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(5) Introduction:

Many gene alterations have been detected in breast cancer, including amplification of oncogene and mutation of tumor suppressor genes (1,2). The alterations of tumor suppressor gene p53 was frequently detected in breast cancer (20%) (3). However, there is a possibility that exposure to mutagens or genetic predisposition influences p53 mutagenesis to a greater extent in one locale more than others (4), so that p53 may be a useful epidemiological tool for identification of mutagen and genetic factors that contribute to breast cancer.

Families with striking histories of breast cancer and other neoplasms, suggested a new familial cancer syndrome of diverse tumors, referred to as Li-Fraumeni Syndrome (LFS) (6). The prospective studies have confirmed the high risk in family members of the tumors types that comprise LFS. A two-step mutation model was proposed. The model is based on the premise that the most cancers are derived from a single cell and that at least two mutational events are required for the development of cancer. In hereditary cancers, the first mutational event is inherited and present in all cells of an individual at birth and can be transmitted through the germ cells, the second event is somatic. In sporadic cancers, both mutations are somatic (8). The alterations of the p53 gene occur not only as somatic mutations in human cancers, but also as germ line p53 mutation in some cancer-prone families. All cells in individuals with LFS also have a single wild-type p53 allele which provides an opportunity to compare the effects of p53 inactivation on the development of cancer in different tissues (7). Germ-line p53 mutations in the LFS are mainly CG-TA transitions at CpG dinucleotides. These may be naturally occurring endogenous events.

Three factors leading to high risk of breast cancer are the family history, early onset (young age) and bilaterality. Patients with diagnosis made during the premenopausal period may have an inherited genetic basis for their disease.

The problem for breast cancer is how to detect which woman have the breast cancer potential or have the inherited genetic factor. The patterns of specific mutations within the p53 gene differ (4). This difference raises the possibility that p53 gene may be a useful epidemiological tool as a gene marker for identification of mutagens and genetic factors that contribute to cancers (5).

This project is focusing on the patterns of p53 gene mutations in breast cancer from a stationary population in order to prove this above possibility by PCR based-SSCP and sequence techniques.

(6) Body:

Tissue Processing:

To determine the tumor/normal composition of the tissue, the first section is deparaffinized, stained with hematoxylin and eosin, and examined immediately. If the section consists of more than 50% normal cells, the areas of the section carrying the normal cells is marked with a pen. The corresponding area is gently scraped off the remaining sections. Last section, scraped for enrichment is stained, examined, in each of the tissue blocks before and after enrichment is noted. Wherever possible, the "normal tissue" that is scraped away will be saved as a source of constitutional DNA from the patients. Again, the extent of contamination of this tissue with tumor cells will be documented. Our pathologist researcher, Dr. Anbazhagan Ramaswamy, will perform all of the examinations, so that there is uniformity in diagnosis and evaluation of the tissues.

PCR-procedure:

This was previously performed on DNA extracted from the paraffin embedded section using a 3-day extraction procedure using detergents and enzyme digestion. The following modifications have resulted in better quality DNA. DNA is extracted from 8-10 micro paraffin section of breast cancer first with xylene, and the pellet is re-extracted twice with 95% ethanol (11). The resultant pellet is dried in a speed-vac, resuspended in PCR buffer, heated to 95°C for 5 minutes, and aliquoted into several tubes for PCR analysis. The polymerase chain reaction is performed as described previously (12)

Analysis of mutations in the p53 gene:

The vast majority of mutations in the p53 in breast cancers have been located in exon 5 to 9 (9, 10). The SSCP approach we have been using with DNA from both tissue and paraffin embedded sections is essentially that described by Gaidano et al (11). Using intron-sequence primers for each exon, exon 5-9 of the p53 are amplified in four PCR reactions, in which one of the four dNTPs is radiolabeled. The sizes of the PCR products

range from 149-249 pb, which are within the range of amplification of fairly degraded DNA. For samples that show mutations, we will clone the 2.9 kb PCR fragment encompassing exon 4-9 in to the TA cloning vector (Invitrogen, San Diego, CA) and sequence both strand using M13 and T7 primers. Internal exon (5, 6, 7, 8) will be sequenced with the 5' and 3' intron primers used in the SSCP analysis

The primers employed in the SSCP analysis (13,14) of the p53 gene are listed below:

Exon 5-5': TTCCTCTTCCTGCAGTACTC	Product	242 bp
5-3' ACCCTGGGCAACAGCCCTGT		
Exon 6-5' ACAGGGCTGGTTGCCAGGGT		194 bp
6-3' AGTTGCAAACCAGACCTCAG		
Exon 7-5' GTGTTGTCTCCTAGGTTGGC		187 bp
7-3' GTCAGAGGCAAGCAGAGGCT		
Exon 8-5' TATCCTGAGTAGTGGTAATC		209 bp
8-3' AAGTGAATCTGAGGCATAAC		
Exon 9-5' GCAGTTATGCCTCAGATTAC		149 bp
9-3' AAGACTTAGTACCTGAAGGGT		

Each 10 μ l PCR reaction contains: 10 pmol of each primer, 2.5 μ M of each dNTP, 1 μ Ci of [α -³²P]-dCTP (3000 Ci/mmol), 100 ng of DNA, and 0.02 units of Taq polymerase (Amplitaq, Perkin Elmer/Cetus) under buffer conditions specified by the manufacturer.

An aliquot of the PCR sample is diluted (1:25) in 0.1% NaDodSo₄, 10 mM EDTA, mixed 1:1 with sequencing stop solution containing 20 mM NaOH, heated to 95 $^{\circ}$ C for 5 min., chilled on ice and loaded (total 6 μ l) onto a 0.5% MDE polymer (AT Biochem) gel containing 10% glycerol. Following fractionation at 8W for 12-15 hours at room temperature, the gel are dried at 80 $^{\circ}$ C and autoradiographed at room temperature for 4-6 hours. Metal plates are attached to the glass plates during the run to prevent rise in temperature of the p53 gene.

Sequencing of the p53 gene:

For the PCR amplification of the 2.9 kb genomic fragment encompassing exon 4-9 the following two primers are used:

Exon 4-5' GACGGAATTCGTCCCAAGCAATGGATGAT

Exon 9-3'GTCAGTCGACCTTAGTACCTGAAGGGTGA

PCR is performed under standard conditions in 100 μ l. The amplified 2.9 kb product is cloned into the TA 1000 cloning vector (Invitrogen, San Diego, CA). When paraffin embedded tissues are used, amplification of the entire 2.9 kb genomic fragment is not possible, therefore, the fragment is amplified in three sections: exons 4-5, exon 7-8, and exon 8-9. The T7 or M13 primers are used to sequence the 5' end of exon 4. An additional primer p53 S4-3': TCAGGGCAACTGACCGTGCA allows the sequencing to be done from the 3' end of the exon to yield the complete 264 bases that comprise the exon. The remainder of the exon are sequenced using the same primers used for SSCP analysis.

Preliminary result of p53 gene mutation pattern in breast cancer:

The principal investigator of this project, Dr. BaoFa Yu, started this project last year for p53 mutation profiles in premenopausal (less than 35 years old women) versus postmenopausal (more than 70 years old women) breast cancer in a stationary population. He hypothesizes that the etiology of breast cancer involves a complex interplay of genetic, hormonal and dietary factors that are superimposed on the physiological status of the host, and that premenopausal women are more likely to have germ-line mutation in p53 tumor suppressor gene, while the breast cancer of postmenopausal women will have somatic mutation in p53 gene. Moreover, the p53 gene will have specific codons or specific nucleotides mutated that might provide clues to the genetic or environmental factors that contribute to breast cancer in this stationary population.

By one year's hard work, Dr. BaoFa Yu found that 3 of 21 breast cancer in postmenopausal women had only mutations in exon 5 of the p53 gene, while the 3 of 21 breast cancer in premenopausal women had mutation in exon 6 and 7 of the p53 gene (tale1).

Table 1 Mutation in the p53 gene in the younger and old groups
Patients No. Age SSCP Shift Condon Sequence Change Amino acid
(years) in exon substitution

A12	71	5	156	CGC to CCC	Arg to Pro
			140	ACC to ATC	
			137	CTG to CCG	
A19	70	5	156	CGC to CCC	Arg to Pro
A15	71	5	undo		
B5	29	6	undo		
B7	33	6	undo		
B13	25	7			

This result is very exciting result because we can see there is a difference perterns of p53 mutations in the pre and post menopausal breast cancer, but it is limited by the numbers of samples, so we need to continue this project by expanding into a large scales of breast cancer samples.

(7). Conclusions:

This preliminary result indicate that p53 mutation patterns in breast cancer in the premenopausal and postmenopausal women is different and comparing the changes between the pre- and post-menopausal breast cancer will allow us to determine if any genetic and /or environmental factors are contributing to breast cancer in this stationary population. Dr. BaoFa Yu will continue this research by increasing number of the studying group in order to confirm his hypothesis.

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- (9). Appendix: No