment of death in the NBSS, and the verification of the cause of death, have been described in detail elsewhere (120).) We collected the benign tissue for 349 of the 460 controls (i.e.an average of about 4 controls per case). As described in (e) above, as a result of the forthcoming linkage, we expect to identify an additional 38 cases and to obtain blocks for

30 of them. On the basis of our experience to date we would expect to obtain about 113 of the 150 controls for these 30 cases. This would yield a total of 462 controls for the expanded case-control study.

Table 2. Numbers of cases and controls anticipated for the expanded case-control study

	Cases	Controls
Blocks received to date	74	349
Additional blocks expected as a result of 1998 linkage	30	113
TOTAL	104	462

(g) Questionnaires: Menstrual and reproductive history, use of oral contraceptives, and dietary factors are thought to be involved in the etiology of BBD (125), and it is possible that some of these factors influence risk of progression from BBD to breast cancer. Therefore, estimates of the association between cyclin D1 and c-erbB-2 overexpression and p53 protein accumulation and risk of breast cancer will, where appropriate, be adjusted for these factors. Information on risk factors for BBD will come from questionnaires completed by the NBSS participants.

At the time of their enrolment in the NBSS, all participants completed a questionnaire which sought identifying information, as well as data on factors such as demographic characteristics, family history of breast cancer, menstrual and reproductive history, use of oral contraceptives and replacement estrogens, and cigarette smoking. Additionally, approximately two-thirds of the 89,835 women enroled in the NBSS completed self-administered diet history questionnaires. The dietary questionnaire was introduced in 1982, at which time some women had already been enrolled in the study (and were not seen again at the screening centres). The diet history contained questions on the frequency of consumption and usual portion size of 86 food items, and also had an open-ended section for describing other food items normally eaten. Photographs of various portion sizes were included in the questionnaire to assist participants to quantify intake. The questionnaire also included questions on current and past height and weight, and on consumption of beer, wine, and spirits. A comparison between the self-administered questionnaire and a full interviewer-administered questionnaire which has been subjected to both validity and reliability testing (126) and used in a number of epidemiologic studies (127) revealed that the two methods give similar results for the major macronutrients, dietary fibre, and vitamin C (128).

(h) Statistical power: This was calculated according to that described by Breslow and Day (129).

(2) CONDUCT OF THE STUDY:

(a) Coding, data entry, and processing: The lifestyle information is available on the computerized NBSS database. The NCIC Epidemiology Unit's standard procedures for quality control is used for coding and data entry.

(b) Collection of paraffin-embedded breast material: For the completed case-control study of p53 and c-erbB-2 protein changes in benign breast disease, we created a database consisting of identifying information, plus details of the location and accession number of the 552 paraffin blocks. This information was used to generate lists for each hospital of the study participants for whom we wished to obtain paraffin blocks. We then wrote to the pathologist-in-chief at the hospital seeking the blocks.

This same approach was used to expand the existing tissue bank. The database was updated to include all 4,888 subjects with a diagnosis of BBD in the NBSS. We propose to collect the blocks of the remaining 4,336 (4,888-552) women.

For the completed case-control study, we collected 423 blocks of benign tissue from the 552 paraffin blocks requested. As a result, we predict that we will obtain 3,322 blocks out of the 4,336 blocks requested.

(c) Histopathological Review: Sections from blocks received for the nested case-control study were reviewed and classified by Dr. Kandel and a collaborator, Dr. W. Hartwick, according to the criteria developed by Page (130), and as described in the recent consensus conference for DCIS (131). Briefly, in benign lesions, the presence or absence of epithelial proliferation was determined, and when epithelial proliferation was present, the lesions were classified further according to the presence or absence of cytological atypia. The cancers were classified by histological type. The histology of the cases and controls from the expanded study will be reviewed similarly.

(d) Experimental methods: In this section we describe the methodology that was used for the cyclin D1 immunostaining. For completeness we also present details of the immunohistochemical staining for c-erbB-2 and p53.

(i) Cyclin D1 Expression in Breast Disease:

Since we do not have access to frozen tissue, immunohistochemical staining was used to detect cyclin D1 overexpression. The antibody that we selected works on paraffin-embedded tissue. There is a good correlation between immunostaining and Western blot analysis which indicates that the positive immunoreactivity is not a false positive (36). Immunohistochemical staining allows cellular localization of the immunoreactivity, so it was possible to ensure that the cyclin expression was occurring in breast epithelial cells. In addition, this approach allowed us to determine whether the immunoreactivity was present in the histopathology considered to be associated with increased malignant potential.

Breast cancers were stained in order to determine whether the expression present in the benign breast disease was maintained in the malignant lesion, or was present in the breast cancer only.

Immunohistochemical staining for cyclin D1: 5um thick section were cut and placed on aminopropyltriethoxysilane (2%, Sigma Chemical Co. MO, USA) coated slides and deparaffinized. The tissue underwent antigen retrieval (microwave pretreatment in 10 mM citrate buffer, pH 6.0, for 15 minutes at a medium high setting). The endogenous peroxidase was inactivated using 3% hydrogen peroxide, and the sections were blocked with normal horse serum (20 µl/ml, Vector Laboratories, Burlingame, CA) containing 5% bovine serum albumin (BDH Laboratory, Poole, England) in TBS buffer (5 mM Tris-HCl, pH 7.6, 0.85% NaCl). The sections were incubated with antibody reactive with cyclin D1 protein (monoclonal, dilution 1:2000, Upstate Biotechnology, Lake Placid, NY) overnight at 4°C. After washing, the sections were incubated with biotinylated anti-mouse IgG (dilution 1:200, Vector Laboratories) for 30 minutes at room temperature, followed by avidin-biotin peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories). Immunoreactivity was visualized with 3',3-diaminobenzidine tetrahydrochloride (DAB, Vector Laboratories) and the sections counterstained briefly with hematoxylin. T47D cells which had been embedded in paraffin served as the positive control (57). This cell line is known to overexpress cyclin D1 protein and was always immunopositive under our conditions. The negative control consisted of replacing the primary antibody with TBS or non-immune mouse serum (DAKO Corp, Carpinteria, CA). Distinct nuclear staining indicated a positive reaction and cytoplasmic staining was considered nonspecific and interpreted as negative. The staining was assessed as + when only occasional cells were positive, ++ when clusters of cells were positive and +++ when there was diffuse staining throughout the pathological area. To assess the accuracy of the immunoreactivity interpretation, a subgroup of cases (n=25) and controls (n=25) was reviewed a second time (without knowledge of the initial grading) to provide a measure of the misclassification of immunoreactive status, which will enable estimates of the association between cyclin D1 immunoreactivity and risk of breast cancer to be adjusted for misclassification (132).

(ii) c-erbB-2 Overexpression and p53 Accumulation in Breast Disease:

c-erbB-2 overexpression and p53 protein accumulation was assessed immuno-histochemically in the case-control study and will be examined in the expanded study when we receive the results of the linkage are made available to us.

Immunohistochemical assessment of c-erbB-2 overexpression: 5um sections will be incubated with antibody reactive with c-erbB-2 (NCL-CB11, Novocastra Lab. Inc. Newcastle upon Tyne, dilution 1/160) overnight at 4°C. NCL-CB11 recognizes an internal epitope in formalin-fixed paraffin-embedded material (133). After washing, the sections will be incubated with biotinylated goat anti-mouse IgG and reactivity detected using the avidin-biotin peroxidase complex and 3'-3' diaminobenzidine. Positive controls will include a paraffin-embedded cell block of the human breast cancer cell line SK-Br-3 which

overexpresses c-erbB-2. As a negative control, the primary antibody will be replaced with PBS or mouse non-immune serum. Immunoreactivity will be indicated by membrane and/or cytoplasmic staining of cells as determined by light microscopy.

Immunohistochemical staining for p53: Tissue sections will be reacted with monoclonal antibody reactive to both wild-type and mutant forms of p53 (clone DO-7, Novocastra Laboratories Ltd. Newcastle upon Tyne, England) following microwave pretreatment (134). Immunoreactivity will be determined using a biotinylated-anti-mouse IgG, followed by avidin-biotin peroxidase complex and 3'-3' diaminobenzidine tetrachloride. Paraffin-embedded tissue, which was shown by genetic analysis to contain a p53 mutation, will be used as a positive control. The negative control will consist of replacing the antibody with PBS or mouse non-immune serum. A positive reaction will be indicated by nuclear staining as determined by light microscopy.

(4) STATISTICAL ANALYSIS:

Essentially, the statistical analysis involves comparison of the frequency (either singly or in combination) of cyclin D1, c-erbB-2 overexpression and p53 protein accumulation in the cases and controls, using conditional logistic regression with multiple controls per case (122). The association between these genetic changes and factors which are thought to be involved in the etiology of BBD and breast cancer (e.g., reproductive, menstrual, and dietary factors, as well as BBD histology) were examined, as is the association of the latter variables with risk of progression to breast cancer.

Further analyses will be directed towards within-individual comparisons of cyclin D1 and c-erbB-2 overexpression and p53 in BBD and breast cancer. One possible interpretation of any changes which are found to be common to both conditions will be that they contribute to the development of breast cancer rather than arise as a consequence of it.

RESULTS

We have updated the data base with respect to identifying details of the remaining individuals in the cohort with benign breast disease.

We have contacted a total of 228 hospitals. The number has changed from our grant proposal because of the ongoing hospital mergers that are occurring in Canada. We are in the process of acessioning the paraffin blocks received to date. 134 hospitals/ laboratories have sent 1574 blocks out of 2019 requested (78%). An additional 51 hospitals have responded that they are willing to send the blocks that we have requested (1283 blocks) and that they are forthcoming. Forty-three hospitals (900 blocks) have replied and informed us that the blocks requested have been discarded. Repeated follow-up phone calls are being made to the lab director or their designate for the outstanding 52 hospitals.

Cyclin D1 immunostaining of tissue sections from the 357 existing blocks of benign breast disease has been completed. Cyclin D1 overexpression was seen in 76 samples of benign breast disease. Seventeen of the immunopositive tissues were cases and 59 were controls. When the analysis was completed the presence of cyclin D1 over-expression was not associated with increased risk of developing breast cancer (unadjusted odds ratio = 1.2, 95% confidence intervals = 0.65-2.29). Twenty-two of 52 cancers showed cyclin D1 overexpression. Ten of the 22 immunopositive cancers had cyclin D1 overexpression in their corresponding benign tissue. Six cancers that were immuno-negative showed cyclin D1 overexpression in their benign tissue. Data analysis is continuing.

In the second year of this grant, the tasks (tasks 1, 2, and 3) as described above have been competed within the timelines indicated in the statement of work.

However, technical objective 3 (tasks 4 and 5) in the statement of work in the grant proposal has not been accomplished yet because of circumstances beyond our control. This task involves extension of the ongoing project by the addition of more cases and their controls. The cases are identified by the linkage of the NBSS database to the Canadian Cancer Database. There was a delay at Statistics Canada and the linkage has been completed. The breast cancer diagnoses were verified and this took more time than anticipated for technical reasons. We hope that the information should be made available to us within the next 6 months. At that time we will begin the c-erbB-2, p53 and cyclin D1 immunostaining studies as described.

