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Impact of Cyclophosphamide to Modulates the Genetic Signature of Epithelial Mesenchymal Transition Markers CK19 & Vimentin in Circulating Tumor Cells Population and DNA Copy Number Variations in Breast Cancer Patients

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Abstract:
Circulating tumor cells are the frontier area of medicine for early prognosis and diagnosis during progression of disease like cancer. Etiopathology of breast cancer is complex due to heterogeneous group of cell population and epithelial-mesenchymal transition markers Sox4, EpCAM, CK19 and vimentin play significant role during onset of tumorigenesis after invasion to the blood vessels and to reach neighboring targeted tissue .Cyclophosphamide, an important antineoplastic drug by the clinicians and also used for auto-immune disease. Present study has been designed to explore the sensitivity (frequency) of cytokeratin and vimentin in circulating tumor cells population and compare after in- vitro exposure with cyclophosphamide at different time intervals using flowcytometric analysis using specific antibodies and compare with controls. The same study further extended to evaluate DNA copy number variations to evaluate the tissue specific genetic susceptibility in breast cancer patients. **:** Flowcytometer, a highly sensitive tool for the qualitative and quantitative analysis of circulating tumor cells using specific monoclonal antibodies of cytokeratin 19 and vimentin, conjugated by PE (phycoerythrin) after gating. The sensitivity of epithelial-mesenchymal transition markers (CK19 and Vimentin) in circulating tumor cells population were further evaluated after in-vitro exposure with cyclophosphamide (1.0 ugm/ml) at three different time interval i.e. 24, 48 and 72 hours in lymphocytes cultures. Further, the tissue specific genetic susceptibility were evaluated by DNA copy number variations. Findings, reveals the mean values of circulating tumor cells population in control group was +vemAbCK19 (3243.667) followed by decreasing trend were observed after exposure at 24 hours (2753.50), 48 hours (2720.00) and 72 hours (1945.50). Statistical analysis showing significant differences ($p<0.001$) with respect to controls using chi square-test. Similarly, vimentin showing decreasing trends at first 24 hours **(**4565.00) and 72 hours (2997.50) with significant ($p<0.001$) differences. PCR based study using specific - primers of CK19 and vimentin on agarose gel (1.5 %) and individual bands density were evaluated for DNA copy number variations. The sensitivity of vimentin (323bp) its isoform (225 bp), and CK19 (573bp) showing decreasing trend significantly ($p<0.05$) with respect to controls. Data was further analyzed on basis of different age - groups of the breast cancer patients i.e an early (25-35 years) age-group that showing highest (42.85%) frequency followed by decreasing trend (28.50 %) were observed in older age groups (65-75 years).

Conclusion: Present study reveals that - 1) discordance in the frequency of circulating tumor cells population may be either due to different pathological stages or tissue specific sensitivity after drug exposure at different time intervals mediated toxicity significantly at an early age-groups patients. 2) long-term exposure of the drug showing positive approach to the clinicians for better management, 3) variations in the frequency of DNA copy number significantly support the above findings of cell population that confirm drug mediated genetic susceptibility, and 4) vimentin isoforms are highly relevant to increase dug binding capacity to the antigen of sub-population of circulating tumor cells population during metastasis. Hence, present study may consider as prognostic, diagnostic biomarker for early detection of breast cancer patients.

Key Words. Circulating tumor cells, DNA Copy Number and Breast Cancer Patients,

1. INTRODUCTION.

World Health Organization (2024) says that breast cancer is one of the most complex disease in women and increasing burden continue to increases in > 50 year of age-groups. Hence, early genetic screening becomes an essential tool for better management and survival of the patients that boost the clinicians. Recent study reveals that circulating tumor cells (CTCs) , play an important role to explore the mechanism of tumorigenesis during migration with epithelial to mesenchymal transition (EMT) markers after activation of proto oncogene to primary tumor sites [1-2]. Importantly, the sub-population of tumor cells shows genetic-heterogenicity during differentiation and proliferation of tumor cells at all the stages. Earlier studies on (CTCs) shows controversies due to poor relation between prognosis and diagnosis in variety of cancer patients [3]. Recently, FDA has approved the screening of phenotypic expression of sub-population for liquid biopsy samples using specific monoclonal antibodies for CD45 and EpCAM [4 -7]. Cytokeratin, a filamentous glycoprotein, is abundant in cytoplasm of malignant cells that play an important role to maintain tissue architecture during progression of disease like cancer. CTCs play a "central role" for all the pathological stages between primary to metastasis and maintaining plasticity during angiogenesis. Vimentin, is a specific transmembrane glycoprotein and constitute intermediate filaments to provide networking for adherence between two cell-junction and maintain cellular integrity during signaling act as genetic biomarker for metastatic event during progression of disease. The differential signature of vimentin gene-expression in CTCs are known to initiate migration and invasion from primary tumor site from epithelial cells to mesenchyme through EMT makers in synchronize fashion (Sox4 & EpCAM) that develop phenotypic aggressiveness in cancer patients [8-10].

Cyclophosphamide (CP), an interesting molecule for the clinicians because of its dual relevance as an anticancer and immunosuppressive drug commonly used for the management in cancer and rheumatoid arthritis patients. Application, of CP by the clinicians either alone or in combination for therapeutics is complicated process due to adverse toxic side effects through drug as such or metabolites reactions after enter in liver monooxygenase 450 enzyme system.. Hence, it's becomes challenging to the scientists as well as clinicians for drug mediated cellular toxicity is still lacking in the literature [11]. The contradictory reports after CP treatment in breast cancer patients and promotes metastasis in ovarian cancer cell-lines (MDA-MB-231) followed by increase expression of proteins [12]. In-vivo or in-vitro studies of the same author reveals that CP causes genetic damage and induces complex chromosome rearrangements (CCRs) including aneuploidy at S-phase of cell-cycle in differentiating tumor cell population. The mutagenic potential were also observed in variety of tissues (bone marrow & testis) and also causes protein profile changes in developing rat testes [13-15]. Importantly, CP also modify qualitative and quantitative reduction of primary germ cells and causes interferences in fertility. Hence, the present study has been designed with the aims to explore the etiopathology after in- vitro exposure with CP at three different time intervals i.e. 24, 48, 72 hours to evaluate the frequency of CK19 & Vimentin expression in CTCs population in different age-groups of the patients. The frequency of DNA copy number variations of the same EMT markers were also evaluated for tissue specific genetic susceptibility in breast cancer patients.

