8. APPENDIX

Appendix-I: Case History and Consent Form of Breast Cancer Patients

Lab. No. G-22

DATE
PGIMS OPD No.
CR NO.

Questionnaire to study risk factors in breast cancer patients

We are requesting your help in evaluating the risk factors in breast cancer patients. Your participation is voluntary. The questionnaire is confidential and will only take a few minutes of your time. Your assistance is greatly appreciated.

(1) Case No  (2) Age/sex

(3) Patient’s Name  (4) Blood group rh factor

(5) Father’s/Husband’s/Guardian’s Name

(6) Address

(7) Contact No  (8) Rural/Urban

(9) Education

(10) Religion  Hindu □  Muslim □  Sikh □  Christian □  Other □

(11) Consanguinity  Yes □  No □

(12) Pedigree

(13) Anthropometric measurements

(i) Height  (ii) Weight
(14) Marital status  (i) Married □ (ii) Unmarried □

(15) Age factor
   (i) At menarche  (ii) At menopause  (iii) At onset of breast cancer

(16) Pregnancy history
   (i) No. of pregnancies  (ii) Age at first pregnancy

(17) Abortion history
   (i) Abortion  (i) Yes □ (ii) No □  (ii) Number of abortions
   (iii) Age at first abortion

(18) Cancer history
   (1) Cancer stage
   (ii) Cancer at any other site

(19) Breast feeding  (i) Yes □ (ii) No □
   (i) Lactation Duration

(20) Using birth control pills  (i) Yes □ (ii) No □

(21) Smoking  (i) Yes (ii) No □

(22) Alcohol consumption  (i) Yes □ (ii) No □

(23) Treatment  (24) Follow Up
Patient’s Consent Form

Statement: I have been explained, all about nature and purpose of this study. I have been asked for clearing any question regarding this protocol. I am giving consent to researcher for releasing information obtained from me to the authorities, Government agencies and ethics committee. My identity will be confidential. I have got decided to be a part of this research and agree to cooperate with the investigator.

Name of patient ______________________

Patients / Parents of the patients / Guardians.

Signature__________  Thumb Impression
APPENDIX-II: REQUIREMENTS

1. Material for Immunohistochemistry staining:
   - Xylene
   - Ethanol
   - Hematoxylin
   - Diaminobenzidine
   - Antigen retrieval system
   - Primary antibody
   - Secondary antibody
   - Incubator
   - Coplin jars
   - Microscope
   - Phosphate buffered saline
   - Streptavidin-HRP
   - Staining jars

   **Preparation of reagents:**

   A.) Citrate Buffer: 10 mM Sodium Citrate Buffer
      Sodium citrate trisodium salt dihydrate (C₆H₅Na₃O₇ • 2H₂O) - 2.94 g
      Distilled water = 1 lit.
      Adjust the pH up to 6.0.

   B.) EDTA: (1mM EDTA):
      EDTA = 0.372 grams
      Distilled water = 1 Liter.
      And then adjust the pH up to 8.0.

   C.) Tris EDTA:
      10 mM Tris/1 mM EDTA and pH 9.0:
      Tris base=1.21 gram
      EDTA= 0.372 g
      Distilled water=950 ml.
      Adjust pH to 9.0, and then adjust volume to 1 L with distilled water.

   D.) 3% hydrogen peroxide:
      10 ml of 30% hydrogen peroxide were added to the 90 ml of distilled water.

   E.) PBS: Added the following reagents:
      10X PBS= (0.1M PBS, Adjust the pH up to pH 7.4):
      Na₂HPO₄ Anhydrous=10.9 grams
      NaH₂PO₄=3.2 grams
      NaCl = Added 90 grams.
      Adjust volume to 1 L with distilled water.

2. Material for hemoglobin concentration:
   - Hemometer
   - Pipette
   - Distilled water
   - Blood sample
   - N/10 HCl
   - Cotton, spirit, needle.
3. Material for W.B.C count:
- Hemocytometer
- Pipette
- Compound Microscope
- Two percent acetic acid
- One percent hydrochloric acid
- Cotton, spirit, needle.

