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Molecular cytogenetic analysis of aneusomies of chromosome 3 and 17 in adenocarcinoma of cervix detected by fluorescence *In situ* hybridization

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Abstract

Cancer is a disease characterized by the accumulation of numerical chromosomal aberrations along with the development of genetic instability. The present study was an attempt to assess the numerical chromosomal changes in the Adenocarcinoma of the cervix with reference to chromosome 3 and 17 through the use of Fluorescence in situ hybridization technique. Centromeric enumeration probes (Alpha satellite DNA) were used to assess the Nullisomies, Monosomies, Trisomies and Polysomies of the said chromosomes. FISH results of CEP 3 and CEP-17 in the present study showed the frequent gains of chromosome 3 over the chromosome 17. Statistical analyses were conducted by using ANOVA and Chi square tests. ANOVA results were found to be significant for both CEP 3 and CEP-17 in adenocarcinoma of the cervix. Chi square test (disease v/s control) was also used to study the significant difference between the patients and the controls and it was found to be significant for both chromosomes 3 and 17. Interphase cytogenetics by FISH has now gained broader use in the detection and diagnosis of both hematological as well as solid tumors. The present however is a very small attempt but the aim of the study is to test the usefulness of FISH as a complementary diagnostic tool for the detection of common numerical changes in malignant cells.

Keywords: Molecular cytogenetic, adenocarcinoma, cervix detected, fluorescence

Introduction

Cervical cancer is one of the major health problems for women in India. Mortality due to cervical cancer is also an indicator of health inequities^[1] as 86% of all deaths due to cervical cancer are in developing, low- and middle-income countries^[2]. Every year in India, 122,844 women are diagnosed with cervical cancer and 67,477 die from the disease. India has a population of 432.2 million women aged 15 years and older who are at risk of developing cancer. It is the second most common cancer in women aged 15–44 years. India also has the highest age standardized incidence of cervical cancer in South Asia at 22, compared to 19.2 in Bangladesh, 13 in Sri Lanka, and 2.8 in Iran. Therefore, it is vital to understand the epidemiology of cervical cancer in India^[3].

Cytogenetic and molecular genetic studies have shown that chromosomal instability involving gains and losses of whole chromosomes or chromosome regions is likely to occur in most human malignancies including breast, cervix, lung and ovary cancer, neoblastoma, leukemia and sarcomas. Solid tumors are commonly associated with an array of orchestrated genetic changes, and the identification of changes causally related to the carcinogenic process has been frustratingly slow, mainly as a consequence of the enormous volume of secondary abnormalities reflecting the phenomenon of genomic instability.

Fluorescence in situ hybridization (FISH) is a cytogenetic technique developed in the early 1980s. FISH uses fluorescent DNA probes to target specific chromosomal locations within the nucleus, resulting in colored signals that can be detected using a fluorescent microscope. Compared to the conventional cytogenetic (CC) metaphase karyotype analysis, FISH does not require cell culturing, and can directly use fresh or paraffin-embedded interphase nuclei for a rapid evaluation. With the discovery of numerous disease-related genes in recent years, the applications of FISH broadened to include more genetic diseases, hematologic malignancies, and solid tumors. Fluorescence *in situ* hybridization (FISH) is a powerful technique used in the detection of chromosomal abnormalities.

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The high sensitivity and specificity of FISH and the speed with which the assays can be performed have made FISH a pivotal cytogenetic technique that has provided significant advances in both the research and diagnosis of hematological malignancies and solid tumors. From a medical perspective, FISH can be applied to detect genetic abnormalities such as characteristic gene fusions, aneuploidy, and loss of a chromosomal region or a whole chromosome or to monitor the progression of an aberration serving as a technique that can help in both the diagnosis of a genetic disease or suggesting prognostic outcomes. FISH can also be applied to such research applications as gene mapping or the identification of novel oncogenes or genetic aberrations that contribute towards various cancers.

Material and Methods

FISH has emerged as an indispensable tool for both basic and clinical research as well as diagnostics, in leukemia and cancers. Fish uses fluorescent labeled DNA probes that target specific DNA sequences. The present study is an attempt to summarize the methodology and utilization of FISH technique in unraveling the chromosomal highlights how the field is moving away from conventional methods towards molecular cytogenetic approaches.

The present study was conducted in the Institute of Human Genetics, University of Jammu in collaboration with the Department of Gynecology and Obstetrics, SMGS hospital, Govt. Medical College, Jammu. The females complaining of the excessive vaginal discharge, post coital bleeding, abnormal vaginal bleeding, suspected cervical lesion or unhealthy cervix were selected for the study. In order to draw the comparison between the confirmed cases of cervical cancer and normal, control samples of the healthy females were also taken up. After detailed history of the patients, they were subjected to general physical, gynecological examination and the cervical biopsies were taken only after taking their written consent.