2. Materials and Methods.

Peripheral blood (3.0 ml) samples (n=149) were collected in EDTA sterile vials after clinical diagnosis of breast cancer patients and (n=100) respective controls belong to the different age - groups from the OPD of department of surgery & radiation oncology, All India Institute of Medical Sciences, Patna, Bihar ,India. The present study is dully approved by the IRC (Institute Research Committee) and Institute Ethical Committee (IEC).The samples were collected after informed consent from either from the patient or relatives for genetics profiling with controls.

2.1 Isolation of Circulating Tumor Cells by Ficoll's Gradient Method.

Short-term lymphocyte cultures (n=150) were setup in sterile RPMI-1640 media, supplemented with 10% foetal bovine serum and phytohemagglutinin (PHA), and antibiotics. Proliferating cells were exposed with cyclophosphamide (1.0 µ/ml) at three different time intervals i.e 24, 48 and 72 hours. Circulating tumor cells were isolated after harvesting the cultures using Ficoll's density gradient centrifugation procedure from periphery mononuclear cells (PBMCs) and ring is formed at 400g after 30 minutes of centrifugation at cooling centrifugation. Cells were washed twice with 1.0 ml PBS and again centrifuge at 1200 rpm (10 min) at 4^0C and stored at -20°C, till further characterization. Morphological characteristic of individual cells were also visualized under phase contrast microscope after staining with 1.0 % Giemsa to check the viability of proliferating cells before flowcytometric analysis of EMT markers CK19 & Vimentin using specific monoclonal antibodies.

2.2 Characterization of Epithelial Mesenchymal Transition Marker- CK19 and Vimentin in Circulating Tumor Cells

The flowcytometer based characterization of EMT markers the monoclonal antibodies were used for cytokeratin19 (A-3) PE Lot # D2919, [Catalog No sc-376126] conjugated to detect intra cellular antigen (Santa Cruz Biotechnology USA). Similarly, for antibodies of vimentin (VI/RE/1), PE [REF-MA1-19656], Lot ZB4223746] were purchased from Thermo fisher (USA) to detect extra cellular (matrix) antigen in tumor cells population. Cell were fixed and permeabilization buffer is used according to the protocol of the kit (PerFix-nc,

Beckman Coulter, France). Staining procedures were strictly followed by the manufacture's protocol and 50 ul of CTCs were stained with 200ug/ml for CK19 PE and 100ug for Vimentin PE monoclonal antibodies, respectively, and cells were incubated for 60 minutes. Cell were fixed for 10 minute in 5ul of PerFix-nc buffer-1 followed by PerFix-nc buffer-2 and then add antibodies , incubated for 30 minutes, wash two times with 3.0 ml of phosphate buffer and centrifuge at 1600 rpm for 5 minutes to remove excess amount of residues. These cell were allow to keep in permeabilization buffer 3 (1:10 dilution) and again centrifuge at 1600 rpm, finally resuspend the pellet in 500 ul of PerFix-nc buffer-3, and finally cells becomes ready for acquisition immediately on flowcytometric analysis [6].

2.3 Sample Acquisition and Data analysis.
Beckman Coulter Life Science DxFLEX software (Serial No BG10007 Model No DxFLEX) equipment is used for detection of circulating tumor cells with blue argon laser at 488nm. FlowClean Cleaning Agent (100 ul) from Beckman Coulter is used for daily clean of instrument and QC/Standardization is passed using DxFLEX Daily QC Fluorospheres (REF C39283, LOT 15AQHF Beckman Coulter) before performing experiments. FSC and SSC data were analysed using CytExpert version 2.2 for DxFLEX software and gating strategy were selected for CK19 and Vimentin positive circulating tumor cells excluding two cell population i.e. lymphocytes & erythrocytes using specific monoclonal antibodies. More explicitly on a CD326/SSC dot plot a gate was drawn to select only CD326 population.CK19/SSC dot plot a gate was drawn to select CK19 population. In another set of experiment, where, the cells were exposed with cyclophosphamide for detection of CK19 and Vimentin positive population of CTCs at different time intervals, compare with controls [16-17]

2.4 DNA Copy Number Variation of Epithelial Mesenchymal Transition Marker - CK19 and Vimentin in Circulating Tumor Cells Population.
PCR products of EMT markers (CK19 & Vimentin) in CTCs were characterize after using with specific forward & reverse primers- CK19 F - 5'-ATTCCGCTCCGGGCACCGATCT; R-CGCTGATCAGCGCCTGGATATGCG and Vim-FP-5'-GACAATGCGTCTCTGGCACGTCTT-3'; Vim-RP-5'-TCCTCCGCCTCCTGCAGGTTCTT-3', after confirmation of sequences from NCBI (BLAST/http://blast.ncbi.nlm.nih.gov.). The total volume of 25µl PCR mixture containing 5x Green GoTaq PCR reaction buffer that consist of dNTPs Mix (10 mM), 1µl each of 10 pmol of primers, 0.2µl of GoTaq DNA polymerase (5U/µl). The reaction profile was consist 35 cycles comprising, initial denaturation at 95°C for 5 minutes. EMT markers showing slightly different PCR protocols CK-19, the denaturation time at 95°C for 45 seconds, annealing at 60.2°C for 30 seconds and elongation at 72°C for 1 minute, followed by final elongation at 72°C for 7 minutes. For vimentin, the cell-cycle conditions remain same i.e. 35 cycles comprising, initial denaturation at 95°C for 5 minutes, followed by denaturation 95°C for 45 seconds, annealing at 60.0°C for 1.0 minutes and elongation at 72°C for 45 seconds, followed by final elongation at 72°C for 8 minutes. The amplified PCR products of CK19 (573bp), Vimentin (323bp) were characterize on agarose gel (1.5%) and individual bands density were evaluated after staining with ethidium bromide for of DNA copy number variations using inbuilt software by Gel Doc systems (Bio Rad USA).

