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Little is known about factors that produce genetic abnormalities in breast tumors; germline mutations explain only a few cases. Environmental determinants as well as unrecognized genetic mechanisms may be involved. Using identical (MZ) and fraternal (DZ) twin pairs concordant for breast cancer, patterns of somatic abnormalities will be compared to other tumor characteristics and to breast cancer risk factors. Breast cancer risk factors will also be compared in breast cancer-discordant identical twin pairs having proband tumors with and without specific somatic abnormalities. In the second year of the study, we have 1) continued to follow-up 207 pairs of MZ concordant twins, and 2) have initiated the procedures to obtain consent and release forms from 131 pairs of DZ concordant pairs and from 549 MZ discordant pairs. At present we have received tissue blocks/slides from 170 cases (147 from MZ concordant pairs, 14 from DZ concordant pairs, and 9 from MZ discordant pairs). Tissue is now available from at least one member of 109 MZ concordant pairs and 14 DZ concordant pairs; for 39 of these pairs, tissue is available for both twins. Immunohistochemistry has been performed on slides from 138 cases for p53 , HER-2/neu, estrogen receptor and progesterone receptor expression. Interpretation of these results is ongoing.

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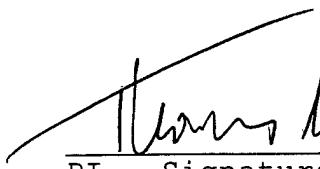

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A. INTRODUCTION

Abnormalities relating to the p53 gene are the most commonly found genetic aberration in breast cancer tumors, and include overexpression of p53 protein, loss of heterozygosity at the p53 locus, and specific mutations in the p53 gene. However, it is unknown why do some tumors have these changes and others do not. Further, little is known about what factors are involved in the interaction of oncogenes such as HER-2/neu with p53.

While investigators in previous studies have attempted to link p53 abnormalities to tumor histology, survival time, estrogen and progesterone receptor status, Her-2/neu, and, in some cases, risk factors for breast cancer, none has studied all of these factors within a large population of twins. These subjects offer great potential for distinguishing the role of predisposing genetic factors from environmental exposures. Specifically we will address the following issues in this study: 1) Are genetically similar tumors more likely to occur among identical twins than among fraternal twins? 2) Do environmental factors predispose to concordance or discordance of genetic abnormalities? 3) Do fraternal twins, concordant for environmental exposures, tend to be discordant for genetic abnormalities, suggesting that other predisposing genetic factors that can be identified? 4) Among identical twins discordant for disease, are specific environmental factors more related to tumors with a genetic abnormality than those without?

Three methods have been commonly used to detect p53 abnormalities: immunohistochemical methods of detecting overexpression of the mutant p53 protein, polymerase chain reaction (PCR) techniques for the detection and sequencing of specific p53 mutations, and Southern blots to detect loss of heterozygosity (LOH) at the p53 gene locus. Studies have indicated that 50-60% of breast tumors may have LOH in the 17p region; there may be overexpression of the p53 mutant protein in 27-54% of all breast tumors (3). Specific mutations in the p53 gene usually occur in the highly conserved exons 5-8 (4,5). Twenty-five percent have been shown to occur in codons 245, 248, 273, and 282 (6). From collaborative efforts of specific p53 mutations in more that 30 types of cancer it has been shown that different types of cancer evince different patterns of DNA base substitutions (7).

Rarely have all types of abnormalities been investigated within the same tumor tissue, but a few studies provide information on the correlations between them. Overexpression of the mutant p53 protein product has been seen in association with mutation of the p53 gene (8) but not invariably (9). LOH and overexpression of the p53 protein have been found to occur independently (9,10,11). The mechanism by which dysfunction in the p53 gene leads to malignant transformation is therefore unclear.

Under one hypothesis it would be necessary for both copies of the p53 gene to be inactivated by loss or mutation to prevent the transcription of the normal or 'wild-type' protein and hence prevent normal function of the gene. The failure by some investigators to demonstrate damage to or loss of both copies of the p53 gene suggests that additional steps or other mechanisms must precede malignant transformation. For example, under a hypothesis of co-dominance, a stable mutant protein might bind

to and inactivate any wild-type protein produced (12). Strong immunohistochemical staining for p53 in normal cells has been found in a mother and daughter with a family history of breast cancer (13). However, no p53 overexpression was found in fibroblasts from individuals from families with the Li-Fraumeni syndrome who had germline DNA mutations of the p53 gene (14). Thus another event (apart from damage to p53) sometimes may be necessary for expression of mutant protein, or only certain mutations in p53 may be related to overexpression of the mutant protein and subsequent malignant transformation.