Publications during the past year include:

1. Rohan TE, Hartwick W, Miller AB, Kandel R. Immunohistochemical-detection of c-erbB-2 and p53 in benign breast disease and breast cancer risk. JNCI 1998;90:1262-1269.
2. Zhu XL, Rohan T, Hartwick W, Kandel R. Cyclin D1 gene amplification and protein expression in benign breast disease and breast carcinoma. Mod Pathol, in press, 1998.
3. Duffy SW, Rohan TE, Kandel R. Misclassification in a matched case-control study with variable matching ratio. Submitted to Statistics in Medicine, 1998.

Copies of these manuscripts are in the accompanying appendix.

CONCLUSIONS

As the collection of blocks and the expansion of our case-control study has not been completed, the conclusions that can be drawn are limited. Our recently published case-control study involving the use of benign breast tissue from individuals enrolled in the NBBS has been called a "paradigm for future studies of additional biomarkers that may identify women with high risk benign breast disease" in a recent editorial about our studies. (135). This supports the approach that we are using to identify biomarkers of increased breast cancer risk and we are continuing to collect the paraffin blocks of the benign breast tissue to be able to do these types of studies.

Cyclin D1 protein overexpression as detected immunohistochemically does not appear to be associated with increased risk of developing breast cancer in the group of individuals studied to date. This study will be expanded when additional cases are identified by the linkage described above.

We also intend to analyze the tissue for cyclin D1 amplification by differential PCR. This methodology is established in the laboratory and we have used it to determine whether there is cyclin D1 amplification in a series of normal, benign breast tissue ranging from proliferative disease to ductal carcinoma in situ which has been associated with increasing risk to develop breast cancer and invasive cancer (63). The individuals analyzed in that study were not part of the NBSS cohort as the tissues were obtained from the Pathology Department at Mount Sinai Hospital, Toronto. We demonstrated that cyclin D1 amplification can occur before histological alterations are seen and that the frequency of amplification are higher in lesions with cellular atypia (atypical hyperplasia and ductal carcinoma in situ) reaching a frequency similar to invasive carcinoma. This suggests that it would be worthwhile examining whether the presence of cyclin D1 gene amplification is a marker of increased breast cancer risk. A letter to modify our research proposal will be submitted as requested by the guidelines.

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Personnel Currently Employed by Grant

Technicians

Alan Roy
Shu-Qiu Li

APPENDIX

ARTICLES

Immunohistochemical Detection of c-erbB-2 and p53 in Benign Breast Disease and Breast Cancer Risk

*Thomas E. Rohan, Warren Hartwick, Anthony B. Miller, Rita A. Kandel**

Background: **We studied the associations between c-erbB-2 protein overexpression and p53 protein accumulation in benign breast tissue and the risk of subsequent breast cancer.** ***Methods:*** **We conducted a case–control study nested within the cohort of 4888 women in the National Breast Screening Study (NBSS) who were diagnosed with benign breast disease during active follow-up. Case subjects were the women who subsequently developed breast cancer (ductal carcinoma *in situ* [DCIS] or invasive carcinoma). Control subjects were matched to each case subject on NBSS study arm, screening center, year of birth, and age at diagnosis of benign breast disease. Histologic sections of benign and cancerous breast tissues were analyzed immunohistochemically. Information on potential confounding factors was obtained by use of a self-administered lifestyle questionnaire.** ***Results:*** **Accumulation of p53 protein was associated with an increased risk of progression to breast cancer (adjusted odds ratio [OR] = 2.55; 95% confidence interval [CI] = 1.01–6.40), whereas c-erbB-2 protein overexpression was not (adjusted OR = 0.65; 95% CI = 0.27–1.53). The findings for c-erbB-2 and p53 did not differ among strata defined by menopausal status, allocation within the NBSS, history of breast disease, and whether the benign breast disease was detected at a scheduled screen or between screens. The results were also similar after exclusion of case subjects whose diagnosis of breast cancer occurred within 1 year of their diagnosis of benign breast disease and after exclusion of subjects with DCIS.** ***Conclusions:*** **p53 protein accumulation, but not c-erbB-2 protein overexpression, appears to be associated with an increased risk of progression to breast cancer in women with benign breast disease. [J Natl Cancer Inst 1998;90: 1262–9]**

Evidence consistent with the multistage model of carcinogenesis has come from the genetic analysis of human tumors *(1)*. Although cross-sectional in design, these studies have suggested that cancers showing multiple genetic abnormalities evolve from earlier morphologic stages showing only a subset of the same abnormalities *(2,3)*. For breast cancer, the relevant histologic stages are unknown, although there is some suggestion that proliferative disease without atypia progresses to atypical ductal hyperplasia and then to ductal carcinoma *in situ* (DCIS) and invasive cancer *(4)*. The accompanying sequence of molecular changes is not well characterized; however, of the many candidate early-molecular events, there is some evidence that changes in c-erbB-2 and p53 protein expression might be relevant to breast cancer progression. This hypothesis is based on the high frequency of alteration of c-erbB-2 and p53 in invasive breast cancers *(5)*, detection of c-erbB-2 *(6–9)* and p53 *(10–17)* proteins in benign breast disease, and animal models that suggest a role for these genes in the early stages of breast tumorigenesis *(18,19)*. If this presumption is correct, individuals identified with these molecular changes in benign breast tissue might be at increased risk of progression to breast cancer. In the cohort study reported here, we investigated this possibility by studying the association between c-erbB-2 protein overexpression and p53 protein accumulation detected in benign breast tissue and the risk of subsequent breast cancer.

Subjects and Methods

The investigation was undertaken as a case–control study nested within the cohort of 4888 women in the National Breast Screening Study (NBSS) who received a histopathologic diagnosis of benign breast disease during the active follow-up phase of the NBSS.

The National Breast Screening Study

The NBSS, which has been described in detail elsewhere *(20,21)*, is a multicenter randomized, controlled trial of screening for breast cancer in 89 835 Canadian women who were recruited during the period from 1980 to 1985 and who were followed actively until 1988 *(21)*. Women were eligible to participate if they were 40–59 years old and had no history of breast cancer (*in situ* or invasive). Those who were 40–49 years old at recruitment were randomly assigned to receive either an annual mammogram and physical examination or usual care after an initial physical examination, whereas those who were 50–59 years old at recruitment were randomly assigned to receive either an annual mammogram and physical examination or an annual physical examination only.

The NBSS was approved by the appropriate ethics committees, and the study described here involved the analysis of material and data from that study in accordance with the approved study design.

**Affiliations of authors:* T. E. Rohan, A. B. Miller, Department of Public Health Sciences, University of Toronto, ON, Canada; W. Hartwick, R. A. Kandel, Department of Pathology and Laboratory Medicine, Mt. Sinai Hospital, and Department of Laboratory Medicine and Pathobiology, University of Toronto.

Correspondence to: Thomas E. Rohan, M.B., B.S., Ph.D., Department of Public Health Sciences, University of Toronto, McMurrich Bldg., 3rd Floor, 12 Queen's Park Crescent West, Toronto, ON M5S 1A8, Canada (e-mail: tom.rohan@utoronto.ca).

See "Notes" following "References."

Diagnosis of Breast Disease in the NBSS

When there was clinical or radiologic evidence of a lesion, patients underwent either a needle aspiration or a biopsy. In the NBSS, each histologic diagnosis was reviewed for study purposes by a reference pathologist. Our study was restricted to subjects who had no evidence of breast cancer (*in situ* or invasive) on their initial surgical biopsy as determined on review by the reference pathologist. Women with a history of benign breast disease were not excluded from participation.

Incident cases of breast cancer were ascertained by record linkage with the provincial cancer registries, and death clearance was performed by linkage to the Canadian National Mortality Database *(21)*. The dates of the linkages varied by province, ranging from late 1988 to early 1991.

Definition of Case Subjects

Case subjects were the 92 women who had a histologic diagnosis of benign breast disease made by a reference pathologist during the active follow-up phase of the NBSS and who subsequently developed breast cancer. (The median interval between diagnosis of benign breast disease and subsequent breast cancer was 767 days.) For the purpose of this study, cancer was defined as any form of breast carcinoma; there were 16 case subjects with DCIS and 76 case subjects with invasive carcinoma.

Definition and Selection of Control Subjects

Control subjects were women with benign breast disease who had not developed breast cancer by (but were alive at) the date of diagnosis of the corresponding case subject. Five control subjects were selected randomly for each case subject from those non-case subjects available within the strata defined by screening center, NBSS study arm, year of birth, and age at diagnosis of benign breast disease. (Implicitly, therefore, control subjects were matched to case subjects on the interval between the date of diagnosis of benign breast disease and the date of diagnosis of breast cancer in the corresponding case subject.)

Questionnaire

At the time of their enrollment in the NBSS, all participants completed a questionnaire that sought identifying information, as well as data on potential breast cancer risk factors, including demographic characteristics, family history of breast cancer, and menstrual and reproductive histories.

Histopathology Review

For this study, hospitals and clinics storing the paraffin-embedded blocks of benign and malignant tissues were contacted and asked to review the histology, to send one representative block per lesion, and to indicate fixative type and whether the tissue had been frozen before fixation. Blocks or sections of paraffin-embedded benign tissue were obtained for 74 (80.4%) of the 92 case subjects and for 349 (75.9%) of the 460 control subjects; blocks or sections of malignant tissue were obtained for 62 (83.8%) of the 74 case subjects. Sections from the blocks received were reviewed and classified according to the criteria developed by Page and Anderson *(22)* and without knowledge of the case–control status of the study subjects.

p53 and c-erbB-2 Immunostaining

Sections (5 μm) were cut from the paraffin blocks, mounted on slides coated with aminopropyltriethoxysilane (2%; Sigma Chemical Co., St. Louis, MO), and deparaffinized. The sections for p53 immunostaining underwent antigen retrieval (microwaved in 10 m*M* citrate buffer [pH 6.0] for 15 minutes at a medium-high setting). In all sections, the endogenous peroxidase was inactivated with the use of 3% hydrogen peroxide, and the sections were blocked with normal goat serum (20 μL/mL; Vector Laboratories, Inc., Burlingame, CA) containing 5% crystallized bovine serum albumin (BDH Laboratory Supplies, Poole, U.K.) in phosphate-buffered saline (PBS). The sections were incubated overnight at 4 °C with antibody reactive with p53 (DO-7, monoclonal, dilution 1 : 40; Novocastra Laboratories, Newcastle Upon Tyne, U.K.) or c-erbB-2 (NCL-CB11, dilution 1 : 160; Novocastra Laboratories) *(23)*. After being washed, the sections were incubated with biotinylated goat anti-mouse immunoglobulin G (dilution 1 : 200; Vector Laboratories, Inc.) for 30 minutes at room temperature (21 °C), followed by incubation with avidin–biotin peroxidase complex (Vectastain Elite ABC Kit; Vector Laboratories, Inc.). Immunoreactivity was visualized with 3′,3-diaminobenzidine tetrahydrochloride (Vector Laboratories, Inc.), and the sections were counterstained briefly with hematoxylin. For p53, the positive controls were sections from a paraffin-embedded breast cancer that was known to have a p53 mutation associated with p53 protein accumulation. Positive controls for c-erbB-2 were sections of SK-Br-3 cells, a human breast cancer cell line that overexpresses c-erbB-2 *(24)* and that had been processed as a cell block in paraffin, and sections of a paraffin-embedded breast cancer known to overexpress c-erbB-2. The negative controls consisted of replacement of the primary antibody either with PBS or with mouse nonimmune serum.