2.5 Statistical Analysis.
Significant differences (p= values) between two CTCs population i.e CK19 and Vimentin were calculated using SPSS 27.0 version software (USA). The data was further analysed using chi square - test (two tailed) to find out the level of significance, confidence interval (C.I.) at 95% and odd ratio (O.R) between cases and controls.

3. Results

3.1 Findings of Circulating Tumor Cells Population after In-Vitro Exposure with Cyclophosphamide.
Table-1 showing the details statistical analysis of two EMT markers - CK19 and Vimentin in CTCs population isolated from breast cancer patients using flowcytometric analysis to evaluate the (%) frequency and their sensitivity. The sensitivity or cellular toxicity were again evaluated after in-vitro exposure of CP at three different time intervals (24, 48 &72 hours) to evaluate the frequency of proliferating cells population and compare the same with controls. The mean ± s.d values (%) frequency in mix circulating tumor cells population showing by forward cell scattering (FCS) and pure population after gating is more than (3243.66 ± 17.51) cells were score in CK19 monoclonal antibodies labeled CTCs population, and decreasing trend were observed significantly ($p<0.05$) cells, after exposure with CP to the proliferating cell and compare with controls as shown in fig. -1A, B, C (controls) and fig.1D, E, F for 24, 48 (fig.1 G,H, I) and for 72 hours as shown in fig. J, K, L). The statistical analysis were carried out for 72 hours, in the CTCs population of CK19 shows significantly ($p< 0.001$) decrease (1945.5) (20.54) with the calculated values of odd ratio (188) and C.I at 95% interval varying between 1679.26 - 917.06 with respect to controls.

The data of vimentin was further analyze for CTCs population that showing highly significant ($p<0.001$) decreasing trends at

24 hour (4565.5 ±14.51) and 72 hours (2997.5±15.0) during exposure with CP and compared with controls (9528 ± 12.19) group. The frequency (%) of mean ± s.d values (1055 ± 10.58) of CTCs population were observed at 48 hour exposure of CP showing lack of significant difference with respect to controls (fig.2 A, B, C) but significance were observed at 24 as shown in fig. 2 D, E, F and 72 hours (fig.2 J, K, L), perhaps due to selective sensitivity towards CP.

3.2 DNA Copy Number Variations of CK 19 & Vimentin in Circulating Tumor Cells

The sensitivity of EMT markers CK19 and vimentin were also characterize by PCR with specific forward and reverse primers on agarose gel (1.5%) electrophoresis followed by individual bands intensity were evaluated for DNA copy number variations using densitometric analysis with the help of inbuilt software by Gel Doc systems (Bio Rad. USA).. The amplified products showing bands of CK19 (573bp) and vimentin (323bp) after staining with ethidium bromide (fluorescence dye). The individual bands showing different- tissue specific sensitivity (frequency) as shown in figure 3A & 3B Bar diagram showing unusual significant variation of vimentin at 24 and 72 hours with respect to controls (fig.3A).This systematic decreasing trend were observed in CK19 at 24, 48, 72 hours intervals, after CP exposure in proliferating cells and compare with controls (figure 3B)..

3.3 Age-Wise Changes in Epithelial Mesenchymal Transition Markers - CK19 and Vimentin Expression in Circulating Tumor Cells Population

Peripheral blood samples of BC patients were divided at different age-groups that showing variation in frequency of the epithelial mesenchymal marker - after combining of CK19 and Vimentin in circulating tumor cells, because of similar physiological chemical nature of (glycoprotein) and abidance in the cytoplasm of the tumor cells. The highest frequency (42.86%) were observed in early (25-35 year) young age-groups, followed by decreasing trend were observed in elderly 65-75 years of age-groups (28.6 %) as shown in figure-4.

4.0 Discussion.

The genetic signature in breast cancer patients are highly relevant because of influence by genetic and epigenetics factors. Besides, this food habit including dietary factors like smoking or alcohol in rural or urban population effects endocrine-dysregulation. In India, Bihar belong to agriculture state and women also work in agriculture fields where exposed to pesticide or insecticide that might have exposed, if continue for longer period of time that increase risk factor for damage genetics factors including chromosome aberrations or DNA either in somatic or germ cells. In tumor bearing patients, earlier diagnosis is essential for better management by the clinicians. Still diagnosis is based on invasive techniques like histopathology, immunochemistry showing lack of accuracy with time consuming, less sensitive resulting high mortality rate and delay in the management for cancer patients. Therefore, scientists have developed and improving most sensitive technology based on (liquid biopsy), application of CTCs using specific EMT markers evaluated by specific monoclonal antibodies using flowcytometric analysis for cancer patients.