Another mechanism by which the normal function of p53 gene may be interrupted is by nuclear exclusion (15). When p53 protein is found in the nucleus of cells, mutations in the gene are usually found, whereas when the protein is found in the cytoplasm, mutations are generally not found. If the protein is sequestered in the cytoplasm (by binding with heat shock proteins) then it may be unable to regulate nuclear division. Some studies have shown p53 protein to occur in the cytoplasm of lobular breast cancers (16).

When p53 mutations in germline tissue were found in members of Li-Fraumeni families (17), efforts to detect germline mutations in other high-risk families were intensified, largely without success (18, 19, 20). While these studies were based on small numbers of families: 5 (18) and 25 (19), or cases: 19 individuals with bilateral disease (20). This failure has led to the presumption that environmental factors or other genes may also determine the abnormalities in the p53 gene that lead to breast cancer (21). In any event, the inactivation or disabling of the p53 gene appears to be an important step in a large proportion of breast cancer cases, and studies have shown it to be an early step, present in *situ* tumors and maintained throughout all stages of tumor progression (8).

Since the etiology of breast cancer appears to be complex and heterogenous, other genes, especially oncogenes, may sometimes interact with p53 in the development and progression of breast cancer. HER-2/neu (or also referred to as c-erbB-2), located on the long arm of chromosome 17 (17q12-21.32) has been shown to occur in 20% of invasive breast cancer tumors and in 50% of all ductal carcinoma in situ (22). Studies that have examined the association of p53 with HER-2/neu have produced mixed results; at least four have found the two to be correlated (23, 24, 25, 26), while others have not (27, 28). Barbareschi et al. (26) suggest that p53 and HER-2/neu alterations may occur independently and at an early stage of tumor progression. Escape from hormonal control may be associated with HER-2/neu overexpression (which has been related to estrogen receptor negative tumors); while alterations in p53 may induce a high proliferation rate, leading to tumor progression and further opportunities for genetic damage.

The association of p53 abnormalities and HER-2/neu overexpression with estrogen and progesterone receptor status, histology, progression, and patient survival may provide insights into the mechanisms of tumor development and progression. While some studies have linked p53 overexpression to tumors with a more aggressive phenotype (28), it may be that LOH is more critical to tumor progression than any specific mutation (11). Nuclear p53 expression has been associated with tumors of aggressive (ductal) as well as less aggressive (medullary) histology (16); however neither LOH nor specific mutation sequences were assessed. HER-2/neu is generally found in association with a

poorer prognosis (29).

The relationship of p53 and HER-2/neu overexpression to environmental and other genetic risk factors has not been extensively studied. A higher proportion of tumors with p53 protein expression in familial than in sporadic cases has been reported (30). p53 has been associated with low levels of estrogen receptors (23, 26, 28) and late age at first full term pregnancy has been linked to the prevalence of estrogen receptors (McTiernan et al., 1986). An effect of breast-feeding on risk has been found to be dependent on expression of HER-2/neu (32).

To assess the interrelationships of tumor suppressor genes, oncogenes, specific mutations, loss of heterozygosity, and protein overexpression, it is essential that all factors be examined in the same material. This study presents the opportunity to study the several characteristics of breast cancer tumors in a large group of familial cases--concordant twin pairs--and relate these findings to genetic identity and to environmental risk factors. Secondly, a large number of disease discordant identical twin pairs offers the opportunity to further study association of environmental factors with specific genetic changes in breast cancer tumors.

B. BODY

Work done during the second year of the project has included the following:

1) Contact with twins from the International Twin Registry to obtain consent and release forms for acquisition of tissue blocks
   a. Ongoing efforts to obtain consent and release forms from concordant MZ twin pairs from the Twin Registry.
   b. Initiation of efforts to obtain consent and release forms from additional subgroups of twins including: concordant DZ twins and discordant MZ twins.
2) Ongoing correspondence with hospitals to borrow tissue blocks.
3) Laboratory procedures
   a. Logging in of received blocks and slides in database.
   b. Ongoing processing of tissue blocks to cut slides and storage of them
   c. p53, HER-2/neu, and estrogen/progesterone receptor immunohistochemistry.
4) Results

Contact with Twins

At our site visit meeting with Mrs. Catherine Smith, Human Review Specialist, our consent forms were reviewed and she determined that they were too detailed and could be revised, since we were only asking the twins for permission to obtain tissue blocks. We revised them according to requirements of the Human Use Review and Regulatory Affairs Division and the revisions were approved as of Feb. 20, 1996. Since that time we have used the revised letters and forms which are included in the Appendix.