For p53, any nuclear staining was considered a positive reaction, and cytoplasmic staining was considered nonspecific and interpreted as negative. For c-erbB-2, any staining—cytoplasmic, cytoplasmic membranous, or membranous—was considered positive. Although the protein is predominantly membrane bound, the significance of cytoplasmic staining is not entirely clear *(24–27)*. For this reason, any staining was accepted as positive, but the localization of the staining was noted. The percentage of immunopositive cells was estimated and categorized into one of two groups: less than 10% or 10% or greater of all epithelial cells. Staining was considered localized if one duct with its associated ductules/lobules showed immunopositivity; all other positive immunostaining was considered diffuse. The slides were reviewed without knowledge of the case–control status of the study subjects.

The immunostaining was performed on the benign tissue of 383 subjects only (74 case subjects and 309 control subjects), since 40 control subjects were eliminated because tissue for the corresponding case subject was not received. For 21 of the 383 subjects (three case subjects and 18 control subjects), there was no breast epithelium in the tissue sections, rendering them unsuitable for immunohistochemical analysis. For an additional three control subjects, there was no epithelium in the p53-immunostained sections only.

For 30 benign and 18 malignant, randomly selected biopsy specimens, the slides were reread by the same reviewer without knowledge of the results of the first reading. For both c-erbB-2 and p53, there was agreement of about 93% for the presence or absence of these changes in the benign specimens. The corresponding values of κ [a measure of agreement beyond chance *(28)*] were 0.72 and 0.64, respectively, indicative of substantial agreement between readings *(29)*. For the cancers, there was 89% agreement between readings for c-erbB-2, with a κ of 0.74; for p53, there was 100% agreement, with a κ of 1.00.

Statistical Analysis

Odds ratios (ORs) and 95% confidence intervals (CIs) for the associations among c-erbB-2 protein overexpression, p53 protein accumulation, and risk of breast cancer were obtained from conditional logistic regression models *(30)*. Adjusted OR estimates were obtained by including terms representing the following potential confounders in the regression models: history of breast cancer in a first-degree relative, age at menarche, age at first live birth, menopausal status (premenopausal, perimenopausal, or postmenopausal), body mass index [weight (kg)/height $(m)^2$], and hyperplasia (ductal or lobular, with or without atypia). Women who reported having had a menstrual period within the last year were defined as premenopausal, as were those who had had a hysterectomy without bilateral oophorectomy and were less than 45 years of age; those who had ceased having menstrual periods within the last 12 months without surgical intervention were defined as postmenopausal, as were those who had had a bilateral oophorectomy and those who had had a hysterectomy only and were more than 55 years of age; the remaining women were classified as perimenopausal. For categorical variables, tests for trend (on 1 degree of freedom) in associations were performed by fitting the categorized variables as continuous variables in conditional logistic regression models. Further analyses involved within-individual comparisons of c-erbB-2 and p53 in benign breast disease and breast cancer. All statistical tests were two-sided.

Results

A summary of the numbers of paraffin blocks requested and obtained and of the subjects included in the analyses is presented in Table 1. Comparisons of those subjects for whom benign tissue was and was not obtained revealed few differences between them in their distributions by breast cancer risk factors (data not shown); similarly, there was little difference between control subjects for whom blocks were received for the corresponding case subjects and those for whom they were not.

Table 1. Summary of number of biopsy specimens sought, obtained, and analyzed

	Benign tissue		
	Case subjects	Control subjects	Malignant tissue
No. of blocks requested	92	460	92
No. of blocks or sections obtained	74	349	62
No. of sections immunostained	74	309	62
No. of immunostained sections suitable for analysis*			
c-erbB-2	71	291	52
p53	71	288	53

*These numbers are less than those for whom immunostaining was performed because there was no breast epithelium in the tissue sections for three case subjects and 18 control subjects for both c-erbB-2 and p53 and for an additional three control subjects for p53 only. The number of case subjects and control subjects included in the matched analyses was less than the number with immunostained sections suitable for analysis because one case subject was in a stratum with no control subjects and 17 control subjects were in strata with no corresponding case subjects.

Table 2 shows the risk of breast cancer in relation to several factors for those subjects in whom immunostaining was performed. None of the variables was strongly associated with altered risk, but there was some suggestion of a reduction in risk with a family history of breast cancer, a relatively late age at menarche, a history of a live birth (regardless of the age at live birth), and postmenopausal status. There was also a small increase in risk with hyperplasia in the benign tissue. (For this analysis, the one control subject with lobular hyperplasia and the three control subjects with atypical ductal hyperplasia were combined with those with ductal hyperplasia without atypia; there were no case subjects with atypical hyperplasia.) When the associations shown in Table 2 were examined in all 552 subjects, they were largely unchanged, but there was less evidence for a reduction in risk with a family history of breast cancer (OR = 0.97; 95% CI = 0.55–1.73).

The tissue from nine case subjects and 40 control subjects showed immunostaining for c-erbB-2 (Fig. 1). There was a small decrease in the risk of progression from benign breast disease to breast cancer in association with c-erbB-2 protein overexpression, although the CIs included one (Table 3). ORs differed little according to the extent, location (cytoplasmic or membranous, with or without cytoplasmic staining), or distribution of immunostaining. For those subjects with hyperplasia and with c-erbB-2 immunostaining anywhere in their biopsy, the unadjusted OR was 1.13 (95% CI = 0.43–2.96) and the adjusted OR was 1.00 (95% CI = 0.34–2.97).

The tissue from 10 case subjects and 19 control subjects showed immunostaining for p53 (Fig. 2). After adjustment for breast cancer risk factors, there was a 2.5-fold increase in risk of breast cancer in association with p53 protein accumulation (Table 4). The increase in risk was evident when less than 10% of cells were immunopositive but was even greater when 10% or more were immunopositive. ORs for localized immunostaining and diffuse immunostaining were similar, but the risk was higher in those subjects where staining for p53 was observed in the same area as that for c-erbB-2 than in those subjects where it was not. In the presence of hyperplasia, p53 immunostaining anywhere in the biopsy specimen was associated with an approximately fourfold increase in risk (unadjusted OR = 4.62 [95% CI = 1.02–20.94]; adjusted OR = 3.87 [95% CI = 0.72–20.64]).

The associations for c-erbB-2 and p53 were similar after ex-

Table 2. Risk of breast cancer in association with family history of breast cancer, menstrual and reproductive characteristics, Quetelet's index, and benign breast disease histology in those in whom immunostaining was performed

Factor	Level	No. of case subjects*	No. of control subjects	OR (95% CI)†
Breast cancer in first-degree relative	No‡	61	242	1
	Yes	10	49	0.77 (0.37–1.62)
Age at menarche, y	≤12‡	32	107	1
	13	18	96	0.59 (0.31–1.13)
	≥14	20	87	0.68 (0.37–1.26)
	Two-sided *P* for trend§			.18
Age at first live birth, y	Never had‡	13	31	1
	≤22	23	99	0.46 (0.19–1.08)
	23–26	23	91	0.53 (0.23–1.22)
	≥27	12	70	0.37 (0.15–0.93)
	Two-sided *P* for trend§			.663
Menopausal status	Premenopausal‡	35	132	1
	Perimenopausal	12	56	0.74 (0.34–1.62)
	Postmenopausal	24	103	.68 (0.25–1.80)
Quetelet's index, kg/m^2	<22.5‡	24	88	1
	22.5–26.2	26	98	1.14 (0.54–2.40)
	>26.2	21	104	0.91 (0.37–2.22)
	Two-sided *P* for trend§			.807
Hyperplasia in benign tissue	Absent‡	42	185	1
	Present	29	105	1.22 (0.72–2.06)

*Unmatched distributions (matched ORs cannot be calculated directly from these numbers); age at menarche was missing for one case subject and one control subject, Quetelet's index was missing for one control subject, and hyperplasia could not be assessed for one control subject for whom tissue was received.

†OR = odds ratio; CI = confidence interval.

‡Reference category.

§*See* "Statistical Analysis" in "Subjects and Methods" section.

Fig. 1. Breast duct showing epithelial hyperplasia with cytoplasmic and membranous immunostaining (arrows) for c-erbB-2 (hematoxylin counterstain, original magnification ×500).

clusion of the 19 case subjects (and their matched control subjects), whose diagnosis of breast cancer was made within 1 year of their diagnosis of benign breast disease. When the analyses were restricted to the matched case–control sets containing case subjects with invasive breast cancer (i.e., after exclusion of the 14 case subjects with DCIS and their matched control subjects), the unadjusted OR for c-erbB-2 immunopositivity was 0.68 (95% CI = 0.24–1.88) and the adjusted OR was 0.60 (95% CI = 0.21–1.73), whereas the unadjusted and adjusted ORs for p53 were 2.85 (95% CI = 1.04–7.82) and 2.51 (95% CI = 0.88–7.21), respectively; with the exception of the unadjusted OR for p53, these ORs were not statistically significant. Also, when the 23 case subjects whose benign and malignant lesions occurred in opposite breasts were excluded, the unadjusted ORs for c-erbB-2 and p53 immunopositivity were 0.90 (95% CI = 0.35–2.27) and 2.11 (95% CI = 0.73–6.06), respectively. The corresponding adjusted ORs were 0.77 (95% CI = 0.29–2.04) and 1.93 (95% CI = 0.61–6.07), respectively. There was no evidence that the patterns for c-erbB-2 and p53 immunostaining differed among the strata defined by age, menopausal status, NBSS study arm, history of breast disease, and whether the benign breast disease was screen detected or interval detected.

When the analyses for c-erbB-2 and p53 were repeated according to whether individuals showed c-erbB-2 protein overexpression or p53 protein accumulation alone or in combination, the unadjusted ORs relative to those for individuals who were negative on both were 0.56 (95% CI = 0.20–1.56) for c-erb B-2 alone, 2.50 (95% CI = 0.86–7.27) for p53 alone, and 2.55 (95% CI = 0.55–11.76) for those who were immunopositive for both. The corresponding adjusted ORs were 0.51 (95% CI = 0.18–1.45), 2.43 (95% CI = 0.79–7.50), and 1.98 (95% CI = 0.40–9.90).

Table 5 shows the concordance between the immunohistochemical findings for the benign and malignant tissues for the case subjects. Of the 48 subjects who were negative for c-erbB-2 protein overexpression in their benign tissue samples, 15 showed evidence of overexpression in their malignant tissue samples; for p53 protein accumulation, the corresponding figure was 23% (10/44). Both of those subjects who showed evidence of c-erbB-2 protein overexpression in their benign tissue samples retained evidence of overexpression in their malignant tissue samples, whereas two subjects whose benign tissue samples showed p53 protein accumulation had p53-negative cancers.