The exact mechanism of tumorigenesis is highly complex and still not clear, but authors believe that after activation of proto oncogene (kras) mediated early transcription factor (Sox4) pass their signaling to EMT markers CK19 and Vimentin that exist in abundance in cytoplasm to initiate the migration and invasion to the primary tumor sites through blood stream by CTCs population at the tumor site of rich ecological niche . Although, the biology of EMT markers like, Sox4, EpCAM, CK19 and Vimentin are quite interesting because of involvement at pre metastasis, metastasis and post metastasis events during tumorigenesis, suggesting, among all EMT markers, Vimentin may be used as the most significant biomarker for early detection because of dual properties- 1) abundance in cytoplasm, and 2) exist of isoform help to strongly bind to the antigen (truncated protein) during proliferating cells of the breast cancer patients. The advancement of CTs is the emerging field in molecular medicine for early diagnosis and timely management by the clinicians may improve the life of the cancer patients. Earlier study based on RT-PCR showing discordance in (%) frequency of EMT markers in variety of tumors like adenocarcinoma of gall bladder, pancreatic tissue and Wilms tumor and breast cancer perhaps either due to tissue specific genetic susceptibility [18] Earlier also support the findings of the present study that reveals flowcytometery is a sensitive and reliable technique for the study of CTCs population , used for the identification of EMT markers like Sox4 and EpCAM gene signature with different expression after exposure with 5-Azacytidine to evaluate the sensitivity of CTCs population [6]. Although, the 5-azacytidine and cyclophosphamide both are antineoplastic agents and discrimination in the frequency of EMT markers in CTCs population may be either different specific genetic susceptibility towards antigen or pharmacodynamics in nature. Similarly, present study also showing significant down-regulation of CK19 and Vimentin in CTCs population after in-vitro CP exposure in different age-group patients due to cytostatic nature of the drug mediated toxicity. The discordance in the frequency of EMT markers between 5-azacytidine and cyclophosphamide due to different pharmacokinetics or tissue specific susceptibility towards drug-protein interaction, suggesting early detection of

EMT markers becomes essential to helpful for the clinicians for better management to the cancer patients [19 - 22].

Interestingly, EpCAM, an intracytoplasmic EMT marker showing more sensitivity (98.98%) for the transition of CTCs population after tag with mAbEpCAM even after CP exposure that again validates the significant role during progression of disease. CP metabolite such as acryl aldehyde (acroline) - a strong mutagenic or carcinogenic might have increased risk factor of the secondary tumorigenesis if exist inside the tissue. Data of the present study showing high sensitivity between two different antigen in CTCs population due to highly significant ($p<0.001$) differences at 72 hours, suggesting that these extracellular cytoplasmic markers are more prone for cellular toxicity or mutagenicity, if exposed for longer period of time. Unequivocally, findings showing lack of consistency in individual frequency (%) in CTCs population between different antigens (CK19 and Vimentin) based on either due to different stages of tissue growth (pre or post metastasis) or may be different age-groups for drug mediated selective sensitivity. Earlier study showing elderly group (>50 year) have high significant +ve CTCs population number as compared to the women belongs to young age group (less than 50 years) of female patients but present study showing just contrast finding may be because of two different nature of antigen EMT markers i.e Sox4 - an early nuclear transcription factor activated by proto oncogene (kras) and over expression is responsible for diversity in signaturing during migration followed by invasion to CTCs population in blood stream that showing aggressiveness in cancer patients Although, high expression of EpCAM in elderly age groups (51-61years) may be due to stability or abundance in cytoplasm during metastasis or post metastatic stages during disease progression [23].

The study of CTC population acts as 'key player' for early diagnosis of cancer patients and their identification is a difficult task because of small quantity (1-2%) or 1-5 cell found in millions of cell per 7.5 ml in the blood samples at all the stages i.e. early metastatic to post metastatic events [6, 8, 9]. Present study showing differential expression of CK19 and vimentin gene signature due to different sensitivity in CTCs population after exposure to CP, at different time interval to determine mutagenic potential. Although, the earlier study reveals that CP, induces variety of chromosomes aberrations, DNA, RNA and protein-synthesis either due to dose or time intervals in-vivo or in-vitro [19]. Similarly, our study also confirm that the systematic significant reduction of +mAb CK19 tagged CTCs population after using CP, 24,48 & 72 hours exposure may be either due to abundance in cytoplasm and selective sensitivity and over- expression (97.0 %) varies in different tissues like colon cancer, but reduce to 41.7 % , in breast cancer patients may be due to tissue-specificity genomic susceptibility either at M0 metastatic or M1 stage of the cell- cycle during progression of the disease. [7, 9]. Interestingly, the variation in the mean values of Sox4 cell-population increases with respect to controls may be due to selective genetic susceptibility towards antigen or over-expression act as growth initiator during metastasis. Side scattering of CTCs is the relevant features for the characterization of three major subset population including polymorphic cells, monocytes and lymphocytes. Now after gating, become necessary to know cell-proliferation rate based on cell-kinetics during therapeutics in disease progression that might be helpful for the clinicians to the breast cancer patients. Most of the studies shows variation in antigen expression using two different approaches either MCF-7 cells or immunochemistry or after collection of 10-15 ml of blood samples for isolation of CTCs population, that seems to raise ethical problems and to confirm genetic heterogeneity. Although, our approach have an advantage to reduce sample size (0.5ml), followed by to reduce heterogeneity in CTCs sub population with increased sensitivity of EMT markers [22, 23].

Interestingly, the data of the present study is dually evaluated that confirm the sensitivity of the EMT markers using specific antibodies to detect two different antigens (+mAbCK19 or +mAbVimentin) and DNA copy number variations after exposure with CP. Earlier study of Sox4 also support the present study is to encourages metastasis through over-expression and responsible for phenotypic aggressiveness in cancer patients [24]. Our study also emphasizes that the behavioral phenotypic changes occurs because of Sox4 over-expression and signaling to other EMT markers i.e EpCAM, CK19 and Vimentin in CTCs population either in in synchronize manner or independently to promotes tumorigenesis unconstitutionally [25] .

Conclusion. In CTCs EMT marker- Vimentin signature is variable due genetic susceptibility under the influence of antineoplastic drugs that conform for metastatic biomarkers in breast cancer patients for early age groups. Present study will be significantly helpful to the clinicians for better management, if diagnosed at an early stage of cancer patients .However, our study also focus on functional aspects to open new avenues of drug mediated research for cellular sensitivity through tissue engineering techniques to reverse the transition i.e from mesenchymal to epithelial cells by developing new inhibitors to reduce the mortality followed by enhance the survival of the patients.

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Availability of Data and Materials
The data presented in this study are available on request from the corresponding author.

Authors' Contributions
AKS executed experiment designed, implementation and Shalini, PhD student help for validation of findings and data analysis of the study during preparation of the manuscript.MK help for clinical diagnosis of the breast cancer patients.