Material and Methods

Collection of the tissue

One part of the excised biopsy tissue was fixed in formalin and submitted to the Pathology Department, Govt. Medical College, Jammu for histopathological examination where as another part was transferred to the vials containing 0.075M KCl solution (Hypotonic solution) for cytogenetic analysis.

Preparation of the slides

Bits of the cervical tissue collected in hypotonic solution were processed for the preparation of the slides for cytogenetic analysis following Atkin & Baker, 1979(a) with slight modification.

Fish study: During the present study, for the Molecular cytogenetic study, Fluorescence *in situ* Hybridization (FISH) was processed on 4 confirmed cases of Adenocarcinoma of the cervix. In order to draw a comparison between diseased and normal condition, 10 control samples were also taken up. Vysis made Alphoid DNA probes (Centromere Enumeration Probes, CEP) were used and the site of the hybridization of probes with cellular DNA was observed under fluorescence microscope Olympus BX61 for fluorescent signals. The probes used were namely:

1. CEP 3 (D3Z1) 3p11.1- q11.1 Alpha satellite DNA Spectrum orange probe
2. CEP 17 (D17Z1) 17p11.1- q11.1 Alpha satellite DNASpectrum orange probe

The protocol for FISH was followed according to Olaharski *et al.*, 2006 [4] with slight modification. In brief, slides were denatured by incubation with formamide (70% in 2X SSC) at 73°C for 5 mins in a water bath. Then the slides were dehydrated through a graded ethanol system (70%, 80%, 90% 1 min for each slide) hybridization solution (5 µl) was applied to each slide which was covered with a coverslip and sealed with rubber cement. After incubation for 16 hrs at 37°C in a humidified chamber, slides were washed with 2X SSC for 5 mins at 73°C. Then DAPI (2 µl) was applied to each spot and again covered with a coverslip. Apart from diseased samples, 10 samples of healthy subjects (controls) were also subjected for FISH where both CEP 3 and CEP 17 probes were used.

Fish Microscopy

FISH signals were visualized by fluorescence microscopy using a light source that illuminates the fluorescently labeled specimens. FISH technique was used to investigate the chromosomal aberrations in cervical cancers that may lead to various genetic changes for the occurrence of chromosomal instability at different levels of tumor progression.

Statistical methods: Statistical tests including ANOVA and Chi square tests were used to analyze the numerical chromosome aberration data obtained by FISH analysis.

Results

Tumorigenesis is a multistep process that involves a series of Genetic and Epigenetic alterations. It has been suggested that cancer cells are genetically unstable and their acquisition of Genetic Instability may represent an early step in the process of carcinogenesis. Cytogenetic and molecular genetic studies have shown that chromosomal instability involving gains and losses of whole chromosomes or chromosomal regions is likely to occur in most human malignancies including breast, cervix, lung and ovary cancer, neoblastoma, leukemia and sarcomas.

Routine chromosome analysis by itself provides only limited information about the identity of structural aberrations. The advent of Fluorescent *in situ* hybridization (FISH) procedures has ushered in an entirely new field of molecular cytogenetics. This technique has aided the identification of structural chromosome rearrangements and the definition of breakpoints in tumor cells. GTG- Banding combined with FISH is especially useful in unraveling complex or subtle structural aberrations.

Since during the present study FISH probes were used on the interphase nuclei, the interpretation was therefore, based upon the signals detected in the FISH treated interphase nuclei. FISH results were interpreted by evaluating the number of the signals detected in the interphase nuclei. Interphase nuclei possessing 2 signals were categorized as the normal one where as nuclei possessing less or more than 2 signals were categorized as the abnormal one (**Fig.1&2**) with the abnormal number of chromosome no. 3 or chromosome no. 17 respectively.

FISH results of CEP 3 and CEP-17 in the present study showed the frequent gains of chromosome 3 over the chromosome 17. Trisomy 3 and Monosomy 17 were the most consistent chromosomal aberration observed by using CEP- 3 and CEP-17 probes in the diagnosed cases of adenocarcinoma (Table 1).

FISH technique was also carried out in 10 healthy samples (control) to make a comparison between the diseased condition and the normal samples. The average percentage frequencies of different types of the cells (Normal,

Nullisomic, Monosomic, Trisomic and Polysomic) by using CEP 3 and CEP- 17 in Adenocarcinoma was found to be the highest 95% each (Table 2).