Discussion

The results of this cohort study suggest that p53 protein accumulation as determined immunohistochemically in benign breast disease is associated with increased risk of progression to breast cancer; c-erbB-2 protein overexpression did not show this association. Since the patient characteristics were collected before 1986, there was insufficient information to determine whether any of the study subjects had Li–Fraumeni syndrome. However, it is unlikely that the presence of this syndrome accounts for our findings for p53 because the syndrome is uncom-

Table 3. Association betwen c-erbB-2 protein overexpression and risk of breast cancer

Aspect of staining	Level	No. of case subjects*	No. of control subjects	OR (95% CI)†	
				Unadjusted‡	Adjusted§
Presence	Absent‖	62	251	1	1
	Present	9	40	0.73 (0.31–1.68)	0.65 (0.27–1.53)
% cells immunopositive	Absent‖	62	251	1	1
	<10	5	20	0.76 (0.25–2.34)	0.74 (0.23–2.39)
	⩾10	4	20	0.69 (0.22–2.17)	0.57 (0.18–1.83)
Location	Absent‖	62	251	1	1
	Cytoplasm	6	26	0.74 (0.25–2.17)	0.59 (0.19–1.82)
	Membrane¶	3	14	0.71 (0.20–2.55)	0.73 (0.20–2.72)
Distribution	Absent‖	62	251	1	1
	Localized	6	23	0.80 (0.29–2.21)	0.74 (0.26–2.12)
	Diffuse	3	17	0.63 (0.17–2.26)	0.53 (0.14–1.96)

*Unmatched distributions (matched odds ratios cannot be calculated directly from these numbers).
†OR = odds ratio; CI = confidence interval.
‡Adjusted for matching factors only (with the use of conditional logistic regression).
§Adjusted for variables in Table 1.
‖Reference category.
¶Membranous staining only or membranous and cytoplasmic staining were combined because only one subject (a control) had purely membranous staining.

Fig. 2. Benign breast epithelium showing cells with nuclear immunoreactivity (arrows) indicative of p53 protein accumulation. Cells with no staining are also present (hematoxylin counterstain, original magnification ×500).

mon and immunostaining for p53 was confined to epithelial cells, which suggests that the abnormalities observed were somatic and not germline *(13)*. Furthermore, only one of the 10 p53-immunopositive case subjects had a family history of breast cancer in a first-degree relative.

There have been two previous follow-up studies of protein changes in benign breast disease. One *(16)* showed no association between p53 protein accumulation and the risk of breast cancer, whereas the other *(31)* suggested indirectly that p53 and c-erbB-2, as part of a group of biomarkers, might be associated with risk. However, both studies were small and adjusted for confounding either incompletely *(31)* or not at all *(16)*. Nevertheless, our findings are in keeping with those of Fabian et al. *(31)*.

It is possible that our findings reflect selection bias resulting from the study of surgical biopsies only and not fine-needle aspirates. However, it seems unlikely that the mode of diagnosis would have been related to exposure (protein status). Furthermore, it is unlikely that bias arose from the selection of subjects from the benign breast disease cohort, given that both case subjects and control subjects were sampled independently of exposure and that those for whom blocks of benign tissue were and were not obtained differed little with respect to other variables. We did observe that older blocks (i.e., those collected in the early years of the NBSS) were more likely to have been discarded, but selection bias resulting from a relationship between the year of collection and immunostaining status seems unlikely. Also, although it is possible that p53 and c-erbB-2 immunopositivity might be more likely in larger lesions and that the larger the benign lesion the greater the breast cancer risk, we observed no relationship between our success in obtaining paraffin blocks and the size of the lesions from which the paraffin blocks were obtained.

Table 4. Association between p53 protein accumulation and risk of breast cancer

Aspect of staining	Level	No. of case subjects*	No. of control subjects	OR (95% CI)†	
				Unadjusted‡	Adjusted§
Presence	Absent‖	61	269	1	1
	Present	10	19	2.79 (1.16–6.73)	2.55 (1.01–6.40)
% cells immunopositive	Absent‖	61	269	1	1
	<10	5	12	2.28 (0.74–7.04)	1.96 (0.60–6.47)
	⩾10	5	7	3.67 (1.03–13.04)	3.60 (0.96–13.49)
Distribution	Absent‖	61	269	1	1
	Localized	6	13	2.68 (0.86–8.32)	2.41 (0.73–7.93)
	Diffuse	4	6	2.96 (0.78–11.28)	2.76 (0.69–11.07)
Same area as c-erbB-2	Absent‖	61	269	1	1
	Not same	2	3	2.27 (0.37–13.90)	1.48 (0.22–9.81)
	Same	8	16	2.98 (1.08–8.18)	3.03 (1.05–8.72)

*Unmatched distributions (matched odds ratios cannot be calculated directly from these numbers).
†OR = odds ratio; CI = confidence interval.
‡Adjusted for matching factors only (with the use of conditional logistic regression).
§Adjusted for variables in Table 1.
‖Reference category.

Table 5. Concordance between immunostaining results for benign and malignant tissues*

Marker	% with negative immunostaining of benign and malignant tissues (No.)	% with negative immunostaining of benign tissue and positive immunostaining of malignant tissue (No.)	% with positive immunostaining of benign tissue and negative immunostaining of malignant tissue (No.)	% with positive immunostaining of benign and malignant tissues (No.)
c-erbB-2	66.0 (33)	30.0 (15)	0 (0)	4.0 (2)
p53	66.7 (34)	19.6 (10)	3.9 (2)	9.8 (5)

*Percentages are percent of all subjects for whom both benign and malignant tissues are available. Of the 62 cases for whom blocks of benign and malignant tissues were obtained, two blocks of benign tissue and 10 blocks of malignant tissue were unsuitable for analysis for c-erbB-2; for p53, the corresponding numbers were two and nine.

Biased assessment of the study exposures is an unlikely source of error, given that protein status of the benign breast tissue was determined without knowledge of the patient outcome (and the slides prepared from malignant tissue were read without knowledge of the findings from the benign tissue) and that the assessments were highly repeatable. Misclassification of the immunohistochemical staining results might have arisen from various sources. First, fixative type and/or duration may affect the immunoreactivity of the p53 and c-erbB-2 proteins *(32–34)*. However, there was little difference between case and control subjects in the distribution of their blocks by fixative type, and additional adjustment for type of fixative had little effect on the OR estimates for p53 and c-erbB-2. We were not able to control for the length of tissue fixation. Second, misclassification might have occurred also for the six case subjects and 14 control subjects for whom slides were obtained rather than paraffin blocks. For example, p53 immunoreactivity has been shown to decrease with the duration of storage of cut slides *(35)*. However, deletion of these case subjects from the analyses had little impact on the results, and it has been shown recently that antigen retrieval, which was used in the present study, diminishes the loss of immunoreactivity *(36)*. Third, it is possible that low levels of overexpression were not detected because of the limited sensitivity of the antibodies *(33,37)*. Fourth, underestimation of immunopositivity might have occurred because only one block per subject was examined. Given that the most likely effect of all of these parameters on marker status is to have induced false-negatives *(38)* and that any misclassification arising from these sources is likely to have been nondifferential, the estimates of association would have been biased conservatively.

Misclassification of the histopathology of the initial (benign) biopsy specimen may have occurred. For example, it is possible that some case subjects had cancers that were not sampled at the time of the initial biopsy or were not represented in the block examined in this study. We addressed these issues in part by excluding those case subjects whose cancer was diagnosed within 1 year of the biopsy for benign breast disease; in so doing, the results were similar to those overall.

Although DCIS is considered by some to be a precursor of invasive breast cancer *(39)*, we included case subjects with this diagnosis in the cancer group because the treatment (surgical resection and radiotherapy) of this condition is similar to that of invasive carcinoma *(40)*, and we could not exclude the possibility of cancer elsewhere in the breast. Further support for differentiating DCIS from benign states and for grouping it with invasive carcinoma comes from the observations that similar proportions of DCIS and invasive carcinoma show cyclin-D overexpression and that these proportions are higher than those for various grades of benign and hyperplastic human breast lesions *(41)*.

Confounding is a relatively unlikely explanation for the study findings, given that the ORs were adjusted for many potential confounding variables, although the possibility of residual confounding by these and other variables cannot be excluded. Nevertheless, since it is not known whether factors such as those adjusted for in the present analyses operate by altering the c-erbB-2 and p53 genes or their expression or whether they influence breast cancer risk by other means, it is not clear that the adjusted ORs are more appropriate than the unadjusted ones. In this regard, however, it should be noted that the adjusted and unadjusted associations were mostly quite similar.

The statistical power of our study was somewhat limited, given the relatively small number of individuals who had disease that progressed to breast cancer by the end of the follow-up period. Although to some extent this problem was compensated for by selecting multiple control subjects per case subject, the statistical power was compromised further by the fact that we did not obtain benign tissue for all of the potentially eligible study subjects. Indeed, given the number of study subjects included in the analyses and the observed proportion of control subjects whose tissues showed immunostaining for c-erbB-2, the statistical power of the study to detect an OR of 2 at the two-sided 5% significance level was about 58%. For p53, the corresponding statistical power was about 37%, given the observed proportion of control subjects whose tissues displayed p53 immunostaining. [These calculations were based on formulae for unmatched case–control studies, so that the power of our study was probably somewhat higher, given the matching *(42)*.] Furthermore, our study had little power to detect differences in the associations between strata defined by menopausal status and other factors.

c-erbB-2 is a normal cellular gene present on chromosome 17q21 *(43,44)*. It encodes a membrane protein (p185), which is tyrosine phosphorylated following interaction with its ligands *(43)*. Overexpression of c-erbB-2 occurs either through changes in amplification and/or through messenger RNA (mRNA) overexpression *(5,44,45)*. Its role in the development of human breast cancer is unknown *(46)*. In the present study, there was little alteration in risk with positive immunostaining overall or by location. These results were essentially unchanged when the analyses were repeated after those with cytoplasmic staining were reclassified as negative (unadjusted OR for positive immunostaining = 0.72 [95% CI = 0.20–2.58]; adjusted OR = 0.74 [95% CI = 0.20–2.76]). The lack of association of risk with cytoplasmic staining is consistent with studies showing that breast cancer cells with cytoplasmic staining are more differentiated than those with membranous staining *(47)*. Our results do not support a role for immunohistochemically detected c-erbB-2 protein overexpression at a relatively early stage of tumor formation. However, they do not preclude roles for c-erbB-2 gene amplification and/or mRNA overexpression or stabilization.

p53 is involved in regulating cell proliferation, inducing apoptosis, and promoting chromosomal stability. Disruption of these functions appears to have a pivotal role in carcinogenesis *(48)*. Our findings are in keeping with a role for p53 protein accumulation in breast cancer development. However, by using immunohistochemistry alone, we may have underestimated the true risk of developing breast cancer in association with p53 changes. For example, not all p53 mutations result in positive immunostaining *(38)*. Therefore, a more complete assessment of the role of p53 in influencing breast cancer risk will come from studies combining both immunohistochemistry and p53 gene sequencing, as well as from studies of other mechanisms by which p53 can be functionally inactivated.

To a large extent, the protein changes observed in the benign tissue were evident also in the corresponding cancers. This observation provides some support for the notion that these changes might occur at a relatively early stage in the disease

process, but it does not necessarily indicate that these changes influence the disease process. The fact that the cancers of two subjects showed evidence of apparent reversion of the p53 protein accumulation in their benign tissue could reflect methodologic problems related either to tissue fixation or to tumor sampling. However, there is evidence from cross-sectional studies for the disappearance from tumors of molecular changes present at an earlier stage. For example, high expression of the progesterone receptor has been observed in proliferative disease without atypia in the absence of expression in a concurrent DCIS *(49)*.

In conclusion, our results suggest that p53 protein accumulation in benign breast disease is associated with increased risk of subsequent breast cancer (DCIS or invasive carcinoma). Since the investigations reported here can be done on cytologic material obtained from fine-needle aspirates of the breast *(31)*, they could possibly be used as a screening tool in conjunction with mammography. In particular, for those women with benign breast disease who are identified as being at increased risk of progressing to breast cancer (on the basis of their status with respect to one or more of the molecular markers), closer follow-up and perhaps early intervention might be warranted.