4. Material for platelet count:
- Two pipettes of 20 μL capacity
- Hemocytometer
- Petri plates
- Microscopes
- Ammonium oxalate about 11.45 grams
- Sorensen phosphate buffer around 1.0 grams
- Thimerosal of about 0.1 gram
- Made up volume with distilled water around 1 liter

Reference Interval - 150-440 x 10^9 /L

5. DNA Isolation:

Equipment -
- UV spectrophotometer
- Incubator
- Vortex mixer
- UV transilluminator with Polaroid lens camera
- Mini gel electrophoresis with power supply
- Microwave oven

Reagents:
1. Red cell lysis buffer: Mixed the following chemicals
   - TRIS = 10Mm
   - MgCl₂ = 5Mm
   - NaCl = 10Mm

2. White cell lysis buffer: Mixed the following chemicals
   - TRIS = 10Mm
   - EDTA = 10Mm
   - NaCl = 50Mm

3. Proteinase K solution
   Dissolved proteinase K solution in distilled water to make a final conc. of about 10 mg/ml.

4. TE solution
   - TRIS = 1Mm
   - EDTA = 0.1 Mm

Storage of DNA
- At 4⁰C for daily use.
- At -20⁰C for several years.
6. Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP):

**Equipments:**
- Thermal cycler
- UV transilluminator
- Mini gel electrophoresis unit
- Vortex mixer
- Water bath, pH meter
- Deep freeze -20°C and Centrifuge machine

**Reagents:**
- Water should be ultra pure
- Taq Polymerase of Applied Biosystem
- 10X PCR Buffer Took 200 mM Tris-HCl (pH 8.4), 500 mM KCl.
- MgCl$_2$ (25 mM)
- dNTPs (2.5 mM)
- Sample Templates about (10ng)
- PCR Primers

7. Microsatellite assay:

**Equipments:**
- Refrigerator of (-80°C) capacity
- Pipettes
- Template Plate with capacity 96 well
- Adhesive Film for PCR plates
- Easy Peel Heat Sealing Foil
- TempPlate Sealing Foil
- PCR
- GeneMapper for mapping purpose

**Reagents:**
- Genomic DNA controls (25 pg/µL)
- Controls sample: Two water blank were used as negative control in experiment
- Primers: primers were used of 100 µM concentration. They were diluted to make 10 µM volumes for use in experiment.
- GeneScan (Applied BiosystemsUSA3730)
- Hi-Di Formamide (Applied BiosystemsUSA)
- Taq Polymerase (Applied BiosystemUSA)
- 10X PCR Buffer (Supplied with the referenced reagent)
- MgCl$_2$ (50 mM)
- DNTPs (100 mM)
- Sample Templates were used (1 µl per reaction)
APPENDIX-III: PRECAUTIONS

1. Precautions for Immunohistochemical Staining
   ✤ Biological specimens, before and after fixation and all materials exposed to them were handled with proper precautions.
   ✤ Never pipetted the reagents by mouth and avoid contacting the skin and mucous membranes with both reagents and specimens.
   ✤ If reagents come into contact with sensitive areas, wash with copious amounts of water.
   ✤ Wear personal protective means while handling any human biological material and while performing the staining procedure.
   ✤ Incubation times or temperatures other than those specified might give erroneous results.
   ✤ Unused solutions were disposed according to local, State laws and regulations.
   ✤ Once the tissue sections have been rehydrated, do not allow them to dry.
   ✤ Dry the slide around the tissue section with an absorbent wipe.
   ✤ Using a diamond pencil, china marking pencil or nail polish, drawn a circle on the microscope slide around the section.

2. Precautions for hematological analysis:
   ✤ Blood column used were free from all the air bubbles while handling.
   ✤ Never touched the tips of the pipette.
   ✤ Holded the pipette very straight.
   ✤ Platelet counts were counted within three hours.
   ✤ If the platelet clumps were seen in the hemocytometer, the whole procedure was repeated.
   ✤ Eating, drinking and smoking were not done in the work area.
   ✤ Hands, pens, and other things were kept away from mucous membranes.
   ✤ Mouth pipetting was prohibited.

3. Precautions for Molecular techniques:
   ✤ Proper sterilization was used for DNA isolation technique.
   ✤ Glass wares were properly washed and baked at 300°C for 4 hours.
   ✤ Ensured that all the equipment was properly calibrated before use.
   ✤ Used the UV goggles while working with the UV light box.
   ✤ Disposed of the biological waste into orange Biohazard bags.
   ✤ Did not use plastic or polycarbonate containers, test tubes, pipettes etc.
   ✤ Did not dispose of hazardous or noxious chemicals in laboratory sinks.
   ✤ Washed the hands before beginning and after doing the work.
   ✤ Disinfected the lab benches at the beginning and end of the work.
   ✤ Disposed the broken glass in the containers.
   ✤ When Agarose gels were stained with ethidium bromide handled them with great care. Ethidium bromide is a mutagen and may cause harm to body.
APPENDIX-IV: STATISTICAL ANALYSIS

1. Percentage frequency
   Number of patients/Total number of patients \( \times 100 \)

2. Mean
   \[ \bar{X} = \frac{\Sigma X}{N} \]
   \( \Sigma X \) = Sum of particular values
   \( N \) = Number of individuals

3. Median
   \( \text{Median} = \frac{N+1}{2} \)th observation if the “N” is odd for patients.
   \( \text{Median} = \text{average of} \frac{N}{2} \text{th and} \frac{N}{2}+1 \text{th observation if “N” is even for patients.} \)

4. Standard deviation
   \[ \sigma = \frac{\Sigma (X - \bar{X})^2}{\Sigma f} \]
   \( \Sigma f \) = Total frequency of patients
   \( \Sigma f (X - \bar{X})^2 \) = Sum of the products of squares of deviations from Arithmetic mean with corresponding frequency of patients.