Three groups of twins have been contacted and results are shown in Table 1. Our procedures for contacting the twins are the same for each group. Beginning with those who were diagnosed after 1975 and for whom we had already obtained pathology reports, we sent a letter explaining the study, the informed consent, and a release form to each twin for her signature. If we determined that a twin was deceased, these forms were sent to her next of kin. If we did not receive a response from a twin after 4 weeks, we have called the twin to be sure they received the forms and to answer any questions. Additional follow-up has been performed as required. For those with diagnosis dates before 1975, we called the hospitals first to determine if the tissue blocks were still available, before initiating the correspondence with the twin. Of the 85 hospitals called, blocks were available for approximately 30%.

1) MZ concordant twins: 207 pairs of identical female twins, concordant for breast cancer, were initially selected to obtain archived tissue blocks. We have continued to follow-up our contacts with these twins and their next-of-kin to obtain consent and release forms. Of the 288 cases for which blocks are assumed to be available (i.e. diagnosis date was after 1975, or before 1975 and hospital indicated that blocks were available), we have received consent forms from 68.7% and are continuing to follow-up an additional 49 cases. Refusals to participate have been low, with 4.8% declining to participate. Another 3.9% have been lost to follow-up.

2) DZ concordant twins: We initiated efforts to obtain consent and release forms from 131 DZ concordant pairs, by sending letters first to twins who were diagnosed after 1975 and known be alive at last contact. We have also recently sent letters to the next of kin to those who were known be deceased. Currently we have received consent and release forms from 37 of the 213 cases (17.4%) for whom blocks are assumed to be available; however we are early on in the process of obtaining consent and expect that we will obtain similar results to the MZ concordant twins.

3) MZ discordant twins: We also selected 549 MZ discordant pairs who met the following criteria: a) they were diagnosed after 1975 and we had obtained their pathology report, and b) they had completed the epidemiologic questionnaire that was sent to all females pairs of twins with at least one member with breast cancer who participated in the International Twin Study Registry. We sent letters initially to 410 of these cases who were known to be alive at last contact. We have received consent and release forms from 205 or 50% of these twins.

Correspondence with Hospitals

Once the signed informed consent and release forms were obtained from a twin, a letter was sent to the hospital along with the release form requesting the tissue blocks, including one that was most representative of the tumor and one that contained normal tissue, such as a lymph node. If the hospital's policies prohibited sending the blocks, we requested that 20 unstained slides be cut from each of the blocks specified, and sent to us. For hospitals not responding follow-up efforts were initiated. Currently we have 80 requests pending with hospitals (42 from MZ concordant pairs, 20 from DZ concordant pairs, and 18 from the MZ discordant pairs, Table 1). We have encountered some delay in obtaining blocks from some hospitals and have had to call them several times to obtain

the blocks. While most have provided the blocks without charge, some have asked for as much as $1,000/twin. When we have explained that this would not be possible we have ususally been able to obtain the blocks for no more that $50-$100/twin.

We have obtained blocks from 170 cases to date (147 from MZ concordant pairs, 14 from DZ concordant pairs, and 9 from MZ discordant pairs, Table 1). Currently we have 185 consent forms received from twins and are in the process of sending these requests to hospitals (9 from MZ concordant pairs, 3 from DZ concordant pairs, and 173 from the MZ discordant pairs). First, we must identify the correct hospital address and enter it into our database.

Laboratory Procedures

Once the blocks (or slides) are received, they are transferred to Dr. Press's Laboratory in padded envelopes which have the Twin ID number, name of submitting hospital, and number of blocks and/or slides provided. This information is logged into a master data file. Variables in this file include information the characteristics of the tissue, number of blocks, number of nodes sampled, and patient information. One H&E slide is cut from each block submitted. Since numerous blocks are sent with some specimens, this enables us to pick a block that is most representative of the tumor and one that is most representative of normal tissue. The 20 unstained slides are then cut from the chosen blocks and are then coated with paraffin so that antigenicity is not lost during storage. After this process has been completed, the blocks are sent back to the hospitals.