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Notes

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Dr. Rita Kandel
Pathology and Laboratory Medicine
Mount Sinai Hospital, Ste. 600
600 University Avenue
Toronto, Ontario M5G 1X5
Canada

RE: MP98-258 "Cyclin D1 Gene Amplification and Protein Expression in Benign Breast Disease and Breast Carcinoma"

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CYCLIN D1 GENE AMPLIFICATION AND PROTEIN EXPRESSION IN BENIGN BREAST DISEASE AND BREAST CARCINOMA

Xin Li Zhu, Warren Hartwick, Tom Rohan,[1] and Rita Kandel

Department of Pathology & Laboratory Medicine, Mount Sinai Hospital and

Department of Public Health Sciences[1], University of Toronto, Toronto, Canada

Please address correspondence to: Dr. Rita Kandel
Pathology and Laboratory Medicine
Mount Sinai Hospital, Ste. 600
600 University Avenue
Toronto, Ontario M5G 1X5
Canada
Tel: (416) 586-8516
Fax: (416) 586-8628
Email: rkandel@mtsinai.on.ca

Running title: Cyclin D1 in Breast Disease

ABSTRACT

Cyclin D1 plays a critical role in regulating cell cycle progression. Gene amplification and protein overexpression of cyclin D1 have been detected in breast cancer but little is known about whether these changes occur in normal breast tissue and in breast lesions associated with increased risk of development of invasive breast cancer. We examined for cyclin D1 gene amplification and protein overexpression in 30 cases of benign breast disease (16 epithelial hyperplasias without atypia and 14 atypical ductal hyperplasias), and 18 cases of ductal carcinoma in situ (DCIS) using differential PCR and immunohistochemical staining. We compared their frequency to that in 15 cases of normal breast tissue and 17 cases of invasive ductal carcinoma. Cyclin D1 gene amplification was detected in 15% of those with normal breast tissue, 19% of those with epithelial hyperplasia without atypia, 27% those with atypical ductal hyperplasia, 35% of those with ductal carcinoma in situ, and 25% of those with invasive ductal carcinoma; corresponding figures for protein overexpression were 13%, 13%, 57%, 50%, and 64%, respectively. The results of this study suggest that cyclin D1 amplification and protein overexpression can occur before histological alterations are seen, but that the frequencies of these changes are higher in histological lesions with cellular atypia (atypical hyperplasia and DCIS) reaching frequencies similar to those observed in invasive carcinoma.

Keywords: differential PCR, immunohistochemistry, cyclin D1, benign breast disease

INTRODUCTION

Cancer has been defined as a proliferative disorder characterized by unregulated cell growth (1). Under normal conditions, progression through the cell cycle is orderly, and is regulated by cyclins and their associated cyclin-dependent kinases (cdk) (2,3). Two major checkpoints exist, one at the G_1-S interface and a second at the G_2-M interface. The first prevents replication of damaged DNA and the latter prevents segregation of structurally altered chromosomes (4). Disruption at either of these points may play a role in the pathogenesis of malignancy (1).

Cyclin D is involved in regulating cell cycle progression from G_1 into the S phase (1). There are three types of cyclin D, namely D1, D2 and D3, each having its own pattern of tissue-specific expression. These cyclins can form complexes with cdk4 or cdk6 which then phosphorylate the retinoblastoma (RB) protein and allow the cells to pass into the S phase. Cells which overexpress cyclin D1 show reduced exit from G_1 to G_0 suggesting a role for cyclin D1 at this regulatory point (5). Several observations have led to the suggestion that cyclin D1 amplification and/or overexpression are tumorigenic. Firstly, cell transformation results when cyclin D1 is transfected with the adenovirus E1A oncogene into BRK cells (6); secondly, rat fibroblasts transfected with cyclin D1 have a shortened G_1 phase and form tumors when injected into nude mice (7); and thirdly, mammary hyperplasia and breast cancer develop in transgenic mice which overexpress cyclin D1 (8). It has been suggested that cyclin D1 is not a dominant oncogene but requires the presence of other oncogenes to induce tumors (9). Other investigators have suggested that cyclin D1 overexpression enhances gene amplification and may contribute to

genomic instability (10).

Cyclin D1 has been studied extensively in breast cancer. Cyclin D1 amplification has been observed in up to 33% of breast cancer cell lines and between 11 and 23% of human breast cancers (11-16). Cyclin D1 accumulation as detected immunohistochemically occurs in up to 81% of breast cancers, although the frequency appears to be dependent on the antibody used (11,12,16). However, there have been relatively few studies of cyclin D1 in normal breast tissue and in breast lesions associated with increased risk of developing invasive breast cancer. Immunohistochemical studies have shown that normal human breast epithelium and breast tissue adjacent to breast cancers demonstrate, at most, occasional cells which express cyclin D1 protein (14,17,18). An in-situ hybridization study demonstrated that 18% of benign breast lesions showed cyclin D1 mRNA overexpression (19). Cyclin D1 gene amplification and overexpression as well as protein accumulation also occur in ductal carcinoma in situ (DCIS) (20).

The human and experimental data suggest that cyclin D1 amplification and/or protein overexpression might have a role not only in breast cancer but also in the putative early stages of breast neoplasia such as epithelial hyperplasia, a histopathological change known to be associated with increased risk of progression to breast cancer (21-26). In this study, we explored the occurrence of cyclin D1 protein expression and gene amplification in a series of normal breast tissue samples, cases with benign breast disease, DCIS, and invasive ductal carcinoma.

MATERIAL AND METHODS

Tissues and Cell Lines

Representative cases of normal breast tissue (15 cases), benign breast disease (16 cases

of epithelial hyperplasia without atypia and 14 cases of atypical ductal hyperplasia), DCIS (18 cases) and breast carcinomas (17 cases) were identified by searching the files of the Department of Pathology at Mount Sinai Hospital for the period from 1990 to 1997. The breast tissue had been fixed in 10% neutral buffered formalin and embedded in paraffin. The hematoxylin and eosin stained sections were reviewed by two of the authors (RK and WH) and classified using the criteria described by Page et al. (27) and the recent consensus conference (28).

The human breast carcinoma derived cell lines; a) ZR-75-1 and MDA-MB-453 which have two to five-fold amplification of cyclin D1 (14), b) MCF-7, MDA-MB-468, and MDA-MB-231, which have no cyclin D1 amplification (13,14), and c) T47D, which shows cyclin D1 overexpression immunohistochemically (14) were obtained from the American Type Culture Collection (ATCC). The cells were grown in culture, harvested using trypsin - EDTA (Sigma Chemical Co., St. Louis, MO), and centrifuged to form pellets. The cell pellets were placed in 3% bacto-agar (Difco Laboratories,Detroit, MI), fixed in 10% buffered formalin and then embedded in paraffin. 5 μm thick sections were cut and used as controls for polymerase chain reaction (PCR) and/or immunostaining.

Microdissection of Tissue

Sections were cut from the paraffin blocks using standard precautions to avoid cross contamination of tissue between cases. This included cutting one case at a time, changing microtome blades between cases, floating the section in its own water bath, and cleaning the work areas of the microtome with xylene between blocks. The sections were dried at 37°C overnight and then deparaffinized. The sections were stained with hematoxylin for 30 seconds,

and the pathological area was dissected out using a dissecting light microscope (Laborlux 6000, Leica, Canada). For the normal breast tissue sections, random ducts and/or lobules were microdissected. 4 µl of mineral oil (Sigma Chemical Co, St Louis, MO) was placed on the microdissected tissue which was then transferred to a microfuge tube in a pipette tip.

DNA Extraction

Genomic DNA was extracted as described by Zhuang (29) with some modifications. Briefly, the microdissected tissue was incubated in 50 µl buffer (50 mM Tris-HCl, pH 8.5, 1 mM ethylenediamine tetracetic acid, 0.5% Tween 20) containing 0.5 mg/ml of proteinase K (Sigma Chemical Co, St. Louis, MO) at 50°C for 24 hours. The proteinase K was then inactivated by boiling at 95°C for 15 minutes.

Cyclin D1 Amplification

Semiquantitative differential polymerase chain reaction was used to determine the presence of cyclin D1 gene amplification and to estimate its extent. As fragmented genomic DNA (<200 bp) may influence the results of differential PCR, γ- interferon (γ-IFN) was analyzed in a multiplex PCR reaction in order to indirectly assess DNA quality first (30, 31). Two sets of primers (see table 1 for the sequences), specific for different exons of γ-IFN gene and which generate PCR products of 150 and 82 bp (γ-IFN 150 and γ-IFN 82) were co-amplified in the same reaction tube as described previously (31). If the γ-IFN82/γ-IFN150 ratio of the PCR products was 3 or less, the tissue was considered suitable for further analysis (31). For such cases, aliquots of the proteinase K digested tissue were then examined for cyclin D1

amplification using PCR. Both asparagine synthetase (Asp) and cyclin D1 (see table 1 for primer sequences) were co-amplified in the same reaction tube. Asp is a housekeeping gene and served as an internal control. The PCR was performed in a total volume of 15 µl. Briefly, 1 µl of the digest was mixed with 14 µl of PCR working solution containing 10 mM Tris HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 100 µM of each dNTP, 1 U of AmpliTaq DNA polymerase (Roche Diagnostic Systems Inc., Branchburg, NJ) and 1 µM of each primer. The samples underwent 30 cycles of amplification in an automated thermocycler (DNA Thermal Cycler, Perkin Elmer, Branchburg, NJ). Each cycle consisted of 1.2 minutes of denaturation at 94°C (except for the first cycle which was 10 minutes in length), 1 minute of annealing at 55°C, and 1 minute of elongation at 72°C. The PCR products were separated by electrophoresis on a 12% polyacrylamide gel at 200V for 2 hours and visualized following ethidium bromide staining. Each tissue was analyzed at least twice in separate polymerase chain reactions. Direct sequencing of PCR products of one control and case were done using the initial sense primers and the Thermo Sequenase radiolabelled terminator cycle sequencing kit (Amersham Life Sciences, Cleveland, Ohio, USA).

Semiquantification of Cyclin D1 Amplification

To determine whether there was cyclin D1 gene amplification, the ratio of the cyclin D1 PCR product to the Asp PCR product was derived from photographic negatives of ethidium bromide stained gels which were quantified by laser densitometry (Computing Densitometer Model 300A, Molecular Dynamics, Sunnyvale, CA). There were at least two gels per sample and each gel was scanned three times. A mean ratio of cyclin D1 to Asp was determined and a ratio

of greater than 1.5 was considered indicative of gene amplification (11,32). The amount of cyclin D1 gene amplification was categorized as + for a ratio of >1.5 to ≤2.5, ++ for a ratio of >2.5 to ≤3.5 and +++ for a ratio of >3.5.