Ethical Approval and Consent to Participate
Study was approved by Institute ethical committee of AIIMS Patna (AIIMS/Pat/IRC/2020/610), and informed consent form was dually signed by patients.

Patient Consent for Publication
Patients consent form was dually signed by patients involved in study.

Competing Interests
There are no competing interests among the authors.

References.
1. Cristofanilli, M., et al. (2004) Circulating Tumor Cells, Disease Progression, and Survival in Metastatic Breast Cancer. The New England Journal of Medicine, 351, 781-791.
2. Jansson, S., Bendahl, P.-O., Larsson, A.-M., Aaltonen, K.E. and Rydén, L. (2016) Prognostic Impact of Circulating Tumor Cell Apoptosis and Clusters in Serial Blood Samples from Patients with Metastatic Breast Cancer in a Prospective Observational Cohort. BMC Cancer, 16, 433.
3. Chen, L.M., Lazcano, O., Katzmann, J.A., Kimlinger, T.K. and Li, C.Y. (1998) The Role of Conventional Cytology, Immunocytochemistry, and Flow Cytometric DNA Ploidy in Evaluation of Body Cavity Fluids: A Prospective Study of 52 Patients. American Journal of Clinical Pathology, 109, 712-721.
4. Schneller, J., Eppich, E., Greenebaum, E., Elequin, F., Sherman, A., Wersto, R. and Koss, L.G. (1987) Flow Cytometry and Feulgen Cytophotometry in Evaluation of Effusions. Cancer, 59, 1307-1313
5. Herrmann, H., Strelkov, S.V., Burkhard, P. and Aebi, U. (2009) Intermediate Filaments: Primary Determinants of Cell Architecture and Plasticity. Journal of Clinical Investigation, 119, 1772-1783.
6. Saxena Ajit K, Shalini and Kumar Manoj.Flowcytometric based Analysis of Sox4 Gene Expression-An Early Transcription Factor Influenced by 5-Azacytidine and Compare with EpCAM in Circulating Tumor Cells Isolated from Breast Cancer Patients. Journal of Oncology Research and Therapy. 9 (03); 1-7; 2024.47-57.
7. Pollard, T.D. and Cooper, J.A. (2009) Actin, a Central Player in Cell Shape and Movement. Science, 326, 1208-12126. Poruk, K.E., et al. (2016) Circulating Tumor Cell Phenotype Predicts Recurrence and Survival in Pancreatic Adenocarcinoma. Annals of Surgery, 264, 1073-1081.
8 Anderson, J.M., Heindl, L.M., Bauman, P.A., Ludi, C.W., Dalton, W.S. and Cress, A.E. (1996) Cytokeratin Expression Results in a Drug-Resistant Phenotype to Six Different Chemotherapeutic Agents. Clinical Cancer Research, 214. 97-1051.
9. Ao, Z; Shah, S.H.; Machlin L.M.; Parajul.i R.; Miller P.C.; Rawal .S.; William A. J.; et al .(2015). Identification of cancer associated fibroblast in circulating blood from patients with metastasis breast cancer. Cancer Res. 75, 4681-4687. 10. Saxena Ajit K**.** Shalini, Pritanjali Singh, Veena Singh. Differential expression of Vimentin gene modulated by MTHFR C677T gene polymorphism in circulating tumor cells isolated from breast cancer patient. Genetics and Molecular Research, 23(1); 1-10, 2024.
11. Gadisa DA, Assefa M, Wang SH, Yimer G. Toxicity profile of Doxorubicin-Cyclophosphamide and Doxorubicin-Cyclophosphamide followed by Paclitaxel regimen and its associated factors among women with breast cancer in Ethiopia: A prospective cohort study. J Oncol Pharm Pract. 2020 ; 26 (8):1912-1920.
12. Hung CM, Hsu YC, Chen TY, Chang CC, Lee MJ. Cyclophosphamide promotes breast cancer cell migration through CXCR4 and matrix metalloproteinases. Cell Biol Int. 2017 ; 41(3):345-352.
13. Saxena Ajit K**.,** and Singh, G. Cyclophosphamide induced chromosomal aberrations and associated congenital malformations in rat. In- Vitro Cell & Developmental Biology. 34(10): 751-752; 1998.
14. Saxena Ajit K and Singh, G. Cyclophosphamide mediated changes in the frequency of chromosomal aberrations in rat testis when exposed in-utero. Proc. Nat Aca Sciences. 68:247-252; 1998.
15 Saxena, Ajit K and Singh G. Cyclophosphamide modulate gene expression in neonatal rat testis following antenatal exposure to the developing rat foetuses during testicular differentiation. Archives of Andrology. 42: 205-210; 1999. ok
16. Unger, K.M., Raber, M., Bedrossian, C.W., Stein, D.A. and Barlogie, B. (1983) Analysis of Pleural Effusions Using Automated Flow Cytometry. Cancer, 52, 873-877.