5. Z-test for proportion
   \[ \left( \frac{\bar{p}_1 - \bar{p}_2}{\sqrt{\bar{p}(1 - \bar{p}) \left( \frac{1}{n_1} + \frac{1}{n_2} \right)}} \right) \]
   \( p = \frac{(p_1 \times n_1 + p_2 \times n_2)}{(n_1 + n_2)} \)
   \( p_1 \) = The sample proportion from population no. 1
   \( p_2 \) = The sample proportion from population no. 2
   \( n_1 \) = The size of sample no. 1
   \( n_2 \) = The size of sample no. 2

6. Student t-test
   \[ \frac{(\bar{X} - \mu)^2}{S\bar{X}} \]
   \( S\bar{X} \) = Standard error of mean
   \( \mu \) = Population mean
   \( \bar{X} \) = Sample mean

7. Odds ratio
   \[ OR = \frac{a/b}{c/d} = \frac{a \times d}{b \times c} \]
   Expermental Events (patients) Odds = a/b
Control Events (controls) Odds = \( \frac{c}{d} \)
Odds ratio = \( \frac{a}{b} / \frac{c}{d} \)

8. **Pedigree symbols**: (Figure 66)

9. **Chi-square test**

\[
E = \sum \frac{(O - E)^2}{E}
\]

a. \( O \) = Observed frequency of cases

b. \( E \) = Expected frequency of cases

10. **logistic regression test**

Logit \( \left( p_{disease} \right) = \log \left( \frac{P_{disease}}{1 - P_{disease}} \right) = \beta_0 + \beta_1 X_1 + \ldots + \beta_j X_j \)

Where:

\( p_{disease} \) = probability that an individual has cancer.

\( \beta_0 \) = Intercept

\( \beta_1, \beta_2, \ldots, \beta_j \) = coefficients of genetic factors.

\( X_1, X_2, \ldots, X_j \) = variables of genetic factors

11. **Hardy-Weinberg equilibrium**

\[
(p + q)^2 = 1
\]

\[
p^2 + 2pq + q^2 = 1
\]

\( p \) = the frequency of the dominant allele

\( q \) = the frequency of the recessive allele

\( p^2 \) = frequency of homozygous dominant

\( 2pq \) = frequency of heterozygous

\( q^2 \) = frequency of homozygous recessive

12. **Linkage and haplotype analysis**

Linkage disequilibrium:

\[
D' = D / D_{max}
\]

Where:

\[
D_{max} = \begin{cases} 
\min \left\{ p_A p_B, (1 - p_A) (1 - p_B) \right\} & \text{when } D < 0 \\
\min \left\{ p_A (1 - p_B), (1 - p_A) p_B \right\} & \text{when } D > 0 
\end{cases}
\]

An alternative to \( D' \) is the correlation coefficient between pairs of loci, expressed as the

\[
r = \frac{D}{\sqrt{p_A (1 - p_A) p_B (1 - p_B)}}
\]

13. **Haplotype diversity analysis**

\[
H = \frac{N}{N - 1} \left( 1 - \sum x_i^2 \right)
\]

Where \( x_i \) is the (relative) haplotype frequency of each haplotype in the cases and \( N \) is the sample size of whole population.

14. **Sensitivity and Specificity analysis (Blair et al., 2009)**

\[
\text{Sensitivity} = \frac{a}{a + b}
\]
Specificity  = \frac{d}{c + d} \\
\text{Disease prevalence} = \frac{a + b}{a + b + c + d}

15. Mann Whitney Test

\[ U = n_1 n_2 + \frac{n_2 (n_2 + 1)}{2} - \sum_{i=1}^{n_2} R_i \]

U = Mann Whitney U test
N1 = sample size one i.e cases
N2 = Sample size two i.e controls
Ri = Rank of the sample size (Total no. of women)

16. Software used

I. WINDOW 2007 EXCEL

II. SPSS: The statistical tests like mean, median, t-test, test of proportion (z-test) were calculated by SPSS version 16.

III. Medcalc: Kaplan-Meier survival curve and long rank test were calculated by Medcalc.

Figure 66: Pedigree symbols used in pedigree analysis of breast cancer patients.