ANOVA results were found to be significant for both CEP 3 and CEP-17 in adenocarcinoma of the cervix (Tables 3 and 4). Chi square test (disease v/s control) was also used to study the significant difference between the patients and the controls and it was found to be significant for both chromosomes 3 and 17 (Table 5 & 6).

Table 1: Average %age frequencies of different types of cells (Normal, Nullisomic, Monosomic, Trisomic & Polysomic) by using CEP-3 and CEP-17 probe in the cases of adenocarcinoma of the cervix.

Cases	Total no. of cells analysed/patient	% of Normal cells	% of Nullisomic cells	% of Monosomic cells	% of Trisomic cells	% of cells with>3 signals
CEP-3 results						
Case no.1	57	8.07	14.03	19.32	42.52	16.06
Case no.2	57	15.78	17.54	10.52	35.08	21.05
Case no.3	52	13.23	25.07	13.46	30.99	17.25
Case no.4	59	10.50	5.08	8.5	48.81	27.11
CEP-17 results						
Case no.1	56	19.64	28.57	30.35	16.07	5.35
Case no.2	52	17.30	28.84	44.23	7.69	1.92
Case no.3	55	12.72	21.18	38.18	18.18	9.74
Case no.4	53	16.98	15.09	37.73	24.52	5.68

Table 2: Results of CEP 3 and CEP-17 probe in controls

Controls	Total no. of cells analysed/patient	% of Normal cells	% of Nullisomic cells	% of Monosomic cells	% of Trisomic cells	% of cells with>3 signals
CEP-3 results						
1	50	94	2	4	Nil	Nil
2	50	94	2	4	Nil	Nil
3	50	96	4	0	Nil	Nil
4	50	94	2	4	Nil	Nil
5	50	94	2	4	Nil	Nil
CEP-17 results						
1	50	97	1	2	Nil	Nil
2	50	94	2	4	Nil	Nil
3	50	96	2	2	Nil	Nil
4	50	95	2	3	Nil	Nil
5	50	96	1	3	Nil	Nil

Table 3: ANOVA analysis for the FISH results by using CEP 3 probe in Adenocarcinoma of the cervix.

	Sum of squares	df	Mean squares	F	Sig.
Between groups (treatment)	271.723	4	67.931	.963	.456
With in groups	1058.157	15	70.544		
Total	1329.879	19			

Table 4: ANOVA analysis for the FISH results by using CEP 17 probe in Adenocarcinoma of the cervix.

	Sum of squares	Df	Mean squares	F	Sig.
Between groups (treatment)	2544.876	4	3136.219	42.174	.000
With in groups	9295.500	125	74.364		
Total	9452.129	129			

Table 5: Specificity and sensitivity of CEP 3 probe in the diagnosed cases and their relative significance with the controls:

	VAR0001
Chi square test	772.669
Df	2
Asym. Sig.	.000

Table 6: Specificity and sensitivity of CEP 17 probe in the diagnosed cases and their relative significance with the controls:

	VAR0001
Chi square test	710.977
Df	2
Asym. Sig.	.000

Discussion

The introduction of fluorescence in situ hybridization (FISH) almost 30 years ago marked the beginning of a new era for the study of chromosome structure and function. FISH gained widespread recognition as a physical mapping technique to support large-scale mapping and sequencing efforts related to the human genome project; however, its accuracy and adaptability were simultaneously, or soon after, exploited in other areas of biological and medical research.

A significant percentage of solid tumors have a very low mitotic index, necessitating culture of the cells, which may not be successful in all cases or may lead to overgrowth by normal (diploid) cells. The morphology of the chromosomes in solid tumors is more often fuzzy thus show low mitotic index with poor quality metaphases and other technical difficulties than the cell morphology encountered in Leukemias. FISH technique helps in revealing the cryptic abnormalities such as small deletions and translocation. It has managed to overcome many of the drawbacks of traditional cytogenetics.

Although solid tumors, particularly carcinoma, play larger part in human morbidity and mortality, cytogenetics of solid tumors develop more slowly than those of hematological malignancies. This situation happens because of several technical limitations. Firstly, the chromosome quality in solid tumors is often suboptimal due to necrotic samples that result in destruction of cancer cells before culturing. Second, in contrast to hematological malignancies, which often contain few cytogenetic changes, most solid tumors have multiple and complex chromosomal changes acquired during tumor progression, which cause difficulties in identifying the primary chromosome changes associated with the specific tumor type. Third, low mitotic index in short term culture of cancer cell that is necessary to avoid overgrowth by normal stromal or supporting cells [5, 6]. In recent years, the development of cytogenetic technique e.g. FISH has led to the description of specific chromosome abnormalities in solid tumor because it can be performed on fresh tumor tissue, exfoliative cells, and embedded specimen [7, 8].