Cyclin D1 Immunostaining

Tissue sections were placed on aminopropyltriethoxysilane (2%, Sigma Chemical Co. MO, USA) coated slides and deparaffinized. The tissue underwent antigen retrieval (microwave pretreatment in 10 mM citrate buffer, pH 6.0, for 15 minutes at a medium high setting). The endogenous peroxidase was inactivated using 3% hydrogen peroxide, and the sections were blocked with normal horse serum (20 µl/ml, Vector Laboratories, Burlingame, CA) containing 5% bovine serum albumin (BDH Laboratory, Poole, England) in TBS buffer (5 mM Tris-HCl, pH 7.6, 0.85% NaCl). The sections were incubated with antibody reactive with cyclin D1 protein (monoclonal, dilution 1:2000, Upstate Biotechnology, Lake Placid, NY) overnight at 4°C. After washing, the sections were incubated with biotinylated anti-mouse IgG (dilution 1:200, Vector Laboratories) for 30 minutes at room temperature, followed by avidin-biotin peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories). Immunoreactivity was visualized with 3',3-diaminobenzidine tetrahydrochloride (DAB, Vector Laboratories) and the sections counterstained briefly with hematoxylin. T47D cells which had been embedded in paraffin served as the positive control (14). The negative control consisted of replacing the primary antibody with TBS or non-immune mouse serum (DAKO Corp, Carpinteria, CA). Distinct nuclear staining indicated a positive reaction and cytoplasmic staining was considered nonspecific and interpreted as negative. In normal tissue, the presence of staining in any of the epithelium was considered

positive. In benign breast disease or cancer, only immunoreactivity in the pathological area was interpreted as positive. The staining was assessed as + when only occasional cells were positive, ++ when clusters of cells were positive and +++ when there was diffuse staining throughout the pathological area.

RESULTS

Semiquantification of Cyclin D1 Amplification

The differential PCR assay was assessed for sensitivity and reproducibility using the cell lines ZR-75-1, MDA-MB-453, MCF-7, MDA-MB-468 and MDA-MB-231. The cyclin D1/Asp ratio in the ZR-75-1 and MDA-MB453 cell lines, which by Southern blot analysis have been shown to have a two to five-fold amplification of cyclin D1, was always greater than 1.5. A ratio as high as 5.4 was obtained in some PCR runs. The other cell lines, which are not amplified for cyclin D1, had cyclin D1/Asp ratios of less than 1.5 in all runs. These results suggest that this method is appropriate for determining whether the cyclin D1 gene is amplified and sufficiently sensitive to detect two-fold gene amplification.

As shown in Figure 1, a 149 bp product consistent with cyclin D1 was detected in 13 normals, 16 hyperplasias, 11 atypical hyperplasias, 17 DCIS and 16 carcinomas. PCR product sequencing was done for one control (ZR-75-1) and one case (C8) which confirmed that the product obtained was cyclin D1. Two normals (N14 and N15), 3 atypical hyperplasias (A12, A13 and A14), 1 DCIS (D18), and 1 invasive carcinoma (C17) exhibited poor DNA quality as no PCR products for either γ-IFN82 or γ-IFN150 were detected (results not shown). As detailed in tables 2 and 3, amplification was detected in two of 13 cases (15%) of normal breast tissue,

but in both of these cases the level of amplification was low. Three of 16 cases (19%) of epithelial hyperplasia without atypia, three of 11 cases (27%) of atypical ductal hyperplasia, six of 17 cases (35%) of DCIS, and four of 16 cases (25%) of cancer showed gene amplification. The relative amount of amplification showed no correlation with the histologic changes, as only low levels of gene amplification were detected in breast cancer. Of the DCIS cases, one of four low grade tumors, three of six intermediate grade and two of seven high grade tumors showed amplification.

Cyclin D1 Protein Overexpression

Protein overexpression was determined by immunohistochemical staining (Fig. 2). Cyclin D1 immunoreactivity was observed in two cases of normal breast tissue (13%) [Tables 2 & 3] and two cases of epithelial hyperplasia without atypia (13%). The proportion of cases showing protein accumulation was higher in the atypical hyperplasias (57%), in the DCIS cases (50%), and in the invasive cancers (64%) than in the cases of normal breast tissue and epithelial hyperplasia without atypia. In the normal breast tissue, only occasional cells were positive. The intensity and extent of immunostaining was more often greater in the cancers. Nonspecific cytoplasmic staining of epithelial cells, nerves, and/or blood vessels was seen in some sections but this was easily distinguished from the nuclear staining indicative of protein overexpression.

Association of Gene Amplification and Protein Overexpression

Gene amplification occurred in the absence of protein overexpression and vice versa (Table 3). One of the 13 normal breast tissue samples had both gene amplification and protein overexpression, whereas one of the 16 epithelial hyperplasias without atypia, two of the 11

atypical ductal hyperplasias, four of the 17 cases of DCIS, and two of the 16 cancers had both of these changes.

DISCUSSION

This study demonstrated that cyclin D1 amplification and protein overexpression occur in normal tissue, breast tissue associated with increased breast cancer risk, and breast cancer. The frequencies of these changes were similar in normal tissue and epithelial hyperplasia without atypia, but were higher in breast tissue showing atypical ductal hyperplasia and DCIS, reaching frequencies similiar to those observed in invasive carcinoma.

Experimental studies, in transgenic mice, have shown that overexpression of cyclin D1 is associated with development of both hyperplasias and carcinomas (8). Our findings are in keeping with those results.

There have been two other studies examining cyclin D1 in benign breast disease in humans (19, 33). Millikan et al. (33), using differential PCR, did not demonstrate cyclin D1 amplification in any of 60 subjects selected from a cohort of women with benign breast disease. There are two possible explanations for this discrepancy with our results. Firstly, in their series, there were only 10 cases of epithelial hyperplasia and one case of atypical ductal hyperplasia. Secondly, in our study only tissue showing the specific pathological change underwent molecular analysis, whereas in the study of Millikan et al., localized molecular analysis was not performed. This could potentially have decreased the sensitivity of their differential PCR, as the pathological cells may have been diluted by non-contributory cells such as stromal, endothelial,and inflammatory cells. In the other report, Weinstat-Saslow et al. (19) examined

cyclin D1 mRNA expression. It is not known whether cyclin D1 mRNA overexpression is the result of gene amplification and so their results are not directly comparable to those of this study. However, the investigators were able to demonstrate that cyclin D1 mRNA overexpression occurs in hyperplasias with or without atypia, and that there was a higher frequency of overexpression in DCIS and invasive cancer than in the hyperplasias.

Cyclin D1 protein expression in breast cancer cells, as detected by immunostaining, has been reported in 28-81% of cases (11,12,16,34-37). Our findings are within this range, as 64% of our cases showed protein expression. There has been one previous study examining cyclin D1 immunopositivity in DCIS and, as did our study, it showed that 50% of cases were immunopositive (20). As well, similiar to our findings, Bartkova et al. (17) showed cyclin D1 immunopositivity in occasional cells in normal breast epithelium. We observed protein overexpression in the absence of gene amplification, suggesting that other mechanisms, most likely post-transcriptional, play a role in cyclin D1 protein overexpression. Conversely, it is not evident why protein accumulation was not detected immunohistochemically in all cases with gene amplification. Others have also observed similar discordances between gene amplification and protein overexpression (16,20). Possible explanations include changes in protein and mRNA stability, increased transcriptional rate, and method and/or antibody insensitivity.

The fact that cyclin D1 amplification and protein overexpression have been detected in normal breast tissue suggests that molecular and protein changes may occur prior to the development of histological changes such as hyperplasia. It is possible that the presence of amplification was an artefact of the methodology or indicative of undetected aneuploidy.

However, these are considered unlikely as there have been several reports describing molecular and protein changes in apparently normal breast tissue (38-40), and the frequency of cyclin D1 gene amplification in invasive carcinomas in this series was similiar to that detected by others using Southern blot analysis which is the standard methodology (11,37).

Cyclin D1 changes were also detected in cases of hyperplasia without atypia, a histological change associated with increased risk of developing breast cancer. The frequency of these alterations was higher in the presence of cellular atypia, although the number of cases was small. As hyperplastic change is only associated with a 2 to 4 fold increase in risk depending on the degree of atypia, it is possible that detection of cyclin D1 gene amplification and/or protein overexpression might enhance our ability to predict breast cancer risk. This is of particular interest as the interobserver variability in the histological diagnosis of atypical hyperplasia has led to questions as to its usefulness as a marker of risk (41).

The role of cyclin D1 in the pathogenesis of breast cancer has not been fully delineated despite intensive study. The low frequencies of gene amplification and protein overexpression in breast tissue showing no or minimal increased risk for breast cancer development as compared to those in breast tissue with a higher risk, suggest that changes in the cyclin D1 gene and/or protein expression may play a role in malignant transformation. However it is possible that any such changes do not contribute directly to the malignant transformation of a cell but rather result in a phenotype that favors or allows the critical alterations to occur (42). For example Zhou et al (10) demonstrated that cyclin D1 overexpression in a transfected rat liver epithelial cell line resulted in increased number of cells with CAD gene amplification. Nevertheless, a better assessment of

the predictive significance of cyclin D1 changes in women with benign breast disease will come from prospective studies in which women with benign breast lesions are followed for the subsequent development of breast cancer. Studies of this type might also help determine which of the presence or absence of gene amplification, the amount of gene amplification, and/or protein overexpression, is the best predictor of risk of progression to breast cancer.

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FIGURE LEGENDS

Figure 1

Ethidium bromide stained gel showing PCR products from normal breast tissue (N1-N13), cases of epithelial hyperplasia without atypia (H1-H16), atypical ductal hyperplasia (A1-A11), DCIS (D1-D17), invasive ductal carcinoma (C1-C16) and controls. The PCR products for cyclin D1 (D1) and asparagine synthestase (Asp) are indicated. The M denotes 123 bp DNA ladder. The ZR, MCF and 453 represents human breast carcinoma derived cell lines ZR-75-1, MCF-7 and MDA-MB-453 respectively, which were the controls.

Figure 2

Immunohistochemical detection of cyclin D1 protein in (A) a case of florid epithelial hyperplasia without atypia and (B) a case of invasive ductal carcinoma. Positive nuclear staining is present (DAB with hematoxylin counterstain, magnification x 400).

TABLE 1

SEQUENCES OF PCR PRIMERS

Gene	Sequences	Sequence Region	Reference
γ-IFN82	sense, 5'-GCAGAGCCAAATTGTCTCCT-3'	(nt 2012-2031)	31
γ-IFN82	antisense, 5'-GGTCTCCACACTCTTTTGGA-3'	(nt 2074-2093)	31
γ-IFN150	sense, 5'-TCTTTTCTTTCCCGATAGGT-3'	(nt 4582-4601)	31
γ-IFN150	antisense, 5'-CTGGGATGCTCTTCGACCTC-3'	(nt 4712-4731)	31
cyclin D1	sense, 5'-ATGTGAAGTTCATTTCCAAT-3'	(nt 722-741)	32
cyclin D1	antisense, 5'-TGGGTCACACTTGATCACTC-3'	(nt 851-870)	32
asparagine synthetase	sense, 5'ACATTGAAGCACTCCGCGAC-3'	(nt 496-515)	43
asparagine synthetase	antisense, 5'-CCACATTGTCATAGAGGGCG-3'	(nt 639-658)	43

TABLE 2

CYCLIN D1 AMPLIFICATION AND PROTEIN OVEREXPRESSION IN NORMAL TISSUE, BENIGN BREAST DISEASE AND BREAST CARCINOMA

Normal Breast			Hyperplasia Without Atypia			Atypical Ductal Hyperplasia			Ductal Carcinoma in Situ			Invasive Ductal Carcinoma		
No.	AMP[a]	IHC[b]	No.	AMP	IHC	No.	AMP	IHC	No.	AMP	IHC	No.	AMP	IHC
N1	-	-	H1	-	-	A1	+++	++	D1	-	-	C1	-	+
N2	-	-	H2	++	-	A2	-	++	D2	+++	+	C2	+	+
N3	+	+	H3	-	-	A3	-	-	D3	++	++	C3	-	-
N4	-	-	H4	+	-	A4	-	-	D4	-	-	C4	-	-
N5	-	-	H5	-	-	A5	-	-	D5	-	-	C5	-	+
N6	+	-	H6	-	-	A6	-	+	D6	-	-	C6	-	+++
N7	-	-	H7	-	-	A7	+++	-	D7	-	+++	C7	-	+++
N8	-	-	H8	-	++	A8	-	++	D8	+++	-	C8	-	-
N9	-	-	H9	-	-	A9	-	-	D9	-	-	C9	-	+++
N10	-	-	H10	+++	++	A10	+	+	D10	++	+++	C10	+	-
N11	-	-	H11	-	-	A11	-	+	D11	-	-	C11	+	-
N12	-	-	H12	-	-	A12	NP	++	D12	-	++	C12	-	++
N13	-	+	H13	-	-	A13	NP	++	D13	-	++	C13	-	+++
N14	NP[c]	-	H14	-	-	A14	NP	-	D14	-	+	C14	-	++
N15	NP	-	H15	-	-				D15	+	-	C15	-	++
			H16	-	-				D16	+	+	C16	+	+++
									D17	-	-	C17	NP	-
									D18	NP	+			

[a]AMP: amplification of cyclin D1 gene as determined by differential PCR scored as negative (-) or +, ++ and +++ representing amplification between > 1.5 and ≤2.5, >2.5 and ≤3.5, and > 3.5 respectively,

[b]IHC: Immunohistochemistry for cyclin D1, scored as negative (-) or + for occasional positive cells, ++ for clusters of positive cells, or +++ for diffuse immunoreactivity.