Immunohistochemistry: p53 and HER-2/neu

When a specimen is selected to be stained, two slides per analysis are taken. One is for the antibody of interest and the other is used as a negative control. A positive control is used for every antibody on each day's run. The antibodies are scored on the basis of intensity of staining. HER-2/neu, being a membrane protein, is scored as low (+), over-expressed (++), or highly over-expressed (+++). p53, a nuclear protein, is scored both by staining intensity and by percentage of cells with that particular intensity, i.e. (27%, +++), (33%, ++), (10%, +).

Results

a) Status of twin participation and acquisition of blocks

Table 1 shows the status of each group of twins (i.e. MZ concordant, DZ concordant, and MZ discordant). Nearly three-quarters of the twins in the first group selected (MZ concordant pairs) have been resolved, which includes not only the receipt of the release and consent forms, but also the acquistion (or not) of the blocks from the hospital. Of the DZ concordant pairs 27.9% have been resolved as have been 5.1% of the MZ discordant pairs.

Table 1: Status of twin participation and acquisition of blocks/slides by category of pair

	Category of Pair					
	MZ Concordant		DZ Concordant		MZ Discordant	
Status	N	%	N	%	N	%
Total pairs	207		131		549	
Total individuals (cases)	414	100.0	262	100.0	549	100.0
Resolved cases:						
Blocks/slides received	147	35.5	14	5.3	9	1.6
Blocks/slides not available*	126	30.4	49	18.7	5	0.9
Twin refused	20	4.8	1	0.4	1	0.2
Lost	16	3.9	9	3.4	13	2.4
(Total resolved)	(309)	(74.6)	(73)	(27.9)	(28)	(5.1)
In process:						
Letter not yet sent to twin/nok	5	1.2	92	35.1	139	25.3
Pending with twin/next of kin	49	11.8	74	28.2	191	34.8
Received consent/need to request blocks from hospital	9	2.2	3	1.1	173	31.5
Pending with hospital	42	10.1	20	7.6	18	3.3
(Total in process)	(105)	(25.4)	(189)	(72.1)	(521)	(94.9)

*largely consists of cases who were diagnosed before 1975

Among concordant pairs for whom we have received blocks, we have 39 pairs with blocks received from both twins (38 MZ and 1 DZ) and 84 pairs with blocks received from one twin (71 MZ and 13 DZ). Thus, in total we have received blocks from at least one twin for 123 pairs (109 MZ and 14 DZ).

b. Immunohistochemistry

To date blocks from 138 twins (individuals) have been processed by Dr. Press's Laboratory, and immunohistochemistry for p53, HER-2/neu, and estrogen (ER) and progesterone (PR) receptors has been completed for all of these samples. The interpretation of these results is in process at this time. Of the 55 specimens which have been interpreted so far, HER-2/neu was overexpressed in 13 and highly overexpressed in 6. Thus HER-2/neu overexpression of any degree was found in a total of 19 samples (34.6% of the 55 tested). Analysis of p53 overexpression in the same samples showed

overexpression in 14 (25.4%), ranging from one case with only 16% of the cells stained with low intensity to a case where 90% of the cells stained and the staining intensity was evenly divided between low, moderate, and high. Twenty-two of the samples have been analyzed for ER and PR; 77.3% (17) were positive for ER and 59.1% (13) were positive for PR. When all of the stainings have been interpreted we will analyze the correlation of these measures within the same tissue and between members of pairs.

C. CONCLUSIONS

We have continued to follow-up the 207 MZ concordant twin pairs selected during the first year of the project and have begun the process of obtaining consent forms and tissue blocks with two additional groups of pairs--131 DZ concordant pairs and 549 MZ discordant pairs. The revised consent form has been helpful. Other than the hospitals who no longer have the blocks, we have had excellent cooperation from most of the hospitals contacted; however some have required numerous follow-up phone calls and faxes to obtain the blocks. Also, while some have initially said they would charge us fees that would cost up to $1,000/twin, we have been able, for the most part, to obtain the blocks at a reduced cost. We have had some delays in identifying next-of-kin for deceased cases; however, once located, nearly all are willing to participate. Once all efforts have been expended to locate the next of kin without success, we will contact the hospitals directly. The laboratory procedures for processing the blocks are in place and immunohistochemistry procedures have been implemented. During the next year of the project we will have more immunohistochemistry results interpreted, and we will perform SSCP on samples that are positive for p53 and do DNA sequencing to identify specific mutations.