A large number of Centromeric enumeration probes (CEP) have also been utilized in the analysis of cells from a variety of tumors like breast [9, 10] hepatocellular carcinoma [11], lung cancers [12]. CEP probes not only make possible to illustrate the nucleus structure in the precondition of preserving the nucleus shape but also allow the quantitative evaluation of target gene amplification by using Centromeric probes. CEPs are available in demonstration of trisomy, monosomy and ploidy level abnormalities. The introduction of automated microscopic FISH scanning systems has also facilitated the developments of FISH based assays, which could serve as surveillance tools in the patients suffering from various diseases including cancers. Therefore FISH becomes an essential tool for relative DNA copy number change estimation and in addition it detects balanced structural rearrangements and assesses ploidy level.

In the study undertaken, Trisomy 3 was also frequently observed. Numerical changes in the form of Trisomy 3 were the most consistent chromosomal aberrations reported in the diagnosed cases of the cervical carcinoma. Our findings have been found to be more or less similar with the findings made [13-16] who used CEP 3(D3Z1) in cervical carcinoma. Uhlig *et al*, 2013 examined the role of in situ hybridization

(ISH) tests, including fluorescence ISH (FISH), to detect chromosomal abnormalities or DNA from high-risk oncogenic HPV genotypes on cervical cytologic specimens to increase the clinical validity of identification of precancerous lesions or cervical cancer. The results of the present study showed the loss of chromosome 17 as the most frequent observation in the diagnosed cases of the adenocarcinoma of the cervix.

Study of the available literature showed that the loss of chromosome 17 is likely to be a relatively an early event in cervical carcinogenesis. Both the structural and numerical chromosomal abnormalities targeting chromosome 17 are often observed in the tumors from a wide variety of tissues and therefore suggest that this loss may represent a common event in carcinogenesis. Andrew *et al*, 2000 used alpha satellite DNA FISH Probes of chromosome 17 on the samples of vulvar squamous cell carcinoma and synchronous vulvar skin. The results so obtained concluded that the loss of chr 17 was identified in 5% of all the samples and was significantly associated with women with SCC in situ. Using the FISH method, a mixture of CEP-3, 7 and 17 and locus specific identifier p16 FISH probes were used by Philips *et al.*, 2006 to enumerate chromosomes 3, 7 and 17 and detect 9p21 locus deletion on chromosome 9 which is a non-invasive strategy for bladder cancer screening.

FISH technique was also carried out in 10 healthy samples (control) by using both CEP 3 and CEP 17 probe. ANOVA test was used to analyze the data on the numerical chromosome aberration data obtained by FISH analysis. ANOVA (analysis of variance) is a collection of statistical models, and their associated procedures, in which the observed variance in a particular variable is partitioned into components attributable to different sources of variation. In its simplest form ANOVA provides a statistical test of whether or not the means of several groups are all equal, and therefore generalizes t-test to more than two groups. ANOVAs are helpful because they possess an advantage over a two-sample t-test. Doing multiple two-sample t-tests would result in an increased chance of committing a type I error. For this reason, ANOVAs are useful in comparing two, three or more means. ANOVA results were found to be significant for both CEP 3 and CEP-17 in adenocarcinoma of the cervix. Chi-square test (also chi squared test or χ^2 test) is any statistical hypothesis test in which the sampling distribution of the test statistic is a Chi-square distribution when the null hypothesis is true, or any in which this is asymptotically true, meaning that the sampling distribution (if the null hypothesis is true) can be made to approximate a Chi-square distribution as closely as desired by making the sample size large enough. Chi square test was also used to study the significant difference between the patients and the controls and it was found to be significant for both chromosomes 3 and 17. Chi square test was used to study the specificity and sensitivity of the FISH technique used during the present study to make a comparison between the disease and normal samples. Chi square statistical test has been used by various workers like [19-21].

In the past decade, the clear advances have been made in the field of cytogenetics and oncology. The experts in both the fields have tried their best to make the diagnosis and detection of cancers by using cytogenetic and molecular genetic techniques more efficient and effective. In an area

like J&K, much effort are being required to establish a more organized methods for both screening and detecting these genetic changes in a variety of the cancers so that the discovery of biomarkers and the development of therapeutic drugs could be made for the treatment of cancers.

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