17. Krishan, A., Ganjei-Azar, P., Jorda, M., Hamelik, R.M., Reis, I.M. and Nadji, M. (2006) Detection of Tumor Cells in Body Cavity Fluids by Flow Cytometric and Immuncytochemical Analysis. Diagnostic Cytopathology, 34, 528-541.
18 Saxena Ajit K, Shalini, Vipul Vaishnava, Pritanjali Singh and Manoj Kumar. Discordance in the frequency of epithelial mesenchymal transition markers Sox4, EpCAM and CK19 gene expression in circulating tumor cells in variety of cancer patients – Significant correlation with p53 and MTHFR C677T gene polymorphism. AS Cancer Biology 8(11) 11-17; 2024 \.
19. Davidson S, Crowther P, Radley J, Woodcock D C. (1992). Cytotoxicity of 5- aza 2'- deoxycytidine in a mammalian cell system. Eur J Cancer 28:362-368.
20. Fucik V, Michaelis A and Rieger R. (1970). On the induction segment extension and chromatid structural changes in Vicia faba chromosome after treatment with 5-azacytidine and 5'-aza-2-deoxycytidine.Mutation Res.9:599-606.
21. Saxena Ajit K, Srivastava AK, Singh G. (2007).Unexpected segregation of chromosome and common fragile site expression induced by 5 Azacytidine exposure in human lymphocytes of Down syndrome patients. Biomed Research 18 (1):31-34.
22.Kiziltepe T, Hideshima T, Catley L ,Raje Noopur , Yasul Hiroshi, et al. (2007). 5-Azacytidine, a DNA methyltransferase inhibitor induces ATH mediated DNA double strand breaks responses, apoptosis and synergistic cytotoxicity with doxorubicin and bortezomib against multiple myeloma cells. Molecular Cancer Therapeutics, 6 (6); 1718-1727.
23. Saxena Ajit K**.** Meenakshi Tiwari, Pritanjali Singh, Veena Singh. Comprehensive Mutational Spectra of Epithelial Mesenchymal Transition Markers-SOX4, EpCAM, CK19 and their Impact of Methylene Tetrahydrofolate Reductase C677T Gene Polymorphism Associated Risk Factor Modified by BRCA and TGFβR1 Genes in Pre/Post-Menopausal Cases of Breast International Journal of Cancer. Medicine, 7; (1)158-182: 2024.
24. Song G, Sun Y, Shen H and Li W (2015). Sox4 over - expression is a novel biomarker of malignant status and poor diagnosis in breast cancer patients Tumour Biol. 36(6):4167-73
25. Konigsberg R, Obermayr E, Bises G, Pfeiler G and Gneist M. (2010).Detection of EpCAM positive and negative circulating tumor cells in metastatic breast cancer patients. Acta Oncologia 20 (1):700-710.

Caption of Figures

Figure-1 A-L. Flowcytometric analysis showing detection of CK19 in CTCs population in breast cancer patients. Graphically represents total circulating tumor cells population +/-ve CK19 (red/pink) (fig.1A). Forward cell scattering showing after gating pure population (red) (fig.1B). Histogram showing mean frequency of population + CK19 (17.51) in controls (fig.1C) and after in-vitro exposure of CP at 24 hour interval, graphically, showing pure cell population of CK19 (fig.1D), and pure population after gating of +veCK19 (red) (fig.1E). Histogram showing decreasing trend of frequency + CK19 (16.58) in cell population (fig.1F) at 48 hour (fig.1G) and pure population (fig.1H). Mean frequency of + CK19 (15.93) (fig.1I) with mix population (fig.1J) and after gating pure population (fig.1K) at 72 hour as shown in histogram (fig.1L).
Figure-2 A-L Graphically represents total CTCs population +/-ve vimentin (red/pink) as shown in fig.2A, forward cell scattering showing cell-size of total cell population and pure population after gating (fig.2B).Histogram showing mean frequency of cell population of +vimentin (12.19) in control (fig.1C). In-vitro cells were exposed with CP at 24 hour interval, graphically, showing total cells population of vimentin (fig.2D) and pure population (fig. 2E) with histogram showing frequency (14.51) of cell population (fig.2F). At 48 hour interval, graphically, showing mix and pure population of vimentin (fig.2G & 2H), and histogram showing mean frequency of + vimentin (10.38) as shown in fig.2I. Similarly, at 72 hours mix and pure + vimentin cell population depicted in fig2 J &2K. Histogram showing mean frequency of + vimentin (15.00) CTCs population (fig.2L).
Figure-3A &B. Bar diagram showing variation in the (%) frequency of DNA copy number variations (CK19 and Vimentin) in CTCs population after single exposure with CP at three different time interval i.e. 24, 48 and 72 in breast cancer patients. Initially, bar diagram showing decreasing trend at 24 hours exposure, suddenly increase of CK19 in CTCs population i.e. at 48 hours and again decrease significantly at 72 hours (fig.3A). Bar diagram of vimentin showing systematic decreasing trend at 24, 48 and 72 hours in CTCs population as compared to controls.
Figure-4.Pie-chart showing frequency (%) distribution of EMT markers in different age-groups in CTCs of breast cancer patients. Maximum frequency of CTCs population were observed in 25-35 age group followed by decreasing trend were observed in 45-55 and 65-75 age groups.

Table -1: Data analysis showing level of significance between CK19 and Vimentin in CTCs population of breast cancer patients after in-vitro exposure with CP and compare with controls.

S.No	EMT markers	Control &Exposure Time intervals	Mean & % (frequency)	Standard Deviation	Standard Error	Confidence Interval @ 95%	p-values
1	CK19	Control	3243.66 (17.51)	863.84 (0.07)	---	---	---
		24hrs CP exposure	2753.5 (16.58)	551.5 (0.032)	223.647	942.17 - 38.16	0.0343*
		48hrs CP exposure	2720.1 (15.93)	719.0 (0.008)	245.258	1019.35- 27.98	0.0389*
		72hrs CP exposure	1945.5 (20.54)	21.5 (0.020)	188.564	1679.26 - 917.06	0.0001**
2	Vimentin	Control	9,528 (12.19)	4002.40 (0.06)	---	---	---
		24hrs CP exposure	4565.5 (14.51)	1885.5 (0.01)	617.722	6913.76 -301.1	0.0001**
		48hrs CP exposure	1055.99 (10.38)	682.1 (0.05)	317.289	719.64 - 2861.64	0.2338
		72hrs CP exposure	2997.5 (15.00)	1073.5 (0.03)	403.921	8358.08 -4702.91	0.0001**