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Appendix

1. Letter to twin describing study-revised
2. Letter to next of kin describing study-revised
3. Informed consent--twin-revised
4. Informed consent--next of kin-revised

INTERNATIONAL TWIN STUDY
USC/Norris Comprehensive Cancer Center
Department of Preventive Medicine
1441 Eastlake Ave. MS #44
P.O. Box 33800
Los Angeles, CA 90033-0800
(800) 421-9631 (213) 764-0448

October 25, 1996

^F1^
^F2^
^F3^

Dear MS. ^F4^:

In the past, you have participated in studies of breast cancer carried out by the International Twin Study. I am writing to ask your participation in an important extension of these studies. We propose to search for and study certain abnormalities of the tumor cells which appear after the tumor has already started. In certain cases, we will also search for inherited genetic abnormalities. This new study is funded by the U.S. Army Department of Defense.

To do this study, we need your permission to borrow the specimen of the tumor from the hospital or clinic and take a small slice of it for study. The tumor specimen will be returned without delay to the health care provider. We will use the slice that is removed only to characterize certain elements of the DNA in the cells.

Participation is entirely voluntary. If you agree to participate, the only thing you need to do is to read, sign and return the enclosed forms. One is the release form that will be sent to the hospital to enable us to obtain the tissue blocks. A second form is a consent form that states that you voluntarily agree to participate in the study and that you relinquish all right, title, and interest to this sample of tissue. We have also included a follow-up form for the Twin Study, on which any changes in your address, telephone number, or disease conditions can be noted.

All results of the study will be kept entirely confidential and information from the study will only be released for publication in statistical form, such that no individual can be recognized. In the very unlikely event that any of the results from the study are of particular importance to your family, that information will be released, but only to you.

After you have signed the forms, **please mail them back to us in the enclosed postage-paid envelope.** Thank you very much for your assistance in our effort to unlock the secrets of this terrible disease. If you have any further questions about the study please feel free to call me.

Sincerely,

Thomas M. Mack, M.D.
Professor

INTERNATIONAL TWIN STUDY
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Department of Preventive Medicine
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October 25, 1996

^F1^
^F2^
^F3^

Dear M . ^F4^:

In the past, your 5~ participated in studies of breast cancer carried out by the International Twin Study. I am writing to you as her next of kin for your assistance in an important extension of these studies. We propose to search for and study certain abnormalities of the tumor cells which appear after the tumor has already started. In certain cases, we will also search for inherited genetic abnormalities. This new study is funded by the U.S. Army Department of Defense.

To do this study, we need your permission to borrow the specimen of the tumor from the hospital or clinic and take a small slice of it for study. The tumor specimen will be returned without delay to the health care provider. We will use the slice that is removed only to characterize certain elements of the DNA in the cells.

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Sincerely,

Thomas M. Mack, M.D.
Professor

UNIVERSITY OF SOUTHERN CALIFORNIA

CONSENT FORM FOR STUDY ENTITLED:

"GENETIC ABNORMALITIES IN BREAST CANCER TUMORS FROM TWINS"

I, _____, voluntarily and freely give permission to release a small slice of my stored tumor specimen to the study for analysis, and thereby relinquish all right, title, and interest to this sample of tissue.

_____

Participant's signature

_____

Participant's name (printed)

_____

Date of signature

UNIVERSITY OF SOUTHERN CALIFORNIA

NEXT OF KIN CONSENT FORM FOR STUDY ENTITLED:

"GENETIC ABNORMALITIES IN BREAST CANCER TUMORS FROM TWINS"

I,_____, as next of kin, voluntarily and freely give permission to release a small slice of her stored tumor specimen to the study for analysis, and thereby relinquish all right, title, and interest to this sample of tissue.

_____
Next of kin's signature

_____
Next of kin's name (printed)

_____
Date of signature

_____
Patient's Name (printed)

USC/Norris Comprehensive Cancer Center, 1441 Eastlake Ave. MS #44, P.O. Box 33800, Los Angeles, CA 90033