[c]NP: No PCR product detected.

TABLE 3

ASSOCIATION BETWEEN CYCLIN D1 GENE AMPLIFICATION AND PROTEIN OVEREXPRESSION

Diagnosis	% gene amplification[a]	% protein overexpression[b]	% of amplified cases showing immunopositivity[c]	% of immunopositive cases showing amplification[d]
Normal breast	15 (2/13)	13 (2/15)	50 (1/2)	50 (1/2)
Hyperplasia without atypia	19 (3/16)	13 (2/16)	33 (1/3)	50 (1/2)
Atypical ductal hyperplasia	27 (3/11)	57 (8/14)	67 (2/3)	33 (2/6)
Ductal carcinoma in situ	35 (6/17)	50 (9/18)	67 (4/6)	50 (4/8)
Invasive ductal carcinoma	25 (4/16)	64 (11/17)	50 (2/4)	18 (2/11)

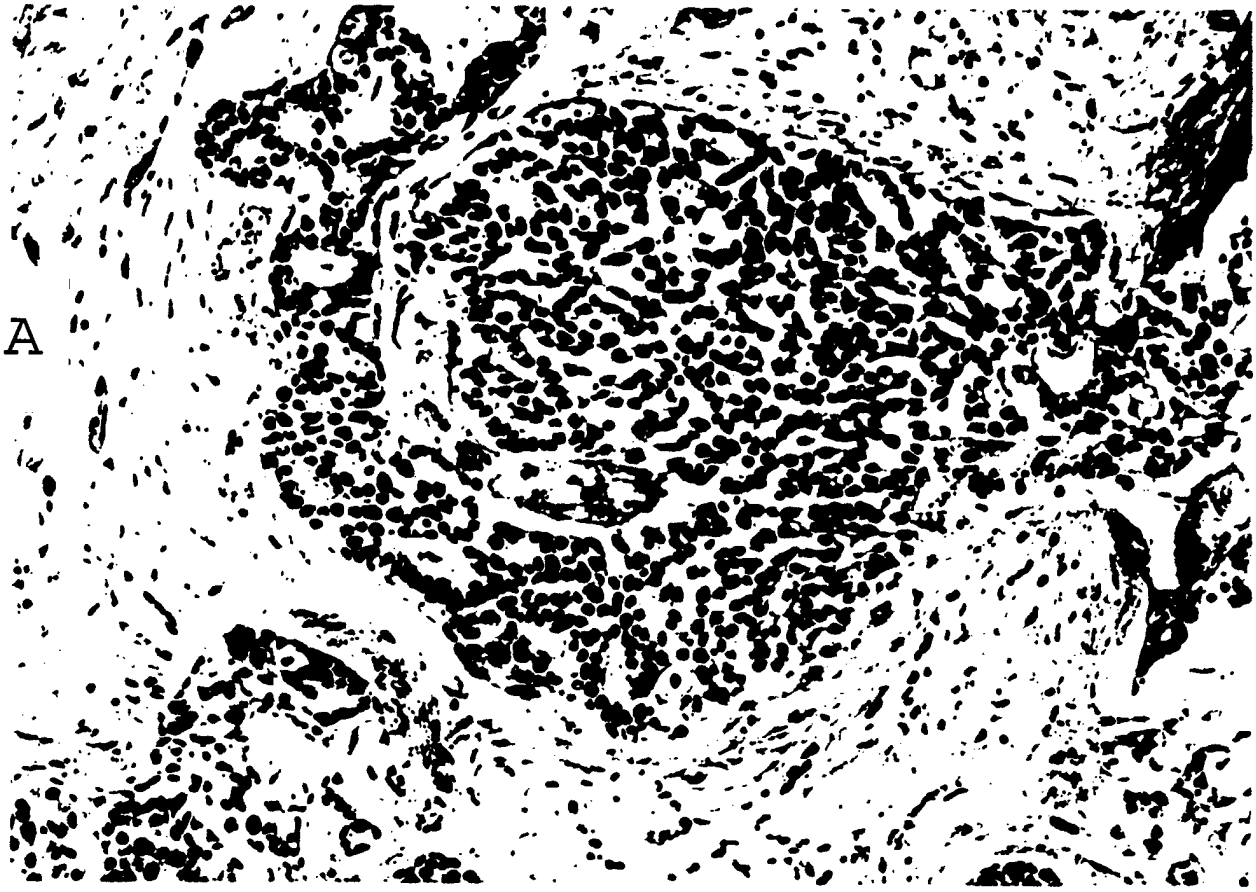
[a] The numbers in parentheses indicate the number of amplified cases over the total number of cases which had a detectable PCR product.

[b] The numbers in parentheses indicate the number of immunopositive cases over the total number of cases analyzed, including the cases which showed no PCR product.

[c] The numbers in parentheses indicate the number of cases with both gene amplification and immunopositivity over the total number of cases with cyclin D1 amplification.

[d] The numbers in parentheses indicate the number of cases with both gene amplification and immunopositivity over the total number of immunopositive cases which had a detectable PCR product.

Figure 2A



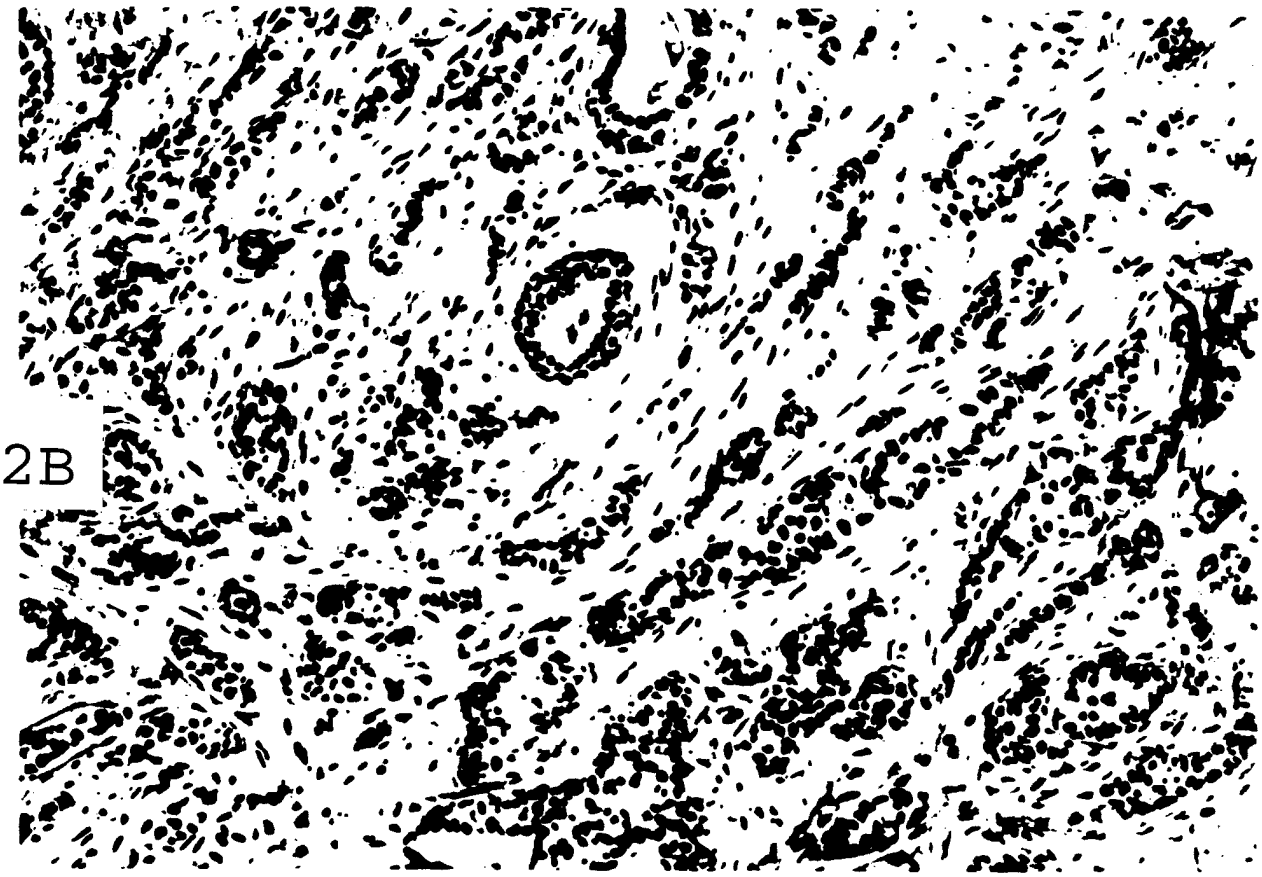


Figure 2B

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MISCLASSIFICATION IN A MATCHED CASE-CONTROL STUDY WITH VARIABLE MATCHING RATIO

STEPHEN W. DUFFY[1], THOMAS E. ROHAN[2] AND RITA KANDEL[3]

[1] *MRC Biostatistics Unit, Institute of Public Health, University Forvie Site, Robinson Way, Cambridge CB2 2SR*

[2] *Department of Public Health Sciences, University of Toronto, Canada*

[3] *Department of Pathology and Laboratory Medicine, Mt. Sinai Hospital, University of Toronto, Canada.*

MISCLASSIFICATION IN A MATCHED CASE-CONTROL STUDY WITH VARIABLE MATCHING RATIO

SUMMARY

We provide a simple analytic correction for risk factor misclassification in a matched case-control study with variable numbers of controls per case. The method is an extension of existing methodology, and involves estimating the corrected proportions of controls and cases in risk factor categories within each matched set. These estimates are then used to calculate the Mantel-Haenszel odds ratio estimate corrected for misclassification. A simple and conservative variance estimate is developed. An example is given from a study of risk factors for progression of benign breast disease to breast cancer, in which the risk factor is a biological marker measured with poor sensitivity.