** Highly significant differences ($p<0.001$ were observed between experimental groups and controls using X^2-test

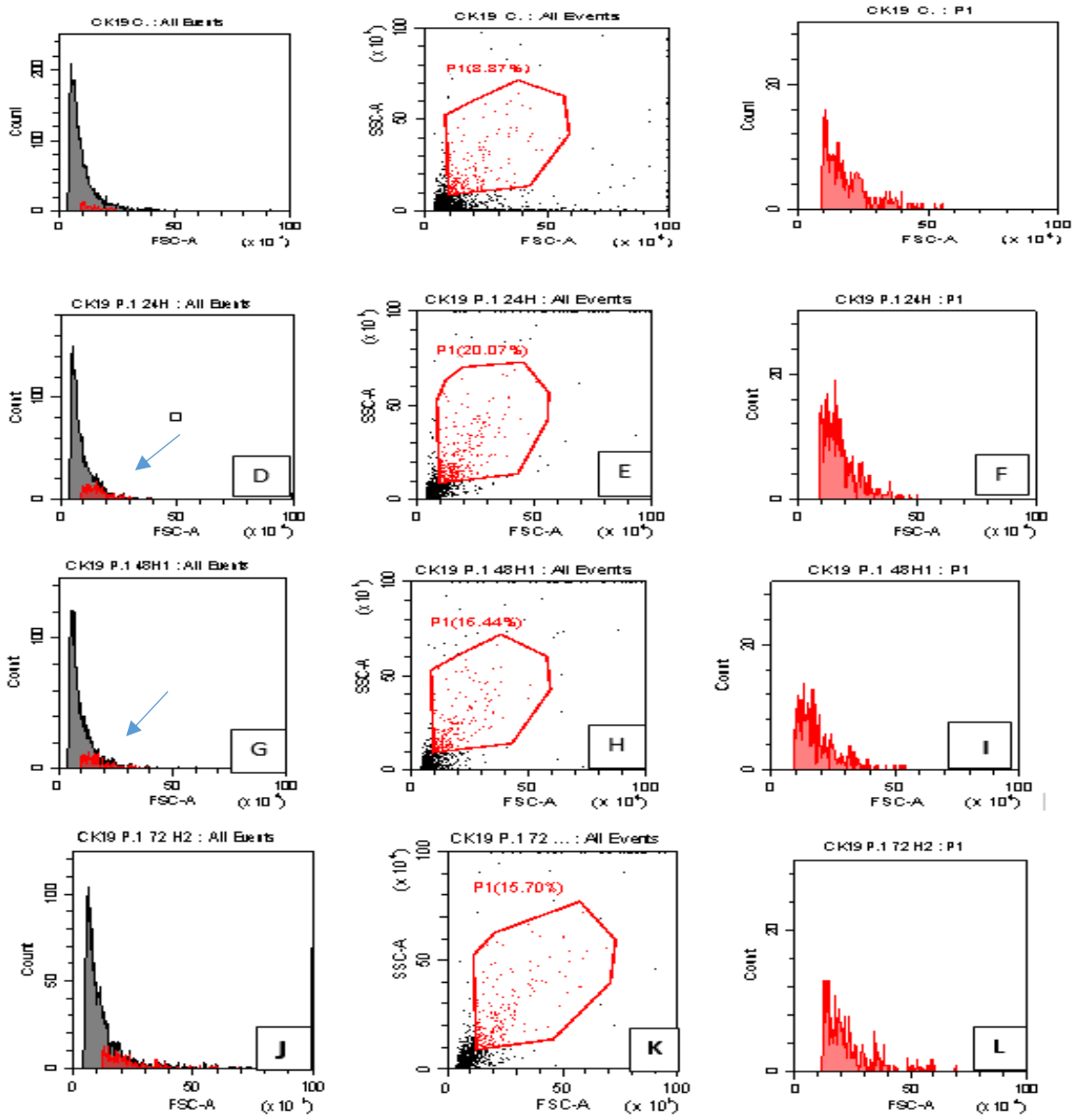


Figure-1 A-L

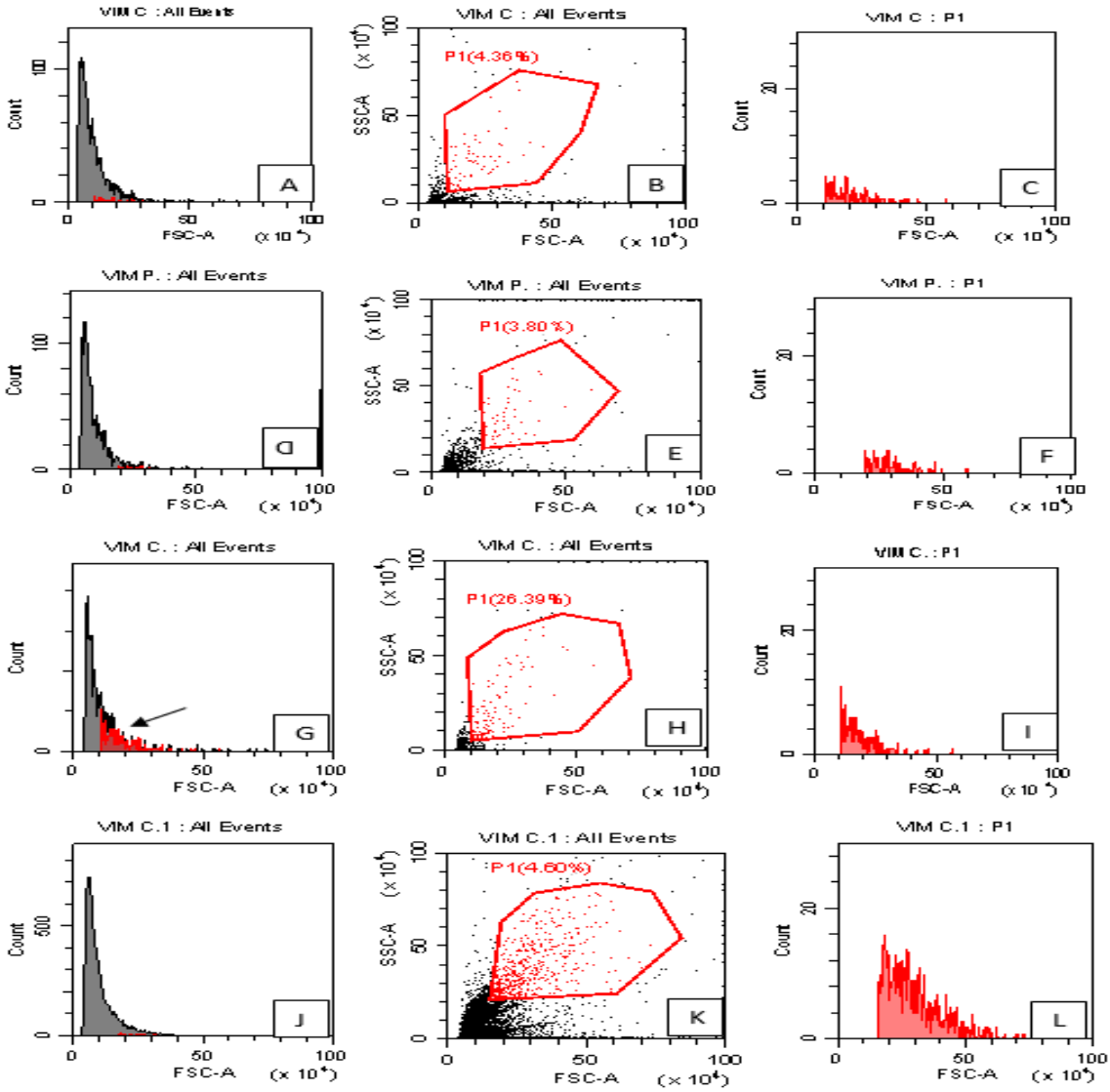
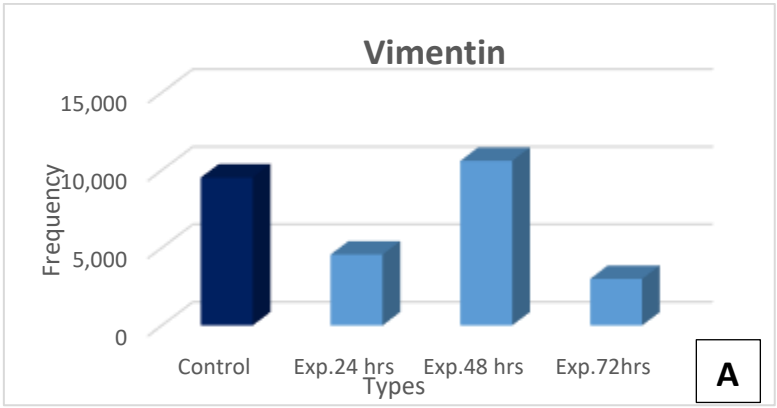