1. INTRODUCTION

There is a considerable literature on the subject of misclassification of risk factors in epidemiological studies. The various methods are reviewed by Bashir and Duffy[1]. Methods have been developed for use in the settings of the prospective study[2], the unmatched case-control study[3-6] and the pair-matched case-control study[7-9]. In the latter case, Greenland[7,8] has developed a linear algebraic correction to the estimated numbers of case-control pairs by categories of discrete risk factors, to yield odds ratio estimates which are corrected for the effect of misclassification.

To our knowledge, a readily usable method has not been developed for the corresponding problem of a matched case-control study, a binary risk factor, and a variable number of controls per case. It is the purpose of this paper to develop such a method, prompted by a case-control study of this design in which we encountered a serious deficiency in sensitivity of detection of the risk factor of interest.

2. THE PROBLEM

Suppose we have a matched case-control study with m matched sets. Within each matched set l (l=1,2,..., m), there is one case and n_l controls. Assume we are interested in the effect of a binary risk factor. Within matched set l, let c_l be the proportion of cases with observed risk factor positive (c_l must equal zero or one), and let r_l be the proportion of controls with observed risk factor positive. If there were no misclassification, we could use conditional logistic regression to obtain the odds ratio estimate of relative risk, or equivalently calculate the Mantel-Haenszel estimate stratified by matched set:

$$OR_{MH} = \frac{(c_l(n_l - n_l r_l))/(n_l + 1)}{((1 - c_l)n_l r_l)/(n_l + 1)}$$

Now suppose the determination of the risk factor is subject to error. Clearly, if we perform the statistical analysis using the observed risk factor data, we may obtain seriously biased results[1]. If estimates of the error probabilities are available, there is scope in principle for estimating true risk factor prevalences and deriving an odds ratio estimate which is corrected for the misclassi-

fication. Greenland[7-9] develops a correction method whereby the matrix of observed cell counts is multiplied by the inverse of the product matrix of case and control misclassification probabilities to obtain estimates of the true cell counts. To expand this to the situation of multiple and variable controls per case, it is easier to lay out the calculations in terms of individual cell probabilities rather than in terms of correction by matrix multiplication. Nevertheless, the principle of back-calculation of the true risk factor prevalences within matched sets is essentially the same.

3. CORRECTING THE MANTEL-HAENSZEL ESTIMATE FOR MISCLASSIFICATION

Let RF=0 correspond to risk factor negative status and RF=1 to risk factor positive. Let OF=0 and OF=1 correspond to observed risk factor status. Using Greenland's[7] notation, we let

$$\pi_{ij} = P(OF = i|RF = j)$$

be the error probabilities for the cases and let τ_{ij} be the corresponding error probabilities for the controls. For the case in any matched set,

$$P(OF = 1|case) = P(RF = 1|case)\pi_{11} + P(RF = 0|case)\pi_{10}$$

Thus the probability that the case is truly positive for the risk factor is

$$P(RF = 1|case) = \frac{P(OF = 1|case) - \pi_{10}}{\pi_{11} - \pi_{10}}$$

Similarly for a control in any given matched set

$$P(RF = 1|control) = \frac{P(OF = 1|control) - \tau_{10}}{\tau_{11} - \tau_{10}}$$

The probabilities of being truly risk factor negative are easily calculated in the same way, as

$$P(RF = 0|case) = \frac{P(OF = 0|case) - \pi_{01}}{\pi_{00} - \pi_{01}}$$

and

$$P(RF = 0|control) = \frac{P(OF = 0|control) - \tau_{01}}{\tau_{00} - \tau_{01}}$$

From the above, we can calculate the expected number of cases positive in stratum l, say, as

$$\frac{c_l - \pi_{10}}{\pi_{11} - \pi_{10}}$$

and the expected number of controls positive as

$$\frac{n_l(r_l - \tau_{10})}{\tau_{11} - \tau_{10}}$$

We can now recalculate the corrected Mantel-Haenszel odds ratio estimate using the expected true numbers instead of the observed:

$$OR_{MHC} = \frac{\sum_l \frac{(c_l - \pi_{10})(1 - r_l - \tau_{01})n_l}{(\pi_{11} - \pi_{10})(\tau_{00} - \tau_{01})(n_l + 1)}}{\sum_l \frac{(1 - c_l - \pi_{01})(r_l - \tau_{10})n_l}{(\pi_{00} - \pi_{01})(\tau_{11} - \tau_{10})(n_l + 1)}}$$

It should be noted that in the absence of error, this simplifies to the usual Mantel-Haenszel es-

timate, and in the case of one-to-one matching to Greenland's estimate [7].

We can re-express the numerator of the above as

$$\left\{\sum_l \frac{n_l c_l (1-r_l)}{n_l+1} + \tau_{01}\pi_{10}\sum_l \frac{n_l}{n_l+1} - \pi_{10}\sum_l \frac{n_l(1-r_l)}{n_l+1} - \tau_{01}\sum_l \frac{n_l c_l}{n_l+1}\right\}$$

$$\times \frac{1}{(\pi_{11}-\pi_{10})(\tau_{00}-\tau_{01})}$$

A similar formula holds for the denominator. Decomposing the summation in this way is useful for computing purposes but has no conceptual value.

4. VARIANCE ESTIMATION

Here we develop a simple, conservative variance estimate. In the absence of misclassification, the usual variance estimate of the logarithm of the Mantel-Haenszel odds ratio is V/QR, where Q is the numerator and R the denominator of the Mantel-Haenszel odds ratio, and V is the sum of the score variances V_l for each individual stratum. The score variance for an individual stratum is in turn equal to the null variance of any arbitrary cell of that stratum[10], so

$$V_l = \frac{n_l 1 (c_l + r_l)(n_l + 1 - c_l - r_l)}{(n_l+1)^2 n_l}$$

In our point estimate after correction for measurement error, c_l is replaced by

$$\frac{c_l - \pi_{10}}{\pi_{11} - \pi_{10}}$$

and other cell values by similar formulae. The variance of c_l may therefore be replaced by

$$\frac{V(c_l)}{(\pi_{11}-\pi_{10})^2}$$

and other cell variances similarly. A conservative approximation to the overall variance would be to replace *V/QR* by *V/QRM*, where

$$M = min((\pi_{11}-\pi_{10})^2, (\tau_{00}-\tau_{01})^2, (\pi_{00}-\pi_{01})^2, (\tau_{11}-\tau_{10})^2)$$

5. EXAMPLE

We have a matched case-control study of breast cancer nested within a cohort of women with benign breast disease, with the aim of establishing risk factors for progression to cancer. We have 70 cases and a variable number of controls per case. The risk factor under consideration is the immunohistochemical marker c-erbB-2. Uncorrected risk factor status is shown tabulated by case-control status in Table 1.

The particular antibody test used for this marker in our study has poor sensitivity, given in a large validation study[11], external to our study population, as 51%. Specificity is quoted as 100%. Assuming non-differential error between cases and controls, this corresponds to $\pi_{00}=\tau_{00}=1$, $\pi_{11}=\tau_{11}=0.51$, $\pi_{01}=\tau_{01}=0.49$ and $\pi_{10}=\tau_{10}=0$. Additionally, a small repeatability study on 29 subjects in this case-control study gave the results shown in Table 2. This, together with the uncorrected control prevalence of 14% gives estimates of sensitivity and specificity of 49% and 100% respectively (details of estimation from SWD), corresponding to $\pi_{00}=\tau_{00}=1$, $\pi_{11}=\tau_{11}=0.49$, $\pi_{01}=\tau_{01}=0.51$ and $\pi_{10}=\tau_{10}=0$.

Results uncorrected for mismeasurement and corrected using the two sensitivity estimates are shown in Table 3. The corrections make little difference to the point estimate, since although they involve substantial alterations to the estimated prevalences, the alterations apply to both cases and controls. Both corrections, however, make a large difference to the interval estimate, as they entail dividing the standard error estimate by 0.51 and 0.49 respectively.

6. DISCUSSION

The method proposed here is a simple adaptation of Greenland's approach[7]. It is relatively easy to apply. While the formula for the overall estimate is awkward, its component parts are simple, and it is easy to compute. A fortran program which performs the correction is available from the authors.

When there is 1:1 matching, our point estimate reduces to that of Greenland[7], although the variance estimate does not. In the case of no mismeasurement, both our point and variance estimates reduce to the usual Mantel-Haenszel estimate and variance. Our variance estimate is relatively primitive, depending on the extent of misclassification rather than on the amount of information in the validation sample, although it is at least conservative. Indeed, it is particularly so for the example considered here, where the sensitivity is poor. The greater the maximum mismeasurement probability, the larger the corrected variance becomes. It could be argued that this is a desirable feature, giving a larger estimate of uncertainty in the case of poorer measurement.

Our example is an interesting one. From Table 3 one can see that there is a large correction to the prevalence estimates (if sensitivity is around 50% and specificity 100%, the true prevalence is likely to be around double the observed). One would normally be reluctant to make any use of a measurement which required such a large correction. It is, arguably, justifiable in this case, that of a biomarker measured by a laboratory test with well-documented false positive and negative error rates.

In principle, this method is extendable to the case of multiple levels of a risk factor and/or the effect of several covariates simultaneously. As before, the most promising approach would be to build on Greenland's method[7]. We express all possible combinations of risk factors as a single vector of dimension $d=\Pi\, d_i$, where the product is over all risk factors and d_i is the number of levels of the ith risk factor. Suppose the d x d matrix of correct and incorrect classification probabilities is M and in any given matched set the observed proportions of controls in all possible d combinations is given by the vector $\boldsymbol{p}$, of dimension d. Then we estimate the vector of

true proportions q as

$$q = M^{-1} p$$

The same formula applies to the case in each matched set, although the vector of observed proportions will have zero as every component except one, which will take the value one. This is simple in theory, but would give rise to practical problems of dealing with very large matrices if there are numerous potential confounders, and development of variance estimates would be likely to be complex.

In our example, we used both external and internal validation data to calculate the misclassificaiton probabilities. In general, it might be considered preferable to use internal validation, but with two caveats. Firstly, the correction for misclassification is applied multiplicatively, assuming independence of the validation and the main study. Secondly, it is frequently the case that internal resources enable only a small validation or repeatability study to be carried out, whereas results of large and therefore more precise validation studies may be available from the literature. In our example, there were 29 subjects in the internal repeatability study, and 187 in the external validation study[11]. Perhaps a reasonable strategy is to use information on the misclassification probabilities from both internal and external sources, as in our example.

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Table 1: Case-control status by c-erbB-2 status, uncorrected for measurement error

c-erbB2 status	No. (%) of cases	No. (%) of controls
Negative	62 (89)	235 (86)
Positive	8(11)	39(14)
Total	70	274

Table 2: First and second determinations of c-erbB-2 status cross-tabulated

First determination	Second determination	
	Negative	Positive
Negative	25	1
Positive	1	2

Table 3: Odds ratios and 95% confidence intervals unadjusted and adjusted for measurement error

Correction	Case prevalence	Control prevalence	OR	95% CI
Uncorrected (100% sensitivity, 100%specificity)	11%	14%	0.72	(0.30,1.69)
External (51% sensitivity, 100% specificity)	22%	28%	0.66	(0.12,3.50)
Internal (49% sensitivity, 100% specificity)	23%	29%	0.66	(0.11,3.71)