VIM C : All Events
VIM C : All Events
P1(4.36%)
VIM C : P1
VIM P. : All Events
VIM P. : All Events
P1(3.80%)
VIM P. : P1
VIM C. : All Events
VIM C. : All Events
P1(26.39%)
VIM C. : P1
VIM C.1 : All Events
VIM C.1 : All Events
P1(4.60%)
VIM C.1 : P1
Count
SSC-A
FSC-A
A
B
C
D
E
F
G
H
I
J
K
L

Figure-2 A-L


Vimentin
15,000
10,000
5,000
0
Frequency
Control
Exp.24 hrs
Exp.48 hrs
Exp.72hrs
Types
A

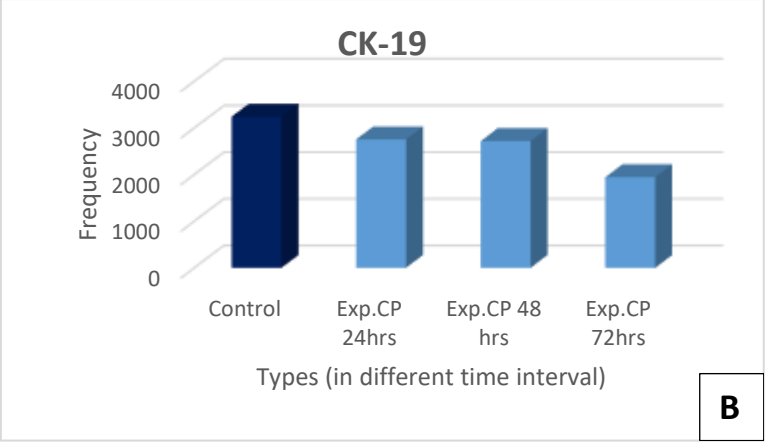


Figure 3A & B:.

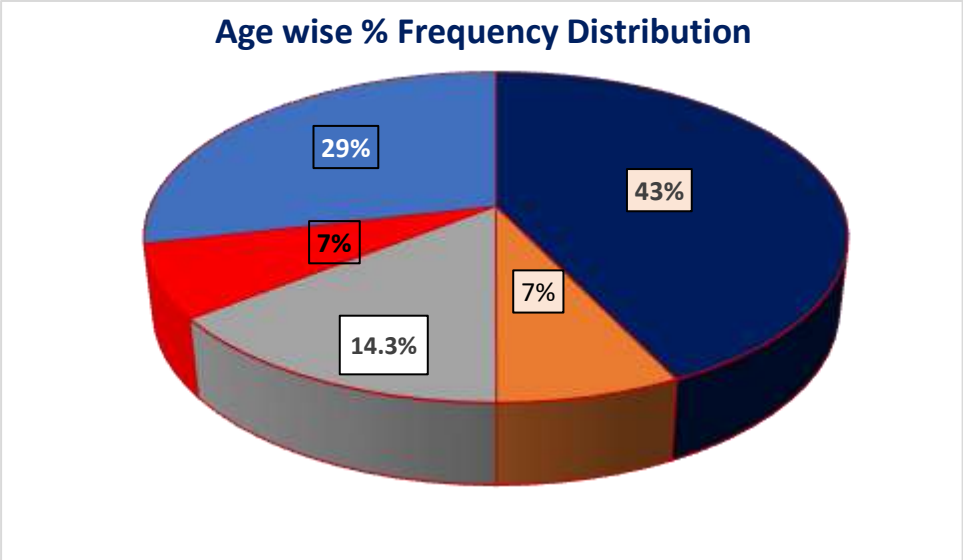


Figure 4-